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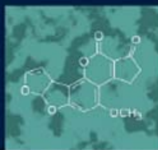
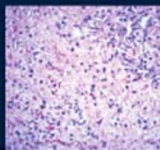
RAMESH C. GUPTA





# VETERINARY TOXICOLOGY

## BASIC AND CLINICAL PRINCIPLES

# VETERINARY TOXICOLOGY

*This book is dedicated to my daughter Rekha, wife Denise, and parents the late Chandra and Triveni Gupta*

# VETERINARY TOXICOLOGY

## Basic and Clinical Principles

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*There are in fact two things, science and opinion; the former begets knowledge, the latter ignorance*

Hippocrates (460BC–377BC)

# Foreword

## WHAT'S THIS ABOUT "VETERINARY TOXICOLOGY"?

"Exciting!" "Challenging!" "Progressive!" "Rewarding!" Certainly not "Dull!" "Routine!" "Old stuff!" "Repetitious!" Just look at the titles of the chapters in this encyclopedic reference, and you'll get turned on to the relevant and cutting-edge science, diagnostics and real-world problem-solving going on in the discipline of "Veterinary Toxicology".

Where did it come from? How did it evolve to get that way? What is its current focus and where is it going? These are all stories worth telling. Let us start at the beginning . . .

In the beginning was folk medicine to deal with sufferings. That evolved into ritualistic "medicine", the use of herbs, plants, and incantations. Studies of how things work and categorizing illnesses organized into formal medicine, and then disciplined further to physiology. With the use and application of natural and synthetic chemicals to challenge body functions, pharmacology grew into its own specialty dealing with using these same compounds for therapeutic purposes. With even more sophistication, recognition of numerous dangerous effects from these products called for a clearer definition and understanding of what separated a "safe" medicinal from one that caused more pain and danger than the illness being treated. The thesis of "dose is everything" was born . . . and with it and its confounding parameters, toxicology was born!

And what a prodigious science that birth produced! Spurred on by the industrial revolution, urbanization, and "Better living through chemistry", the unfortunate ill effects of "too much" became obvious. Instances of chemical misuse, finding chemicals in unexpected locales, and the lack of the knowledge needed to deal with environmental, human, and animal toxic misadventures spurred public outcries. Government actions followed, but were also led by concerned and inquiring scientists. Their recognition that there is strength and opportunities in bonding together to encourage and share the development of this necessary and energetic science flowed into the growth of international organizations fostering and sponsoring the growth of toxicology in all its important ramifications.

As a specialty, veterinary toxicology was an early player in the toxicology arena. At first it was closely related to veterinary clinical medicine and pathology, particularly concerned with animal losses associated with the large scale use of open ranges for livestock grazing and then later the application of various insecticides to control insect-borne diseases in animals. But expanding agricultural needs triggered by the growing US population and scientific advances in chemistry, biochemistry and diagnostic techniques "pushed the envelope" to understand and resolve these losses and improve animal health. If animals were being challenged by these risks, could humans be far behind?

Increased sophistication of human pathology and forensic medicine stimulated similar developments in veterinary medicine. With the ever-increasing expansion of livestock populations on western ranges, concern developed for the death losses from consumption of poisonous plants. Poor grazing conditions and access to often unrecognized toxic range plants stimulated a wide range of investigations by federally employed as well as private veterinarians and resulted in large numbers of publications in the 1920s and the 1930s to inform livestock owners and to reduce losses. With this came the need for clearly identifying what toxic components were present and how they might be effectively treated. Such studies recognized the value of the veterinarian's training and utilized his multifaceted skills in toxicologic studies for clinical evaluations. This was rapidly recognized by industrial institutions and the veterinarian's talents were

sought for application to commercial drug trials in laboratory animals. These veterinary scientists wearing the hat of toxicology began expanding their responsibilities to biochemical separations, electron microscopy and studies of the cellular and molecular mechanisms of these chemical actions.

World War II and the extensive use of insecticides for pest control and speculative gas warfare resulted in veterinarians being employed by the armed forces in other experimental animal studies. After the war, these and newer chemicals were widely applied to problems in agriculture. Their use required skilled veterinary supervision and all too quickly veterinary treatment when the misuse of these potent chemicals occurred. Facilities at universities, such as Texas A&M, and at governmental research institutions, such as the Poisonous Plant Laboratory at Logan, Utah, focused efforts on the growing hazards and clinical problems resulting in domestic animals. The growth of the pesticide field, coupled with the intensive land use encroaching on plant and animal habitat, required increasing chemical and biological knowledge to understand and identify the disease processes involved.

Veterinary pathologists, such as C.C. Morrill, W.L. Sippel, K. McEntee, and P. Olafson, became increasingly interested in the toxic problems now being seen in expanding numbers. J.L. Shupe concerned himself with detail studies of fluorine intoxication associated with industrial pollution problems. Veterinary pharmacologists began to investigate specific toxicants and their effects on domestic animals; W.G. Huber studied toxic effects of chemotherapeutic agents and antiseptics; R.P. Link identified dicumarol as the anti-clotting factor in sweet clover poisoning and spoke out warning against insecticide poisonings; P.B. Hammond investigated heavy metal toxicities with particular interests in utilizing chelating agents to treat lead poisoning; O.H. Muth studied trace minerals and their interactions in animal intoxications; R.D. Radeleff worked extensively with insecticides and their harmful effects in domestic animals; J.S. Palmer worked closely with Radeleff performing similar investigations on herbicide and pesticide toxins; W. Binns and J.W. Dollahite studied the pathology and biochemistry of numerous poisonous plant intoxications in livestock. Information describing the specific pathology and biochemistry produced by the increasingly recognized number of xenobiotics and naturally occurring materials were coupled with the veterinary experiences discovered in diagnosing clinical cases and effectively providing treatment.

By the mid-1950s, toxicology was a highly active area of veterinary medicine. Biochemical and molecular interactions were discussed and the tools of other disciplines were brought to focus upon the problems of domestic animal poisonings. With it, a new breed of veterinarian emerged. The developing veterinary toxicologist had to understand physiology and pathology, but equally important he had to be a chemist with wide knowledge of separative and quantitative instrumental techniques. Professional judgment of clinical episodes and a working knowledge of metabolic and excretory processes were needed. He had to become intimately familiar with pharmacology and the molecular action of a wide variety of chemicals. Understanding treatments to be administered for specific intoxications was necessary, and finally he had to logically and scientifically put into perspective the often confusing and confounding assortment of signs, lesions and analytical results to reach rational interpretations and conclusions for the numerous problems being solved. Since increasing knowledge was being sought, this well-grounded veterinarian had also to be able to conduct significant independent research in well-equipped facilities. More and more veterinarians now were conducting toxicological research investigations as their primary mission.

In the presence of this increasing need and professional situation, a small group of veterinarians specializing in toxicology united to focus attention on the needs of veterinary toxicology and to assist the progress and growth of this discipline. The formation of the American College of Veterinary Toxicologists (ACVT) in 1958 was the beginning of formal development and recognition of veterinary toxicology. At a meeting in Salt Lake City, Utah, on January 15, 1958, the ACVT was formed by 11 veterinarians stimulated by, and engaged in, toxicology. The organizing committee consisted of Doctors Chapman, Christofferson, Furgeson, Harris, Hayden, Holmes, Jones, Phelps, Shupe, Spencer, and Vinsel. The group's objectives were: "To further the educational and scientific progress in veterinary toxicology and to encourage education, training and research in veterinary toxicology; To establish standards of training and experience for . . . specialists in veterinary toxicology; To further recognition of such qualified specialists . . .; To arrange meetings to promote discussion and interchange of ideas in the following fields of veterinary toxicology: teaching, research and development, diagnosis, nomenclature, public health . . .; To provide all possible aid and assistance to its members by the interchange of ideas and scientific information; To review manuscripts . . .; To review published material and keep a file on such reviews . . .; To accumulate and disseminate information in the field of veterinary toxicology . . .; To encourage adoption . . . of uniform clinical and laboratory reporting methods . . .; To suggest or direct basic research in those areas of deficient knowledge . . . " (Constitution, *American College of Veterinary Toxicologists*, Adopted 1958, Salt Lake City, Utah.)

By 1968 the ACVT grew to over 100 Fellows and Associate Fellows. It has worked efficiently and had stimulated national and international recognition of veterinary toxicology as a progressive and dynamic specialty.

This vitality was further stimulated in 1964 by the New York Academy of Sciences publishing a volume devoted to veterinary toxicology based on the proceedings of an international meeting held in New York City (Gabriel, K.L., editor. 1964. Veterinary toxicology. *Annals of the New*

*York Academy of Science* III, Art. **2**: 559–812). This symposium provided basic information on the energetic activities in veterinary toxicology at that time and had the effect of stimulating further growth and multidisciplinary efforts in the field. The increasing demand for specialized training in veterinary toxicology also encouraged academic training programs. Early efforts were established in universities at Cornell, Utah State, Iowa State, Florida, Kansas State and others. This proliferation has continued with training centers established in other universities and institutions, in veterinary diagnostic laboratories and including research training in molecular and genomic toxicology investigations throughout the United States and around the globe. These early centers and their offsprings have fostered the talents to understand and deal with numerous environmental and clinical problems in veterinary medicine.

Formal recognition of veterinary toxicology was initiated with the American Board of Veterinary Toxicology (ABVT) being formally recognized by the American Veterinary Medical Association (AVMA) in the mid-1960. Largely through the efforts of R.D. Radeleff during his term as president of the ACVT, an application for approval of the specialty was accepted by the AVMA Council on Specialty Organizations. A Certifying Board of W. Binns, J.W. Dollahite and R.D. Radeleff was designated to conduct the first examination leading to Diplomate status in the ABVT. Specific training and experience requirements were established for applicants and approval of each applicant's credentials was necessary before the candidate was admitted to the examination. Satisfactory completion of a comprehensive written examination was the final requirement for certification and the privilege of adding "DABVT" behind the successful candidate's name. The first ABVT certifying examination was held in July 1967 in Dallas, Texas. The five successful candidates joined the three original members of the Certifying Board to form the initial group of certified, i.e. "Boarded" Veterinary Toxicologists (Oehme, F.W. 1970. The development of toxicology as a veterinary discipline in the United States. *Clinical Toxicology* **3**: 211–20).

Since that time, annual certifying examinations of the ABVT have been given associated with the Annual Meeting of the AVMA. This certifying body has continued to set and maintain standards of qualification for veterinary toxicologists, and has complimented the continuing growth of veterinary toxicology experience and knowledge. By 2007 a total of 115 veterinarians have successfully completed the examination challenge and become Diplomates. Their special talents and skills continue to be professionally applied in academia, in industrial roles, as regulatory officials, at poison control centers and within diagnostic laboratories, and in consulting responsibilities throughout the world.

In the years since the discipline's early embryonic period, veterinary toxicology has evolved into a multidisciplinary focus that embraces all of basic and clinical sciences. Its unique focus is not only the diversity of its embracing activities, but also the many talents and energies of its participants. It harbors a true global theme and is proud of its recognition and membership in "the only medical profession licenced for treating more than one animal species".

*Veterinary Toxicology: Basic and Clinical Principles* is an encyclopedic documentation of the developments in veterinary toxicology the past four decades and glimpses into the promises of exciting future growth. In a logical and well organized fashion, the contributors cover the vast and dynamic field of veterinary toxicology. Of special interest is the initial chapter on "General Principles of Veterinary Toxicology" by R.O. McClellan, one of the initial ABVT certified veterinarians from the 1967 examination in Dallas. The appropriateness of the first contributor to this volume being a 40-year boarded veterinary toxicologist should not be lost to the readers or the general toxicological community.

The initial part highlights the intensity and diversity of the veterinary contributions to toxicology. Pharmacokinetics, testing models, epidemiology and regulatory concerns, backed up by the timely heightened awareness of terrorism and the increasing necessity for legal compliance and actions are well documented.

Any toxicology text would be remiss if it did not focus on individual organ systems and their respective toxicological effects and clinical manifestations. Part 2 moves through each biological system and ends with immunotoxicology and the disastrous effect that various chemicals can have by upsetting this balance of nature.

Of more recent origin are the veterinary efforts of exploring nanoparticles, radiation, and the mechanisms and models of investigative carcinogenesis utilizing various animal species. The veterinary toxicologist is foremost in working with such models and evaluating study results. Of additional current importance are the chapters on over-the-counter drug toxicity and the prevalent potential of various drugs of abuse to affect animal health.

The traditional group of toxic elements are intelligently and dramatically discussed in Part 5, where metals and micronutrients ranging from aluminum through zinc are laid out in all their toxicity. No group of toxic elements is more historically relevant to toxicology than compounds such as arsenic, copper, fluoride, lead, mercury, selenium, and zinc, and when interspersed with some of the minor minerals a complete array of metal and mineral animal intoxications is provided in this part.

The original emphasis for development of veterinary toxicology comes to the forefront in the middle of this volume. The organochlorines and the organophosphates/carbamates are extensively reviewed. Rotenone sneaks in, but the more recent toxic developments with pyrethroids, fipronil, imidacloprid, amitraz, and ivermectin and selamectin are prominently presented. The part on

rodenticides and avicides, as well as the brief part on herbicides and fungicides, highlight the array of agricultural chemicals that have spurred not only the long-term developments in toxicology but also the environmental impact of widespread use of these groups of compounds.

The environmental areas of veterinary toxicology are discussed by reviewing industrial toxicants and the residual impacts of the biphenyls, dioxins and dibenzofurans. The environmental impact of these and other chemicals found in the environment are highlighted by extensive chapters dealing with their toxicity in birds, an introduction to ecotoxicology, and the distribution of chemicals in the global marine environment through aquatic toxicology, and the adverse effects of cyanobacterial toxins and others affecting marine animals.

Although reviewed in only two chapters, the extensive information on botulinum neurotoxin and the enterotoxins are not overlooked. Neither are the poisonous and venomous compounds generated by animals in the terrestrial environment. The chapter on "Caterpillars and mare reproductive loss syndrome" presents up-to-date information on this event's disastrous effect on equine breeding stables and the puzzling origin of these problems. An in-depth discussion on chemically induced estrogenicity brings readers current with this unique toxic hazard in all animal species including humans.

Part 14 is another expansive discussion of the still important poisonous plant concerns that contributed to and continue to stimulate the interests and skills of veterinary toxicologists. The groups of important United States' poisonous plants are reviewed, and then specific categories of plant toxins are presented: cyanide; nitrate/nitrite; oxalates; *Datura* and related plants; fescue; mushrooms; cottonseed toxicity; and the *Taxus* alkaloids. All these are common and highly concerning dietary risks for livestock and other animal species existing in the natural environment.

Fungal toxins are grouped under the "Mycotoxins" part where aflatoxins, trichothecenes, zearalenone, fumonisins, ochratoxins/citrinin, slaframine, ergot, and the interestingly and dynamic tremorgenic mycotoxins are nicely presented. These compounds present not only animal hazards, but are also important public health concerns for the dietary contamination of grains and other human food sources. Other dietary contaminants are reviewed in the part dealing with "Feed and water contaminants". Ionophores and nonprotein nitrogen dietary supplements are highlighted. Not to be overlooked, water quality and contaminants of water sources alert diagnosticians to the hazards and often animal-threatening risks involved with these aqueous contaminants.

The concluding parts in this book of facts and knowledge address how current methodology allows confirmation of specific poisonings and the appropriate means by which poisoned animals may be treated and managed. After reviewing the basic concepts of analyses, appropriate sample submission requirements for such procedures, the use of proteomics for diagnostic application, the application of microscopic analyses of feeds and animal ingesta for toxic components, and the complementing role of pathology in the diagnostic process are presented. To wrap it all up, a concluding part on therapeutic measures offers recommendations on how to prevent poisonings and, if necessary, what treatments may be applied to treat individual intoxications.

In a full circle, the basic principles of veterinary toxicology have been utilized to understand the mechanisms of toxicology, to relate to the numerous and challenging individual chemical constituents that offer risk and produce injury to animals and indirectly to humans, and to offer current information and recommendations for identifying such problems and specifically managing their animal and public health effects.

It should be apparent that Veterinary Toxicology is about everything – from initial concerns of animal illness to specific molecular and genomic impacts in all of society. The veterinary toxicologist is well equipped and active in identifying the opportunities and challenges presented. The discipline stands increasingly ready to contribute to medical science by utilizing its broad talents to have significant impacts for the health of all animals on this globe.

What's Veterinary Toxicology all about? Those answers are what this encyclopedic volume offers! Enjoy them and use the information to the benefit of society and science!

*Frederick W. Oehme*

# Preface

Veterinary toxicology is a very complex, yet fascinating, subject as it deals with a wide variety of poisons of chemical, mineral, plant, fungal, and animal origins. Presently, synthetic compounds constitute the largest class of chemicals that are most frequently encountered in animal poisonings. Veterinary toxicology is greatly complicated by the wide variations in responses of domestic, aquatic, wild, and exotic animal species to toxicants. In the last few decades, veterinary toxicologists have faced the enormous task of dealing with a flood of new farm chemicals and household products. Understanding the complete profile, especially the mechanism of toxicity, of each toxicant is the biggest challenge for today's veterinary toxicologists. At the present time, toxicologists are facing many new problems. For example, during the incident of September 11, 2001, a large number of pets died in the collapse of the World Trade Center in New York City, while the survivors continue to suffer from respiratory illnesses (Ground Zero Illnesses) caused by dust, debris, and toxic chemicals. In 2005, Hurricanes Katrina and Rita, devastated the lives of many animals in the Gulf coast states (Louisiana and Mississippi). Thousands of animals died, while a large number of others suffered from intoxication with high levels of metals, pesticides, mold, and other toxic substances. Recently, a fatal food from Diamond Pet Foods Company has sparked concern as more than 125 dogs died in more than 25 states in the United States. Aflatoxin was proven to be the culprit. From time to time, unusual toxicological problems are encountered on a large scale, and this trend is likely to continue in the future. Around the world, animals and humans are living in a more polluted environment today than ever before. Many of the toxicological problems are global, while others are regional. Unfortunately, antidotes for common poisons are not readily available, resulting in either delayed or no treatment. Thus, veterinary toxicologists have the tremendous task ahead of facing new challenges of the 21st century.

The primary objective of this book, *Veterinary Toxicology: Basic and Clinical Principles*, is to offer a comprehensive text/reference source to research veterinary toxicologists, students, teachers, clinicians, and environmentalists. The volume is organized into 18 parts, with a total of 91 chapters, in order to offer a stand alone chapter on as many topics as possible. Although the book is heavily focused on target organ toxicity (Part 2), it has many novel chapters on timely topics, such as veterinary toxicology and the law, physiologically based pharmacokinetic modeling, *in vivo/in vitro* toxicity testing models, neurotoxic oxidative stress, nanoparticles, radiation, immunotoxicity, reproductive/endocrine/placental toxicity, chemical terrorism, and carcinogenesis. Poisonous plants, mycotoxins, feed, and water contaminants are covered extensively. Several chapters provide the latest information on problems related to industrial, environmental, aquatic, marine, avian, and zoo toxins. A significant part of the book (Part 16) is devoted to diagnostic toxicology, which includes basic principles, method validation and QA/QC, sample submission, current diagnostic criteria, toxicoproteomics, pathology, and microscopic analysis of feed. Finally, the last part of the book emphasizes prevention and therapeutic measures of common poisonings.

In the past few years, veterinary toxicologists from many parts of the world have realized the need for a standard book that can provide a detailed coverage of the basic and clinical principles of veterinary toxicology. This book addresses global as well as regional toxicological problems, and offers practical solutions. A stand alone chapter is provided on every major topic, with major references for further reading. This book represents the collective wisdom of more than 75 authors, and offers a unique text/reference source for those involved in

veterinary medicine in general and toxicology in particular. Contributing authors for chapters of this book are the most qualified and well-experienced authorities in their respective areas of veterinary toxicology.

The editor is deeply indebted to all the authors for their sincere and dedicated contributions. Technical assistance of Joan Jenkins, Debra Britton and Robin Doss is immensely appreciated. The editor and the authors would like to thank Renske Van Dijk, Keri Witman, Mara Conner, and Tari Broderick, the editors at Academic Press/Elsevier, for their vital input in preparation of this book.

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# Part 1

## General

C H A P T E R

# 1

# Concepts in veterinary toxicology

*Roger O. McClellan*

## INTRODUCTION

Toxicology, from the Greek words toxicon for poison and logos for scientific study, is the study of poisons. Veterinary medicine is that branch of medical science concerned with the diagnosis, treatment and prevention of diseases of animals. The adjective veterinary is derived from Latin – veterinae, beasts of burden. Obviously, the modern field of veterinary medicine extends beyond the "beasts of burden" to include all the domesticated animal species, both livestock and companion animals, as well as non-domesticated species. Indeed, it has expanded to include non-mammalian species. While the focus of toxicology remains on chemicals, it is generally acknowledged that the study of effects of ionizing radiation is a part of the field or at least a closely related specialty. Pharmacology, from the Greek words pharma for drugs and logos for scientific study, is a closely related field concerned with the science of drugs: their preparation, properties, effects and uses in the diagnosis, treatment and prevention of disease.

The field of toxicology is very broad including the identification and characterization of poisons, their physical and chemical properties, their fate in the body and their biological effects. In addition, toxicology is concerned with the treatment of disease conditions caused by poisons. The terms toxicant and poison are used interchangeably. A toxicant is a material that when it contacts or enters the body via ingestion, inhalation, dermal contact or injection, interferes with the normal biological processes and causes adverse health effects. The term toxin is used to describe poisons originating from biological processes. The term toxic is used to describe the effects of a poison on biological systems. Toxicosis is the term used to describe the syndrome of adverse health effects that result from exposure to a toxicant. During the last several decades, increased concern has developed for the effects of long-term low-level exposures to toxicants. With these exposures, adverse health effects, if they occur, may be manifest in a non-specific manner as an increase in the incidence of common diseases in a population.

A wide range of materials produces toxic effects when exposure occurs at sufficiently high levels. Indeed, with extreme levels of exposure most agents can produce adverse effects. For example, while both water and oxygen are required to sustain life, they are toxic when the level of intake is excessive. The nature of the toxic responses depends not only on the toxicant, but also the route of exposure, the duration and intensity of the exposure and the characteristics of the exposed individual, i.e. species, gender, age, pre-existing disease states, nutritional status and prior exposure to the agent or related compounds. The exposure may be brief or prolonged. The response may occur acute or chronic and occur soon after exposure or much later and only after prolonged exposure. The response may be relatively unique to the toxicant, i.e. a specific toxicosis, or distinguishable from common diseases caused by natural processes or exposure to other agents. In many cases, sophisticated statistical methods are required to associate some excess health risk, over and above that caused by other factors, with a particular toxicant exposure. This is especially true today after much progress has been made in controlling exposure to toxic materials.

In this chapter, I first provide a brief historical perspective on the development of veterinary toxicology as a subspecialty of the veterinary medical profession and as a specialized area within the general field of toxicology. This

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is followed by a section on the evolution of veterinary toxicology from an observation-based profession and science to one that places increasing reliance on science developed through experimentation. This includes a discussion of the risk paradigm which has become an integral part of toxicology in recent decades. In the next section, I offer several related paradigms for acquiring, organizing and using knowledge in veterinary toxicology so as to maximize its potential impact. Next, there is a section on the sources of knowledge that may be obtained either through observation or experimentation. These sources may include studies on the species of interest, i.e. people or some other specific animal species, controlled exposure studies in the species of interest, studies in other species, investigations using tissues and cells and structure–activity analyses. This is followed by a section discussing the design of experimental studies to optimize the interpretation and use of the results. This chapter concludes with a discussion of key toxicological descriptors and a brief conclusion section.

## HISTORICAL PERSPECTIVE

### Historical events

The father of modern toxicology is generally acknowledged to be Auroleus Phillipus Theostratus Bombastus Von Hohenheim (1493–1541), who referred to himself as a Paracelsus, from his belief that his work was beyond the work of Celsus, a first century Roman physician (Pagel, 1958). Paracelsus is credited with the well-known statement: "All substances are poisons; there is none which is not a poison. The right dose differentiates a poison from a remedy." Paracelsus advanced many views that were revolutionary for his time that are now accepted as fundamental concepts for the field of toxicology. In contrast to earlier emphasis on mixtures, he focused on the toxicon as a specific primary chemical entity that was toxic. Paracelsus advanced four fundamental concepts:

1 Experimentation is required for examining responses to chemicals.
2 A distinction should be made between the therapeutic and toxic properties of chemicals.
3 The therapeutic and toxic properties are something closely related and distinguishable by dose.
4 It is possible to ascertain a degree of specificity for chemicals and their therapeutic or toxic effects.

It is obvious from the foregoing that toxicology and pharmacology are closely related fields of scientific endeavor. Pharmacology is focused on drugs, including both their effectiveness and safety. Toxicology is concerned with all kinds of chemicals and other agents that may, at some level of exposure, cause adverse health effects. As will be noted at several places in this chapter, toxicology is increasingly concerned with low-level exposures for which the effects, if any are observed, may not be specific to a particular chemical.

Toxicology, in a sense, dates back to the earliest activities of humans. By observation, people came to learn that which could be ingested without harm and, by contrast, the foodstuffs to be avoided because of their harmful properties. They also came to know which animal venoms, plant extracts and other materials could be used for hunting, warfare and assassination. No doubt as animals were domesticated, it became apparent that the human observations and practices could be extended to domestic animals. Unfortunately, domestic animals are not always as astute as people in learning to avoid poisonous plants and other harmful situations. Thus, veterinary practitioners still encounter toxicoses involving animals ingesting poisonous plants.

The history of toxicology has been well documented by several contemporary authors (Milles, 1999; Borzelleca, 2001; Gallo, 2001). The history of veterinary toxicology has not been as well documented, although it is apparent that veterinary toxicology has been an integral part of veterinary medicine since the origins. Veterinary medicine is a specialized branch of medical science with formal programs of study leading to a professional degree. The history of veterinary medicine has been reviewed by several authors (Smithcors, 1957; Stahlheim, 1994; Swabe, 1999; Wilkinson, 2005). The role of veterinary toxicology in the veterinary curriculum is well documented for one of the earliest veterinary medical colleges, that at the Free University of Berlin. Wilsdorf and Graf (1998) provide an account of the development of veterinary toxicology at that University from 1790 to 1945. Oehme (1970) has briefly reviewed the development of veterinary toxicology as a discipline in the United States.

### Textbooks

In the English language, the earliest veterinary toxicology publication I could find was a synopsis of *Veterinary Materia Medica, Therapeutics and Toxicology* (Quitman, 1905) apparently used at Washington State University College of Veterinary Medicine in the early part of the 20th century. I am uncertain of the extent to which this synopsis is based on a French text by Kaufmann (1901). The earliest English language veterinary toxicology textbook I was able to locate was that authored by an Englishman, Lander (1912). This book was also prepared in a second edition (1926) and a third edition was prepared by an Irishman, Nicholson (1945). I am uncertain how widely it was used in the United States. The text included four sections: a brief introduction to toxicology followed by sections on

classes of toxicants; mineral or inorganic poisons; organic poisons and drugs; and poisonous plants. This last section represented half of the book.

Many early students in veterinary medicine in the United States used textbooks prepared for physicians such as Kobert (1897), *Practical Toxicology for Physicians and Students*. It was also common to use either textbooks in pharmacology or veterinary pharmacology that contained a brief coverage of toxicology. Indeed, few veterinary medical colleges prior to the 1950s had full-time veterinary toxicologists on their faculty. Lectures on toxicology were usually included in courses in pharmacology, pathology and clinical medicine.

The first veterinary toxicology text I personally used was authored by Garner (1957) who was then a Senior Lecturer in Chemical Pathology (Veterinary) at the University of Bristol in the United Kingdom and later Head of the Radiobiology Department at the Agricultural Research Council Field Station, Compton, Berks, UK. The text by Garner (1957) was intended as a successor to the third edition of Lander's *Veterinary Toxicology*. A second edition was prepared by Garner (1961) after he became Head of the Public Health Section, Radiological Protection Division, UK Atomic Energy Authority, Harwell, Berks, UK. Later, Garner came to the United States where he was initially associated with Colorado State University directing studies of the long-term effects of radiation on beagle dogs. I recall asking Garner in the early 1970s about the possibility of preparation of a third edition of his *Veterinary Toxicology* text. He indicated that the field of veterinary toxicology had become so broad that it was not readily feasible for a single individual to author a text in veterinary toxicology and he was not interested in "shepherding" a herd of individual chapter authors with specialized knowledge of various aspects of veterinary toxicology.

Radeleff (1964) authored one of the first veterinary toxicology texts published in the United States. A second edition appeared in 1970. This was followed by a text prepared by Osweiler *et al.* (1985). Several books published in the 1960s became classics on the effects of poisonous plants (Kingsbury, 1954, 1964; Hulbert and Oehme, 1968). Recent books on poisonous plants have been authored by Garland and Barr (1998), Burrows and Tyrl (2001, 2006) and Knight and Walter (2001). Murphy (1996) has authored a field guide to common animal poison. It is organized by the organ system affected and then by toxicant.

Osweiler (1996) has authored a text focused on toxicology as part of the National Veterinary Medical Series for Independent Study. It has been widely used by individuals preparing for the National Board Examinations for Veterinary Medical Licensing. Roder (2001) has prepared a text, *Veterinary Toxicology*, as part of a series *The Practical Veterinarian*. Plumlee (2004) has edited *Clinical Veterinary Toxicology* and Peterson and Talcott (2001, 2006) have edited two editions of *Small Animal Toxicology*. The present multi-authored text promises to be the most comprehensive text on veterinary toxicology published to date. A *Veterinary Toxicology* text edited by Chapman (2007) is in preparation.

There are a number of comprehensive general toxicology texts available today. I will note four that the serious student of toxicology will find useful to have in their reference library. *Casarett and Doull's Toxicology: The Basic Science of Poisons* edited most recently by Klaassen (2001) was first published in 1975 and is now in its sixth edition. Hayes (2001), *Principles and Methods in Toxicology*, is now in its fourth edition. *Toxicology*, edited by Marquadt *et al.* (1999), built on an earlier German text by Marquadt and Schafer. *Biological Concepts and Techniques in Toxicology: An Integrated Approach* edited by Riviere (2006) was just released. Serious students will also want to be aware of a 13 volume comprehensive set of toxicology text edited by Sipes *et al.* (1997). Moreover, there are numerous text and reference books available now covering various sub-specialty areas such as *Inhalation Toxicology*, *Reproductive and Developmental Toxicology* and *Dermal Toxicology*.

In addition to text and reference books, there are numerous journals published in the field of toxicology that regularly contain articles that relate recent findings in veterinary toxicology. Many clinically oriented veterinary medical journals contain articles on veterinary toxicology. The on-line search capabilities serving the medical sciences including toxicology and veterinary toxicology are expanding at an exponential rate. Of special note are those maintained under the auspices of the National Library of Medicine, MEDLINE and TOXLINE.

## Organizations

A number of professional scientific organizations have been created as the field of toxicology, including veterinary toxicology, has matured. The most noteworthy include the American College of Veterinary Toxicology (ACVT), American Board of Veterinary Toxicology (ABVT), Society of Toxicology (SOT), American Board of Toxicology (ABT) and Academy of Toxicological Sciences (ATS). The ACVT was one of the earliest scientific societies in the field being founded in 1958. It now exists as the American Academy of Veterinary and Comparative Toxicology (AAVCT). The ACVT was instrumental in fostering the creation of the ABVT and its recognition by the American Veterinary Medical Association (AVMA) as the approved certifying specialty organization for veterinary toxicology. Three well-known veterinary toxicologists, W. Binns, J.W. Dollahite and R. Radeleff, were accepted by the AVMA as Charter Members of the ABVT. They prepared the first certifying ABVT examination which was given in 1967 (see www.abvt. org). I was pleased to be one of seven

individuals in the first class certified, based on examination, as Diplomates of the ABVT.

The SOT, with the world's largest membership of toxicologists, was organized in 1961 (see www.sot.org). Many of the organizers of the SOT were members of the American Society for Pharmacology and Experimental Therapeutics (ASPET) who felt toxicologists needed a "home" of their own. I recall attending an organizational meeting of the SOT held in conjunction with an ASPET meeting at the University of Rochester and the excitement and enthusiasm of the attendees for creating the SOT. As an aside, it would be a few years before I felt my credentials were sufficient that I could apply for membership in the SOT. The SOT fostered the creation of the ABT which held its first certifying examination in 1980 (see www.abtox.org). I was pleased to be one of the first class of individuals certified, based on examination, as Diplomates of the ABT. The SOT includes a number of specialty sections including the Comparative and Veterinary Specialty Section.

A third certifying entity, the ATS, which accepts individuals as Fellows based on a review of credentials, was created in 1981 (see www.acadtoxsci.org). Many veterinary toxicologists belong to all of the organizations noted above and some have been certified by one or more of the certifying organizations: the ABVT, ABT and ATS. Veterinary toxicology has evolved greatly over the past several decades.

# EVOLUTION OF VETERINARY TOXICOLOGY

## Roots in veterinary medicine and toxicology

The evolution of veterinary toxicology occurred concurrently with evolution of its two roots: the profession of veterinary medicine and the science of toxicology. The veterinary medicine profession was initially focused on domestic animals, particularly those used for food, fiber, transportation and to provide power for agricultural endeavors and transportation. With the growth of more specialized agriculture and production practices, the profession with its linkage to domestic livestock stimulated growth of the profession. Veterinary toxicology focused on poisonous plants and then on antidotes for various toxins. The early part of the 20th century presented a special challenge for veterinary medicine as the use of horses and mules in agriculture decreased in favor of the use of equipment powered by internal combustion engines. During this period of time, there must have been considerable uncertainty as to the future of the profession.

By the mid-20th century three movements transformed veterinary medicine. The first related to the traditional roots of the profession in animal agriculture and related to the increasing emphasis given to large-scale highly specialized livestock endeavors. The second related to the increased attention given to providing veterinary medical services to a growing population of companion animals. In both areas the science of veterinary medicine was strengthened as observation-based medical practice was complemented and, ultimately, supplemented by science-based medicine. During this period, veterinary toxicologists began to play an important role in veterinary medical diagnostic laboratories, both in veterinary medical colleges and in state and federal agencies. With the strengthening of the science base of veterinary medicine, including the quality of the science in the veterinary medical curriculum, the third movement, the emergency of the comparative medicine character of veterinary medicine, became more apparent and was enhanced (Wilkinson, 2005). These changes in the profession were accompanied by increased involvement of veterinarians in research on the species of traditional concern to the profession, domestic and companion animals (Stahlheim, 1994), and to participation in a broader range of biomedical research activities, involving use of the traditional laboratory animal species, driven largely by concern for human health (Wilkinson, 2005).

## Emergence of science-based toxicology

Toxicology, like veterinary medicine, was also rapidly changing and evolving in the mid-20th century. The previous strong emphasis on field observations was first complemented and then supplemented by experimentation. This led to the current strong mechanistic orientation of toxicology. With this shift in toxicology came an increased awareness of the utility of a comparative medicine orientation in research directed primarily toward improving human health (Wilkinson, 2005). With this comparative medicine orientation came increased opportunities for individuals educated in veterinary medicine, including veterinary toxicology, to contribute to general toxicology and biomedical science.

These changes in the veterinary medical profession and the emergence of toxicology as a science came during a period when the public was giving increased attention to the health risks, and its counterpoint safety, of new technologies and products. A landmark of the era was publication of Rachel Carson's book, *Silent Spring* (Carson, 1962). She focused on both human health impacts and impacts on the total ecosystem of which people were just a part. Her book was certainly one of the key stimuli to a tidal wave of legislative actions in the United States that focused broadly on the environment with concern for clean air and water; safe food, pharmaceuticals, pesticides, fungicides, rodenticides and consumer products; and a safe working environment.

The legislative actions and related administrative actions in the 1970s created the US Environmental Protection

Agency (USEPA), the Consumer Product Safety Commission, the National Institute of Occupational Safety and Health (NIOSH), the National Center for Toxicological Research, the National Institute of Environmental Health Sciences and the Cancer Bioassay Program within the National Cancer Institute, which evolved into the National Toxicology Program (NTP) now administered by the National Institute for Environmental Health Sciences. This was also a period of rapid expansion of research activities in the pharmaceutical food, chemical and petroleum industries. The chemical industry in 1976 started the not-for-profit Chemical Industry Institute of Toxicology, which now exists as the CIIT Center for Health Sciences, to test commodity chemicals, investigate the mechanisms of chemical toxicity and train additional toxicologists. The Food and Drug Administration (FDA) continued its traditional dual emphasis of ensuring both the efficacy and the safety of drugs and medical devices continued. Increased emphasis was given by the FDA to veterinary drugs and to the potential for veterinary drugs to contaminate meat and milk.

Increasing public concern for safety/risk and the resulting legislation led to the development of increasingly formalized approaches to both safety and risk analysis. This included more clearly defined roles for using the results of toxicological studies, including studies with laboratory animals, to assess the safety, or conversely risk, to humans of the use of pharmaceuticals, other products in commerce, and technologies.

## Toxicology joined to the risk paradigm

As noted earlier, federal legislation passed in the 1970s focused on the health impacts of environmental and occupational exposures and led to more formalized approaches to evaluating the risks and safety of various exposures. The risk paradigm built on the long-standing paradigm linking sources to exposure to dose to adverse health outcomes that had guided toxicology from its earliest days (Figure 1.1). I have reviewed elsewhere the development of the risk analysis paradigm (McClellan, 1999). The risk analysis paradigm originally proposed by the National Research Council (NRC, 1983) and used by the USEPA is shown in Figure 1.2. A later report *Science and Judgment in Risk Assessment* (McClellan, 1994; NRC, 1994) and reports from the Risk Commission (1997) re-affirmed use of the risk paradigm which continues to be a cornerstone of activities not just at EPA but in other national and international agencies and in the private sector.

The original key elements of the risk paradigm were (1) hazard identification, (2) exposure–response assessment, (3) exposure assessment and (4) risk assessment. The NRC (1994) report emphasized the importance of a fifth element – using the results of the risk analysis to guide future research and, thus, reduce uncertainty in future risk estimates. In addition, I have added a sixth over-arching element - risk communication. The hazard identification element has been a source of contention and confusion both with the public and in the scientific community, especially with regard to cancer as I will discuss later.

Hazard is defined as the potential for an agent under some conditions of exposure to cause an adverse effect (NRC, 1983, 1994; McClellan, 1999). With this definition the level of exposure or dose required to produce an adverse health effect is not considered. An agent may be classified as a hazard irrespective of whether or not the exposure conditions required to elicit adverse effects are relevant to human situations. The exposure–response assessment involves characterization of this relationship as it may pertain to likely levels of human exposure. The exposure assessment quantifies, either retrospectively or prospectively, the likely duration and intensity of human

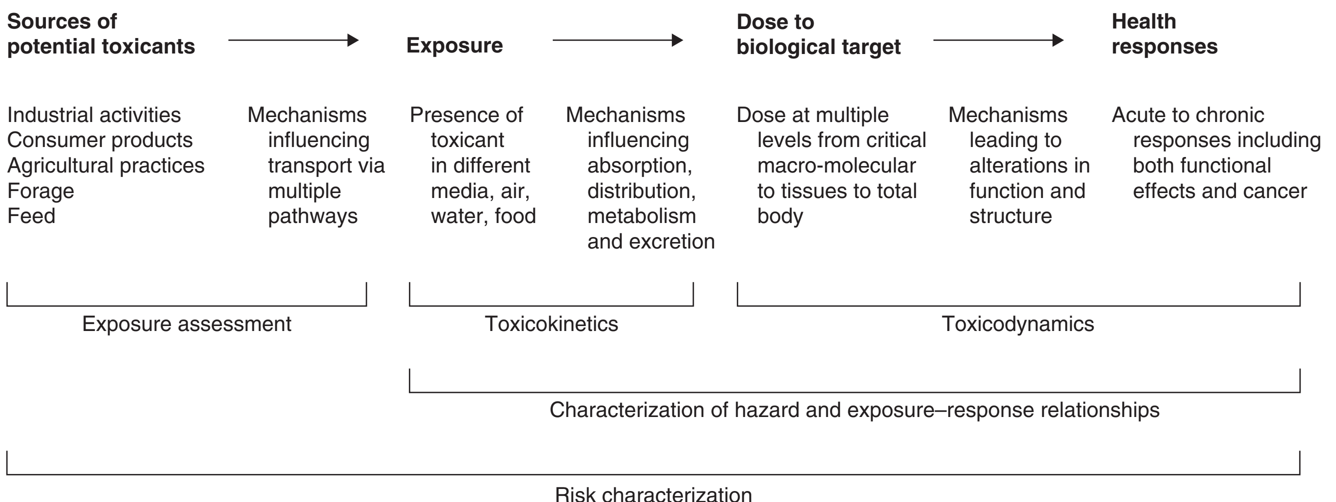


**FIGURE 1.1** Critical linkages for integrating information from sources of toxicants to the development of adverse health effects.

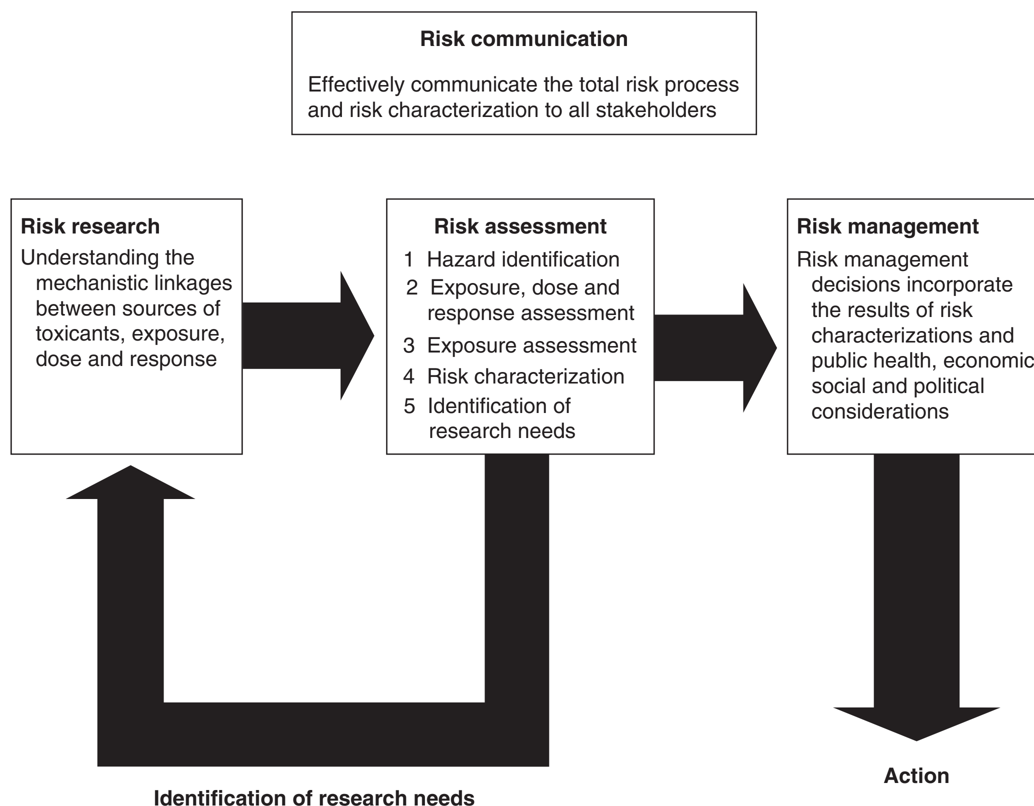


**FIGURE 1.2** Risk paradigm for evaluating potential health impacts of a toxicant.

exposure to the hazardous agent. The risk assessment element brings together information from the other three elements to characterize risk as illustrated in Figure 1.1. Risk is defined as the probability of occurrence of an adverse health effect from exposure to a hazardous agent at a specified duration and intensity of exposure. As an aside, especially in Europe, the word hazard is used as risk has been defined in the United States. Safety is defined as being a condition with a high probability of freedom from any increase in adverse health outcome when the agent is used in a specified manner. Obviously, both safety and risk are relative recognizing that it is not possible to ensure absolute freedom from some small level of risk.

The more formalized risk analysis approaches developed starting in the 1970s built on approaches developed earlier for providing guidance for controlling occupational exposures, the intake of contaminants in food and the safety of pharmaceutical agents. Pre-World War II, the primary focus was on adverse health outcomes that caused functional impairment such as decreased respiratory function. As will be discussed later, the issue of carcinogenic responses received limited attention before World War II. The approach to developing guidance for the control of toxicants was based on the assumption that a threshold exists in the exposure (dose)–response relationship – just as discussed by Paracelsus. The threshold exposure–response

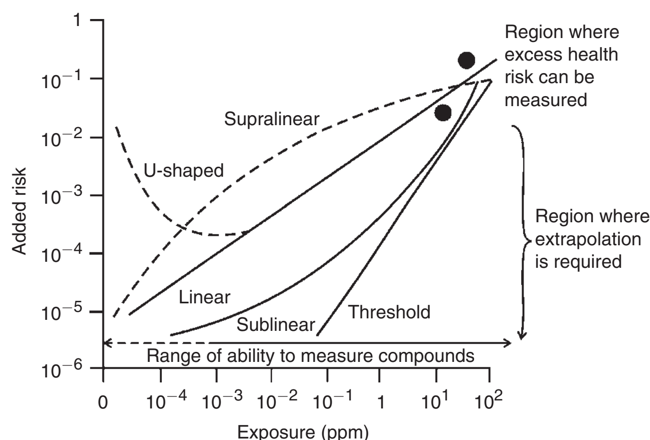


**FIGURE 1.3** Schematic rendering of exposure–response relationships for various toxicants.

relationship is shown in Figure 1.3 along with four other relationships: sub-linear, linear, supralinear and a U-shaped or hormetic function. Note that both scales in this schematic rendering are logarithmic.

Technically, in hormesis there is a beneficial effect at some low level of exposure which decreases with increasing exposure/dose and at yet higher levels adverse effects become apparent. During the last decade, there has been

increased discussion of the concept of hormesis in which very low-level exposures have positive effects with negative effects observed only at higher exposure levels (Calabrese and Baldwin, 2003; Calabrese and Blain, 2005). The concept of hormesis is well known to veterinarians who are aware that certain agents, such as vitamins and minerals, are essential for life at low concentrations and can produce toxicity with excess intake.

As an aside, there has been on-going debate for decades as to whether linear exposure–response relationships, especially for cancer, are realistic, i.e. an added level of exposure, regardless of how small, results in a calculable monotonic increase in cancer risk. It has been argued by some that the linear exposure–response model is appropriate for regulatory purposes for assessing cancer risks because every dose of a new agent is added to a background of genetic damage in somatic cells arising from multiple agents and endogenous factors.

The early development of threshold limit values (TLVs) for control of occupational exposures by the American Conference of Governmental Industrial Hygienists (ACGIH), organized in 1938, assumed the existence of thresholds in exposure–response relationships. The initial data were provided primarily by opportunistic studies of exposed human populations. In the absence of human data, data from controlled exposure studies in laboratory animals were used. This necessitated the use of safety factors to account for inter-individual variability, inter-species extrapolation and duration of the study as will be discussed later. The original safety factors were formally proposed by Lehman and Fitzhugh (1954) of the FDA. Later, the USEPA was organized and began using the same factors. However, the EPA identifies them as uncertainty factors apparently out of a desire to avoid use of the potentially contentious word – safety.

Post-World War II increased public concern developed for the occurrence of cancer. This was stimulated by multiple factors. Extensive research conducted during and after the war on the effects of both external ionizing radiation and internally deposited radionuclides emphasized the importance of cancer as a radiation-induced disease. Concern for radiation-induced cancer was further heightened when the intensive follow-up of Japanese A-bomb survivors revealed an increase, first in hematopoietic neoplasms, and, later in solid cancers. These findings soon led to abandoning a threshold approach to evaluating radiation risks in favor of using a probabilistic approach to assess the health risks of using radiation devices in space and nuclear power. The probabilistic approach using the linear exposure–response model discussed earlier was convenient to use because it could be readily applied to assessing the risks to individuals or populations. My first experience with probabilistic risk assessment came in the mid-1960s when I was on a temporary assignment with what was then the US Atomic Energy Commission (AEC). I worked with a joint AEC–National Aeronautical and Space Administration assessing potential human cancer risks of accidents involved with the launch of spacecraft containing plutonium-238 fueled thermal electric power systems.

Another factor influencing public concern was the increasing incidence of total cancers being observed in all of the economically developed countries including the US driven largely by lung cancer. It is now well known that the increase in lung cancer, first observed in men and then in women, was largely related to cigarette smoking. Rachel Carson's book also helped to create concern for exposure to man-made chemicals contributing to the increasing incidence of cancer. It is now known that this is not the case (Gold *et al.*, 2003).

The experience with radiation soon resulted in its use as a proto-typical carcinogen in developing approaches to risk analysis and risk regulation. Albert (1994) documented the development of the USEPA's approach to assessing cancer risks. Key assumptions in the approach were (a) cancer-causing chemical agents acted like radiation in causing cancer; (b) there was a linear relationship between exposure (dose) and increased risk of cancer extending to the lowest levels of exposure; (c) agents causing cancer in laboratory animals could be viewed as also causing cancer in people and (d) exposure–response relationships could be extrapolated between species by considering differences, body weight and surface area, i.e. metabolic activity. These assumptions were viewed as default options to be used in the absence of specific scientific data to the contrary (McClellan, 1994, 1999, 2003; NRC, 1994).

In response to public concern for chemicals causing cancer, the International Agency for Research on Cancer (IARC) became the first organization to propose a scheme for classifying agents as to their carcinogenic potential (IARC, 1972). The view was that if cancer-causing chemicals or other agents, such as radiation, or workplace conditions involving exposure to chemicals or other agents could be identified, then these could be controlled and the occurrence of cancer in people reduced. The IARC carcinogen classification scheme considers human, laboratory animal and supporting data to classify agents or workplace conditions as (1) carcinogenic to humans, (2) probably carcinogenic to humans, (3) possibly carcinogenic to humans, (4) not likely to be carcinogenic to humans or (5) not classified as to carcinogenicity. The IARC classification is strictly hazard oriented, it does not formally evaluate the potency of these agents for causing cancer at a specific level of exposure. The USEPA, the NTP and other organizations have developed similar carcinogen classification schemes (EPA, 1986, 2005a, b; NTP, 2005). In recent years, IARC (1991) has made provision for increased use of mechanistic data in classifying chemicals as human carcinogens. Both the EPA and NTP now also give increased emphasis to the use of mechanistic data in classifying chemicals as carcinogens

(EPA, 2005a, b) unlike IARC and the NTP, the EPA does develop estimates of cancer-causing potency for some agents classified as having cancer-causing potential. This in turn, using measurements or estimates of exposure, provides the basis for calculating lifetime cancer risks for individuals or populations.

It should be apparent that the cancer classification of a given agent is insufficient for characterizing cancer risk since the hazard-based classification does not include an estimate of the agent's potency. The USEPA has estimated the carcinogenic potency for a number of chemicals. The results are usually related as the concentration of a chemical in water or air that will result in a calculated one in a million probability of cancer occurring above the background incidence (EPA/IRIS, 2006). To estimate the cancer risk for any agent and exposure situation, it is also necessary to estimate the exposure to the agent, both as to intensity and duration. In short, risk is a product of exposure and the potency of the agent for causing the effect.

There has been a tendency for regulatory agencies, such as the USEPA, to use their experience with classifying chemicals as to their carcinogenic potential as a template for also classifying chemicals as to their potential for producing other non-cancer hazards. Thus, there has been a trend toward classifying chemicals as to their potential hazard for causing different health outcomes and labeling them as such, i.e. neurotoxins, reproductive toxins, hepatic toxins, etc. Indeed, some even broader classifications have emerged, i.e. endocrine disrupting chemicals. In my view, this short-hand approach to identifying and classifying hazardous agents as to their potential to cause cancer or other effects is confusing to the public. In my view, the labeling approach has contributed to both radiation reactions and chemical phobia and sometimes irrational actions. It certainly flies in the face of the fact that for many chemicals the admonishment of Paracelsus that "the dose makes the poison" remains true for many chemicals. For many chemicals, even when toxic effects are apparent at high doses, these same adverse effects are no longer manifest at sufficiently low doses. Gold *et al.* (2003) have discussed the challenge of using high exposure (dose) animal studies to identify either man-made or natural chemicals as human carcinogens.

## A FRAMEWORK FOR ACQUIRING INFORMATION

### Linkages from sources to health impacts

The purpose of this section is to provide a conceptual framework for using information to evaluate specific cases of actual or alleged toxicosis and to facilitate the acquisition of new knowledge that will have impact in understanding potential toxic effects. Earlier, in Figure 1.1, a conceptual framework was provided for evaluating the linkages extending from a source of a toxic material to manifestation of an adverse health outcome in an individual or a population. The conceptual framework is equally applicable to humans or other animal species.

The source to exposure linkage has been expanded in Figure 1.4 (Paustenbach, 2001). In this example, an industrial plant is illustrated as the source. The figure serves to illustrate the complex nature of the exposure pathways that may be encountered including the role of livestock. The focus in the figure is on the multiple pathways by which a potential toxicant may reach a human population: inhalation, drinking water, dermal absorption, ingestion of soil, and ingestion of a variety of foodstuffs including milk and meat from domestic animals. All of these pathways might also serve to expose the cow in the figure to the toxicant. The quantities of the toxicant taken in by the cow could cause toxicity in a herd of cows. Equally as important is the role of the cow as a pathway for the toxicant to reach people. For example, the figure illustrates that a toxicant could be present in cow's milk and the milk could be consumed by people. The cow could also be slaughtered and the meat

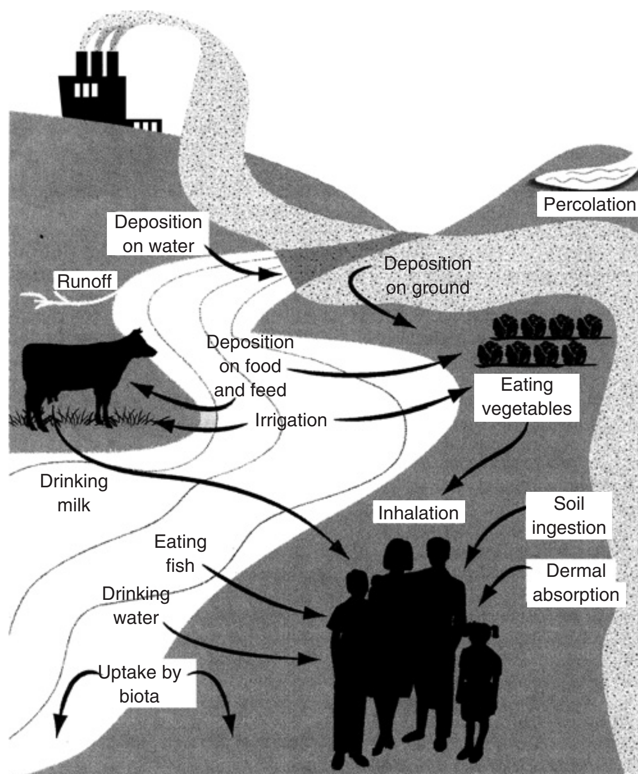


**FIGURE 1.4** Schematic rendering illustrating exposure pathways extending from a source of toxicants to exposure of livestock and people (from Paustenbach, 2001).

ultimately consumed by people. Thus, it is important to recognize that the cow, or any other food animal species, can both manifest toxic effects and serve as a pathway for toxicants to reach people via the food supply.

It is readily apparent that the schematic rendering shown in Figure 1.4 can be expanded or contracted. In natural ecosystems, multiple species might be involved as a toxicant moves from a source or multiple sources via various pathways. In some cases, various species in the ecosystem may be impacted as individuals. Moreover, natural populations may be impacted. In addition, these pathways may ultimately result in the toxicant reaching people. An example is mercury in fish. In practice, veterinarians may encounter situations where poisonous plants in the pasture or in harvested forage may be the source of the toxicant. Feed may be contaminated at a mill and serve as the pathway by which a toxicant reaches the livestock. In other cases, the potential human toxicant may be a pharmaceutical purposefully given to the cow.

## Toxicokinetics

The simple schematic rendering shown in Figure 1.1 can be used to illustrate several important concepts. First, it is important to recognize that contrary to common usage, exposure and dose are not the same. The exposure environment is characterized by the concentration of the toxicant in the media, be it water, air or feed, the quantities taken in and the time course of the intake. Dose is the concentration, over time, of the toxicant in the various tissues of the subject, whether it be a cow, a human or a laboratory rat. The characterization of the kinetics linking exposure with dose is referred to as toxicokinetics (for a toxic agent) or pharmacokinetics (for a pharmaceutical). In actual practice, the term pharmacokinetics is frequently used when it would be more appropriate to use the term – toxicokinetics. Several chapters in this book deal specifically with kinetics of toxicants and pharmaceuticals.

Toxicokinetics (see Figure 1.1) are used to describe the movement and disposition of the toxicant in the organism. This includes consideration of the route of entry: ingestion, inhalation, dermal or purposeful administration by injection. A complete description of the toxicokinetics of a toxicant will take into account (a) the intensity and duration of the exposure, (b) the rate and amount of absorption of the toxicant from the site of entry, (c) the distribution of the toxicant within the body, (d) potential biotransformation to less, equal or more toxic form and (e) the rate of excretion by route (urine, feces or exhalation). All of these aspects of toxicokinetics may be influenced by species differences in physiological and biochemical characteristics. Modern approaches to modeling toxicokinetics attempt to take account of both species differences and similarities in influencing the fate in the body of toxicants. It is also important to recognize that the exposure or dose level may influence the kinetics of a toxicant and its metabolite(s). This is an especially important consideration in extrapolating from laboratory studies that may be conducted at high doses to lower more environmentally relevant exposures/doses.

## Toxicodynamics

The linkage between dose and adverse health outcome shown in Figure 1.1 involves multiple mechanisms as various toxicants may potentially impact all the cells and organ systems of the body. Increasingly, scientists have attempted to model these relationships which, in parallel to the nomenclature for the kinetic phase, are called toxicodynamic or pharmacodynamic models. It is obvious that multiple pathways may be involved in a toxicant producing disease and that knowledge of the individual steps will increase as knowledge of basic biological mechanisms increases. For example, the explosion of knowledge of basic biology at the level of the genome (genomics), proteins (proteomics) and metabolism (metabolomics) has provided a basis for exploring the mechanistic basis of toxicant-induced disease with a degree of refinement that could not even be envisioned even a short time ago.

A later chapter reviews the basic mechanisms of toxicity. In addition, many of the chapters on organ toxicity and specific toxicants contain detailed information on mechanisms of toxicity. As the reader reviews this material, and especially the detailed discussion of biochemical mechanisms of action, it will be important to place those in the context of processes at the cellular and tissue level; i.e. inflammation, cell death, cell proliferation, hypertrophy, hyperplasia, metaplasia and neoplasia. A strength of the veterinary medical curriculum, as with the human medical curriculum, is the emphasis given to understanding both normal and disease processes extending from the molecular level to cells to tissues to organs and, ultimately, to the integrated mammalian organism. A special opportunity exists for medically trained personnel, both veterinarians and physicians, to put the expanding knowledge of molecular and cell processes into the context of overt disease. After years of emphasis on a reductionist approach to basic biomedical science, it has become recognized that this approach needs to be complemented by an integrative approach. This has recently been termed systems biology. In my view, this is not really a new concept. It is more a rediscovery and refinement of the concepts of integrated biology and pathobiology used in veterinary medicine for decades.

There has been great enthusiasm for the use of mechanistic information in safety/risk evaluations as will be discussed later. Recognition of the difficulty of characterizing of all the individual mechanistic steps has given rise to a new term – mode of action. The mode of action has

been defined as the dominant step(s) involved in producing a given toxic endpoint. An example is the role of cell killing as the mode of action for large intakes of chloroform (Butterworth *et al.*, 1995) or formaldehyde (Conolly *et al.*, 2004), over extended periods of time causing tumors in rodents. The exposure–response relationship for cell killing may likely have a threshold which must be considered in extrapolating the findings from high exposure level studies in rodents to humans exposed to low concentrations of these chemicals.

It is my contention that understanding the basic concepts conveyed in Figures 1.1, 1.2 and 1.4 can be very useful in investigating a range of situations where the objective is to establish or refute a causal association between a given source and toxic agent and an increased incidence of an adverse health outcome. I use the term, increased incidence, advisably recognizing in most situations involving domestic animals, either as commercial herds or as companion animals, the situation is one of presence or absence of a given disease and the "ruling out" of other differential diagnoses. However, in situations involving human populations the issue frequently encountered is whether a given toxicant exposure has caused an increase in a disease recognizing that most diseases may have multiple etiologies, e.g. hypertension and diabetes. This is especially the case in evaluating diseases that typically occur late in life, such as cancer and chronic diseases, and with exposure to toxicants that may occur at low levels over long periods of time. In some cases, such as lung cancer and cardiorespiratory disease in humans, a risk factor such as cigarette smoking is so substantial, it is a challenge to determine if low-level exposure to other toxicants such as air pollutants has chronic effects at low exposure concentrations.

## Veterinary toxicology is multi-faceted

It will be apparent to the reader of this book that veterinary toxicology is multi-faceted. Thus, there are many ways to organize and synthesize the knowledge base that we call veterinary toxicology. One dimension is the various classes of toxicants. Another dimension of the field relates to the media that contains the toxicant: air, water, soil and feed. Another dimension considers the various routes of exposure of toxicants: inhalation, ingestion, dermal or purposeful injection. It is also convenient to consider the various organ systems and processes that may be affected by toxicants. This is the basis for organization of a major section in this book. It is also important to consider the individual toxicants or classes of toxicants. This approach is used in organizing another major section of this book. Finally, veterinary toxicologists recognize the necessity of considering the various species of concern. Increasingly veterinary medical practitioners have become more specialized with many focusing their clinical skills on a single species. This book does not include a section addressing the toxicology of individual species. To have done so would have substantially increased the size of this text. However, chapter authors have endeavored to discuss species variations in responses to toxic agents. It is noteworthy that at least one textbook, that of Peterson and Talcott (2006), focuses on small animals. Some of the major comprehensive veterinary medicine texts that focus on other species include chapters on toxicology related to that species such as the *Current Veterinary Therapy* series.

# SOURCES OF INFORMATION

## Case observations in the species of interest

There are multiple sources of scientific information for characterizing the relationship between exposure to a toxicant and toxicant-induced response. Figure 1.5 is a schematic rendering of the multiple sources of information that may be used to understand the toxicity of a given agent.

As discussed earlier, the origins of veterinary toxicology and toxicology, in general, are both rooted in observations. An adverse health effect, either a pattern of morbidity or death in an individual or population, is observed and the disease linked to exposure to a toxicant. Typically, the time interval between exposure and the adverse health outcome was brief which aided in deducing an association. Because the causal association was identified in the species of interest, whether it be a person, a horse or a cow, it was

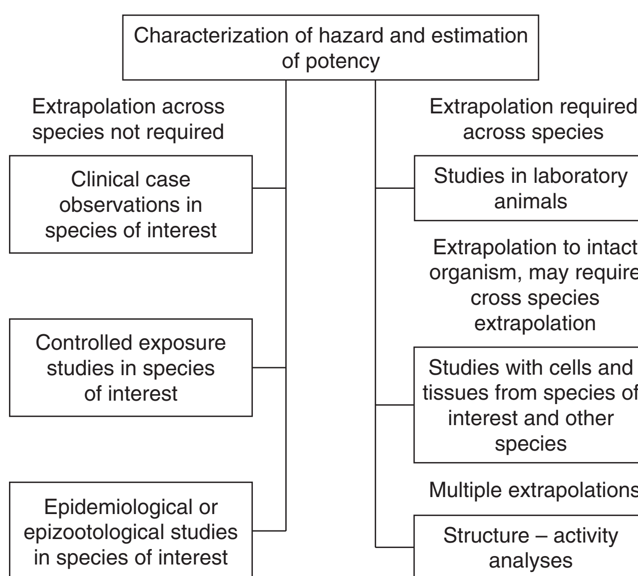


**FIGURE 1.5** Sources of information for evaluating potential toxicants.

not necessary to extrapolate between species. Nor was it necessary to explore in depth the mechanistic basis for the causal association to either diagnose a particular case or prevent future cases. Action to prevent exposures and, thus, prevent disease, could be based on empirical observations.

As you read many of the chapters in this book, you will note that details of the mechanism by which a particular toxicant causes disease have been elucidated to a variable extent. When the toxicant is exclusively of concern in veterinary medicine and has no implications for human health, there has been limited impetus for developing a mechanistic understanding of how a toxicant causes disease. Concern for human health has been a major driver of the biomedical research agenda. An obvious exception is when the toxicoses observed in veterinary medicine have large economic impact or toxicants can reach people via animal products.

There are many circumstances where observational knowledge is not adequate and it is necessary to conduct experiments to characterize the toxicology of an agent. It is obvious that if concern for the potential toxic response is in a non-human species, controlled experiments can be conducted using the species of interest. This is obviously the case for domestic livestock as well as companion animals.

A much more common situation is when concern focuses on potential toxicity of a newly developed agent for use in people or animals. For example, it is necessary to establish the safety of a potential new pharmaceutical or consumer product before it is introduced into commerce. In these instances experimental animals are used as a "first approximation" of the safety of the new compounds to humans. In the case of products intended for use in animals, studies on both efficacy and safety can be conducted in the species of interest. This remains an imperative step in the safety evaluation of new products. There are also circumstances in which it is desirable to extend limited observations from opportunistic studies on people or animals that have been exposed. When a new product is developed and marketed, either a pharmaceutical or a consumer product, various post-marketing surveillance systems should be put in place to attempt to detect any unexpected adverse outcomes.

## Epidemiological/epizootological studies

If a particular chemical has been used for an extended period of time and human exposure has occurred either in the workplace or from the environment, it may be feasible to conduct epidemiological studies. Epidemiology is the study of how disease is distributed in the population and the factors that influence or determine this distribution. The design of a particular epidemiological study will be guided by the hypothesis being tested and the nature of the population(s) available for study. As an aside, the term epidemiology (epi for across, dem for people and ology for scientific study) is applicable to people while the more appropriate related term for studies on animals would be epizootology (epa for across, zoo for animal and ology for scientific study). The details of conducting epidemiological or epizootological studies are beyond the scope of this chapter. A relevant reference for basic concepts in epidemiology is the text by Gordis (2005).

Retrospective epidemiological studies may be feasible for previously introduced agents for which prior exposure has occurred or prospectively for a newly introduced agent. If the agent is new it is obvious that it is not feasible to conduct epidemiological studies to retrospectively evaluate the potential safety/hazard of the agent. If the ultimate interest is in the effects on people, it may be feasible to conduct controlled exposure studies with human volunteers. It is advisable for the planning of such studies to be based on a solid database on the potential toxicity of the agent acquired from studies in laboratory animals. The design and conduct of such human studies must be guided first and foremost by ethical considerations (NRC, 2004). If a non-human species is the target species of concern, then it is obvious that the most relevant information is that acquired from studies conducted in that species.

## Experimentation

An additional option for acquiring information is to conduct toxicological studies in the typical laboratory animal species. Such studies are the cornerstone of research conducted to evaluate the safety/risk of newly synthesized agents whether they be a potential new pharmaceutical, pesticide or herbicide, a significant consumer product or a new chemical or intermediate to be used in commerce. It is well recognized, certainly by veterinarians, that no single laboratory animal species is a miniature version of the human species, i.e. 15 cm in height, weighing 180 g and sharing all of the common biological traits of humans. Fortunately, humans and laboratory animals do share many common biological traits. Knowledge of the extent to which there are similarities and differences between humans and a given laboratory animal species can be used to guide the selection of a species to serve as a surrogate for humans in developing data for safety/risk evaluations for humans. It is encouraging that some veterinary medical schools are recognizing the importance of extending the range of species studied in the core curriculum from the usual companion animal and domestic livestock species to include the common laboratory animal species.

At this juncture, it is appropriate to note the importance of animal welfare issues. The Animal Welfare Act (AWA), initially enacted in 1966 and amended in 1970, 1976, 1985, 1990 and 2002, is the principal federal statute in the United

States governing the sale, handling, transport and use of animals. The AWA applies to all species of warm-blooded vertebrate animals used for research, testing or teaching excluding animals used for agricultural research. The US Department of Agriculture, Animal and Plant Health Inspection Service has responsibility for implementing the AWA. The 1985 Amendments to the AWA clarified the importance of humane care, minimization of pain and distress, consideration of alternatives, the role of institutional animal care and use committees, the psychological well-being of primates and exercise for dogs. The primary reference on animal care and use is the *Guide for the Care and Use of Laboratory Animals* prepared under the auspices of the Institute of Laboratory Animal Resources of the National Academy of Sciences/National Research Council (ILAR, 1996). All toxicologists involved with laboratory investigations should be familiar with the contents of the guide irrespective of the species they use for their research.

An additional matter the experimentalist should be aware of is the need for use of good laboratory practices (GLPs) in the conduct of research intended to be used for regulatory decisions. Both the FDA (FDA, 2001) and the EPA (TSCA, 1985; FIFRA, 1991) have requirements for the use of GLPs. The FDA GLP requirements do not extend to exploratory, mechanism of action or efficacy studies. The basic elements of GLPs are (1) the appointment by the institution of a study director, (2) the use of an independent quality assurance unit, (3) the use of documented standard operating procedures, (4) a written protocol for each study and (5) preparation of a final report containing a GLP compliance statement for each study. The use of GLPs is not required by FDA for studies with domestic livestock. However, investigators conducting studies using domestic livestock would be well advised to attempt to adhere to the basic principles that under-gird GLPs to help ensure the quality and reproducibility of the data being generated.

Another option for acquiring useful toxicity data is to conduct investigations in *in vitro* using tissues or cells from mammalian species, both humans and laboratory animals, and using bacteria and yeasts. An additional option is to conduct structure–activity analyses on the new agent using the large data bank of structure–activity information already available on other related chemicals.

All of the options outlined, to some extent, create extrapolation issues. Even if studies are conducted in the species of interest, it is typically necessary to extrapolate from the high levels of exposure or administered doses studied experimentally to lower exposures or doses anticipated to be representative of intended use. It may also be necessary to extrapolate from a relatively short period of study, say days or a few weeks, to the intended period of use, over months or years. If the studies are not conducted in the species of ultimate interest, there is need to extrapolate between species. It may also be necessary to extrapolate observations made in a population of healthy individuals to a population that includes individuals with pre-existing disease. Some aspects of the extrapolation between species and across exposure/dose levels may be facilitated by physiologically based toxicokinetic and toxicodynamic modeling. However, toxicodynamic modeling is still in its relative infancy.

It is important to recognize that even with today's level of knowledge of these extrapolation issues, it is not possible to estimate, with absolute certainty, the precise numerical level of human exposure to a given agent that may be without any risk of potential harm or will produce a specific level of harm. This is generally recognized in contemporary safety/risk evaluation methodology such that conservative approaches are used in estimating safe levels of human intake of chemicals. By taking a conservative approach to setting standards or providing guidance to limit exposures, there can be a high degree of confidence that an agent can be used safely if used as intended. Ultimately, all processes that develop guidance or standards to limit exposures and thus limit disease require judgments to be exercised. In short, science can inform the standard or guidance development process; however, it cannot prescribe specific standards.

# SCHEMATIC EXPERIMENTAL DESIGNS

The experimental design for testing of any specific hypothesis must be matched to the hypothesis, the desired statistical power and the resources available. Inevitably, decisions on an experimental design involve making difficult choices among options because of resource constraints. In this section, two schematic experimental designs will be discussed to illustrate some of the key issues that must be addressed in planning toxicological studies. The discussion in both cases will assume that the species to be used in the study has already been selected.

## Acquiring toxicokinetic data

The first design, Figure 1.6, illustrates an approach to acquiring data for understanding the link between exposure and internal dose, the kind of data that can be used for toxicokinetic modeling. Recall the toxicokinetic linkage in Figure 1.1. The design shown is based on a single brief intake of the test agent. However, the design can be modified for studying chronic intake of an agent. A critical decision is the choice of the route of administration or intake of the test material. Obviously, such studies are most readily carried out with parenteral administration of the agent. This may be the most appropriate route for a pharmaceutical

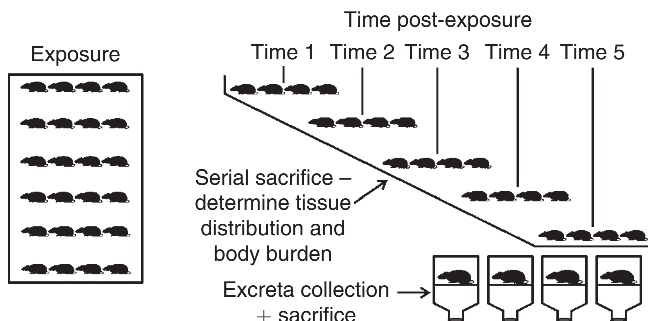


**FIGURE 1.6** Schematic rendering of an experimental design for evaluating the kinetics of an administered toxicant.

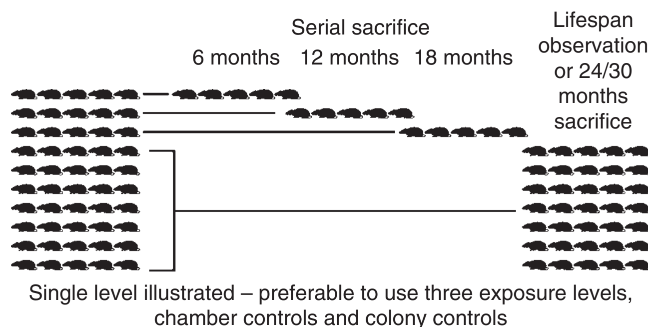


**FIGURE 1.7** Schematic rendering of an experimental design for evaluating exposure (dose)–response relationships for a toxicant.

agent that is to be parenterally administered. However, the resulting data may be of limited relevance to other routes of intake. For example, it may not appropriately mimic oral intake since only a small fraction of some toxicants may be absorbed from the gastrointestinal tract. In short, the route of administration should be matched to the route of concern for real-world exposure to the agent.

With inhalation, the particle size distribution of the airborne toxicant will influence what portion of the inhaled material will be deposited and where it is deposited in the various regions of the respiratory tract. The pattern of retention and subsequent translocation of the deposited material will depend on the size, chemical composition and dissolution properties of the deposited material.

Another key decision is whether conduct of the toxicokinetic studies may be facilitated by using a test agent labeled with radioactive or stable element tracers. Analytical considerations for the initial toxicant as well as any metabolite are of major importance in the conduct of toxicokinetic studies.

The schematic design (Figure 1.6) shows a group of animals maintained for collection of excreta and, perhaps, even sampling of expired air. Data from these analyses can be used along with tissue analyses to obtain a mass balance between the quantity administered and the quantity recovered. The schematic design shows multiple times at which animals will be euthanized and tissues collected for analysis. This allows the development of a dynamic profile of how the body handles the administered material. For organic compounds, provision needs to be made for analyzing for both the parent compound and potential metabolites.

The selection of the sacrifice times will be guided by the anticipated kinetic profile of the agent and its metabolites. It may be useful to obtain preliminary information on retention kinetics from pilot studies. Some organic compounds may be rapidly metabolized leading to the need to schedule all of the sacrifices over a few hours. On the other hand, certain inhaled relatively inherent materials may have long-term retention in the lungs extending over hundreds of days. It is important to recognize that the quantity of material administered may influence the kinetics of the material. Hence, it is desirable to use multiple administered exposure/dose levels as an experimental variable. Without question, the design of any particular toxicokinetic study requires the exercise of considerable professional judgment. Toxicological research is not a “cookie cutter” or “check the box” science.

## Acquiring exposure (dose)–response data

A schematic experimental design for a study to evaluate exposure (dose)–response relationships for toxicants is shown in Figure 1.7. Recall the exposure–response linkage shown in Figure 1.1. The design shown is typical of that which might be used in the conduct of a 2-year bioassay, typically to evaluate carcinogenicity, in rats and mice. The same design, and indeed the same experiment, can be used to evaluate other endpoints and to conduct shorter-term studies. The study should involve administration of the material by a route matched to likely exposure conditions to be encountered with the agent. Administration of an agent by gavage may be acceptable as a surrogate for ingestion, especially when it is desirable to administer specific quantities of material. However, I am not enthusiastic about the repeated use of gavage as a substitute for ingestion of an agent in feed. The use of intratracheal instillation as a surrogate for conducting inhalation exposures to an agent remains controversial. It is my professional opinion that intratracheal administration is a non-physiological mode for delivery of materials to the respiratory tract. It may result in exaggerated quantities of material being deposited in some regions of the respiratory tract while other regions are spared any exposure. This unusual pattern of distribution of the agent is very likely to influence the toxic response of the airways and alveoli. Thus, I am hesitant to even recommend intratracheal instillation for mechanistic studies; the mechanistic information acquired may be irrelevant to the inhalation exposure situations that are of concern for people.

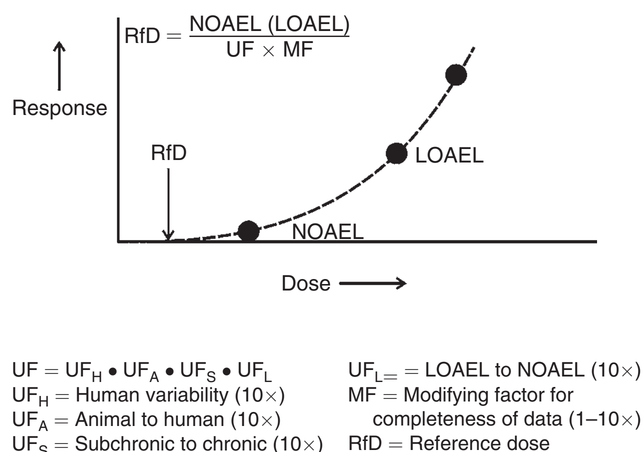


**FIGURE 1.8** Schematic rendering of a threshold exposure–response relationship.

It is critical that exposure–response studies utilize multiple exposure levels, perhaps three or four exposure levels. The choice of the specific exposure levels is one of the most important decisions to be made in planning such studies. One consideration relates to the potential level(s) of exposure to be encountered with intended use. Higher additional levels can be selected above this base level. Selection of exposure/dose levels can also be informed by the results of the kinetic studies. For example, it would not be desirable to use only exposure levels above a level at which metabolic processes are saturated. Another consideration emphasized by the EPA and NTP, especially when cancer is an endpoint, is to select a maximum tolerated dose (MTD) level as the highest exposure/dose level and establish lower levels by some fraction of the MTD level, perhaps 1/2 and 1/4 or 1/3 and 1/9. The use of an MTD has been justified on the grounds that it is necessary to maximize exposure to potentially observe carcinogenic responses recognizing the blunt experimental approach (NRC, 1993).

The extent to which animal bioassays are a blunt approach to detecting the carcinogenic potential of agents is illustrated in Figure 1.8. It can be noted that for a species and train of animals with a background incidence of 1%, a study of 50 animals will require a 20% response to detect a statistically significant effect. As an aside, a population of non-smoking people will experience about a 1% lifetime incidence of lung cancer. A population of two pack a day cigarette smokers will experience about a 20% lifetime incidence of lung cancer compared to the 1% expected in non-smokers. Consideration of statistical information such as these emphasizes the importance of using care in interpreting the results of cancer bioassays using the typical 100 animals per exposure level. The interpretation of the relevance of the results of animal studies for estimating human hazards will be greatly enhanced by knowledge of the mechanisms involved in the toxicant causing disease in the animals.

A key feature of the exposure–response experimental design illustrated in Figure 1.7 is the use of multiple sacrifice times for all exposure levels. In some cases it may be possible to evaluate the functional status of organs at these times, i.e. pulmonary function. In animals with inhalation exposure, when a respiratory tract response is of concern, it may be feasible to collect bronchoalveolar lavage fluid samples for analysis of biochemical and cellular parameters. Most importantly, tissue samples can be collected for histopathological evaluation. The information obtained from the serially sacrificed animals, combined with that obtained from the terminal sacrifice animals, can provide valuable insight into the progression of disease processes over the course of the study. Without question, insight into the pathogenesis of toxicant-induced disease processes will be much more complete when serial sacrifices are conducted than that obtained only from an evaluation of the terminal sacrifice animals. Another option in the design of exposure–response studies is to include a group of animals at each level that are removed from further exposure at one or more times post-inhalation of exposure for maintenance without further exposure. These animals may be euthanized at later times and evaluated for evidence of recovery or reversibility of earlier toxicant-induced changes.

The basic guidance for using multiple exposure (dose) levels and making experimental observations at multiple times is as applicable to the conduct of studies examining hypotheses on the mechanisms of action of toxicants as it is to studies developing information for regulatory decisions. I remain disappointed at the number of published articles on mechanisms of action of specific toxicants that fail to use multiple exposure (dose) levels and multiple observation times. It is only when exposure (dose) level and duration of exposure are included as experimental variables that a true understanding of the mechanisms of toxicity for an agent can be elucidated. Mechanisms are frequently exposure (dose) level and exposure duration dependent.

As the science of toxicology has advanced, increasing attention has been given to developing specialized approaches for evaluating toxicity induced in different organ systems. The various guidelines developed by the USEPA, FDA and NTP are useful references for these specialized approaches. For example, the EPA has published guidelines for evaluating carcinogenicity (EPA, 1996a), gene mutation (EPA, 1996b), reproductive toxicity (EPA, 1996c), developmental toxicity (EPA, 1991) and neurotoxicity (EPA, 1995). The EPA is continually reviewing and updating its guidelines for toxicity testing. Forty-nine harmonized health effects test guidelines used in the testing of pesticides and toxic substances have been developed and can be found on the EPA Office of Prevention, Pesticides and Toxic Substances website (EPA/OPPTS, 2006).

The FDA has provided specific guidance for evaluating the safety of compounds used in food-producing animals (FDA, 1994) and principles for evaluating the safety of

food ingredients (FDA, 2000). The EPA has provided guidelines for evaluating the safety of products intended for use with cats and dogs (EPA, 1998) and domestic livestock (EPA, 1996d).

The various guidelines are useful for planning safety evaluation studies. However, the guidelines should not be used as a substitute for the use of professional judgment in planning, conducting and interpreting toxicological investigations. As noted earlier, toxicology is not a "cookie cutter" or "check the box" science.

# TOXICOLOGICAL DESCRIPTORS

## Toxicology rooted in observations

The results of toxicological investigations, either from clinical case observations or planned experimentation, involve describing the exposure, the dose, the response and interrelationships between these parameters. Exquisite knowledge of exposure or dose or response is not sufficient. Ultimately, it is necessary to understand their interrelationships. With clinical case observations, the initial emphasis is on the clinical findings – what is the response and the need, on the basis of a differential diagnosis, to establish that a toxicant is or is not involved. The evidence for a specific toxicant may be based initially on clinical findings complemented by gross necropsy findings potentially buttressed by histopathological findings. The differential diagnosis of a toxicosis may be strengthened by evidence of a marker of dose, i.e. urine, blood or tissue levels of suspected toxicant. The diagnosis may be further strengthened with evidence of exposure, i.e. the presence of the toxicant in the feed or identification of a poisonous plant. At each step, the qualitative evidence of a toxicosis and a specific toxicant is enhanced as qualitative findings are supplemented by quantitative findings. The analysis is not completed there, though. Other reasonable differential causes of the same or similar clinical signs must also be "ruled out" if the animals or humans are in a real world or field setting.

## Quantifying exposure

Quantitation is paramount in evaluating exposure. In the experimental setting, quantitation is considered beginning with the design of the study and continued through all aspects of the experimentation. To the extent feasible, exposure to the toxicant should be rigorously characterized. This starts with physical and chemical characterization of the test material, be it an alleged pure compound or a mixture, including identification of any contaminants. The exposure circumstances need to be rigorously characterized. This, of course, is easiest to do when the test material is administered by injection. Even with injection, care must be taken to ascertain that the desired quantity of toxicant was actually injected. The quantity administered is typically related to the body weight of the subjects.

With administration by routes other than injection, the situation becomes more complicated. This may involve providing the experimental subjects' feed to which the toxicant has been added. If this approach is used, samples of the contaminated feed should be collected periodically for analysis of the test agent. In some cases, the concentration of the test agent in the feed will be used as a measure of the exposure. To accurately quantify exposure, it will be necessary to know the concentration of the test agent in the feed and also determine the quantity of the contaminated feed containing the test agent that has been ingested. For dermal administration, it is necessary to know the concentration of the test agent in the liquid media applied to the skin and the quantity of the media applied to the skin.

The situation is much more complex for a test agent in the air, whether it is a diluted gas or suspended particulate material. In both cases, it will be necessary to sample and measure the concentration of the test agent in the air at a location as close to the breathing zone of the experimental subjects as possible. For both particulate material and reactive gases, there may be substantial loss of the test agent in the delivery system between the generator used to create the test atmosphere and the breathing zone of the subject(s). Care needs to be taken to minimize such losses. For a toxic agent in a particulate matter form, it is essential to know not only the concentration of the test agent, but also the size distribution of the particulate matter since the aerodynamic particle size distribution will influence the fraction of the inhaled material that will be deposited and where it deposits in the respiratory tract. In some experiments, it may be possible to use a plethysmograph to measure respiration of individual subjects during inhalation exposure. This is most readily accomplished when the exposure period is relatively brief as in a study of the toxicokinetics of the agent. The total quantity of test agent inhaled can be estimated from knowledge of the air volume inspired and the concentration of the test agent in the air. In many studies the air concentration of the test agent may be used as a surrogate measure of exposure. As indicated earlier, exposure and dose are not synonymous. However, in many studies it may be necessary to use the concentration of the test agent in the feed, water or air as a surrogate measure of dose.

## Describing absorption, distribution, metabolism and excretion

A number of different parameters may be evaluated in assessing the kinetics of a test agent (recall Figure 1.6). Some

of the common parameters and terms used are shown in Table 1.1 adapted from Spoo (2004). The four key events involved are absorption, distribution, metabolism and excretion. It is important to recognize that species differences may exist for each of these events. Absorption is the amount of the material that enters the body. As already discussed, the concept is simple. However, in reality it becomes complex as one moves from parenteral administration to oral intake, to dermal uptake or inhalation exposure. Distribution of the material will be influenced by the route of entry and the physicochemical properties of the test agent. Metabolism for compounds varies dependent on the physicochemical properties of the material. In some cases, the material may be very inert and simply be transferred mechanically within the body with some portion excreted over time. In other cases, especially with organic compounds, the metabolism may be quite complex and result in metabolites that are either more toxic, less toxic or have toxicity similar to the parent compound.

Excretion or elimination of the material and its metabolites, if metabolized, may occur via the kidney (urine), gastrointestinal tract (feces) or the lungs (exhalation of volatile compounds). In addition, the agent or metabolites may appear in tears, sweat or exfoliated skin. Some species, such as the rat, may engage in coprophagy, ingestion of feces, such that the test material in the feces is ingested and some portion passes through the body multiple times. Animals may be euthanized at various times during the course of the study and samples of various tissues collected and analyzed for the test agent or metabolites. With small experimental subjects, it may be possible to analyze all the tissues and obtain an estimate of the total body burden of the test agent and metabolites.

In some short-term studies it may be possible to collect and analyze excreta and expired air, if the compound is metabolized to a form that will be present in expired air. This information, along with the results of tissue analyses, can provide an estimate of the total quantity in the body, excreta and expired air for comparison with an estimate of the quantity administered. This kind of mass balance approach is obviously most feasible when radioactive or stable isotope tracers are used. One should not be surprised to find the estimated quantity recovered varying from 75% to 125%; there will be a high degree of experimental variability when multiple samples are being collected and analyzed. Obviously, one should view with suspicion data tables showing recovery of exactly 100% of the administered dose. Such values are typically the result of an over zealous investigator normalizing the data to 100% recovery. For chronic exposure studies, it may be possible to use kinetic modeling to estimate the quantity of the test agent or metabolites present in the experimental

**TABLE 1.1 Common terms used to describe the ADME characteristics of chemicals (Adapted from Spoo, 2004)**

| Term | Abbreviation | Definition |
|---|---|---|
| Concentration | $C_p$ | Concentration of a chemical in plasma (p) at a specific time ($t$) |
| Time | $t$ | Chronological measurement of a biological function |
| Half-life | $t_{1/2}$ | Time required for exactly 50% of a drug to undergo some defined function (i.e. absorbed, distributed, metabolized or excreted) |
| Volume of distribution | $V_d$ | Unitless proportionality constant that relates plasma concentration of a chemical to the total amount of that chemical in the body at any time after some pseudoequilibrium has been attained |
| Volume of distribution (steady state) | $V_{d(ss)}$ | Same as $V$?, except measured when the chemical has reached a steady state in the body |
| Area under the curve | AUC | Total area under the plasma chemical concentration curve from $t = 0$ to $t = \infty$ after the animal receives one dose of the chemical |
| Body clearance of a chemical | $Cl_B$ | The sum of all types of clearance from the body |
| Renal clearance of a chemical | $Cl_R$ | Volume of chemical that is completely cleared by the kidneys per unit of time (ml/min/kg) |
| Non-renal clearance of a chemical | $Cl_{NR}$ | Volume of chemical that is completely cleared by organs other than the kidneys per unit of time (ml/min/kg) |
| Dose | D | The amount of chemical that is administered to an animal; can be further defined as the total dose, that total dose the animal was exposed to, or the absorbed (effective) dose, that being the fraction of the total dose that was actually absorbed by the animal |
| Bioavailability | F | Also known as systemic availability of a chemical. The quantity of percentage portion of the total chemical that was absorbed and available to be processed (CME) by the animal, in the case of intravenous administration, $F = 100\%$ |

ADME: absorption, distribution, metabolism and excretion; CME: chemical metabolism and excretion.

subjects at each exposure concentrations at various times after initiation of exposure.

## Toxicant–induced responses

The types of studies typically used by toxicologists to investigate exposure–response relationships can be placed in four categories related to the duration of the studies: acute, sub-acute, sub-chronic and chronic (recall Figure 1.7). Acute studies are usually of a day or less and may involve intraperitoneal, intravenous or subcutaneous injection, gavage, dermal application or inhalation. Injections may be given once or several times in the 24-h period. Acute inhalation exposures are typically 4-6 h in duration. In all cases, the observations are made over a 24-h period. Sub-acute studies typically involve repeated exposures made on a daily, or 5 days/week, basis for 2-4 weeks with observations over the same period of time. Sub-chronic studies are usually conducted over a period of 1-3 months. In the case of inhalation exposures, these are typically conducted for 4-6 h/day, 5 days/week. Chronic studies are usually conducted for more than 3 months and, most typically, for 2 years. I personally view the use of the terms acute, sub-acute, sub-chronic and chronic as jargon and prefer to communicate the duration of studies in a specific manner, i.e. number of days or months, or as short or long term. I prefer to use the terms acute, sub-acute or chronic as descriptors of a medical condition.

The kinds of toxicant-induced responses that may be encountered are broad, essentially mirroring the range of disease processes that may occur in humans and other animal species. In any well-conducted toxicity study, the investigator should use as broad an array of observational techniques as are reasonably available to characterize the pattern of morbidity and mortality that may develop. Inevitably, cost constraints will influence the choice of endpoints evaluated. It will be useful to prioritize the potential endpoints as to their likely value in terms of the information gained. It is crucial that detailed necropsies be conducted on subjects euthanized at prescribed times and at termination of the study. Tissues should be collected from any gross lesions and tissues identified in the protocol as likely target tissues and processed for histopathological evaluation. It is now routine to establish a defined set of criteria for evaluating the various tissues and characterizing lesions. This approach allows the quantitative evaluation of any pathological findings on a group basis rather than restricting the evaluation to qualitative descriptions of responses in individual subjects.

Toxicity studies to evaluate exposure (dose)–response relationships may extend from minutes to hours when biochemical and physiological responses are being evaluated, to hours to days when acute morbidity and mortality are being assessed, to weeks to months and finally to a significant portion of the lifespan of the species, e.g. 2 years for mice and rats when chronic effects, including cancer induction, are being evaluated. With increased attention given to animal welfare considerations, emphasis is being given to using as few animals as possible to define the acute morbidity and mortality of test materials. Rather than use a traditional approach to attempt to precisely define a lethal dose 50% ($LD_{50}$), it has become customary to use approaches with many fewer animals to define an approximate $LD_{50}$. In some cases, it may be desirable to determine the concentration of a test agent in water or air that produces 50% lethality over a defined period of time, a lethal concentration, $LC_{50}$. This approach remains in common use when studying aquatic organisms.

In modern toxicology, increasing attention is given to conducting studies with exposures that are defined by the anticipated conditions of use of the test material. This may involve initially conducting a study of 2-week duration, perhaps with up to five exposure levels anchored by a level related to anticipated use. The results of this study are then used to select exposure levels, perhaps three or four, and to sharpen the focus of a 90-day study. The results of the 90-day study, in turn, are used to select the exposure levels and sharpen the focus of a study of 2-year duration. Although it has become customary to conduct chronic exposure or 2-year studies with three exposure levels, it should be recognized that use of a control group and three exposure levels spanning a range of concentrations differing by a factor of 2, i.e. 1, 1/2 and 1/4, or a factor of 3, i.e. 1, 1/3, and 1/9, does not provide a robust data set for characterizing the shape of the exposure (dose)–response relationship. On the other hand, the use of exposure levels differing by a factor of 10, i.e. 1, 1/10 and 1/100, may provide an excessively broad range of exposure levels for identifying a lowest observed adverse effect level (LOAEL) or no observed adverse effect level (NOAEL) as will be discussed later.

In chronic studies, major attention is directed to evaluating any toxicant-induced changes in animals at the several exposure levels compared to controls over a 2-year period or until a defined mortality level is reached, such as 20% surviving. Any changes in various indices of morbidity or pathological alterations will be evaluated compared to controls as well as tested for trends across the exposure levels. In many cases, the primary endpoint of concern will be cancer which should include evaluation of all stages of tumor development up to sarcomas and carcinomas. It has become customary to use life table statistical methods such as that of Kaplan–Meier (1958) to evaluate the incidence of key changes. This approach allows for the use of data not only from the survivors at the end of the study, but also animals that have died or been euthanized at interim times. This situation is analogous to that encountered in human epidemiological studies when subjects may be lost to follow up.

It has become customary when the results of chronic studies will be used for regulatory purposes to convene a pathology peer review panel of expert veterinary pathologists, typically Diplomates of the American College of Veterinary Pathology (ACVP), to evaluate histological specimens from representative cases and the diagnoses of the original pathologist to verify that the diagnoses are appropriate and consistent with the scientific norm. As an aside, I encourage veterinary toxicologists to personally review the pathology findings in studies with the study pathologist so as to be familiar with the nature of the pathology findings. However, I discourage veterinary toxicologists from taking on a dual role of toxicologist and pathologist for a study. Indeed, this approach would be unacceptable for a study to be submitted for regulatory purposes unless the toxicologist was also an ACVP Diplomate.

## Describing exposure–response relationships for non-cancer endpoints

It is appropriate to now consider how the data generated from toxicological investigations can be used. Let us first examine a threshold exposure-response relationship as shown in Figure 1.3 and shown now in an expanded form in Figure 1.9. The first step is to examine the data set from critical exposure–response studies to identify key parameters to be used to describe the results. Key determinations are the no observed effect level (NOEL), the highest exposure level for which no effects are observed and the NOAEL, the highest exposure level that produces no adverse effects. Obviously, characterization of an effect as adverse or not adverse is a matter of professional judgment. For example, in a cholinesterase inhibitor study, is a reduction in blood cholinesterase in the absence of salivation or other clinical signs an adverse effect or merely an effect?

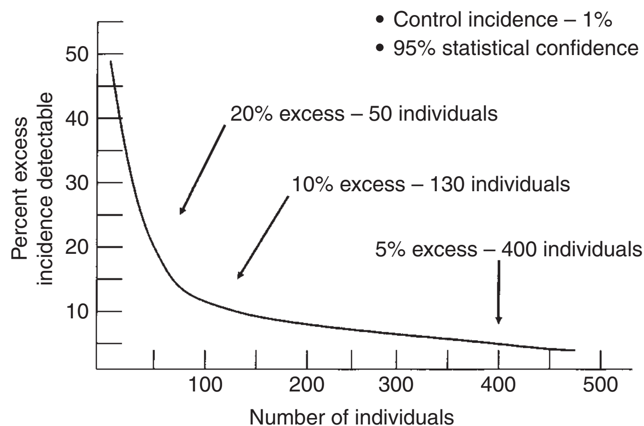


**FIGURE 1.9** Relationships between number of subjects required to detect excess risk and the level of detectable excess risk.

In the absence of the identification of an NOAEL, there is a need to identify the LOAEL, the highest exposure level at which an adverse effect is observed. The specific NOAEL and LOAEL that can be identified are a function of the exposure levels originally selected for studies. To state the obvious, observations can only be made at the exposure levels studied. For example, if the exposure levels studied did not extend to a sufficiently low level, the lowest level might produce an effect thereby precluding observation of an NOAEL. Alternatively, the study might be designed with three exposure levels separated by a factor of 10 with the lowest exposure level identified as an NOAEL and the next higher exposure level identified as producing some modest adverse effects and, thus, identified as the LOAEL. In retrospect, in such a study it is not known whether the "true" LOAEL might have been a factor of 3 or 5 above the NOAEL since these levels were not investigated.

Another consideration is the nature of the effects identified at the NOAEL, was there evidence of enzyme induction or hyperplasia, hypertrophy or atrophy with no evidence of a change in organ weight? Likewise, at the LOAEL was hyperplasia, hypertrophy or atrophy present resulting in modest or substantial changes in organ and body weight? Were histological changes observed that were reversible? Were the changes sufficiently profound that the level would be identified as a functional effect level (FEL)? These questions serve to emphasize the extent to which professional judgment is involved in interpreting the results of all toxicological investigations.

For non-cancer effects a reference dose (RfD) for oral intake or a inhalation reference concentration (RfC) for airborne materials is calculated using the NOAEL or LOAEL as a starting point (Jarabek *et al.*, 1990; Jarabek, 1994). An RfD or RfC may be defined as an estimate (with uncertainty spanning perhaps an order two magnitude) of a continuous oral or inhalation exposure to the human population (including sensitive subgroups) that is likely to be without appreciable risk of deleterious non-cancer effects during a lifetime. The RfD and RfC values are developed from the experimentally determined NOAEL or LOAEL values as shown in Figure 1.9 (Jarabek, 1994) and normalized to continuous exposure. For a more complete description of the process, the reader is referred to a recent book chapter by McClellan *et al.* (2006). The EPA maintains an Integrated Risk Information System that includes comprehensive summaries of the toxicological information available on specific chemicals including RfD and RfC values and estimates of cancer-causing potency. These profiles are available on line (EPA/IRIS, 2006).

A somewhat similar approach for non-cancer effects has been used by the ACGIH to develop TLVs (ACGIH, 2006). A TLV is defined as airborne concentrations of substances that represent conditions under which it is believed that nearly all workers may be repeatedly exposed day after day without adverse health effects. Since the ACGIH TLVs

apply to healthy workers they may not always incorporate an SF or UF of 10 for human variability. The exposure duration for TLVs is based on a 40-h/work week and, thus, the results of animal studies will be normalized to 40 h/week.

The Agency for Toxic Substances and Disease Registry (ATSDR) develops minimal risk levels (MRLs) using a similar methodology. An MRL is an estimate of the daily human exposure to hazardous substance that is likely to be without appreciable risk of adverse non-cancer effects over a specified duration of exposure. For example, MRLs are derived for acute (1–14 days), intermediate (14–365 days) and chronic (365 days and longer) exposure durations. The MRLs are intended to serve as a screening tool to help public health professionals decide to look more closely at particular exposure situations. The ATSDR has prepared toxicological profiles on many chemicals including their MRLs. More than 200 profiles are available on line (ATSDR, 2006).

The NIOSH develops recommended exposure levels (RELs). RELs are set at levels such that virtually all persons in the working population (with the possible exception of hypersensitive individuals) would experience no adverse effects. The Occupational Safety and Health Administration (OSHA) sets permissible exposure levels (PELs) based on consideration of the NIOSH RELs. However, the OSHA values are legally enforceable limits unlike the NIOSH RELs which are guidance.

The International Program on Chemical Safety (IPCS) prepares authoritative reviews on the environmental health impact of various chemicals. The reports are available on line (IPCS, 2006). The exposure limiting values developed by the IPCS are guidance values and not legally enforceable limits. The United States makes extensive use of legal enforceable exposure limits. Many other countries emphasize the use of guidance values. This distinction is important when comparing standards versus guidance originating from different countries.

In considering all of the foregoing guidance or regulatory levels, it is important to recognize that they are set to control exposures for workers or the general public. In each case, they are set to be health protective and, thus, are set at levels below where human effects have been observed or are expected to occur. These values should not be interpreted as being equivalent to levels producing adverse effects in humans.

## Cancer as an endpoint

For cancer as an endpoint, animal exposure–response studies may provide two kinds of input. First, the results may be used in carcinogen classification processes such as those of the IARC, the EPA or NTP. As discussed earlier, these are hazard-based classification schemes – Is a given agent capable of causing human cancer without consideration of the potency of the agent? These schemes have been described elsewhere (McClellan, 1999; McClellan *et al.*, 2006).

If a positive cancer outcome is observed in animal studies, the quantitative exposure–cancer response data may be used in a second way – to develop a risk coefficient, lifetime cancer risk per unit of exposure, for the potency of the agent for causing human cancer. Such extrapolations typically involve linear statistical extrapolations from high levels of exposure used in the animal studies to potential human exposure levels several orders of magnitude lower (recall Figure 1.3). In addition, they may purposefully be calculated based on upper 95% confidence limit on some level of risk, for example, with a probability of a one in one million occurrence. In my opinion, these extrapolated values are highly uncertain. It is quite possible that for some agents classified as possibly or probably carcinogenic to humans based on high exposure level animal study results there is no added cancer risk at very low levels of exposure (Gold *et al.*, 2003). The EPA (2005a) has recently issued guidance for alternative approaches to estimate cancer risks when information is available on the mode of action of the agent, for example, if the cancer arises as a result of the toxicity and secondary cell proliferation rather than a direct effect of the chemical or metabolite on DNA. For example, chloroform has been shown to cause cancer by this mode of action (Butterworth *et al.*, 1995). The EPA (2005b) has also provided guidance for considering the impact of susceptibility of early life exposures for causing cancer.

Information on the cancer-causing potential of various chemicals is included in the material summarized in the USEPA's Integrated Risk Information System (EPA/IRIS, 2006). The IARC monographs on the evaluation of carcinogenic risks to humans are all available on line (IARC, 2006). The monographs cover the carcinogen classification reviews of over 800 compounds. The NTP publishes, on a biannual basis, a *Report on Carcinogens*. The 11th report contained 246 entries, 58 of which were listed as "human carcinogens" with the remaining 188 being listed as "reasonably anticipated to be human carcinogens" (NTP, 2005). The potency of the various agents for causing cancer is quite varied. When examining this literature, many in the public, including some scientists, are surprised to learn how few agents have been conclusively identified as "human carcinogens." The facts stand in sharp contrast to the view conveyed in the popular media and some scientific publications that people live in a "world of carcinogens."

## New potential endpoints

In recent years, the expansion of knowledge at the molecular and cellular level has provided the opportunity for considering the addition of a myriad of new endpoints to toxicological evaluations. This includes an array of new molecular biomarkers which have received substantial

attention. Although biomarkers are frequently discussed as new approaches, it is well known to the veterinary clinician and toxicologist and to physicians that biomarkers have been used in both human and veterinary medicine for centuries.

In some cases, measurement of the biomarkers present in body fluids, urine or exhaled breath serves as an indicator of exposure or, even, dose of a toxicant. Recall the report of the individual arrested for "driving while intoxicated" based on a breathalyzer test for exhaled alcohol which has been converted to a blood alcohol level. In other cases, the biomarker is an indicator of a disease process. Recall individuals being evaluated for prostate cancer based on an elevated level of prostate specific antigen in serum samples.

New biomarkers of exposure will continue to be proposed. For each potential biomarker of exposure, it will be necessary to conduct experiments to validate the utility of the biomarker. A special challenge relates to recognizing the dynamics of the toxicokinetics of various toxicants and establishment of quantitative relationships between exposure and dose at any particular time over the course of the intoxication.

The potential list of biomarkers for toxic responses is seemingly endless. In all fields of medicine, from different kinds of cancer to various functional diseases of every organ system, new molecular markers are being identified on a regular basis. The challenge for toxicologists is to consider from among this array of opportunities which biomarkers are sufficiently well validated with regard to their linkage to diseases and sufficiently reasonable in cost to warrant their use in exposure–response studies. This includes consideration of the new and highly sophisticated genomic tools. There is a special challenge in designing validation studies to make certain that the experimental design is directed toward identifying specific disease-related endpoints or toxicant-related effects rather than merely being another, albeit more sophisticated, marker of non-specific toxic effects. A serious issue in many previous validation studies has been the use of a single high exposure level and a few short-term observation times. Such studies are unable to evaluate exposure-related changes in biomarkers and may not be able to identify toxicant specific changes.

## CONCLUSIONS AND SUMMARY

Veterinary toxicology is a multi-faceted hybrid that draws on and contributes to the veterinary medical profession, the scientific field of toxicology and, broadly, to medical science. Some have characterized toxicology as a distinct scientific discipline. I view toxicology as an applied area of science addressing important societal issues by drawing on multiple scientific disciplines and professions. Veterinary toxicology, as a sub-specialty in veterinary medicine, had a very applied origin – the diagnosis and treatment of toxicoses in domestic animals and companion animals. That important role continues today. However, the field has broadened to include concern for contaminants in human food products originating from animals and for contributing to the conduct and interpretation of safety/risk evaluations for pharmaceuticals, food additives, consumer products and specific chemicals. Veterinary toxicologists who understand both normal and disease processes extending from the molecular level to the integrated mammalian organism and, indeed, populations, have an array of opportunities for making significant contributions to society. The prospects for the future of veterinary toxicology and the opportunities for veterinary toxicologists have never been brighter.

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## REFERENCES

ATSDR, Agency for Toxic Substances and Disease Registry (2006) Toxicological Profiles (http://www.atsdr. cdc.gov/toxpro2. html) and Minimal Risk Levels for Hazardous Substances (http://www.atsdr.cdc.gov/mrls.html).

Albert RE (1994) Carcinogen risk assessment in the US Environmental Protection Agency. *Crit Rev Toxicol* **24**: 70–85.

ACGIH, American Conference of Governmental Industrial Hygienists (2006) *Threshold Limit Values and Biological Exposure Indices for Chemical Substances and Physical Agents*. ACGIH Cincinnati, OH.

Borzelleca JF (2001) The art, the science and the seduction of toxicology: an evolutionary development. In *Principles and Methods of Toxicology*, 4th edn, Hayes AW (ed.). Taylor and Francis, Philadelphia, PA, pp. 1–22.

Burrows GE, Tyrl RS (2001) *Toxic Plants of North America*. Iowa State University Press, Ames, IA.

Burrows GE, Tyrl RS (2006) *Handbook of Toxic Plants of North America*. Blackwell Publishing, Ames, IA.

Butterworth BE, Conolly RB, Morgan KT (1995) A strategy for establishing mode of action of chemical carcinogens as a guide for approaches to risk assessments. *Cancer Lett* **93**: 129–46.

Calabrese EJ, Baldwin LA (2003) The hormesis model is more frequent than the threshold model in toxicology. *Toxicol Sci* **61**: 246–50.

Calabrese EJ, Blain R (2005). The occurrence of hormetic dose responses in the toxicological literature, the hormesis database: an overview. *Toxicol Appl Pharmacol* **202**: 289–301.

Carson R (1962) *Silent Spring*. Houghton Mifflin, Boston, MA.

Chapman M (2007) *Veterinary Toxicology*. Blackwell Publishing, Ames, IA.

Conolly RB, Kimbell JS, Janszen D, Schlosser PM, Kalisak D, Preston J, Miller FJ (2004) Human respiratory tract cancer risks of inhaled formaldehyde: dose–response predictions derived from biologically-motivated computational modeling of a combined rodent and human dataset. *Toxicol Sci.* **82**: 279–96.

EPA, US Environmental Protection Agency (1986) Guidelines for carcinogen risk assessment. *Fed Reg* **51**: 33992–4003.

EPA, US Environmental Protection Agency (1991) Guidelines for developmental toxicity risk assessment. *Fed Reg* **56**: 63798–826.

EPA, US Environmental Protection Agency (1995) Proposed guidelines for neurotoxicity risk assessment. *Fed Reg* **1995**: 60–52032–56.

EPA, US Environmental Protection Agency (1996a) Health effects test guidelines carcinogenicity. OPPTS 870.4200, EPA 712-C-96-211, Public draft.

EPA, US Environmental Protection Agency (1996b Health effects test guidelines. OPPTS 870.5300, EPA 712-C-96-221, Detection of gene mutations in somatic cells in culture.

EPA, US Environmental Protection Agency (1996c) Proposed guidelines for reproductive toxicity risk assessment, EPA-630-R-96-009.

EPA, US Environmental Protection Agency (1996d) Health Effects Test Guidelines, OPPTS 870.7200, Domestic Animal Safety, June 1996. (http://www.epa.gov/opptsfrs/publications/OPPTS_Harmonized/870_Health_Effects_Test_Guidelines/Drafts/870-7200.pc).

EPA, US Environmental Protection Agency (1998) Health effects test guidelines. OPPTS 870.7200, companion animal safety prevention, pesticides, and toxic substances, August 5, 1998 (http://www.epa.gov/OPPTSHarmonized/870).

EPA, US Environmental Protection Agency (2005a) *Guidelines for Carcinogen risk Assessment*. Risk assessment forum USEPA, Washington, DC.

EPA, US Environmental Protection Agency (2005b) *Supplemental Guidance for Assessing Susceptibility for Early-Life Exposure to Carcinogens*. Risk assessment forum. Health Effects Test Guidelines. USEPA, Washington, DC.

EPA, US Environmental Protection Agency, Integrated Risk Information System (EPA/IRIS) (2006). http://www.epa.gov/iris/subst/index.html (accessed on August 1, 2006).

EPA, US Environmental Protection Agency, EPA/OPPTS, Office of Prevention, Pesticides and Toxic Substances (2006) OPPTS harmonized test guidelines (http://www.epa.gov/opptsfrs/publications/OPPTS_Harmonized/870_Health_Effects_Test Guidelines).

FIFRA, Federal Insecticide, Fungicide and Rodenticide Act (1991) Enforcement Response Policy for the Federal Insecticide, Fungicide and Rodenticide Act Good Laboratory Practice (GLP) Regulations.

FDA, Food and Drug Administration (1994) General Principles for Evaluating the Safety of Compounds Used in Food-Producing Animals Guidelines. http://www.fda.gov/cvm/fda/TOCs/guideline3toc.html, (accessed on August 1, 2006).

FDA, Food and Drug Administration (2000) Toxicologic Principles for the Safety of Food Ingredients (Redbook 2000). www.cfsan.gov/redbook/red.toct.html (accessed on August 1, 2006).

FDA, Food and Drug Administration (2001) Good Laboratory Practices for Non-clinical Laboratory Studies. Code of Federal Regulations, Title 21, Part 58. http://www.access.gpo/gov/nara/cfr/waisidx_ o1/21cfr58_01.html (accessed on August 1, 2006).

Gallo MA (2001) History and scope of toxicology. In *Casarett and Doull's Toxicology: The Basic Science of Poisons*, Klaassen CD (ed.). McGraw-Hill, New York, pp. 3–10.

Garland T, Barr AC (eds) (1998) *Toxic Plants and Other Natural Toxicants*. CAB International, New York.

Garner RJ (1957) *Veterinary Toxicology*. Williams and Wilkins, Baltimore, MD.

Garner RJ (1961) *Veterinary Toxicology*, 2nd edn. Williams and Wilkins.

Gold LS, Slone TH, Monley NB, Ames BN (2003) *Misconceptions about the Causes of Cancer*. Fraser Institute, Vancouver, BC.

Gordis L (2004) *Epidemiology*. W.B. Saunders.

Hayes AW (2001) *Principles and Methods of Toxicology*. Taylor and Francis, Philadelphia, PA.

Hulbert LE, Oehme FW (1968) *Plants Poisonous to Livestock*. Kansas State University, Manhattan, KS.

IARC, International Agency for Research on Cancer (1972) *IARC Monographs on the Evaluation of Carcinogenic Risks of Chemicals to Man*, vol. 1. IARC Lyon, France.

IARC, International Agency for Research on Cancer (1991) A consensus report of an IARC monographs working group on the use of mechanisms of carcinogenesis in risk identification. IARC Internal Technical Report No. 91/002, IARC Lyon, France.

IARC, International Agency for Research on Cancer (2006) IARC monographs on carcinogenic risks to humans (http://www.iarcfr/IARCPress/general/mono.pdf).

ILAR, Institute of Laboratory Animal Resources (1996) *Guide for the Care and Use of Laboratory Animals*. National Academy Press, Washington, DC.

International Programs on Chemical Safety (2006) Environmental health criteria monographs (http://www.inchem.org/pages/ehc.html).

Jarabek AM (1994) Inhalation RfC methodology. Dosimetry adjustments and dose–response estimation of noncancer toxicity in the upper respiratory tract. *Inhal Toxicol* **6**: 301–25.

Jarabek AM, Menache MG, Overton Jr JH, Dourson ML, Miller FJ (1990) The US Environmental Protection Agency's inhalation RFD methodology: risk assessment for air toxics. *Toxicol Ind Health* **6**: 279–301.

Kaplan EL, Meier P (1958) Non-parametric estimates from incomplete observations. *J Am Stat Assn* **53**: 457–481.

Kaufmann M (1901) *Thérapeutique et Matiére Médicale Vétérinaire*, 3rd edn, Asselin et Houzeau, Paris.

Kingsbury JM (1954) *Poisonous Plants of the United States and Canada*. Prentice-Hall, Englewood, NJ.

Kingsbury JM (1964) *Poisonous Plants of the United States and Canada*. Prentice-Hall, Englewood, NJ.

Klaassen CD (2001) *Casarett and Doull's Toxicology: The Basic Science of Poisons*, 6th edn. McGraw-Hill Medical Publication Division, New York.

Knight AP, Walter RG (2001) *A Guide to Plant Poisoning of Animals in North America*. Teton New Media, Jackson Hole, WY.

Kobert R (1897) *Practical Toxicology for Physicians and Students*. Translated by Friedburg LH. W.R. Jenkins, New York, 201 pp.

Lander GD (1912) *Veterinary Toxicology*. A. Eger, Chicago, IL, 312 pp.

Lehman AJ, Fitzhugh OG (1954) 100-Fold margin of safety. *Q Bull Assoc Food Drug Official* **XVIII**: 33–5.

Marquadt H, Schafer SG, McClellan RO, Welsch F (eds) (1999) *Toxicology*. Academic Press, New York.

McClellan RO (1994) A commentary on the NRC report "Science and Judgment in Risk Assessment". *Regul Toxicol Pharmacol* **20**: S142–68.

McClellan RO (1999) Human health risk assessment: a historical overview and alternative paths forward. *Inhal Tox* **11**: 477–518.

McClellan RO (2003) Risk assessment: replacing default options with specific science. *Human Ecol Risk Assess* **9**: 421–38.

McClellan RO, Medinsky MA, Snipes MB (2006) Inhalation toxicology, Chapter 16. In *Biological Concepts and Techniques in Toxicology: An Integrated Approach*, Riviere JE (ed.). Taylor and Francis, New York, pp. 295–361.

Milles D (1999) History of toxicology. In *Toxicology*, Marquadt H, Schafer SG, McClellan RO, Welsch F (eds). Academic Press, San Diego, CA, pp. 11–24.

Murphy M (1996) *A Field Guide to Common Animal Poisons*. Iowa State University Press, Ames, IA.

Nicholson JA (1945) *Lander's Veterinary Toxicology*. Bailliere, Tindall & Cox, London.

NRC, National Research Council (1983) *Risk Assessment in the Federal Government: Managing the Process*. National Academy Press, Washington, DC.

NRC, National Research Council (1993) *Issues in Risk Assessment. I. Use of the Maximum Tolerated Dose in Animal Bioassays for Carcinogenicity*. National Academy Press, Washington, DC.

NRC, National Research Council (1994) *Science and Judgment in Risk Assessment*. National Academy Press, Washington, DC.

NRC, National Research Council (2004) *Intentional Human Dosing Studies for EPA Regulatory Purposes: Scientific and Ethical Issues*. National Academy Press, Washington, DC.

NTP, National Toxicology Program (2005) *Report on Carcinogens*, 11th edn. US Department of Health and Human Services, Public Health Service, Washington, DC.

Oehme FW (1970) The development of toxicology as a veterinary discipline in the United States. *Clin Toxicol* **3**: 211–20.

Osweiler GB (1996) *Toxicology*. Williams and Wilkins, Philadelphia, PA.

Osweiler GD, Carson TL, Buck WB, Van Gelder GA (1985) *Clinical and Diagnostic Veterinary Toxicology*, 3rd edn. Kendall Hunt, Dubuque, IA.

Pagel W (1958) *Paracelsus: An Introduction to Philosophical Medicine in the Era of the Renaissance*. Karger, New York.

Paustenbach DJ (2001) The practice of exposure assessment. In *Principles and Methods of Toxicology*, Hayes AW (ed.). Taylor and Francis, Philadelphia, PA, pp. 387–448.

Peterson ME, Talcott PA (eds) (2001) *Small Animal Toxicology*. Elsevier Saunders, St. Louis, MO.

Peterson ME, Talcott PA (eds) (2006) *Small Animal Toxicology*, 2nd edn. Elsevier Saunders, St. Louis, MO.

Plumlee KH (2004) *Clinical Veterinary Toxicology*. Mosby, St. Louis, MO.

Quitman EL (1905) *Synopsis of Veterinary Materia Medica, Therapeutics and Toxicology*, A. Eger, Chicago, IL, 277 pp.

Radeleff RD (1964) *Veterinary Toxicology*. Lea and Febiger, Philadelphia, PA.

*Risk Commission, Presidential/Congressional Commission on Risk Assessment and Risk Management*, vol. 1 (1997) *A Framework for Environmental Health Risk Management*, vol. 2. Risk Assessment and Risk Management in Regulatory Decision-Making, Government Printing Office, Washington, DC.

Riviere JE (ed.) (2006) *Biological Concepts and Techniques in Toxicology: An Integrated Approach*. Taylor and Francis, New York.

Roder JD (2001) *Veterinary Toxicology*. Butterworth-Heinenmann, Boston, MA.

Sipes IG, McQueen CA, Gandofi AJ (1997) *Comprehensive Toxicology*, 13 vols. Pergamon Press, Oxford, UK.

Smithcors JF (1957) *Evolution of the Veterinary Art*. Veterinary Medicine Publishing, Kansas City, MO.

Spoo W (2004) Toxicokinetics. In *Clinical Veterinary Toxicology*, Plumlee KH (ed.). Mosby, St. Louis, MO, pp. 8–12.

Stahlheim OHV (1994) *The Winning of Animal Health: 100 Years of Veterinary Medicine*. Iowa State University Press, Ames, IA.

Swabe J (1999) *Animal, Disease, and Human Society: Human–Animal Relations and the Rise of Veterinary Medicine*. Routledge, London/New York.

TSCA, Toxic Substance Control Act (1985) Good Laboratory Practices Regulations Enforcement Response Policy.

Wilkinson L (2005) *Animals and Disease: An Introduction to the History of Comparative Medicine*. Cambridge University Press, New York.

Wilsdorf G, Graf C (1998) Historical review of development of veterinary toxicology in Berlin – 1945 (in German). *Berlin Munch Tierarztl Wochenschr* **111**: 21–6.

C H A P T E R

2

# Pharmacokinetics and toxicokinetics: fundamentals and applications in toxicology

*Rakesh Dixit*

## INTRODUCTION

The combined and well coordinated processes of the absorption (A) of a drug or a xenobiotic into the systemic circulation, its distribution (D) to organs and tissues, metabolism (M) to other active or inactive chemical species, and its elimination (E) from the body is collectively known as ADME (Gibaldi and Perrier, 1982; Voisin *et al.*, 1990; Shargel and Yu, 1993; Rowland and Tozer, 1995; Medinsky and Valentine, 2001). The pharmacokinetics refers to the kinetics of ADME processes employed at relevant low pharmacological doses where pharmacokinetics generally follow first-order kinetics and kinetic processes are expected to be linear (Gibaldi and Perrier, 1982; Shargel and Yu, 1993; Rowland and Tozer, 1995). Toxicokinetics unlike pharmacokinetics represent the study of kinetic processes of ADME under the conditions of preclinical toxicity/safety testing where depending on the doses employed both first- and zero-order kinetics are expected and kinetic process can substantially change between low and high doses (Medinsky and Valentine, 2001; Dixit *et al.*, 2003). During the last 20 years, the application of toxicokinetics has evolved in the pharmaceutical and chemical industry and toxicokinetics often refer to exposure assessment in drug or chemical safety assessment studies. In contrast to pharmacokinetics, the pharmacodynamics refer to effects elicited by the drug and active metabolites at relevant pharmacological doses while toxicodynamics refer to toxic effects related to doses (systemic exposures) used under the conditions of toxicity testing (Gibaldi and Perrier, 1982; Shargel and Yu, 1993; Rowland and Tozer, 1995).

With a full understanding of dose response, including dose administered and circulating drug levels (systemic exposure) and their relationship to toxicity, the toxicity and safety of xenobiotics, including drugs can be better assessed. In contrast to pharmacokinetics studies where ADME processes are generally first order and linear, toxicokinetics especially at higher doses encompass zero-order and non-linear processes. Non-linearity in toxicokinetics typically result from saturable metabolic clearance processes, saturable transporters, drug-specific biopharmaceutical factors, and toxicodynamics. Biopharmaceutical factors may include alterations in drug absorption at different doses (e.g. from low solubility of drug), differences in blood or tissue distribution (e.g. due to saturable protein binding, changes in tissue pathology), differences in metabolism (e.g. saturable metabolic enzymes kinetics), and in drug elimination (e.g. urinary and fecal excretion) (Medinsky and Valentine, 2001; Dixit *et al.*, 2003). In practice, the most practical surrogate or measure of dosimetry is the determination of the time course of drug and its major metabolite(s) in easily accessible body fluids, including blood or plasma or urine. With a good quantitative understanding of the time course of plasma drug concentration, one can gain information on the kinetics of absorption, distribution, metabolism, and elimination of a given drug. Because of the difficulty in quantifying drug and metabolites concentrations in target organs of toxicity, it is expected at the steady state the concentrations of a drug in blood or plasma are likely to be in equilibrium with concentrations in tissues. At the steady state, the plasma/blood can be considered a reasonable practical

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surrogate for tissue(s) exposure to drug and changes in plasma drug concentrations may reflect changes in tissue drug concentrations over time, and relatively simple pharmacokinetic calculations or models can be extremely useful to describe the behavior of that drug in the body. In order to integrate preclinical animal toxicology data into human risk assessment, it is imperative to have some comparative human pharmacokinetics data at relevant exposures. In veterinary risk assessment, toxicokinetics/pharmacokinetics data from multiple species need to be incorporated into risk assessment.

The fundamental objective of the toxicokinetics is to obtain information on the relationship between the dosage administered and circulating levels of xenobiotics (systemic exposure) under the conditions of toxicity testing. Toxicokinetics data when appropriately obtained may provide the following additional useful information:

1 Relationship between increasing doses and exposures attained (linear, non-linear, or plateau).
2 Sex differences in exposures and their relationship to any potential sex-related differences in toxicity.
3 Effect of repeated administration on exposures and if increase or decrease in toxicity is related to changes in ADME toxicokinetics.
4. Safety of the proposed initial doses in clinical trials or proposed acceptable daily intake (ADI), tolerable daily intake (TDI), or reference dose (RfD).
5 Support dose escalation in subsequent clinical trials.

In pharmaceutical risk assessment, the most important use of the toxicokinetics data has been in assessing safety margins based on interspecies comparison of plasma $AUC$ or $C_{max}$ at no-observed adverse-effect level (NOAEL) and observed adverse-effect level from animal toxicology studies and expected/observed exposures at relevant clinical doses. Unlike the pharmaceutical industry, the human risk assessment of exposures to chemicals present in food, water, or air has traditionally relied on safety or uncertainty factor approach. The ADI or TDI or RfD on mg/kg basis is determined by dividing the NOAEL in mg/kg/day from most sensitive animal species by a factor of 100 which includes a factor of 10 each for extrapolation of safe doses: interspecies (animals to humans) and intraspecies (within humans). The use of this approach has often given low ADI or TDI and RfD for chemicals which are not easily achievable or enforceable. In recent years, there has been an increased emphasis on the use of toxicokinetics to reduce uncertainty in extrapolation of doses across species.

This chapter provides a general introduction to toxicokinetics, description of toxicokinetics parameters, and their assessment using simple equations. The chapter also discusses the applications of toxicokinetics in veterinary risk assessment and drug development.

# FUNDAMENTALS OF XENOBIOTIC DISPOSITION

Animals and humans may be exposed to xenobiotic chemicals present in our air, water, and food through multiple routes, including oral, dermal, and pulmonary. Intentional therapeutic drug exposure may occur through multiple routes, including oral, intravenous, intraperitoneal, subcutaneous, intramuscular, buccal, pulmonary, ocular, and direct regional administration. Therefore, a good understanding of the fundamental processes of ADME processes is critical to understanding toxicokinetics.

# ABSORPTION

Absorption is collectively defined as all processes that comprise the transfer of an unchanged xenobiotic introduced into the body into systemic circulation or site of measurement may be exposed to xenobiotic chemicals present in our air, water, and food through multiple routes, including oral, dermal, and pulmonary. Intentional therapeutic drug exposure may occur through multiple routes, including oral, intravenous, intraperitoneal, subcutaneous, intramuscular, buccal, pulmonary, ocular, and direct regional administration. Therefore, a good understanding of the fundamental processes of ADME processes is critical to understanding toxicokinetics. Some basic concepts in absorption following extravascular routes of administration are demonstrated in Figure 2.1.

## Gastrointestinal absorption

Oral route is the predominant route of exposure to drugs and chemicals. A prerequisite for the absorption through membrane is that the chemical must be dissolved in gastrointestinal (GI) fluids, have enough lipophilicity, and lack charge to pass through the lipid layers. Chemicals that are water soluble, metabolically resistant, and have sufficient lipid solubility are generally rapidly absorbed with a rapid peak concentration. If a chemical is not appropriately soluble in GI fluids, it will have difficulty in making it available to membranes and may face slow and/or sustained dissolution-limited absorption. This may occur when sparingly soluble chemicals/drugs are administered as suspensions. From biopharmaceutical perspectives, drug absorption across biological membranes is governed largely by the p*K*a, pH at the site of absorption, molecular size, molecular weight, and dissociation constant, degree of ionization, aqueous and lipid solubility, partition coefficient, chemical reactivity. Other factors that may impact the biological absorption process include

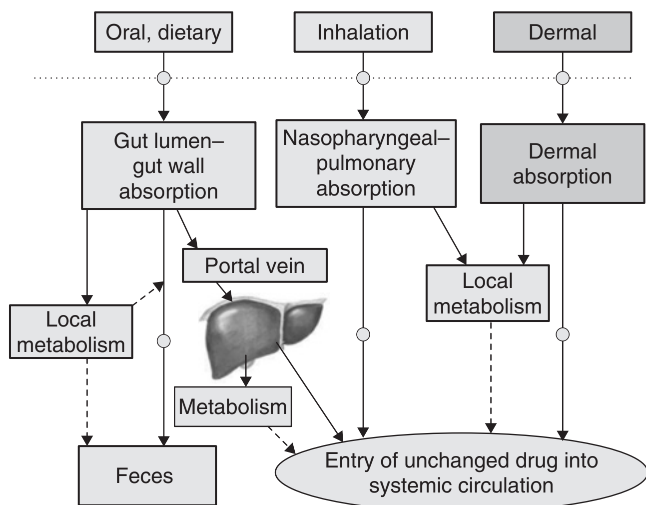


**FIGURE 2.1** *Concepts in extravascular absorption.* When a xenobiotic such as a drug is administered via non-intravenous route, the absorption at the local site is the key process in the systemic delivery of the active substance. Some of the most important extravascular routes include oral/dietary, inhalation (nasopharyngeal–pulmonary), and dermal. With extravascular routes of administration, significant portion of drug can be lost as a result of local site metabolism. In addition, when a drug is administered through oral route, hepatic first-pass metabolism plays a pivotal role in controlling the systemic availability of drug (see text for additional discussion).

gastric emptying time, intestinal transit time, presence or absence of food, gut microflora, and specific drug transporters (Dethloff, 1993).

Route-specific/site of delivery-specific factors includes cell types, surface area at the absorption site, blood flow to and from site of absorption, pH at site of absorption, and site-specific metabolic/transporter effects (Dethloff, 1993). Chemicals, including drugs, are absorbed across lipid-rich membranes principally by (a) diffusion, (b) membrane pores (aqueous channels) and (c) energy-dependent active carrier mediated saturable processes. The majority of xenobiotics pass through membranes through a diffusion process, a process largely controlled by Fick's law. Very small molecules (chemicals up to $4 \times 10^{-4}$ μm in diameter with a molecular mass of less than approximately 200 Da) can filter through the membrane pores without much difficulty.

According to Fick's law (Dethloff, 1993), the rate of diffusion is proportional to concentration gradient (C1−C2) across the membrane, the surface area available for diffusion ($a$), and a diffusion constant ($k$). Overall the diffusion rate is inversely proportional to the thickness of the membrane ($d$):

$$\text{Diffusion rate} = k \times \frac{a(\text{C1} - \text{C2})}{d}$$

Most chemicals or drugs are weak acids or weak bases and therefore exist as ionized or non-ionized species. Because non-ionizable form is lipid soluble, it facilitates diffusion through lipid membranes. In contrast, the ionizable form is poorly lipid soluble and is generally unable to pass through the lipid membranes.

The pH and p*K*a on both sides of microenvironment largely determine the extent of ionization. The Henderson–Hasselbach equation to determine the extent of ionization can be as follows:

$$\text{pH} = \text{p}K\text{a} + \log \frac{[\text{conjugated base}]}{[\text{conjugated acid}]}$$

The pH at the absorption sites in relation to p*K*a has been exploited for improving the absorption and/or increasing urinary elimination of many drugs by reducing renal reabsorption of drugs. For example, when poisoned with salicylate, urinary excretion of salicylate can be substantially increased due to increased alkalinization of urine (from pH 6.5 to 8.0) by giving sodium bicarbonate. Because the p*K*a of salicylate is 3.5, the alkalinization of urine (pH 3.8) decreases the non-ionization form substantially (approximately 25-fold decrease) which reduces the renal reabsorption of salicylate and increases (approximately 5-fold) its urinary excretion. *It can be generally concluded that weak acids are likely to be better absorbed in the low pH of stomach whereas weak bases are likely to be better absorbed in the intestine.* It is not surprising to note that strong organic acids and bases are generally incompletely absorbed in the GI tract due to very strong ionization at all absorption sites. The absorption of most xenobiotics occurs in small intestine because this is well suited for absorption owing to its large surface area afforded by millions of villi and their specialized absorptive epithelium. The absorption through villus is also greatly supported by hepatic portal circulation and central lacteal (absorption into lymphatic system).

### *Factors impacting GI absorption*

There are a large number of factors that limit the availability of xenobiotics. Extreme pH and hydrolytic enzymes (e.g. proteases and lipases) in the GI tract substantially impact the stability of the xenobiotics. The GI tract microflora is known to metabolize a large number of xenobiotics and this process can reduce the availability of xenobiotics absorption, and in some cases, may activate fairly benign xenobiotics into toxic metabolites. Despite very good absorption through the small intestine, many drugs fail to reach systemic circulation in sufficient amounts. Small intestine is very rich in both phase I and II metabolizing enzymes which can actively biotransform a

well absorbed drug. Overall, metabolism by the small intestine can prevent a well absorbed drug getting into portal circulation. In addition to the first-pass effects by intestine, the liver is the chief site of metabolism and contributes greatly to the first-pass metabolism leading to poor circulation of well absorbed drugs. Efflux and influx transporters present on the intestinal wall and liver can also modulate the systemic availability of drugs. Food present in the gut lumen can also substantially impact the absorption of drugs.

## NON-ORAL ROUTES AND XENOBIOTIC ABSORPTION

### Intravenous

For drugs that are poorly absorbed through oral route, intravenous route is often used to bypass absorption and deliver drug directly into systemic circulation. Intravenous route can also deliver very high peak concentrations very rapidly. Continuous intravenous infusion is often used to deliver the desired concentration in a well controlled manner.

### Intramuscular, subcutaneous, and intradermal

These alternate routes are used to deliver drugs rapidly into systemic circulation because drugs delivered through these routes are not susceptible to first-pass metabolism. However, these different routes will deliver drugs with varying rates due to local site-specific absorption influenced largely by blood flow at local sites.

### Buccal and sublingual

Oral mucosa has significant ability to absorb certain drugs. Because these routes are convenient ways to attain desired plasma concentrations, efforts have been made to actively exploit these routes for rapid delivery of drugs into systemic circulation. These routes offer a distinct advantage in delivering drugs into systemic circulation because they can bypass first-pass hepatic metabolism. Major limitations for these routes are local irritation and limitations of the doses.

### Intraperitoneal

The advantage of the intraperitoneal route is that poorly absorbed drugs can be directly delivered to liver by bypassing intestinal absorption and without being subjected to intestinal first-pass metabolism. Drugs when given by the intraperitoneal route can attain a very rapid absorption when compared to the oral route.

### Dermal

For environmental and industrial chemicals, the dermal route is a significant route of exposure. When compared to the oral route, the skin serves as an important barrier for absorption because of the substantially lower surface area, membrane thickness, and poor blood flow. Despite these limitations, many chemicals when presented at large doses can be substantially absorbed. Outer layers of skin (epidermis or stratum corneum) provide a significant barrier to absorption; however, hydration, epidermal erosion, or abrasion can greatly enhance absorption. Unlike the outer layers of skin, the inner layers such as dermis are well perfused. One additional advantage of the dermal route is that drugs given by the dermal route are not subject to significant metabolism, though epidermis has been shown to metabolism certain xenobiotics.

## INHALATION

The inhalation route is one of the major routes of xenobiotic exposure. In recent years, the pulmonary delivery of drugs has become an important route of drug delivery. Lungs receive 100% of the cardiac output and the repetitive branching of the airways from trachea to terminal alveoli provides an enormous surface area for absorption. Absorption from lungs is quite rapid because there is little barrier (alveolar region thickness is only 0.2 μm) and up to 90% of the alveolar surface is exposed to capillary bed. Inhaled drugs are deposited on lungs epithelial surface and deposition of aerosols is influenced by lung-specific anatomical regions. Particles less than 1 μm are typically excluded from alveolar absorption. In certain regions such as nasopharyngeal, tracheal, bronchial and upper bronchiolar, xenobiotics are likely deposited by impactation while in deeper regions of lungs, xenobiotics are deposited by diffusional and sedimentation processes. Passive diffusion plays an important role in xenobiotic absorption and the alveolar region because its enormous surface area coupled with high blood flow is the major site for pulmonary absorption. Although the lung has capacity to metabolize chemicals which will likely decrease the systemic availability, chemicals delivered through lung absorption process bypass hepatic first-pass metabolism and directly enter systemic circulation through heart.

## SPECIES DIFFERENCES IN ABSORPTION

This topic has been discussed in great depth by Beasley (1999). Of over 4000 species of mammals, there are large numbers of species differences in absorption. The food habits of carnivores, herbivores, and omnivores differ greatly and this can be responsible for differences in bioaccumulation of potentially toxic chemicals. It is not too surprising that the accumulation of fat-soluble xenobiotics is higher in carnivores than in herbivores and the extent of accumulation of food chain derived xenobiotics or bioaccumulation factor (concentration in animal tissues divided by concentration in environment/food chain) may vary a log order of magnitude. Ruminants show age-dependent absorption of xenobiotics. Young calf and lamb behave like monogastric animals until maturity when they adapt to high roughage diet. These species also dilute xenobiotics exposure with a longer GI transit time. Additionally consumption of foods rich in fiber tends to lower the bioavailability of toxic compounds. Anaerobic environment in the rumen tends to reduce xenobiotics much more efficiently than in non-ruminants.

Monogastric animals, including carnivores and omnivores, have a lower stomach pH. For example, the gastric pH in dogs and pigs is between 1 and 2. Unlike other species, horses have a higher gastric pH of about 5.5 and their stomach size is smaller in relation to their overall body size. This means they need to eat more often.

## DISTRIBUTION

Organ distribution of xenobiotics is mainly controlled by three different factors: (1) diffusion rate, (2) perfusion rate, and (3) relative affinity to various components (e.g. enzymes, receptors, transporters) in a given organ. When the diffusion rate across membrane is poor, the physicochemical properties of xenobiotics control the rate of tissue penetration. The perfusion rate (rate of delivery to an organ) becomes rate limiting when diffusion is rapid. The well perfused organs include lungs, liver, kidneys, heart, intestines, and brain. Poorly perfused organs include skin, skeletal muscle, connective tissue, and fat. Compounds that are highly plasma protein bound may show a lower tissue distribution and vice versa; however, it is to be stressed that it is the balance between the relative affinity of a chemical to tissue components and protein binding association and dissociation rates that control overall tissue distribution. Protein binding alone does not necessarily control the entire tissue distribution. For example, beta blocker propranol shows a high protein binding and yet has a high tissue distribution because it has a higher affinity for many tissues. Extensive plasma protein binding may decrease the unbound fraction available for tissue distribution; however, in rapidly perfused organs this can increase the diffusion rate from blood to organs. This may happen due to an increase in the concentration gradient due to build up of the protein bound drug in blood which in turn can increase the off rate of protein bound drugs/chemicals to release more drug for tissue distribution. This illustrates that protein binding is a dynamic process and highly protein bound may indeed show high tissue distribution if relative affinities to tissue components are high.

## METABOLISM AND ELIMINATION

Metabolism of xenobiotics is very complex and diverse. It shows differences in occurrence, function, and rates. Species differences in quantitative metabolism are fairly common and it is important to appreciate this when interpreting toxicology data from one species to another species. For a detailed review of metabolism, refer to DeBethizy and Hayes (2001). Primitive species like microbes also have significant metabolism capacity, though microbes generally have very different pathways than mammals and may modify drug toxicity in an unexpected manner. Metabolism generally occurs through both phase I and II pathways. Phase I reactions result in functionalization, which result in the addition or uncovering of functional groups that are needed for subsequent metabolism by phase II pathways. Phase I reactions include oxidation (e.g. cytochrome P-450 (CYP) isoenzymes, xanthine oxidase, peroxidases, amine oxidase, monoamine oxidase, dioxygenases), reduction (e.g. CYP isoenzymes, ketoreductase, glutathione peroxidases), hydration (epoxide hydrolase), and dehydrogenases (alcohol dehydrogenase, aldehyde dehydrogenase). Phase II reactions are biosynthetic in nature and a common goal of all phase II reactions is to make xenobiotics more water soluble, more polar, and more easily excretable. Phase II enzymes include glutathione-*S*-transferases, UDP-glucuronyltransferase, thioltransferase, sulfotransferase, amide synthesis transacylase, *O*-, *N*-, *S*-methyltransferase, acetyltransferases, and thiosulfate sulfotransferase (rhodanese).

Excretion of xenobiotics and their metabolites usually occurs via urine, feces, and expired air for volatile substances. Three major processes within kidney control urinary excretion. These processes are glomerular filtration, reabsorption, and tubular excretion. Glomerular filtration because of the limits of pore size of 70–80 Å filters anything smaller than molecular weight of 20,000 Da.

All high molecular proteins and protein bound chemicals or their bound metabolites are not filtered and remain in blood and will likely be excreted through fecal excretion. Reabsorption of filtered components from urine to

blood occurs through specific active transport process occur in tubules. With the reabsorption of water urine may become concentrated and when this occurs, the reabsorbed components may diffuse back from tubules to blood. Many foreign compounds may also be secreted back into renal tubules against concentration gradient through both cation and anion carrier processes. Because these carrier processes are energy-dependent active process, they are saturable and with increasing doses renal secretion may get decreased causing accumulation of xenobiotic metabolites. Fecal excretion of xenobiotics and their metabolites is an important route of excretion. There are two sources for the excretion of compounds into feces. Unabsorbed drug and drug excreted through bile constitute the fecal excretion. For large molecular weight compounds and their metabolites biliary transport occurs through cationic transporters from hepatocytes into bile. Generally, large molecular weight drugs and their conjugative metabolites are excreted through bile. Based on the evaluation of molecular weight versus biliary excretion of a large number of molecules and their metabolites, it has been estimated that the molecular weight (Da) cutoff for the biliary excretion of chemical moieties in rats, guinea pigs, rabbits, and humans is 325 (±50), 440 (±50), 475 (±50), and 550, respectively (Hirom and Birch, 1976). Chemical moieties of molecular weights ranging from 350 to 450 Da are generally excreted via both urine and feces (Hirom *et al.*, 1972). The estimate of fecal excretion is often complicated by the fecal excretion of the unabsorbed drug when given by the oral route. Bile duct ligation studies can be helpful in evaluating the contribution of bile versus unabsorbed drug in fecal excretion of drug-related moieties.

## CLASSICAL TOXICOKINETIC MODELS

The classical toxicokinetic models represent the most simple form of the pharmacokinetic models (Medinsky and Valentine, 2001; Dixit *et al.*, 2003). The principal component of a classical toxicokinetic model is a central compartment consisting of plasma (systemic) and tissues into which drug and its metabolites equilibrate. The basic principle of a simplistic one central compartment model is that it is assumed that all tissues, including both rapidly and slowly equilibrating tissues, and plasma will attain a rapid equilibrium and the kinetic profile of tissues can be described by measuring drug and its major metabolite(s) in plasma. Similarly, in the multiple compartmental models, drug is present into the central compartment and distributes rapidly between the central and peripheral compartments and the distribution between central and peripheral compartments follows a first-order process. In this model, drug elimination occurs from the central compartment, which is assumed to contain all major rapidly perfused drug eliminating tissues (e.g. kidneys and liver). The major advantages of the classical compartmental toxicokinetic models are that the models do not require information on disposition characteristics of drug-based tissue physiology or anatomical structure (Dethloff, 1993; Dixit *et al.*, 2003). These models, because of their simplicity, provide valuable information in describing and predicting the time course of drug concentrations in the systemic circulation at different doses, the extent of drug accumulation with multiple doses, and aid in selecting effective doses and dose regimens in efficacy and toxicity studies to achieve specific exposures.

One major disadvantage of classical models is their simplistic description of the kinetics of ADME processes in the body since these models are simple mathematic solutions for goodness of fit (Clewell and Andersen, 1985; Dixit *et al.*, 2003). Therefore, the classical models are not able to assess or reflect (1) the biology of specific tissues; (2) individual rates of transfer between compartments; (3) individual contributions and rates in routes of elimination; (4) the time course and exposure information in a specific tissue; and (5) specific drug disposition and elimination which involves dose-dependent non-linear and zero-order processes (Beasley, 1999; DeBethizy and Hayes, 2001).

## FUNDAMENTAL NON-COMPARTMENTAL TOXICOKINETIC PARAMETERS

The non-compartment methods are simplistic and represent the most practical way to describe the kinetic behaviour of the drug. The most relevant pharmacokinetic parameters in the non-compartmental models typically include plasma or tissue area under the concentration versus time curve ($AUC$), maximum concentration achieved ($C_{max}$), time to maximum concentration ($T_{max}$), apparent volume of distribution ($V_d$), systemic clearance ($CL_s$), and terminal half-life ($T_{1/2}$). Figure 2.2 shows the visual concepts in toxicokinetics.

### Area under the plasma/tissue concentration versus time curve

The AUC is considered the most important kinetic parameters in pharmacokinetics and is the quantitative measure of the exposure to drug over the sampling period. The linear trapezoidal rule is the most frequently used method to calculate AUC. Typically plasma concentration versus time curve is constructed. The overall curve is then divided into a series of trapezoids, typically indicated by observed time points and achieved concentrations. Overall AUC is calculated by the summation of the area within each individual trapezoid.

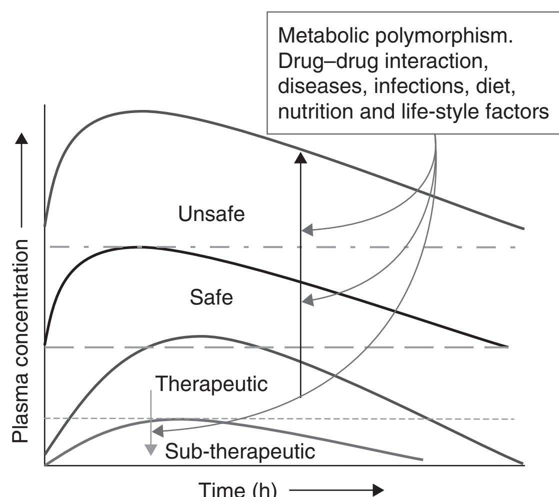


**FIGURE 2.2** *Pharmacokinetics–pharmacodynamics and impact of intrinsic and extrinsic factors.* The use of pharmacokinetics data in clinical settings to maintain therapeutically efficacious concentrations. This is based on a thorough analysis of pharmacokinetics versus pharmacodynamics (effects associated with drug) during clinical trials. It is to realize that there are a large number of intrinsic (patient derived) and extrinsic factors that may increase drug levels which may be unsafe or decrease drug levels that may be sub-therapeutic and may decrease drug's efficacy (see text for additional discussion).

The area under each trapezoid can be calculated as follows:

$$\text{Area}_{\text{Trap}} = 0.5 \times (C_n + C_{n+1}) \times (t_{n+1} - t_n)$$

where $C_n$ is the concentration at the earlier time; $C_{n+1}$ is the concentration at the next later time; $t_{n+1}$ is the later time; and $t_n$ is the earlier time. The overall $AUC_{0-t_{\text{last}}}$ is then calculated as follows:

$$AUC_{0-t_{\text{last}}} = \sum 0.5 \times (C_n + C_{n+1}) \times (t_{n+1} - t_n)$$

The area calculated above is $AUC_{0-t_{\text{last}}}$ when the concentration at time zero is the first concentration (typically immediately prior to dosing); $t_{\text{last}}$ is the time when the last sample was collected and concentration measured. In toxicokinetic studies supporting drug safety evaluation, $AUC_{0-t_{\text{last}}}$ is often measured from time 0 to 24 h. This is a measure of the daily systemic exposure during a repeated dosing. When steady-state toxicokinetics are expected (typically after attaining 5 half-lives), zero-hour concentrations are assumed to be equal to the 24-hour plasma concentrations. The measurement of $AUC$ from time 0 to time $\propto$ represents the most accurate assessment of total systemic exposure following a single dose. When calculating, the remaining $AUC$ from $t_{\text{last}}$ to time $\propto$ is calculated with the terminal elimination rate constant, and is added to $AUC_{0-t_{\text{last}}}$. $AUC_{0-\infty}$ is then estimated as follows:

$$AUC_{0-\infty} = AUC_{0-t_{\text{last}}} + \frac{C_{\text{last}}}{k_{\text{el}}}$$

- $C_{\text{last}}$: Concentration measured at the last time point; $k_{\text{el}}$ is the terminal elimination rate constant and is a measure of the fraction of drug removed from the site of collection per unit of time. The $k_{\text{el}}$ has units of reciprocal time (e.g. $\text{min}^{-1}$ and $\text{h}^{-1}$) and can be determined from the slope of the straight-line portion of the terminal phase of the concentration versus time curve when the concentration data are log-transformed as follows:

$$k_{\text{el}} = -2.303 \times slope$$

The multiplier 2.303 is a conversion factor from log units to natural log (ln). The first-order elimination rate constant $k_{\text{el}}$ is independent of dose.

- $C_{\max}$: This refers to the maximum drug concentration ($C_{\max}$) attained during the time course of the measurement of drug levels. In the non-compartment methods, the information on $C_{\max}$ is often used to determine the extent of drug absorption.
- $T_{\max}$: This refers to the time to attain maximum concentration. This parameter is often useful to assess the rate of drug absorption.
- *Trough levels* ($C_{\min}$): This refers to the minimum drug concentration ($C_{\min}$) attained during the elimination phase in time course of the plasma drug versus time curve. In the non-compartment methods, the ratio of trough level to maximum concentration can often provide valuable information on the rate of drug elimination.
- *Half-life* ($T_{1/2}$): It is the time required for the blood or plasma drug concentration to decrease by one-half, and can be determined from the terminal elimination rate constant by the following calculation:

$$T_{1/2} = \frac{0.693}{k_{\text{el}}}$$

The numerator 0.693 is the natural log of 2. It is to be emphasized that the accuracy of $k_{\text{el}}$ and $T_{1/2}$ estimates is dependent on selection of time points. As a general rule, time points covering up to 5 half-lives should be taken. Both $k_{\text{el}}$ and $T_{1/2}$ are dependent on both volume of distribution and clearance by the following relationship:

$$T_{1/2} = \frac{0.693 \times V_{\text{a}}}{CL_{\text{s}}}$$

## Clearance

Drugs and their metabolites are removed from body via a variety of routes that may include fecal and urinary excretion, excretion in tears or sweat, metabolism in liver, kidneys, lungs, intestinal or other tissues, or by exhalation. Clearance is defined as the volume of drug removed from the body per unit of time with units as ml/min. For example, a $CL_{\text{s}}$ value of 30 ml/min means that 30 ml of blood or

plasma containing drug is removed each minute. This parameter is often normalized to body weight, and thus clearance values are often reported in units of ml/min/kg. The $CL_s$ means clearance as measured from the systemic circulation. $CL_s$ is best calculated from concentration versus time data after an intravenous bolus or infusion dose because there is no absorption and nearly 100% of the drug is bioavailable following intravenous administration. Following extravascular administration (e.g. oral), estimates of clearance should be normalized to bioavailability and can be calculated as follows:

$$CL_s = \frac{F \times dose}{AUC_{0-\infty}}$$

where $F$ is the fraction of the drug dose that entered the systemic circulation following extravascular administration. When constant intravenous infusion to steady state is used, $CL_s$ may be calculated as a function of the infusion rate and the achieved steady-state concentration:

$$CL_s = \frac{k_0}{C_{ss}}$$

where $k_0$ is the rate of intravenous infusion and $C_{ss}$ is the steady-state concentration.

*Total body clearance* can be estimated as the sum of clearances by individual eliminating organs such that:

$$CL_s = CL_r + CL_h + CL_i + \cdots$$

where $CL_r$ is the renal clearance, $CL_h$ the hepatic clearance, and $CL_i$ the intestinal clearance. It is worthy to note that clearance of compounds from a particular organ cannot be higher than blood flow to that organ.

## Volume of distribution

The apparent volume of distribution ($V_d$) relates the total amount of drug in the body to the plasma concentrations in the body. $V_d$ is essentially the volume into which the drug distributes in the body. $V_d$ is considered an indicator of extravascular distribution and has units of liters or liters/ kg of body weight.

$V_d$ can be calculated as follows:

$$V_d = \frac{F \times dose}{k_{el} \times AUC_{0-\infty}}$$

where $F$ is the fraction of dose that enters the systemic circulation. Following intravenous administration, $F$ will have a value of 1 because the bioavailability is 100%.

$V_d$ is considered the *"apparent" volume of distribution* and it has very little or no physiological significance because it usually does not relate to a real biological volume. $V_d$ term is typically drug specific and represents the distribution of drug out of the central plasma compartment. In this context, a drug with a larger $V_d$ will have high extrasystemic tissue distribution and if the drug binds to tissue extensively, $V_d$ may exceed the tissue volume. Once the $V_d$ for a compound is known, it can be used to estimate the amount of drug in the body as follows:

$$X_{drug} = V_d \times C_p$$

where $X_{drug}$ is the amount of drug in the body and $C_p$ is the plasma drug concentration.

# BIOAVAILABILITY

The oral route is the predominant route of administration of pharmaceuticals and the fraction of dose that is available after absorption and first-pass clearance in the systemic circulation is termed *bioavailability* ($F$). Bioavailability can be essentially described as the amount of a drug that enters the systemic circulation and is considered a measure of drug absorption. The route of drug administration, intestinal first-pass effect, hepatic first-pass effect, transporters, formulations, and dissolution rate characteristics can greatly impact the bioavailability. In simplest terms, to determine bioavailability one needs to know the blood/plasma systemic exposure ($AUC_{0-\infty}$) values following intravenous and extravascular (e.g. oral, intramuscular, subcutaneous, intraperitoneal) dosing at the same doses; however, bioavailability can also be determined at varying doses provided the drug pharmacokinetics is linear and follows first-order pharmacokinetics. The bioavailability following an oral exposure is determined as:

$$F\% = \frac{dose_{iv} \times AUC_{0-\infty,ev}}{dose_{ev} \times AUC_{0-\infty,iv}} \times 100$$

$dose_{iv}$ is the intravenous dose; $dose_{ev}$ the extravascular dose administration; $AUC_{0-\infty,iv}$ the area under the curve after the intravenous dose; and $AUC_{0-\infty,ev}$ the area under the concentration versus time curves for the extravascular dose.

Relative bioavailability is often necessary to evaluate the impact of different dose forms (e.g. particle size, solubility, dissolution, vehicle delivery, etc.) on the systemic bioavailability of a drug. To assess bioavailability, intravenous data is not essential, as one extravascular dose form can be compared against another extravascular dose form, where one of the dose forms may be used as the reference material.

# CLASSICAL PLASMA/TISSUE TOXICOKINETICS: APPLICATIONS IN DRUG DISCOVERY AND DRUG DEVELOPMENT

Prior to the testing of promising new therapeutics in healthy humans and/or sick human patients, non-clinical safety studies in laboratory animal species and/or appropriate *in vitro* models are conducted. Figure 2.2 shows a pharmacokinetics guided dosing strategy to improve therapeutic efficacy. Maintenance of plasma drug within a defined window is critical to the success of attaining the therapeutic efficacy. However, a large number of intrinsic and extrinsic factors, including metabolic polymorphism, drug–drug interaction, diseases, infections, diet, nutrition, and life-style factors could influence the therapeutically effective concentrations. Although it is not possible to evaluate many intrinsic and extrinsic factors in animal toxicology studies, it is believed that the use of safety factors/uncertainty factors (10 or greater) will allow the safe conduct of clinical studies.

Non-clinical toxicology studies when conducted properly in two species, typically in one rodent and one non-rodent, provide critical information on dose response in toxicity and its reversibility under a variety of exposure scenarios. The major objective of each toxicity study is to determine an NOAEL and adverse-effect level. Additionally, it is critical to know if toxicity is reversible or irreversible in nature and how long it will take to recover completely from toxicity. With the knowledge of quantitative species differences in ADME and susceptibility to toxicity, the risk assessment of preclinical safety data for human safety has been challenging. There has been a steady progress in integrating toxicity mechanisms, toxicology–pathology data, toxicokinetics, and ADME data into risk assessment. This has greatly helped to reduce the uncertainty regarding the extrapolation of animal toxicology data to estimate the probably of harm at relevant clinical exposures.

# APPLICATIONS OF ADME AND TOXICOKINETICS IN DRUG DISCOVERY AND DEVELOPMENT

## Early optimization of promising discovery candidates

Drug discovery process starts with a hypothesis, including the identification of biological target related to a given disease or illness, and strategies to block the specific target without ensuing toxic effects. A specific disease target may involve an up-regulated target enzyme or receptor, and its blockage may lead to effective treatments. Once a suitable target is characterized, molecules are screened to identify hits with desired biological activity. Prior to undertaking *in vivo* investigations, adequate *in vitro* and *in vivo* pharmacokinetics and metabolism characteristics are usually obtained.

## Toxicokinetics information and its utility in toxicity studies

Because of the limitations associated with the frequency of sampling and total blood withdrawn in a given time, toxicokinetic information from an integrated toxicity-toxicokinetic protocol is limited to an assessment of *AUC*, $C_{max}$, $T_{max}$, $C_{min}$, and the ratio of $C_{min}/C_{max}$. Figure 2.3 shows the potential utility of toxicokinetics in toxicity studies. Below is a scientific discussion on the basics of toxicokinetic information (Figure 2.4).

### *Extent of drug absorption: rate and extent of drug exposure*

The rate and extent of drug entering the blood stream following drug administration is extremely important. A drug may be very rapidly absorbed (rapid attainment of $C_{max}$ with $T_{max}$ of generally less than 2 h) at the low dose; however, solubility- or dissolution-limited drug absorption at

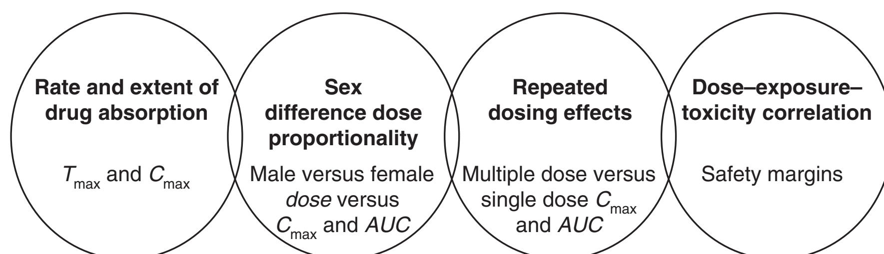


**FIGURE 2.3** *Applications of toxicokinetics in non-clinical toxicology studies.* Preclinical toxicokinetics data when generated along with toxicology studies may have multiple applications. The toxicokinetics data have many applications including the following: (1) rate and extent of drug absorption by knowing how fast a drug enters plasma and to what extent it achieves maximal concentrations; (2) sex differences in exposures help to interpret any potential sex differences in toxicity or differences in dose proportionality in exposures; and (3) repeated dosing effects on exposures by comparing single and multiple dose (typically at presumed at steady state) toxicokinetics (e.g. $C_{max}$ and *AUC*) (see text for additional discussion).

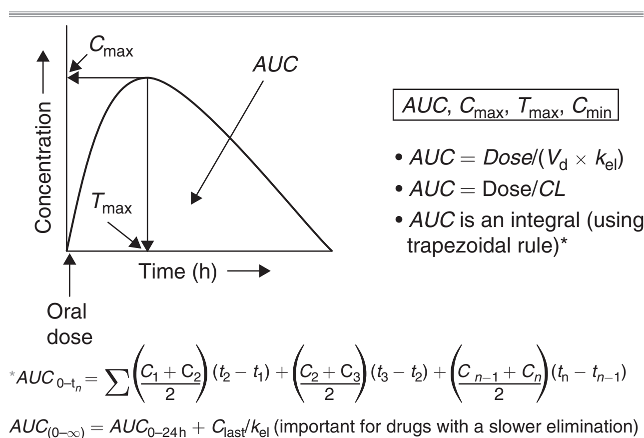


**FIGURE 2.4** *Basic toxicokinetics parameters*. In toxicokinetics studies, the assessment of toxicokinetics is often limited to the assessment of some very limited parameters, such as $C_{max}$, $T_{max}$, and *AUC*. This is due to limited blood sampling along with toxicity evaluation. The *AUC* is the most important pharmacokinetic parameter which can be estimated by statistical methods (trapezoidal rule) or by equations using $V_d$, $k_{el}$ (elimination constant), and drug clearance (*CL*). Because the correct estimate of $k_{el}$, $V_d$, and drug clearance is difficult to attain in oral toxicokinetics studies, it is a common practice to use trapezoidal rule to estimate the *AUC*.

the middle and high doses may lead to slower rise in concentration with longer $T_{max}$ and sustained concentrations. The rate of drug exposure has significant impact on overall safety profile of a drug. There are a large number of biological and drug derived factors that may impact the overall process of drug absorption (Curatolo, 1998). The total amount of drug circulating through blood relative to the administered dose in mg/kg is critical in interpreting the dose response in toxicity and establishing thresholds for adverse effects. Drugs that are poorly or incompletely absorbed due to solubility/dissolution-related problems may leave a large amount of unabsorbed drug in the GI tract. Chronic accumulation of large amounts of unabsorbed drug in the intestinal tract following chronic repeated oral dosing may result in adverse effects on GI homeostasis (e.g. nutrient absorption, GI emptying, changes in GI microflora, chronic GI irritation, and inflammation) and toxicity may arise secondary to these local adverse effects. The local untoward effects due to unabsorbed drug may not be relevant for humans because most drugs are administered at very-low-dosage strengths. In oral studies conducted at high doses for safety testing it is not possible to obtain information on percent bioavailability because of the lack of exposure data at relevant intravenous doses. The extent of absorption can be roughly estimated by comparing the ratio of $C_{max}$ to *AUC*; however, when the absorption is slow and sustained, it is difficult to know the extent of absorption. Drugs that have an effect on GI homeostasis, including pH, changes in gut microflora, gastric and intestinal secretions and enzymes, GI motility, and first-pass intestinal metabolism can substantially alter the rate and extent of drug absorption. Additionally, drug induced GI toxicity can also have a detrimental influence on the systemic availability of drug and its major metabolites. The time ($T_{max}$) to reach $C_{max}$ may be used to monitor certain adverse effects which may be peak concentration dependent and may include CNS adverse effects (e.g. tremors, convulsions), cardiovascular effects (e.g. ECG changes, blood pressure changes), and certain hormonal effects. For certain target organ toxicities that require a sustained presence of drug-related substances, it is critical to fully establish plasma concentration versus time profile and its relationship to target organ toxicity. This information is extremely important when oral dietary route of administration is chosen. Differences in treatment regimens, dosing schedules, or oral routes can produce differences in toxicities because of the mechanistic differences in patterns of drug exposures and adverse effects. Because of the advances in high throughout screening and tendency to generate metabolically stable molecules, newer drug molecules tend to be bulky and poorly soluble in water. Increasingly it is being recognized that these large lipophilic molecules are often poorly absorbed by animal species providing very limited drug exposure for toxicity testing.

Increases in solubility with lipophilic vehicles, including polyethylene glycols (PEG) 300/400, Imwitor, propylene glycol, sorbitol, Tween (polysorbate 80), acidified carboxymethyl cellulose, hydroxypropylcellulose/sucrose/sodium lauryl sulfate (SLS), cremophor, cyclodextrin, and span (sorbitan monoester), have been successfully obtained (Dressman, 1989; Crowley and Martini, 2001). Many of these lipophilic vehicles have been successfully used to enhance absorption for poorly soluble drugs. It is to be emphasized when attempting to enhance exposure maximum caution must be taken to assure that the selected vehicles and their dosing volumes will be well tolerated by preclinical animal species and the toxicity of the formulated drug will not be enhanced by the direct adverse effects of formulation other than what is expected from the increase in exposures.

The rate of drug elimination has an important effect on the extent of drug exposure. Because of the difficulty in knowing when absorption is complete and when elimination begins after oral dosing, it is often difficult to precisely calculate elimination half-life. Drug elimination can be semi-quantitatively estimated by examining the ratio of trough ($C_{min}$) and maximal concentration ($C_{max}$). Drugs with rapid elimination (e.g. short half-life, low $C_{min}/C_{max}$ ratio) generally tend to be less toxic; however, drugs with slower elimination (e.g. longer half-life, high $C_{min}/C_{max}$ ratio) can result in large accumulation of drug after repeated chronic dosing leading to unexpected toxicities. It is to be emphasized however, when a drug is largely cleared via metabolism and the

metabolite(s) is toxic, significant adverse effects can occur with a drug that has a short half-life.

### *Species-specific sex differences in metabolism leading to exposure differences*

It is not an uncommon finding in toxicology studies to have males and females responding to a drug differently. Therefore, it is usually of significant interest to find out if differences in toxicity are due to toxicokinetics differences or related to differences in susceptibility. Sex-related differences in drug metabolism are generally more common in laboratory rats than in other species, including humans, mice, dogs, and monkeys (Skett, 1988; DeBethizy and Hayes, 1994; Shapiro *et al.*, 1995). As early as 1937, Holck *et al.* demonstrated that female rats slept longer than male rats when given hexobarbital (Holck *et al.*, 1937). Subsequently it was proven that prolonged sleeping time was due to a slow metabolism of hexobarbital in female rats than in male rats. Generally, male rats tend to have more CYP per gram liver and greater rate of CYP-dependent metabolism than female rats which may markedly impact the metabolic clearance and overall exposure of the drug. Sex-related differences are developmentally regulated (Dressman, 1989) and appear more frequently in sexually mature rats. Generally, male rats have higher activities of certain important most abundant CYP enzymes than females; however, female rats have higher activities of certain specific CYP enzymes than males (Lin and Lu, 1997). For example, CYP2A2, CYP3A2, and CYP2C11 are male-dominant; however, CYP2A1, CYP2C7, and CYP2C12 are generally female-dominant (Holck *et al.*, 1937; Skett, 1988; Dressman, 1989; DeBethizy and Hayes, 1994; Shapiro *et al.*, 1995). Differential expression of sex-dependent CYPs leads to sex differences in drug exposure. The sexual dimorphic secretion pattern of growth hormone (constant low-level secretion in females versus pulsatile secretion in males) and sex hormones (e.g. testosterone and estrogen) directly regulates the expression of certain hepatic CYPs (CYP2C11 and CYP2C12) in male versus female rats (Kato and Yamazoe, 1990; Legraverend *et al.*, 1992a, b; Waxman *et al.*, 1985, 1990). It is also important to note that as male rats age, their metabolism declines to resemble young female rats (i.e. sexual dimorphism declines). This is due to changes in growth hormone patterns with older male rats exhibiting a sustained release of growth hormone (versus pustule pattern) similar to young female rats. When considering the fact that male rats predominately have higher activity of many CYP enzymes, it is not too surprising that male rats tend to have lower drug exposures than female rats and may show a reduced toxicity.

Other safety species show substantially less and infrequent sex-related differences in metabolism, including species like mice (Macleod *et al.*, 1987), ferrets (Ioannides *et al.*, 1977), and dogs (Dogterom and Rothuizen, 1993). Humans tend to show a relatively less frequent sex difference in drug metabolism. When sex differences in pharmacokinetics are observed in humans, these differences appear to be specifically related to anatomical and physiological differences (e.g. body weight, height, etc.) that may indirectly impact the ADME processes.

### *Dose proportionality in toxicokinetics*

A clear understanding of changes in systemic exposures with increasing doses is critical to interpreting the toxicity response. Given the species differences in rates and extent of ADME process, the comparison of systemic exposure versus doses administered is one of the most practical means of assessing margins of safety (exposure at NOAEL in animals/desired therapeutic exposure). In toxicity studies where doses may vary over several log orders of magnitude, the non-linearity in exposure with increasing doses is relatively a common finding. In interpreting dose proportionality, one also needs to carefully look at the variability across the mean or median values because preclinical animal species tend to vary a lot in ADME processes. These data are best compared when normalized for dose. For example, a dose comparison of AUC/dose will give the best assessment of dose proportionality.

Several possible scenarios may exist with regard to dose proportionality. In scenario A, the increase in exposure is proportional to increase in dose. This usually happens with drugs that follow a linear ADME process. A plot of dose versus AUC/dose will show no or little change with increasing doses. In case B, the increase in exposure is clearly greater than dose proportional and with increasing doses, AUC/dose will increase. This process generally occurs when the kinetics of the drug is dependent on one or multiple saturable processes. The saturable processes may include the following: (a) plasma and tissue protein binding; (b) metabolic enzymes, including saturation by substrate, depletion of cofactors, and product inhibition; (c) renal tubular secretion and tubular reabsorption; and (d) biliary excretion. All saturable processes typically follow Michaelis–Menton kinetics. With increasing concentrations the processes can get saturated (i.e. when concentration exceeds $K_m$ (plasma concentration needed to cause 50% saturation)), and the elimination follows a zero-order reaction ($dC/dt = V_{max}$). Under these circumstances, drug accumulation with high trough concentration may occur with increasing doses. When absorption is limited by dissolution and the saturable clearance occurs in parallel, a sustained plasma concentration versus time profile is often observed with increasing doses. In case C, with increasing doses there is a less than dose proportional increase in exposure. A plot of dose versus AUC/dose will decrease with increasing dose. With increasing doses, the rate and extent of absorption decreases and/or rate of elimination increases. The

absorption can decrease due to (1) poor solubility, (2) poor dissolution, (3) rapid transit time, and (4) saturation of active processes (i.e. influx transporters) (Schanker, 1960; Leahy and Lynch, 1989). In case D, the drug exposure remains similar with increasing doses showing a plateau in absorption. A plateau in exposure is usually observed with drugs that are poorly soluble in GI fluid and rate of dissolution is limited. To demonstrate toxicity at high doses, it is not an uncommon practice in toxicity studies to use doses between 1000 and 5000 mg/kg or 5% maximal in diet without a full understanding of dose proportionality in ADME processes. However, when the availability of drug to potential target sites is limited by poor absorption, the increasing amount of unabsorbed fraction of drug residing in GI tract could cause local GI toxicity (e.g. irritation, inflammation, interference with the absorption of nutrients and minerals) which may not be relevant for humans.

### *Duration of repeated dosing effects on toxicokinetics*

Duration of repeated-dosing-related effects on exposures/toxicokinetics is of considerable interest in toxicology. When toxicity develops during the progress or the end of subchronic or chronic dosing, it becomes important to know if toxicity is due to chronic repeated injury or due to changes in toxicokinetics leading to drug accumulation. Some toxic effects may decrease with repeated dosing due to induction of drug clearance or due to development of adaptive response to toxicity. Typically metabolic induction and a subsequent increase of clearance may occur with repeated dosing; however, in recent years, time-dependent effects on P-glycoprotein efflux transporters, renal tubular reabsorption/secretion, and biliary excretions have been identified as additional potential causes for changes in drug exposure with repeated dosing. Metabolic-induction-related decrease in exposure is normally accompanied by increase in metabolites levels formed due to the induction with the repeated dosing. In certain cases, autoinhibition of drug clearance may lead to drug accumulation that in turn may cause induction leading to a decrease in drug exposure. *Steady-state toxicokinetics* is an important concept in repeated dose toxicity studies. At steady state it can be expected that ADME processes are stable (rate of entry = rate of elimination) and there will be no further increase in exposures with duration of repeated dosing. For drugs following linear pharmacokinetics, 95% steady state can be expected to attained after 5 half-lives. For example, if the elimination half-life is 8 h, then the time to reach steady state will be $5 \times 8$ or 40 h. Therefore, if a drug is dosed every 8 h or three times daily, then steady state will be expected to be attained by 40 h or by fifth dose. Drugs with slower rate of elimination relative to dosing interval will accumulate with repeated dosing. When assuming linear kinetics and one compartment open model, the accumulation factor ($AF$) can be determined as follows:

$$AF = 1/1 - 2^{-e}$$

where $e$ is the dosing interval/half-life.

Therefore, if dosing interval and half-life are equal, a 2-fold accumulation is expected. If the values of $e$ (dosing interval/half-life) are 0.25, 5, 1, 2, 3, and 4, the $AF$ will be 6.3, 3.4, 2.0, 1.33, 1.14, and 1.07, respectively. In simplest terms, the extent of accumulation can be described as follows:

$$\begin{aligned} \text{Accumulation} &= 1/q^{\text{ue}} \times T(\text{doping } \textit{intervalle}); \\ q^{\text{ue}} &= \text{elimination constant} \\ &= 1/[0.693 \times t_{1/2}] \times T \\ \text{or } &= \frac{1.44 \times t_{1/2}}{T} \end{aligned}$$

### *Interanimal variability/genetic polymorphism in drug metabolism in preclinical species*

It is rare to find that all animals in a particular dose group will show a similar toxic response or lack of it when exposed to a test agent. The variability in toxic response has been observed in both inbred and outbred laboratory animals. When high variability in toxic response is observed, toxicokinetics data can help to assess if the variability in response is due to differences in metabolism/toxicokinetics properties or due to individual-based variability in toxicodynamic response. Genetic polymorphism in metabolism is not well characterized in preclinical species, although genetic polymorphism may be similar to humans, which include polymorphic enzymes and other targets. Given the small number of animals per group in toxicology studies, it is often difficult to assess polymorphism in metabolism or toxicodynamics; however, preclinical species can show significant metabolic polymorphism which may impact the assessment of systemic exposure and the interpretation of drug toxicity data. In the case of a celecoxib (anti-inflammatory agent), a polymorphism in the drug metabolism of beagle dogs was demonstrated (Paulson *et al.*, 1999). There were at least two distinct populations of dogs that cleared celecoxib from plasma at either a poor rate or fast rate. The poor metabolizers were designated as PM and extensive metabolizers were designated as EM. In a population of 242 beagle dogs and irrespective of sex, about 45.0% and 53.3% of the dogs were identified as the EM and PM phenotypes, respectively. The EM and PM phenotypes were attributed to be differences in the rate of metabolism of celecoxib by liver CYPs where a high correlation ($r > 0.90$) between *in vivo* clearance and *in vitro* hepatic metabolism rate of celecoxib was determined. In EM dogs, the mean elimination half-life and

clearance of celecoxib were 1.72 and 18.2 ml/min/kg, respectively. When compared to the EM dogs, the PM dogs had a longer half-life (5.18 h) and a slower clearance (7.15 ml/min/kg). Further studies identified CYP2D15 as an important CYP involved in the metabolic clearance of celecoxib. Interestingly four alleles of CYP2D15 designated as CYP2D15*2, CYP2D15*3, CYPD15WT2, and CYPD15 delta in beagle dogs have been identified by Roussel *et al.* (1998). Unlike dogs, CYP2C9 was identified as the major human CYP involved in the metabolism of celecoxib.

When a large interanimal variability in toxicokinetics and metabolism is observed in animals, individual values are relatively more important than mean values because they can be useful in the interpretation of the individual animal toxicity data. In some cases, appropriate statistical tests may be necessary to interpret dose proportionality, sex difference in toxicokinetics, and duration-dependent changes in toxicokinetics.

***Assessment of safety margins with toxicokinetics data***

Doses used in preclinical toxicity studies often utilize log orders of magnitude in excess doses in mg/kg than used in the clinical studies. Traditional approaches for estimation of safety margins have been based on a comparison of mg/kg doses or doses adjusted for species-specific surface area factor:

$$\text{Safety margin} = \frac{\text{No-observed-adverse-effect dose level in animals in mg/kg or mg/m}^{2*}}{\text{Maximal clinical efficacious dose level in humans in mg/kg or mg/m}^{2*}}$$

$$^{*}\text{mg/m}^2 = \text{dose in mg/kg} \times \text{species-specific body surface area factor.}$$

Although the above approach can often provide impressive safety margins, this approach fails to recognize the critical importance of quantitative species differences in ADME processes which are fundamental to the extrapolation of animal toxicology data for humans. ADME processes, especially metabolism, generally do not correlate well with the body weight or body surface area (Voisin *et al.*, 1990). Therefore, safety margins based on administered doses (mg/kg or mg/m$^2$) for drugs that are metabolized are likely to overestimate safety margins. Within the scope of this framework, it is assumed that equal plasma drug exposure will result in equal drug toxicity across species, including humans. This assumption, however, has some important limitations which are as follows: (1) species differences in susceptibility to adverse effects, (2) tissue retention of the drug poorly predicted by plasma concentrations, and (3) differences in elimination pathways. In some cases, the concentration of drug in urine may be more important than in plasma. This is best illustrated by the example of bumetanide, a potent diuretic. While dog and rat showed similar plasma concentrations, the diuretic effects were greater in dogs than in rats. This was due to a higher urinary excretion of unchanged drug in dogs than in rats which were likely to be in a better equilibrium at the site of action than plasma levels (Yu, 1999).

Despite the limitations of the classical toxicokinetics, plasma AUC is a powerful means to extrapolate toxicity from animal species to humans. Generally, plasma AUC is a good surrogate for all ADME processes and safety margins based on the ratio of animal AUC to the human AUC is a key endpoint in predicting the human safety of drug:

$$\text{Safety margin} = \frac{\text{Mean AUC at no-observed-adverse-effect dose level in animals}}{\text{Mean AUC at the maximal clinical efficacious dose level in humans}}$$

It is to be emphasized that for repeated dosing, the AUC should be measured at steady state within one interval of time and with same routes of administration. If routes of administration differ between animals and humans, it is critical to correct AUC for the differences in bioavailability. The AUC data should be assessed at a range of doses and the exposures should not be extrapolated from one dose to another without a full understanding of linearity in kinetic processes. A greater than dose proportional increase in exposure due to saturable metabolism can lead to gross underestimation of toxicity. Species differences in GI first-pass metabolism can lead to poor understanding of the risk of GI toxicity. For this assessment, it is important to consider the exposure to the total drug, including exposure to absorbed versus unabsorbed drug and potential precipitation in GI fluids as a cause for local effects.

Another important factor in interpretation of safety margins based on AUC margins is the impact of plasma protein binding. This is based on the fact that protein bound active substance is unlikely to cross cell membrane and the exposure based on total drug may not be a true representation of the drug at the active target site. Protein binding is a dynamic process and protein drug complexes often involving hydrophobic or hydrophilic interactions are often dissociable. Until a steady state is attained protein–drug complexes are subject to a dynamic process of dissociation and association dependent on total drug and unbound concentrations in tissues. To fully appreciate the value of protein binding it will be important to know the kinetics of association and dissociation of protein–drug complexes. Importance of plasma protein binding in the assessment of safety margins has been controversial since there is no good experimental way to determine the extent of protein binding "*in vivo*" as well as the kinetics of

protein binding and dissociation. Nevertheless, a correction for the protein binding may be necessary when protein binding is very high (e.g. >99%) and a clear species difference in protein binding exists. The AUC of free drug (unbound) can be calculated as follows:

$$AUC_f = AUC_t \times f_u$$

where $AUC_t$ is the total drug $AUC$ and $f_u$ the fraction unabsorbed.

$$\text{Safety margin} = \frac{\text{Mean } AUC_f \text{ at no-observed-adverse-effect dose level in animals}}{\text{Mean } AUC_f \text{ at clinical efficacious dose level in humans}}$$

In some cases of species differences in protein binding, safety margins based on unbound drug can be improved. Safety margins can be greatly improved by taking into consideration differences in protein binding across species. In contrast if protein binding saturates at low drug concentrations, higher doses can result in unexpected toxicity due to increase in free fraction of the drug. With 6,8,-diethyl-5-hydroxy-4-oxo-4*H*-1-benzopyran-2-carboxylic acid a saturable protein-binding-related increase in free drug systemic exposure occurred at doses exceeding saturable absorption and saturable protein-binding leading to unexpected hepatic toxicity (Lin and Yu, 1998). This stresses that an understanding of protein binding, especially at high plasma drug levels, is critical to interpretation of toxicity across species.

### *Application of toxicokinetics in species selection and dose selection for toxicity and carcinogenicity studies*

Good bioavailability and adequate exposure to parent drug and its major metabolites in preclinical species is an important goal of any well designed toxicology study. This helps to assure that toxicity was evaluated at multiples of human exposures in animals and adequate safety margins exist for potential adverse effects in humans. Preclinical animal species that have an adequate plasma half-life and/or clearance for a given drug are likely to show adequate bioavailability and adequate systemic exposures. Based on experience, drugs that are well absorbed and have a moderately sufficient half-life (e.g. 5–10 h) will likely provide an adequate systemic exposure. Because of the saturation of metabolic clearance and protein binding or in absorption, systemic drug bioavailability can change at high doses. Therefore, it is important to have the toxicokinetic exposure data at toxicologically relevant doses in one rodent and one non-rodent species prior to conduct of preclinical toxicology studies.

## SPECIES SELECTION FOR TERATOGENICITY

Evaluations should consider the adequacy of fetal exposure to the test drug and its major metabolites. The human safety evaluation of development and reproductive toxicity (DART) relies exclusively on preclinical animal developmental and toxicity data. One approach is to compare fetal and maternal plasma drug levels at specific stages of gestational development or at the end of gestation in pregnant animals. In practical terms, if fetal levels are at least 5–10% of the maternal levels, the selected species may be adequate for teratology testing. Similar to general toxicity studies, the information about similarity in metabolism between selected DART animal species and humans is necessary to justify the choice of species. Additionally, the adequacy of good systemic exposures relative to potential human exposures is necessary to justify the choice of species, study design, and dosing schedules for DART studies. Most importantly, the species selection for DART studies must consider the relevance of the selected species for identifying reproductive and developmental toxicities for humans. Generally, rats are preferred for fertility studies because of the practicality and a large body of information is available as a reference for reproductive toxicants in rats. Rats and rabbits are preferred species for teratology testing because of the existence of a large amount of background data and the suitability of these species for laboratory teratology studies. Monkeys and dogs have also been used for teratology testing; however, their use for teratology testing should be justified on the basis of specific objectives that cannot be met by standard teratology studies in rats and rabbits. From the regulatory perspective, one rodent and one non-rodent species are required for DART evaluation; however, in some cases a single specie may be sufficient if it can be shown that the selected species have relevance for humans on the basis of pharmacology, toxicokinetics/metabolism, and the biological characteristics.

## DOSE SELECTION FOR PRECLINICAL EARLY DOSE RANGE-FINDING TOXICITY STUDIES

Typically exploratory range-finding studies are conducted to assist in dose selection for the Food and Drug Administration (FDA) mandated Good Laboratory Practice (GLP) toxicity studies needed to support phase I clinical trials. For a large number of drugs, dose selection is difficult for the main toxicity studies, therefore, the dose range-finding studies are of a great value. These early studies

can be from a minimum of 5 to 15 days in duration and depending on the availability of drug typically three to five doses may be used. Selected doses may range from low doses to high doses ranging up to the limit dose of 2000 mg/kg/day. Typically, the low dose should be selected to provide a 2- to 5-fold multiples of the projected clinical efficacious exposure and the high dose should be high enough to produce some toxicity or at the limit dose of 2000 mg/kg/day. Early pharmacokinetic/toxicokinetic data can be very helpful in selecting dose levels, especially the low and middle doses. To conserve the drug, these studies can be conducted in a very small number of animals (5/group for rats) of one sex provided there are no sex-related differences in metabolism. In non-rodents, an ascending 5-day dosing may be conducted at 1 × (e.g. low dose corresponding to a 2- to 5-fold multiples of expected clinically efficacious exposures adjusted for differences in *in vitro* pharmacodynamic activity), 2.5×, 5×, 10×, 20×, etc. or until dose-limiting adverse effects are observed. Toxicity end points may involve histopathology of only major organs of toxicity (typically liver, kidneys, heart, pancreas, lung, brain, GI tissues, sex organs, limited primary and secondary immune organs), limited clinical pathology, and toxicokinetics investigations.

## DOSE SELECTION FOR REPEATED DOSE TOXICITY STUDIES

### High dose

Prior to exposing humans to a new drug entity, especially belonging to a new pharmacological mechanism and/or structure, it is critical to know what adverse effects may occur and if they are reversible. In principle, the high dose should be selected principally based on toxicological considerations. Ideally, the high dose should produce frank toxicity, identify target organ toxicity, and establish a positive correlation between clinical-pathology-based biomarkers and target organ toxicity. Identification of toxicity helps to formulate a better safety monitoring program in phase I–II clinical trials. This also provides assurance that if humans (healthy human volunteers and targeted sick patients) are more sensitive than animals to certain toxicities, appropriate safety monitoring is included in the clinical plans.

Given the dissimilarity in quantitative metabolism, pharmacological mechanisms, and sensitivity to certain toxicity, it is expected that preclinical safety species may not respond to certain pharmacological mechanisms. When toxic effects are not observed with increasing doses, toxicokinetics can be very useful in setting the high dose. If a drug is not getting absorbed with increasing doses, then the lowest dose that provides maximal exposure to drug and its metabolites can be considered as the high dose.

Saturable absorption- or dissolution-limited drug absorption can be demonstrated by establishing that systemic exposure to parent drug and major metabolites has plateaued over the range of doses. In practice, a plateau may be considered when the plasma AUC for the drug and each of its major metabolites does not increase by more than 20% with a 2-fold increase in dose. When there is a large interanimal variability in exposures with increasing insoluble doses, appropriate statistical tests may be necessary to establish a plateau in exposures. If saturable absorption cannot be demonstrated, then the high dose may be based on *maximal feasible dose* or the *limit dose* (generally not to exceed 2000 mg/kg/day).

### Middle dose

In designing the toxicity study, it is also important to fully understand the dose response in toxicity, including the threshold dose for inducing the toxicity. The middle dose selection should consider multiples of projected human maximally efficacious dose, threshold in toxicity response, pharmacodynamic response, and mechanisms of toxicity.

### Low dose

Given the importance of NOAEL in toxicity studies, the low dose should be selected to provide a NOAEL. Ideally, the low dose should be set to provide 2- to 5-fold safety margin over the estimated/projected maximal clinical efficacious dose/exposure.

## CARCINOGENICITY BIOASSAY STUDIES IN RODENTS

The conduct of the 2-year carcinogenicity bioassays remains a major challenge. The US FDA has placed a high importance to the process of dose election and protocol approvals for the carcinogenicity studies. The FDA document has provided details of the information that is needed to seek approval of dose selection and protocol approval prior to initiating carcinogenicity studies (McClain and Dammers, 1981) (Pharmacology/Toxicology, Procedural, Carcinogenicity Study Protocol Submissions). Additionally, the International Conference on Harmonization (ICH) has provided guidance on dose selection for the carcinogenicity studies.

### Use of systemic exposure margins in selecting the high dose for carcinogenicity bioassays

For drugs that are non-genotoxic and well tolerated, a maximum tolerated dose (MTD) may not be attained until excessive unrealistic doses are used. To alleviate the problem of selecting excessive doses, US FDA scientists analyzed carcinogenicity databases available from the US FDA, National Toxicology Program, International Agency for Research on Cancer (IARC), and scientists. Based on the evaluation of carcinogenicity studies of 35 compounds belonging to various pharmacological mechanisms, it was concluded that in the absence of an MTD, an exposure margin of at least 25-fold between systemic exposure in rodents and humans at the maximally recommended clinically efficacious dose will allow an adequate margin of safety. The ratio of 25 will likely allow the detection of a positive neoplastic response in the vast majority of 2-year bioassays for all IARC Class I definitive human carcinogen and 2A possible human carcinogens.

High-dose selection based on the 25-fold AUC margin should consider the following criteria: (1) the drug should have low degree of toxicity; (2) the drug must not be non-genotoxic; (3) the drug must be metabolized similarly in both rodents and humans; (4) if the protein binding is greater than 80%, the AUC should be corrected for protein binding in both humans and in animals; (5) exposure margins should be justified on the basis of the parent drug, parent drug plus major metabolite(s) or solely on metabolites; and (6) exposure data in humans must be attained at the maximum recommended human daily dose.

In practice, maximum recommended human dose (MRHD) is often not established at the time of selecting dose levels for the carcinogenicity studies. Under the conditions of the uncertainty in prediction of MRHD, it is advisable to select an uncertainty factor of 2–5 when using the 25-fold margin. If extrapolations to human suggest that the bioavailability will be good and drug will be metabolically stable, it will be practical to consider an AUC margin of 50- to 125-fold (2- to 5-fold greater than the targeted 25-fold) over the projected exposures at the MRHD. The factor of 2–5 may depend on the following factors: (1) medical need for the targeted therapy, (2) chronic exposure versus intermittent exposure, (3) chronic safety of drug in preclinical species, (4) previous experience with the class of drugs, (5) structure–activity relationship, and (6) inter-human variability in drug exposures.

When the systemic availability of the test drug does not increase with increasing doses, there will be very little benefit in testing the drug at high doses. There are multiples factors that can limit the exposure to drug-related substances, including limited solubility and poor dissolution rate in GI fluids, a very high first-pass metabolism, and saturation of influx transporters. In the vast majority of cases, the solubility and rate of dissolution are major limiting factors in drug absorption. In principle, the drugs that are poorly absorbed will likely attain a plateau in exposures and will offer no benefits to the use of high doses in carcinogenicity studies. The plateau in exposure to the parent drug must be demonstrated over the range of doses to assure that there is no substantial benefit (e.g. 20% or less increase in exposure with increasing doses) in testing the carcinogenicity of high doses. It is also important to establish that the limitations to the increase in exposure with increasing doses are not due to increased metabolic clearance. This can be demonstrated by showing a plateau in exposure to major metabolites with increasing doses.

## CONCLUSIONS

Classical toxicokinetics and drug metabolism studies when conducted to support non-clinical safety assessment studies have greatly reduced the uncertainty in interpretation of preclinical toxicity data and its utility in human health risk assessment. The classical non-compartmental toxicokinetics and drug-metabolism-based data have been particularly useful in selecting the dose levels for short- to long-term toxicity and carcinogenicity studies. Furthermore, these assessments have allowed great improvements in selection of relevant species, formulation, and doses for safety studies. The greatest utility of preclinical toxicokinetics data has been in the interspecies comparison of product toxicity. It is now widely accepted that toxic effects can be better extrapolated from animals to humans when these comparisons are based on toxicokinetics and disposition (absorption, distribution, metabolism, and excretion) data in preclinical species and humans. In this context, safety margin that is based on ratio of animal AUC at NOAEL to human AUC at an efficacious dose is the key predictor of human toxicity risk. It is generally accepted that when the AUC ratio to support safety margin is large, the expected risk of toxicity in humans is low. Although model independent or compartment-based plasma/blood toxicokinetics have served well as a practical means of assessing systemic exposure, they provide no information on the time course of exposure of target organs to drug or metabolites. Overall, toxicokinetics have greatly enhanced our understanding of interspecies differences in toxicity and significance of safety margins.

## REFERENCES

Beasley V (1999) Absorption, distribution, metabolism and elimination. In *Veterinary Toxicology*, Beasley V (ed.). International Veterinary Information Service (www.ivis.org), Ithaca, New York.

Clewell III HJ, Andersen ME (1985) Risk assessment extrapolations and physiological modeling. *Toxicol Ind Health* **1**: 111.

Crowley PJ, Martini LG (2001) Enhancing oral absorption in animals. *Curr Opin Drug Disc Develop* **4**: 73.

Curatolo W (1998) Physical chemical properties of oral drug candidates in the discovery and exploratory development setting. *Pharm Sci Technol Today* **1**: 387.

DeBethizy JD, Hayes JR (1994) Metabolism: a determinant of toxicity. In *Principles and Methods of Toxicology*, Hayes AW (ed.). Raven Press, New York, pp. 59–100.

DeBethizy JD, Hayes JR (2001) Metabolism: a determinant of toxicity. In *Principles and Methods of Toxicology*, 4th edn, Hayes AW (ed.). Taylor and Francis Press, Philadelphia.

Dethloff LA (1993) Route of administration and toxicity. In *Drug Toxicokinetics*, Welling PG, De La Igelsia FA (eds). Marcel Dekker, Inc., New York.

Dixit R, *et al.* (2003) Toxicokinetics and physiologically based toxicokinetics in toxicology and risk assessment. *J Toxicol Environ Health B Crit Rev* **6**: 1.

Dogterom P, Rothuizen J (1993) A species comparison of tolbutamide metabolism in precision-cut liver slices from rats and dogs. Qualitative and quantitative sex differences. *Drug Metab Dispos* **21**: 705.

Dressman JB (1989) Kinetics of drug absorption from the gut. In *Drug Delivery to Gastrointestinal Tract*, Hardy JG, Davis SS, Wilson, CD (eds). Ellis Harwood, Chichester, p. 195.

FDA, *http://www.fda.gov/cder/guidance/index.htm.* (2005).

Gibaldi M, Perrier D (1982) *Pharmacokinetics*, 3rd edn. Marcel Dekker, Inc., New York.

Hirom PC, Milburn P, Smith RL, Williams RT (1972) Species variations in the threshold molecular weight factor for the biliary excretion of organic anions. *Biochem J* **129**: 1071–7.

Hirom RA, Birch CG (1976) Bile and urine as complimentary pathways for the excretion of foreign organic compounds. *Xenobiotica* **6**: 55–64.

Holck HGO, *et al.* (1937) Studies upon the sex difference in rats in tolerance to certain barbiturates and to nicotine. *J Pharmacol Exp Ther* **60**: 323.

Ioannides C, *et al.* (1977) Drug metabolism in the ferret: effects of age, sex and strain. *Gen Pharmacol* **8**: 243.

Kato R, Yamazoe Y (1990) Sex-dependent regulation of cytochrome P-450 expression. In *Principles, Mechanisms, and Biological Consequences of Induction*, Ruckpaul K, Rein H (eds). Taylor and Francis, New York, p. 82.

Leahy DE, Lynch J (1989) Mechanisms of absorption of small molecules. In *Novel Drug Delivery*, Prescott LF, Nimmo WS (eds). John Wiley & Sons, New York, p. 33.

Legraverend C, *et al.* (1992a) Hepatic steroid hydroxylating enzymes are controlled by the sexually dimorphic pattern of growth hormone secretion in normal and dwarf rats. *FASEB J* **6**: 711.

Legraverend C, *et al.* (1992b) Transcriptional regulation of rat P-450 2C gene subfamily members by the sexually dimorphic pattern of growth hormone secretion. *Mol Endocrinol* **6**: 259.

Lin JH, Lu AY (1997) Role of pharmacokinetics and metabolism in drug discovery and development. *Pharmacol Rev* **49**: 403.

Lin JH, Lu AY (1998) Inhibition and induction of cytochrome P450 and the clinical implications. *Clin Pharmacokinet* **35**: 361.

Macleod JN, Sorensen MP, Shapiro BH (1987) Strain independent elevation of hepatic mono-oxygenase enzymes in female mice. *Xenobiotica* **17**: 1095.

McClain RM, Dammers KD (1981) Toxicologic evaluation of bumetanide, potent diuretic agent. *J Clin Pharmacol* **21**: 543.

Medinsky MA, Valentine JL (2001) Toxicokinetics. In *Casarett and Doull's Toxicology: The Basic Science of Poisons*, Klaassen CD (ed.). McGraw-Hill, New York.

Paulson SK, *et al.* (1999). Evidence for polymorphism in the canine metabolism of the cyclooxygenase 2 inhibitor, celecoxib. *Drug Metab Dispos* **27**: 1133.

Roussel F, *et al.* (1998) Expression and characterization of canine cytochrome P450 2D15. *Arch Biochem Biophys* **357**: 27.

Rowland M, Tozer TN (1995) *Clinical Pharmacokinetics*, 3rd edn. Lippincott Williams & Wilkins, Philadelphia.

Schanker LS (1960) On the mechanism of absorption of drugs from the gastrointestinal tract. *J Med Pharm Chem* **2**: 343.

Shapiro BH, Agrawal AK, Pampori NA (1995) Gender differences in drug metabolism regulated by growth hormone. *Int J Biochem Cell Biol* **27**: 9.

Shargel L, Yu A (1993) *Applied Biopharmaceutics and Pharmacokinetics*, 3rd edn. Appleton & Lange, Norwalk.

Skett P (1988) Biochemical basis of sex differences in drug metabolism. *Pharmacol Ther* **38**: 269.

Voisin EM, *et al.* (1990) Extrapolation of animal toxicity to humans: interspecies comparisons in drug development. *Regul Toxicol Pharmacol* **12**: 107.

Waxman DJ (1992) Regulation of liver-specific steroid metabolizing cytochromes P-450: cholesterol 7-hydroxylase, bile acid 6-hydroxylase and growth hormone-responsive steroid hormone hydroxylase. *J Steroid Biochem Mol Biol* **43**: 1055.

Waxman DJ, Dannan GA, Guengerich FP (1985) Regulation of rat hepatic cytochrome P-450: age-dependent expression, hormonal imprinting, and xenobiotic inducibility of sex-specific isoenzymes. *Biochemistry* **24**: 4409.

Waxman DJ, *et al.* (1990) Pituitary regulation of the male-specific steroid 6 beta-hydroxylase P-450 2a (gene product IIIA2) in adult rat liver. Suppressive influence of growth hormone and thyroxine acting at a pretranslational level. *Mol Endocrinol* **4**: 447.

Yu DK (1999) The contribution of P-glycoprotein to pharmacokinetic drug–drug interactions. *J Clin Pharmacol* **39**: 1203.

CHAPTER

# 3

# Physiologically based pharmacokinetic modeling

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## INTRODUCTION

Physiologically based pharmacokinetic (PBPK) models are mathematical simulations of physiological processes that determine the rate and extent of xenobiotic chemical absorption, distribution, metabolism and excretion. Such models can be used for predicting internal doses at target organs and tissues due to their conformity to actual organs, tissues and physiological processes. Internal dose predictions are useful for dose–response analyses and risk assessment involving specific mechanisms and sites of toxicity. Successes in the application of PBPK models to predict xenobiotic concentrations at target sites have led to its acceptance as a modeling technique in risk assessments. It is also used in mechanistic studies of the underlying processes that determine pharmacokinetic profiles and dose-response relationships.

In veterinary toxicology PBPK methods can be used to improve the accuracy of predictions of toxicity across species by applying data obtained in one species to predictions in another species. It can be used to predict the effects of changes in physiological conditions, environmental conditions, activity levels and pathological changes on xenobiotic concentrations in target tissues. This allows for more accurate assessment of risk in varied individual animals and populations. PBPK approaches can be used to study and understand the effects of mechanisms that determine the internal exposure of animals to potential toxins – such as dermal absorption and xenobiotic metabolism. PBPK models can also be used to address problems associated with the exposure of food producing animals to drugs and chemicals that may result in potentially harmful or undesirable residues in meat, milk and other foods of animal origin (Brocklebank *et al.*, 1997; Riviere, 1999; Craigmill, 2003; Buur *et al.*, 2005, 2006; Van der Merwe *et al.*, 2006).

## BACKGROUND

Adverse effects due to exposure to potentially toxic xenobiotics are dependent on the rate and extent of absorption of the chemical into the body, its distribution to susceptible tissues and the specific interactions with biological targets. Predicting tissue concentrations over time is essential for the prediction of adverse effects (Andersen *et al.*, 2006).

Pharmacokinetic models describe the concentration/time profiles of chemicals in the body. Models are predictive tools that can be used to analyze risk due to xenobiotic chemical exposure. Models can also be used as an aid to understanding the mechanisms of chemical absorption, transport and elimination. Various types of pharmacokinetic models have been developed and the appropriateness of a specific model type depends on the available data and the model's purpose (Riviere, 1999). One of the key challenges to the successful use of PBPK models is identifying the appropriate conditions for its application.

The various membranes that form the body's interface with the environment and separates internal organs and tissues have to be permeable to a wide variety of chemicals so that nutrients can be absorbed and waste products eliminated. Depending on physical–chemical characteristics, concentration, solvent systems and environmental conditions; exposure to xenobiotic chemicals through dermal contact, ingestion and inhalation may lead to potentially harmful absorption of chemicals through permeable

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membranes. Living organisms are adapted to xenobiotic exposure through the development of transport and elimination mechanisms that prevent the build-up of harmful chemical concentrations in organs and tissues. Pharmacokinetic parameters, such as duration of internal exposure and tissue concentrations are useful for estimating risk values when the body's transport and elimination mechanisms are overwhelmed or inadequate to prevent the build-up of harmful chemical concentrations.

Traditional compartmental pharmacokinetic models describe the aggregate result of all the processes involved in determining the concentration/time curve of a compound in a reference compartment – most often the venous blood (also called the central compartment). It uses single or multiple compartments and first-order rate equations chosen to optimally describe experimental data, with no physiological relevance or implied fidelity to anatomical structure or physiology. The purpose of these models is to predict plasma concentrations after similar exposure conditions. PBPK models also make use of compartments, but the compartments are derived from mathematical descriptions of actual body compartments or tissues. Links between the compartments simulate physiological processes of partitioning, transfer, metabolism and excretion (Krishnan and Andersen, 2001). The concentration/time curves of chemicals in specific organs and tissues can be estimated. This offers an advantage over traditional compartmental models, because indirect estimations of concentrations in target organs/tissues based on administered dose, exposure conditions or concentration/time curves in a reference compartment may not be directly related to target organ/tissue concentrations and biological effects. Since PBPK model parameters are derived from actual physiological systems, differences in physiology, anatomy, environment, metabolism and the effects of chemical-induced changes and pathology can be simulated. Parameters can also be scaled to reflect different dose ranges, species, breeds, genetic polymorphism and life-stages.

The advantages of PBPK models are, however, often difficult to realize because the necessary anatomical and physiological parameters are not available and/or the relevant pharmacokinetic processes are not well understood. Ideal, complete PBPK models are not possible because complete knowledge of the modeled system is not attainable. Most PBPK models are, therefore, simplified representations of reality, based on assumptions regarding the most important processes and structures that determine the pharmacokinetic profile of the chemical in question. Typical PBPK models represent the body as a series of well-stirred compartments representing major organs and tissues of interest, a single dose and route of exposure and the major route of excretion. The compartments are linked by blood flow and the movement of chemicals between compartments is determined by tissue/blood partitioning and blood flow rates (Krishnan and Andersen, 2001). However, highly detailed, multiple compartment models can be constructed that include complex dosing regimens or exposure scenarios, detailed organ structures and physiological processes, as well as specific processes of metabolism and simulations of metabolite pharmacokinetics. Models of varying complexity focusing on specific organs and routes of absorption/elimination – such as the skin and respiratory organs have also been developed (McCarley and Bunge, 2001; Andersen *et al.*, 2002; Frederick *et al.*, 2002).

## MODEL CONSTRUCTION

The first step in the construction of a PBPK model is determining the purpose of the model and what internal tissue doses are needed to answer the specific scientific questions being asked. Once that is done, a schematic diagram is constructed that consists of each of the tissue compartments of interest, a plasma compartment, and a compartment or compartments that represent the rest of the system in question. It is often necessary to include more than one compartment to represent the remaining portions of the body to reflect the differences in high and low blood flow tissues. Tissue blocks can be further subdivided into vascular, extracellular and intracellular spaces (Colburn, 1988). Tissue blocks and their subcompartments can be combined as necessary to make the model as simple or as complex as needed. A schematic diagram for a generic whole body model is shown in Figure 3.1. Figure 3.2 depicts a generic tissue block with subcompartments as well as possible binding sites and sources of elimination. In contrast to the full body model, Figure 3.3 presents a schematic diagram used in the prediction of tissue residues for sulfamethazine (SMZ) in swine (Buur *et al.*, 2005). This model has several simplifications including the reduction of number of tissue blocks and the simplification of the tissue blocks into a single compartment. Since this model was designed for the prediction of SMZ drug residues in edible swine tissues, only the edible tissues of muscle, adipose, kidney and liver were incorporated into the model. The rest of the body was lumped into a single tissue block labeled carcass. Elimination is by renal filtration and is schematically diagramed using arrows that are not directed into the plasma compartment. The main metabolite is also modeled and the two xenobiotics are linked together via the liver tissue block. For the purposes of this model, the only tissue of interest for the metabolite is the liver, which is the site of the metabolic pathways. Thus the rest of the body is lumped into a single body component. This is a good example of how complexity can be added or removed from a model in order to accomplish specific scientific aims.

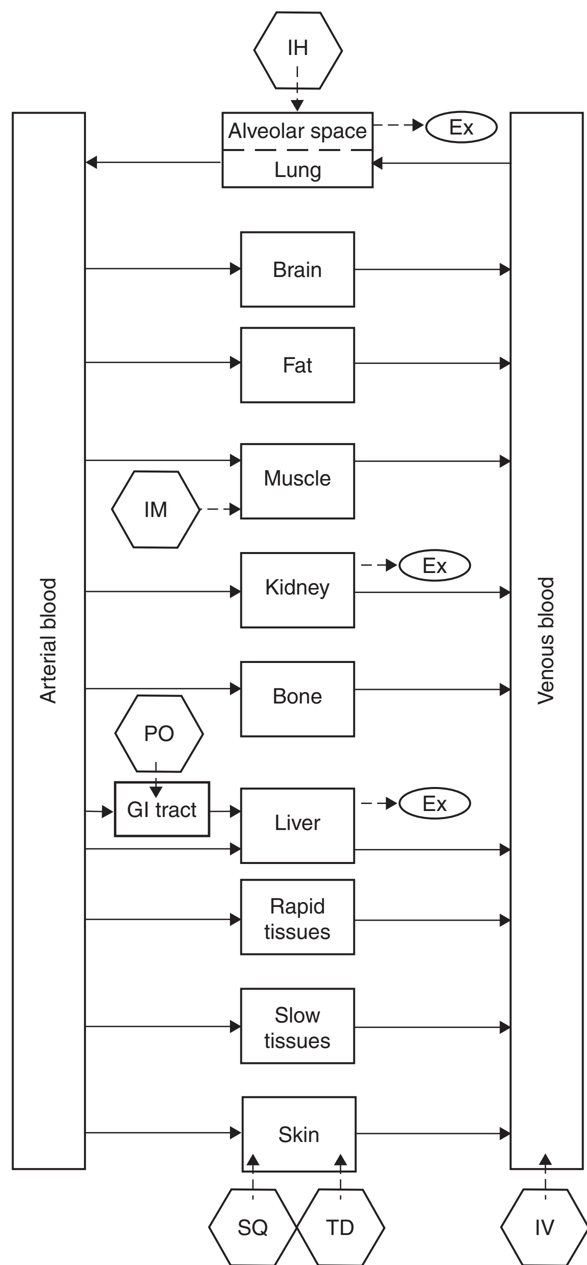


**FIGURE 3.1** Full body model for a generic mammal that includes standard dosing routes of intravenous (IV), intramuscular (IM), subcutaneous (SQ), transdermal (TD), inhalational (IH) and oral (PO). Ex represents excretion and removal of the xenobiotic from the body system. Solid line arrows represent blood flow. Dashed line arrows represent excretion processes. Dash-dot line arrows represent absorption processes.

After a schematic representation is achieved, the next step is to write mass balance equations that represent the changes in xenobiotic concentration in the tissues over time. In order to do this it must be decided if the rate-limiting step for xenobiotic partitioning is due to blood flow (flow-limited model) or diffusion through the cellular membrane (diffusion- or membrane-limited model). Flow-limited models are often for xenobiotics that are small and/

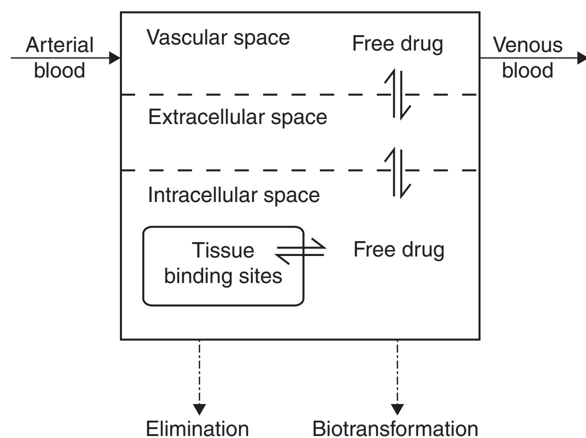


**FIGURE 3.2** Schematic diagram of a generic tissue block. Solid line arrows represent blood flow. Dashed line arrows represent excretion processes.

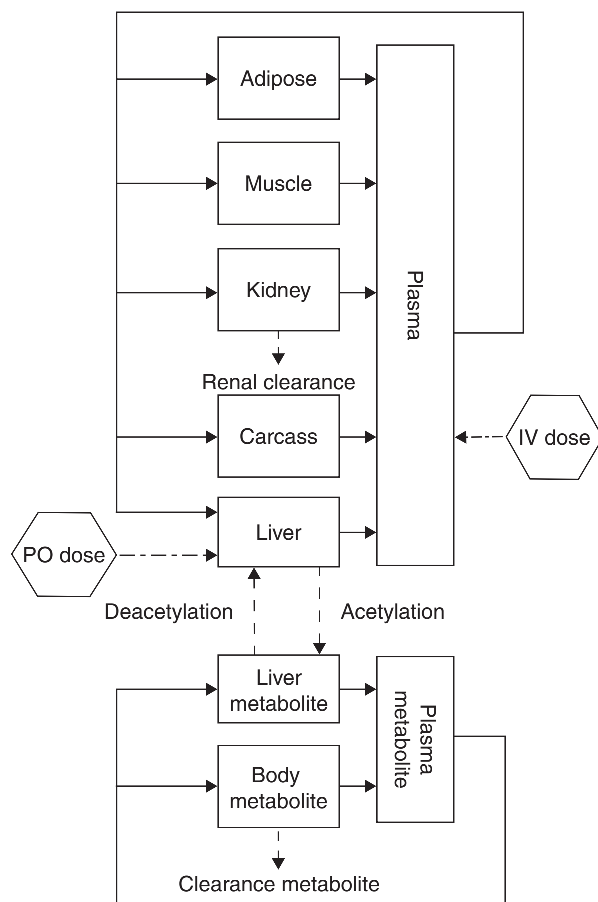


**FIGURE 3.3** PBPK model for SMZ in swine with both oral and intravenous routes of administration. Solid line arrows represent blood flow. Dashed line arrows represent excretion processes. Dash-dot line arrows represent absorption processes.

or lipophilic as well as for organ systems with small volumes or high blood flow to mass ratios. Membrane-limited models, on the other hand, often correspond to large molecules that are polar in nature or large volume organ

systems that have a small blood flow to mass ratio (Ritschel and Banerjee, 1986).

The basic mass balance equation for a flow-limited tissue compartment is:

$$V_t dC_t/d_t = Q_t\,(C_a - C_v) \qquad \textbf{3.1}$$

where $Q_t$, $V_t$ and $C_t$ are the blood flow, anatomic volume and concentration of the xenobiotic in tissue t and $C_a$ and $Cv$ are the concentration of the xenobiotic in the arterial and venous circulations perfusing tissue t, respectively. A basic assumption in flow limited models is that the xenobiotic is in instantaneous equilibrium and is homogenous throughout the tissue block. This is known as a well-mixed model and allows for the relationship between venous blood concentration and the vascular space to be defined according to the ratio:

$$C_v = C_t/P_t \qquad \textbf{3.2}$$

where $P_t$ is the tissue-to-blood partition coefficient for tissue t. Therefore, the final mass balance equation would be:

$$V_t\, dC_t/d_t = Q_t(C_a - C_t/P_t) \qquad \textbf{3.3}$$

This equation can be used in models where all tissue compartments are simplified into a single compartment, like that seen in our example model of SMZ. In contrast to that simplified model, a membrane-limited model does not assume that $C_v$ is in equilibrium with the vascular space. Instead it uses multiple subcompartments of extravascular and intracellular space. The assumption here is that the vascular space is assumed to be instantaneously in equilibrium with the extracellular space. The mass balance equation is then defined by rate of change in the extracellular space per unit of time and can be written as:

$$V_e\, dC_e/d_t = Q_t\,(C_a - C_e) - K_t\,(C_e - C_i) \qquad \textbf{3.4}$$

$$V_i\, dC_i/d_t = K_t\,(C_e - C_t/P_t) \qquad \textbf{3.5}$$

where $V_e$ and $C_e$ are the anatomic volume and xenobiotic concentration of the extracellular space in tissue $t$, $V_i$ and $C_i$ is the volume of the intracellular space and the concentration of xenobiotic in the intracellular space respectively, and $K_t$ is the membrane permeability coefficient for tissue t.

While the above equations do well for tissue blocks that do not alter the xenobiotic, further modifications are necessary for those tissue blocks that metabolize or excrete the xenobiotic. This is most often accomplished by the addition of a mass removal term, Rex. The mathematical definition of Rex can be a simple or as complex as needed and can include anything from a simple first-order equation to detailed Michaelis–Menton equations for multiple enzymes. The resulting equation for a flow-limited tissue block is:

$$V_t\, dC_t/d_t = Q_t\,(C_a - C_t/P_t) - \text{Rex} \qquad \textbf{3.6}$$

For example, if Rex is defined as the clearance of a specific organ the equation would be:

$$Vt\, dCt/dt = Qt(Ca - Ct/Pt) - Ct\, Cl_{organ} \qquad \textbf{3.7}$$

where $Cl_{organ}$ is the clearance of the eliminating organ.

Tissue blocks can be further refined in many cases by the addition of modification terms. You could add terms to describe processes such as protein binding, tissue binding, active transport, billiary excretion, enterohepatic circulation and metabolism.

For example, if Rex is governed by metabolism and the appropriate parameters of enzyme activity are available it may be described using Michaelis–Menton concepts and the equation would take the form of:

$$\text{Rex} = (V_m f_1)/(K_m + f_1) \qquad \textbf{3.8}$$

where $V_m$ is the maximum rate of metabolism, $K_m$ is the concentration at which the rate of metabolism is 50% of maximum and $f_1$ is the free blood concentration in the metabolizing organ.

Once mass balance equations have been written for tissue blocks, the final step is to write the mass balance equation for the plasma compartment. Recall that the inputs of xenobiotic into this compartment are from the venous blood streams from the various tissue blocks. The rate of change in the plasma space per unit of time is then:

$$V_p\, dC_p/d_t = \Sigma Q_t\, C_v - Q_p\, C_p \qquad \textbf{3.9}$$

where $V_p$, $Q_p$ and $C_p$ are the anatomic volume, the total blood flow, and the concentration of the xenobiotic in the plasma compartment respectively. $C_v$ represents the venous concentration of the xenobiotic from tissue t. You can also modify this compartment to incorporate such mechanisms as protein binding or partitioning into red or white blood cells by the addition of modification terms just like you would in the other tissue compartments. The final mass balance equations for the SMZ tissue blocks in our example model are listed in Table 3.1.

## PARAMETER ESTIMATION AND IDENTIFIABILITY

Accurate parameters are essential in PBPK models. As can be seen in the above equations, these parameters are often physiologic (blood flow, organ volume, vascular space,

**TABLE 3.1 Mass balance equations used in the SMZ PBPK model for the parent compound of sulfmethazine.**

| Tissue compartment | Equation |
|---|---|
| Muscle | $V_m \frac{dC_m}{dt} = [C_a - (C_m/P_m)]Q_m$ |
| Adipose | $V_{ad} \frac{dC_{ad}}{dt} = [C_{ad} - (C_{ad}/P_{ad})]Q_{ad}$ |
| Carcass | $V_c \frac{dC_c}{dt} = [C_a - (C_c/P_c)]Q_c$ |
| Kidney | $V_k \frac{dC_k}{dt} = [C_a - (C_k/P_k)]Q_k - (C_a CL_{renal})$ |
| Liver | $V_l \frac{dC_l}{dt} = [C_a - (C_l/P_l)]Q_l - (C_l CL_{acytlation}) + (C_{liver\ met} CL_{deacytlation})$ |
| Plasma | $Vp \frac{dC_p}{dt} = [(C_m/P_m)Q_m] + [(C_{ad}/P_{ad})Q_{ad}] + [(C_k/P_k)Q_k] + [(C_l/P_l)Q_l] + [(C_c/P_c)Q_m] + \text{IV dose} - (C_p Q_{tot})$ |

**V** is tissue volume; **C** is SMZ concentration; **P** is blood:tissue partitioning coefficient; **Q** is blood flow; **CL** is clearance; ***IV*** dose is total ***IV*** dose. Subscripts represent tissues (m: muscle; a: arterial; ad: adipose; c: carcass; k: kidney; l: liver; p: plasma), mechanisms (renal: renal clearance; acytlation; deacytlation), total blood blow (tot) or metabolite concentration (liver met).

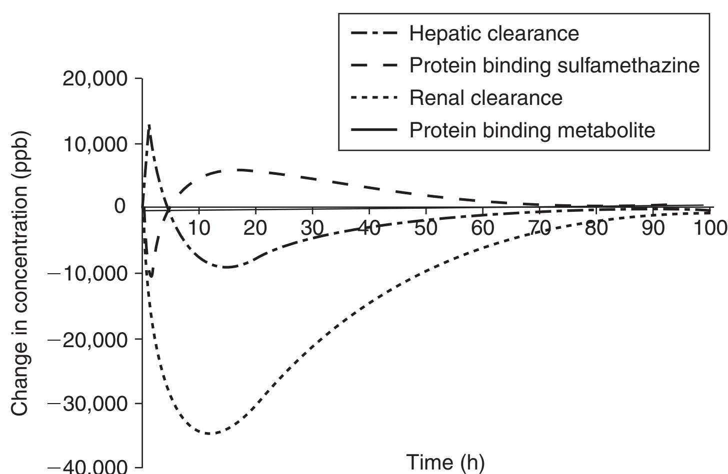


**FIGURE 3.4** Sensitivity analysis of a few parameters in the SMZ PBPK model showing the relative contributions to venous blood concentration of SMZ from protein binding of SMZ, hepatic clearance, renal clearance and protein binding of acetyl-SMZ.

etc.), physiochemical (partitioning coefficients, membrane permeability coefficients, rate of absorption, etc.) and even *in vitro* (protein binding, Michaelis–Menton constants, etc.) in nature. Most of the parameters can normally be found in the published literature with some degree of confidence. Some parameters can be derived using various *in vitro* and *in vivo* experimentation. However, there will be some parameters that are still not accounted for. In this case, you can estimate the value of those parameters using a curve fitting process against known data points (Sheiner, 1985). Several curve fitting software packages are available for this use and most use some function of a least likelihood ratio to estimate the parameters.

It is important to emphasize that a weakness of PBPK models is their dependence upon a large number of parameters. The large numbers of parameters also makes identifiability an issue in some cases. Identifiability is the ability to specifically determine a unique volume for each parameter based on an ideal data set. As you increase the number of tissue compartments, the ability to uniquely identify all parameters is diminished without the inclusion of additional data points. To correct this type of error, multiple tissue compartments can also be sampled. Thus it is not just the number of samples taken, but also the number of tissue compartments sampled that is important for accurate parameter estimation (Audoly *et al.*, 2001). When identifiability is a problem, you can decrease the number of parameters you are trying to estimate by using sensitivity analysis. A sensitivity analysis compares the relative contributions of a parameter to an output of interest (Evans and Andersen, 2000). For example, in the SMZ model above, you may look at the relative contributions to plasma disposition from the parameters of renal clearance, hepatic clearance and tissue partitioning coefficients. Figure 3.4

shows a sensitivity analysis of various parameters for the SMZ model. The parameters of protein binding, hepatic clearance and renal clearance have the greatest effect at early time points. If you have only enough data to identify two parameters, the sensitivity analysis can help narrow the scope of important parameters.

Parameters can also be estimated using statistical distributions rather than single numbers. Just like with single point estimations, the accuracy of the model is directly proportional to the accuracy of the distribution. In many cases, distributions of populations can be found in the literature. There are also several software programs that can estimate distributions given a shape (normal, log-normal, beta, etc.), mean and range.

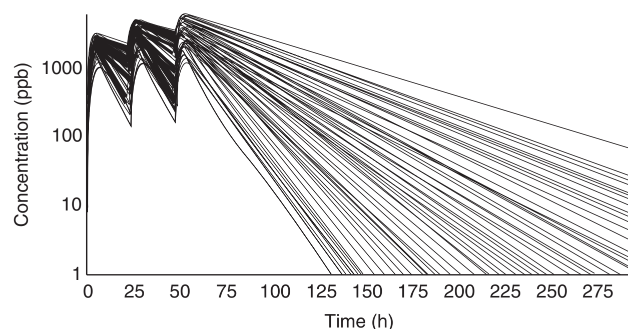


**FIGURE 3.5** Representative Monte Carlo analysis for SMZ PBPK model after multiple oral dosing. Parameters varied included gastric emptying time, absorption rate, protein binding of SMZ, protein binding of acetyl-SMZ and hepatic clearance.

## MODEL VALIDATION

The final step in building a PBPK model is confirming that your model can actually be predictive. This type of validation occurs by comparing model simulations to an external data set. It is important to exclude any data used in the estimation of parameters in your external data set. Simulations of the model are typically plotted along side observed data points (simulation plots); predicted values plotted versus observed values (correlation plots) and residuals plotted against time (residual plots). Results are then subjected to qualitative and quantitative analysis for goodness of fit. Unlike traditional pharmacokinetic modeling approaches, there is currently no standardized way to evaluate the goodness of fit for PBPK models. Often, a combination of visual examination of residual plots and simulation plots along with the quantification of regression correlation values ($R^2$ values) are used. In general, residual plots should have even distributions around zero without noticeable time bias. Correlation plots should have regression lines with $R^2$ values close to 1 and intercepts close to your starting value (in most cases, this is zero). Simulation plots also look for time and concentration bias.

If a complex model were created by the incorporation of population distributions, then model validation becomes even more qualitative in nature. In this case, a sampling method such as Monte Carlo or bootstrapping is used to generate specific numbers for the parameters in question. This is repeated a large number of times and the output becomes a set of simulations all plotted alongside each other. This gives a visual representation of what a population may look like (Thomas *et al.*, 1996; Sweeney *et al.*, 2001). Figure 3.5 shows a Monte Carlo analysis using the SMZ model after multiple oral dosing (Buur *et al.*, 2006). The parameters varied to get this output included oral absorption rate, rate of gastric emptying, protein binding and both renal and hepatic clearances. Validation of this data is performed by plotting the multiple simulation runs along side the external data points just like the simulation plots described above. However, confidence in the distributions and in the model is determined, not by correlation coefficients or residual plots, but by visual inspection alone. The more data points covered within the spread of the output, the higher the confidence in the predictive ability of the model.

## APPLICATIONS

PBPK models are most often used in toxicology to predict the concentrations of toxic chemicals and/or their toxic metabolites in target tissues. Target tissue concentrations predict the time course of toxic effects better than exposure concentrations or concentrations in a reference compartment such as venous blood. The adaptability of PBPK models makes them suitable for extrapolations across different exposure scenarios and routes, species, breed, age, physiological state, pathological changes and sex differences. PBPK models are also used in fundamental research to understand the effects and interactions between anatomical structure, tissue composition and physiological processes that influence the concentration/time course of xenobiotic chemicals.

Toxicologists are often faced with situations where the risk of chemical exposure must be estimated in the absence of observed data under the specific conditions of the exposure being analyzed. An advantage of PBPK models is that different exposure scenarios can be simulated using the same base model by varying the mathematical descriptions of the dosing regimen. These may include single, repeated or continuous oral ingestion, single or repeated intravenous boluses, constant intravenous infusion, single or repeated injections at various body sites, inhalation and topical exposures under various conditions.

When a good PBPK model is available, this approach result in more accurate predictions of risk compared to estimates based solely on exposure concentration and time (Simmons *et al.*, 2005).

Changes in physiological processes, environmental conditions, activity levels and pathological changes typically result in changes in the parameters used to describe a physiological system. If the extent and direction of parameter changes are known or predictable, those parameters can be altered in a PBPK model to simulate its effects on tissue xenobiotic concentrations. An example is the effects of body size, body fat content and sex on the pharmacokinetic behavior of trichloroethylene (Sato *et al.*, 1991). Similar approaches have been used to simulate the effects of pregnancy (Gabrielsson *et al.*, 1984), lactation (Fisher *et al.*, 1990) and species differences (Gabrielsson, 1991). The key requirement is that the parameters used, such as metabolic rate constants, physiological descriptors and partition coefficients, must be available or scaleable. It is also essential that the most important kinetic processes, such as pathways of metabolism, routes of excretion, active transport mechanisms and protein-binding potential, are similar or that the differences are well understood and describable.

PBPK models may be used to determine a minimum toxic dose in a species for which adequate data is not available based on toxicological data obtained in routine laboratory animal studies. This is achieved by linking tissue concentrations with pharmacodynamic effects. A dose metric, such as the area under the curve (AUC) of the tissue concentration over time, can be associated with a specific toxicological endpoint – such as a no-observed-adverse-effect level (NOAEL). Uncertainty factors can be applied to the dose metric to determine maximum acceptable exposure levels, also known as a reference dose, for risk assessment and regulatory purposes. The advantage of using well-validated PBPK models to obtain a reference dose is that PBPK models can eliminate some of the uncertainty associated with classical toxicological studies because variability associated with study design and interspecies variation can be accounted for. This can reduce the need for uncertainty factors while maintaining an acceptable margin of safety when estimating maximum acceptable exposure levels. An example of this process was the use of a PBPK model for a NOAEL of acetone in the rat and the human to increase the reference dose of human exposure to acetone (Gentry *et al.*, 2003). This was achieved by reducing uncertainty associated with pharmacokinetic, pharmacodynamic and interspecies variability. It resulted in a reduction of the uncertainty factor from 300 to 60. The new uncertainty factor retained a factor of 10 for human variability and a factor of 6 for the uncertainty associated with using a subchronic study to predict chronic effects. The new reference dose increased from 0.3 mg/kg/day, obtained from classical risk assessment methods, to 16 mg/kg/day.

The capacity of PBPK models to include non-linear processes, such as enzymatic processes typical of xenobiotic metabolism, makes it suitable for extrapolations across wide dose ranges. For example, the non-linear effects of high doses of ethylene oxide on DNA and hemoglobin adduct formation in the rat could be successfully simulated by using *in vitro* estimated rate and saturation parameters of hemoglobin and DNA binding, glutathione conjugation and hydrolysis (Krishnan *et al.*, 1992). However, predictions outside of the range of concentrations at which a model was validated may be misleading due to differences in xenobiotic metabolism rate parameters at widely varying concentrations. An example is the over-prediction of perchloroethylene metabolite excretion when the metabolism rates estimated using relatively high-dose exposures were used to predict rates of metabolism at the low concentrations relevant to cancer risk assessments. Some PBPK models overestimated rates of metabolite excretion at low parent compound concentrations 5–15-fold (Clewell *et al.*, 2005).

PBPK models can be applied to problems associated with the exposure of food-producing animals to potentially toxic chemicals and drugs and the resulting residues in meat, milk and other edible tissues of animal origin. Parameters describing the terminal phase of concentration/time curves are traditionally obtained using classical compartmental or non-compartmental pharmacokinetic methods. These parameters are then used to estimate residues at time points outside the time range of experimental data or below the detection limits of analytical techniques. These methods assume linearity outside the time and concentration ranges of experimental data. They also assume that the simulated exposure scenarios, study design and study animals are similar to that of the observed data used to obtain the parameters. Validated PBPK models, however, can overcome some of these disadvantages to provide more realistic and accurate predictions at lower concentrations and at extended time points. PBPK model predictions are less dependent on the dosage and route of administration used to validate the model and PBPK models can be adapted to account for variation in study designs and study animals. Monte Carlo analysis can be used to account for population variability in the food animal and can help to isolate sources of uncertainty in risk assessment.

Specific processes involved in the absorption, distribution, metabolism and elimination of xenobiotics can be isolated and modeled using PBPK methods. This approach makes it possible to describe specific processes and structures in greater and more physiologically accurate detail without the models being too cumbersome. To achieve the most benefit from this approach, degrees of freedom should be protected by estimating most parameter values independently from the concentration/time curve of observations in a reference compartment. When the

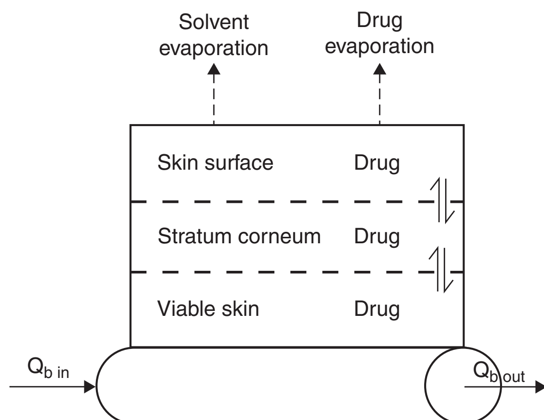


**FIGURE 3.6** A PBPK model of organophosphate pesticide dermal absorption in a flow-through diffusion cell (Van der Merwe *et al.*, 2006).

influence of any dependent parameters on the reference concentration/time curve can be reliably identified, the model can be used to simulate the effects of key parameters. It can also be used to do limited hypotheses testing related to key parameters. An example of this approach is the use of a PBPK model to describe the *in vitro* dermal absorption of organophosphate insecticides in flow-through diffusion cells (Figure 3.6) (Van der Merwe *et al.*, 2006). Extensive independent parameter estimations reduced the dependent parameters to three – each with an identifiable influence on the concentration/time curve in the receptor chamber of the flow-through cell. Sensitivity analyses were used to identify important parameters and to generate hypotheses regarding the effects of changes in the skin related to those parameters. For example, the number of cell layers in the stratum corneum was identified as an important parameter and the effect of varying numbers of cell layers was simulated. The simulation could then be verified by using skin with varying layers of cells in the stratum corneum in flow-through cell experiments.

## CONCLUSIONS

PBPK models provide a continuously evolving new frontier in toxicokinetic modeling. As our understanding of the systems and processes involved in toxicokinetics improve and expand – so does our ability to use new knowledge in PBPK models. It allows for the adaptability needed to simulate varied physiological processes and biological system conditions. PBPK models are likely to assume an ever more important role in our efforts to understand and predict the consequences of exposure to toxins and its application in veterinary toxicology can be expected to expand.

## REFERENCES

Andersen ME, Green T, Frederick CB, Bogdanffy, MS (2002) Physiologically based pharmacokinetic (PBPK) models for nasal tissue dosimetry of organic esters: assessing the state-of-knowledge and risk assessment applications with methyl methacrylate and vinyl acetate. *Regul Toxicol Pharm* **36**: 234–45.

Andersen ME, Lutz RW, Liao KH, Lutz WK (2006) Dose-incidence modeling: consequences of linking quantal measures of response to depletion of critical tissue targets. *Toxicol Sci* **89**: 331–7.

Audoly S, Bellu G, D'Angio L, Saccomani MP, Cobelli C (2001) Global identifiability of nonlinear models of biological systems. *IEEE Trans Biomed Eng* **48**: 55–65.

Brocklebank JR, Namdri R, Law FC (1997) An oxytetracycline residue depletion study to assess the physiologically based pharmacokinetic (PBPK) model in farmed Atlantic salmon. *Can Vet J* **38**: 645–6.

Buur JL, Baynes RE, Craigmill AL, Riviere JE (2005) Development of a physiologic-based pharmacokinetic model for estimating sulfamethazine concentrations in swine and application to prediction of violative residues in edible tissues. *Am J Vet Res* **66**: 1686–93.

Buur J, Baynes R, Smith G, Riviere JE (2006) The use of probabilistic modeling within a physiological based pharmacokinetic model to predict drug residue withdrawal times in edible tissue: sulfamethazine in swine. *Antimicrob Agents Chemother* **50**: 2344–51.

Clewell HJ, Gentry PR, Kester JE, Andersen ME (2005) Evaluation of physiologically based pharmacokinetic models in risk assessment: an example with perchloroethylene. *Crit Rev Toxicol* **35**: 413–33.

Colburn WA (1988) Physiologic pharmacokinetic modeling. *J Clin Pharmacol* **28**: 673–7.

Craigmill AL (2003) A physiologically based pharmacokinetic model for oxytetracycline residues in sheep. *J Vet Pharmacol Ther* **26**: 55–63.

Evans MV, Andersen ME (2000) Sensitivity analysis of a physiological model for 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD): assessing the impact of specific model parameters on sequestration in liver and fat in the rat. *Toxicol Sci* **54**: 71–80.

Fisher JW, Whittaker TA, Taylor DH, Clewell III HJ, Andersen ME (1990) Physiologically based pharmacokinetic modeling of the lactating rat and nursing pup: a multiroute exposure model for trichloroethylene and its metabolite, trichloroacetic acid. *Toxicol Appl Pharmacol* **102**: 497–513.

Frederick CB, Lomax LG, Black KA, Finch L, Scribner HE, Kimbell JS, Morgan KT, Subramaniam RP, Morris JB (2002) Use of a hybrid computational fluid dynamics and physiologically based inhalation model for interspecies dosimetry comparisons of ester vapors. *Toxicol Appl Pharmacol* **183**: 23–40.

Gabrielsson JL (1991) Utilization of physiologically based models in extrapolating pharmacokinetic data among species. *Fundam Appl Toxicol* **16**: 230–32.

Gabrielsson JL, Paalzow LK, Nordstrom L (1984) A physiologically based pharmacokinetic model for theophylline disposition in the pregnant and nonpregnant rat. *J Pharmacokinet Biopharm* **12**: 149–65.

Gentry PR, Covington TR, Clewell III HJ, Anderson ME (2003) Application of a physiologically based pharmacokinetic model for reference dose and reference concentration estimation for acetone. *J Toxicol Environ Health A* **66**: 2209–25.

Krishnan K, Andersen ME (2001) Physiologically based pharmacokinetic modeling in toxicology. In *Principles and Methods of Toxicology*, Hayes AW (ed.). Taylor & Francis, Philadelphia, pp. 193–241.

Krishnan K, Gargas ML, Fennell TR, Andersen ME (1992) A physiologically based description of ethylene oxide dosimetry in the rat. *Toxicol Ind Health* **8**: 121–40.

McCarley KD, Bunge AL(2001) Pharmacokinetic models of dermal absorption. *J Pharm Sci* **90**: 1699–1719.

Ritschel WA, Banerjee PS (1986) Physiological pharmacokinetic models: principles, applications, limitations and outlook. *Methods Find Exp Clin Pharmacol* **8**: 603–14.

Riviere JE (1999) *Comparative Pharmacokinetics: Principles, Techniques, and Applications*. Iowa State University Press, Ames.

Sato A, Endoh K, Kaneko T, Johanson G (1991) A simulation study of physiological factors affecting pharmacokinetic behaviour of organic solvent vapours. *Br J Ind Med* **48**: 342–47.

Sheiner LB (1985) Analysis of pharmacokinetic data using parametric models. II. Point estimates of an individual's parameters. *J Pharmacokinet Biopharm* **13**: 515–40.

Simmons JE, Evans MV, Boyes WK (2005) Moving from external exposure concentration to internal dose: duration extrapolation based on physiologically based pharmacokinetic derived estimates of internal dose. *J Toxicol Environ Health A* **68**: 927–50.

Sweeney LM, Tyler TR, Kirman CR, Corley RA, Reitz RH, Paustenbach DJ, Holson JF, Whorton MD, Thompson KM, Gargas ML (2001) Proposed occupational exposure limits for select ethylene glycol ethers using PBPK models and Monte Carlo simulations. *Toxicol Sci* **62**: 124–39.

Thomas RS, Lytle WE, Keefe TJ, Constan AA, Yang RS (1996) Incorporating Monte Carlo simulation into physiologically based pharmacokinetic models using advanced continuous simulation language (ACSL): a computational method. *Fundam Appl Toxicol* **31**: 19–28.

Van der Merwe D, Brooks JD, Gehring R, Baynes RE, Monteiro-Riviere NA, Riviere JE (2006) A physiological-based pharmacokinetic model of organophosphate dermal absorption. *Toxicol Sci* **89**: 188–204.

C H A P T E R

# 4

# Toxicological testing: *in vivo* and *in vitro* models

*Magda Sachana and Alan J. Hargreaves*

## INTRODUCTION

The large number of new chemical entities in human and veterinary pharmaceutical industry has led to increased demand for safety screening to ensure successful drug development. Part of this screening process includes the determination of the toxic potential of these new compounds by applying internationally recognized *in vivo* or *in vitro* toxicological tests. The importance of toxicological testing is critical, as many pharmaceutical and agrochemical products have to be assessed for their safety, before they become available for general use. Furthermore, in some cases, basic research can produce data that eventually leads to the ban of certain chemicals because they were proved to be unsafe.

Animal studies and validated *in vitro* models are extensively used for screening of agents in order to identify and predict potential ill-effects to humans, domestic and farm animals. Although the detection of adverse health effects of xenobiotics is the main objective of toxicity testing, it can be complemented by more sophisticated biomolecular approaches aimed at the elucidation of the mechanisms of action of certain chemicals.

Another area of veterinary interest regarding toxicological testing is the evaluation of the safety of veterinary drugs for food animals, as well as the safety assessment of veterinary drug residues in human food of animal origin, in order to determine the doses that cause non-observed effect level (NOEL) and consequently to establish the acceptable daily intake (ADI) in human diet.

Toxicological testing laboratories now have to comply with strict official controls and inspections on animal use at any time that can be reinforced by relevant legislation. In most European countries, veterinarians play a pivotal role in toxicity testing and are required by law to be employed by designated establishments that undertake experiments on animals, including toxicological studies. More specifically, a Named Veterinary Surgeon is responsible not only for the health and well being of laboratory animals but also to advise on the selection of adequate *in vivo* models and ensure that priority is given to the use of alternative methods during the local ethical review process. Furthermore, the refinement of experimental techniques and husbandry approaches should be one of the priorities of a veterinary surgeon working in animal units, especially where regulated procedures take place, and distressed animals should be kept under very close supervision by a veterinarian. A similar approach is used in the US, where all institutes are also obliged to establish an Institutional Animal Care and Use Committee (IACUC), where the participation of a trained and experienced veterinary surgeon is essential to ensure that all aspects of the 3 "R"s (replacement, reduction and refinement) (Russell and Burch, 1959) have been followed before approving animal use for toxicological studies.

A veterinarian with specific professional or scientific interest in toxicology should be aware of the alternative methods in toxicity testing, advise on replacement and refinement of laboratory animals used in toxicological studies and safeguard animal welfare. The main aims of this chapter are to outline the major aspects of *in vivo* and *in vitro* models in toxicity testing and to give a brief overview of endpoint determination. The principal focus will be to highlight some of the current *in vivo* and *in vitro* models available in toxicity testing and inform on new technologies and approaches used in this field.

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# *IN VIVO* MODELS IN TOXICITY TESTING

## Introduction

With the continuous development of new chemicals and pharmaceutical products, laboratory animals have become important and well-established tools for the generation of *in vivo* toxicological data. Originally, *in vivo* experiments were aimed at the prediction of acute systemic toxicity usually in rodents. Currently more sophisticated, targeted and multi-species approaches with well-defined endpoints and experimental protocols are applied to toxicological studies, especially for regulatory testing.

As the science of toxicology evolves, an increasing number of *in vitro* alternative tests have been validated or they are currently under development. However, in some cases, animal models in toxicity testing are irreplaceable, especially in the tests required by the regulatory authorities to protect human and animal health. Furthermore, there is public demand to know the toxicity risks posed in every day life, which necessitates the use of animal models comparable to humans.

Although animals are relatively expensive experimental models compared to the alternative methods discussed later, there are several important reasons for their continued use. Firstly, there is extensive information available on their normal biochemical and physiological properties. Secondly, the published data from the measurement of toxicological endpoints *in vivo*, using models of relevance to humans and domestic/farm animals, makes animal testing a valuable tool to predict toxicity.

The animals that are most commonly used in toxicological testing are rodents and rabbits (Table 4.1). Cats and dogs are used less frequently in toxicity testing (and mostly in preclinical toxicology or phase I pharmacological studies), whereas non-human primates are rarely used and mainly to study metabolism of toxic compounds. In this table are not included the studies conducted on companion animals to determine safety limits in products that are directly applied to cats or dogs (EPA, 1998a). The interested reader is referred to some more specialized books regarding animal toxicity testing (e.g. Arnold *et al*., 1990; Gad and Gad, 2006) and some useful websites relevant to regulatory toxicological testing (e.g. http://www.epa.gov/opptsfrs/publications/OPPTS_H

**TABLE 4.1 Examples of animal models used in selective toxicity tests**

| Order | Species | Toxicity tests | References |
|---|---|---|---|
| **Rodentia** | **Rat** | Developmental toxicity | (EPA, 1998b, 2000; OECD, 2001b) |
| | | Carcinogenicity | (OECD, 1981a, b; Chhabra *et al*., 1990) |
| | | Cutaneous toxicity | (OECD, 1987) |
| | | Genotoxicity | (OECD, 1984; EPA, 1998c) |
| | | Immunotoxicity | (IPCS, 1996; EPA, 1998g) |
| | | Neurotoxicity | (OECD, 1997c; EPA, 1998d, e) |
| | | Developmental neurotoxicity | (EPA, 1998f; OECD, 2003) |
| | | Reproductive toxicity | (EPA, 2000; OECD, 2001a) |
| | **Mice** | Carcinogenicity | (OECD, 1981a, b; Chhabra *et al*., 1990) |
| | | Skin sensitization | (EPA, 2003) |
| | | Genotoxicity | (OECD, 1984; EPA, 1998c) |
| | | Immunotoxicity | (IPCS, 1996; EPA, 1998g) |
| | | Neurotoxicity | (OECD, 1997c; EPA, 1998d, e) |
| | | Reproductive toxicity | (OECD, 2001a) |
| | **Hamsters** | Carcinogenicity | (Gad, 1998) |
| | | Genotoxicity | (Loomis and Hayes, 1996) |
| | **Guinea Pigs** | Cutaneous toxicity/skin sensitisation | (OECD, 1987; EPA, 2003) |
| | | Developmental neurotoxicity | (Kaufmann, 2003) |
| **Lagomorpha** | **Rabbit** | Developmental toxicity | (EPA, 1998b; Foote and Carney, 2000; OECD, 2001b) |
| | | Cutaneous toxicity | (OECD, 1987; Auletta, 2004) |
| | | Reproductive toxicity | (Foote and Carney, 2000) |
| **Avian** | **Hen** | Neurotoxicity | (OECD, 1995a, b) |
| **Swine** | **Minipigs** | Cutaneous toxicity | (Auletta, 2004) |
| | | Developmental neurotoxicity | (Kaufmann, 2003) |
| **Canine** | **Dog** | Carcinogenicity | (Loomis and Hayes, 1996) |
| | | Cutaneous toxicity | (Vail *et al*., 1998) |
| | | Neurotoxicity | (EPA, 1998e) |
| | | Developmental neurotoxicity | (Kaufmann, 2003) |
| | | Reproductive toxicity | (FDA, 1982) |
| **Non-human primates** | **Monkey** | Developmental toxicity | (Buse *et al*., 2003) |
| | | Cutaneous toxicity | (deBlois and Horlick, 2001) |

armonized/870_Health_Effects_Test_Guidelines/Series/ and http:// lysander.sourceoecd.org/vl=23534020/cl=14/nw=1/rpsv/cw/vhosts/oecdjournals/1607310x/v1n4/contp1-1.htm).

## Animal welfare in toxicity testing

Animal welfare legislation is currently applicable in most countries to prevent misuse of animals in toxicological testing. In the US, the Animal Welfare Act as amended (7 USC, 2131-2156) provides guidelines and protection to animals used for scientific purposes including toxicity testing. In Europe, because of new developments in alternative approaches to animal testing and the increased public concern about animal welfare issues, Directives 86/609/EC and 2003/65/EC will be shortly amended or replaced by new directives, in order to upgrade the minimum standards for laboratory animals by introducing appropriate animal welfare indicators and better training and information for animal handlers. Furthermore, it is necessary that there is explicit and easy to follow documentation in each project licence involving toxicological testing in animals, where the steps followed in case of animal distress are described. Testing new chemical compounds on *in vivo* models can lead sometimes to pain, discomfort or animal distress. Animals exhibiting signs of suffering should be examined by a veterinarian or trained personnel and, if there is a good scientific reason to continue the study, administration of analgesics should be considered. Other options available are the euthanasia of the distressed animals and the decrease or the discontinuity of the test compound dose.

In general, there is a trend for improvements to enhance animal welfare, not only in animal housing (e.g. breeding, handling and feeding) but also in the same regulatory toxicity testing guidelines, by applying humane endpoints and decreasing the number of animals required (Combes *et al*., 2004).

Handling laboratory animals and administering toxicological compounds is a stressful procedure for both the animals and the designated personnel. In recent years, there has been an attempt to apply the highest ethical standards not only by introducing an excellent environment and a social and complex housing but also by reducing and improving any invasive techniques used on laboratory animals.

Implanted biosensors are now available that permit the continuous telemetric monitoring of physiological and biochemical parameters in experimental animals, and have been proved useful in toxicological studies to minimize not only the artefacts due to animal handling and restraint but also to improve animal welfare. In addition, non-invasive and currently expensive methods such as magnetic resonance imaging (MRI) and nuclear magnetic resonance spectroscopy are available for the visualization of pathological findings and determination of the distribution of a test chemical in laboratory animals, respectively, which contribute to improved animal welfare.

## Routes of test compound administration

Toxicity testing in animal models is most useful if it imitates the human or domestic/farm animal route of exposure to chemical agents. Based on the medium of exposure in human and domestic animals, it is possible to decide on which is the administration route of choice in animal toxicological tests (Table 4.2). Depending on the route of administration, experimental evaluation may differ because of variation in the absorption, metabolism and elimination of a compound. Oral exposure can lead to absorption by the digestive system and metabolism by the liver, whereas following inhalation a toxic compound is more likely to be absorbed by the respiratory system. Metabolism of xenobiotics can also occur in placenta, in the test dam and foetus. This can lead to changes in the balance of parent compound and metabolites, complicating the picture even more in case of developmental toxicity tests.

This metabolism of administered chemical should be relevant to human or domestic animals and is critical for risk assessment exercises. The final toxic effect will depend on a balance between the level of toxic agent reaching the target tissue and its rate of elimination and/or bioinactivation by mixed function oxidases, serum hydrolases or binding to serum proteins. Furthermore, there are interspecies differences regarding the metabolism of xenobiotics (Nebbia, 2001). For example, cats are at high risk to develop hepatotoxicity especially after paracetamol administration. This is due to differences in bioactivation of paracetamol, which occurs only in cats through *N*-hydroxylation with the help of cytochrome P450 2E1, during the oxidative reactions in phase I transformation (Nebbia, 2001). Ruminants are less susceptible to organophosphates (OPs) such as parathion than monogastrics, because the rumen microflora plays an important role by reducing the nitro group of OPs to an amino group (Nebbia, 2001).

The majority of toxicological studies commonly employ administration of the agent in animal feed or water or by

**TABLE 4.2 Proposed administration routes of test compounds in laboratory animals based on the medium of exposure**

| Medium of human and domestic/ farm animal exposure to toxicant | Administration route |
|---|---|
| Food commodities | Oral |
| Water | Oral |
| | Inhalation |
| | Dermal |
| Air | Inhalation |
| Household/environmental surfaces | Oral |
| | Dermal |

stomach intubation (i.e. by gavage) in order to imitate a known or potential human or domestic animal exposure. The use of oral gavage is commonly used in administration of high doses of xenobiotics and in developmental toxicity tests, but is less practical in case of long-duration studies. Inhalation is used when there is a need to duplicate industrial or environmental exposure to dusts, aerosols and fumes. In this case, nose, head or whole body exposure chambers are used, depending on the exposure time. For cutaneous administration, a toxic agent may be injected intradermally or simply applied topically on the skin or ears and sometimes covered with bandages. In case of experimental studies where the need for complete absorption of a tested compound is considered essential, parenteral routes of administration (intraperitoneal, intramuscular, intravenous and subcutaneous) are selected. However, the solubility and bioavailability of the tested agent can also influence the degree of absorption and how much of it is directly available in a laboratory animal.

Toxicokinetic and pharmacokinetic information on tested compounds and their comparison among laboratory animals and humans are also important to determine dosing parameters and improve the toxicological data obtained. The administered dose in toxicological studies should be decided taking into account many physicochemical parameters of chemical agents, biological differences between species, previously published data and after careful planning or preliminary experimentation.

## Reproductive toxicity tests

Reproductive toxicity testing is based on the measurement of reproductive functional and structural defects caused by toxic agents in both males and females. The toxicity endpoints most frequently studied in reproductive *in vivo* testing are summarized in Table 4.3. Rats and, to a lesser extent, mice are the species of choice in reproductive toxicological testing because they are considered inexpensive compared to bigger mammals (EPA, 2000; OECD, 2001a). On the other hand, small rodents need to be euthanized even for simple endpoints such as collection of sperm, whereas rabbits can be sampled regularly without being sacrificed, making them an alternative in reproductive toxicological tests (Foote and Carney, 2000). Some of the disadvantages of using rabbits are the higher cost due to greater amounts of chemical compounds administered and the increased cases of abortions because of the relatively high incidence of gastrointestinal dysfunction. For example, rabbits are poor models for veterinary residue testing and, more specifically, for antibiotics because these compounds have been found to cause diarrhoea and consequently abortion (Barlow *et al.*, 2002).

**TABLE 4.3 Selective endpoints applied to laboratory animals during reproductive toxicity testing**

| Female | Male |
|---|---|
| Reproductive tract morphology | Sperm structure/morphology |
| Reproductive tract receptors | Sperm motility/viability/count |
| Ovum properties | Sperm DNA integrity |
| Recovery of blastocysts | Hormonal balance/Receptor interactions |
| Hormonal balance/receptor | Fertility testing interactions |
| Length and normality of oestrus cycle | |
| Fertility testing | |
| Uterine condition | |
| Implantation | |
| Lactation | |
| Maternal behaviour | |

Dogs have been occasionally used for reproductive toxicity testing, because the physiology of their reproductive system in both sexes has been extensively studied, contributing to significant background knowledge (FDA, 1982). However, the dog is not the model of choice in reproductive toxicity testing because it is a rather expensive animal model and the number of litters is not as high as in rodents. The main difficulty in running reproductive toxicological tests is the requirement for an oestrous-synchronized population. Both the induction of ovulation by hormonal treatment and artificial insemination mainly in rabbits and dogs can overcome this problem and allow advanced planning for animal fertility studies.

Since exposure to chemicals can occur throughout life, a multi-generation study which extends over at least two generations, using a single type of laboratory rodent, is desirable in order to reveal reproductive toxicological data (OECD, 2001a; Barlow *et al.*, 2002), although this approach requires large numbers of animals and it is time consuming.

## Developmental toxicity testing

Developmental toxicity testing is primarily used to determine hazard regarding the potential effects of prenatal exposure on the developing foetus. These studies focus on functional and structural changes that can be observed throughout the development from zygote to neonate. The most important developmental phase is the organogenesis period that is always taken into account in developmental toxicity testing. Based on these studies, chemical compounds can be categorized as teratogenic and/or foetotoxic by recording structural malformations, developmental retardation and/or mortality, respectively. The vast majority it teratogenic chemical agents have been identified using rodent experimental models (EPA, 1998b, 2000;

OECD, 2001b). However, the failure of rodents to detect teratogenic signal on some occasions and the similarities in placentation and pregnancy physiology between humans and rabbits, led to the use of the rabbit as a second model for assessing the effects of toxic compounds on development (EPA, 1998b; Foote and Carney, 2000; OECD, 2001b). Furthermore, although non-human primates have been suggested as models for teratological testing (Buse *et al.*, 2003), they have several limitations such as a long gestation period, only single or twin offspring, high rates of abortion and ethical constraints.

The detection rates for veterinary pharmaceutical agents demonstrated to be teratogenic/foetotoxic were found to be 55–79% using individual species (Hurtt *et al.*, 2003). However, when the rat and rabbit data were both considered, there was a significant increase in detection rate to almost 100%, suggesting that in the absence of teratogenicity in rat, a second species developmental test in lagomorpha is required to provide high standards of public protection (Hurtt *et al.*, 2003).

## Cutaneous toxicity testing

The aim of *in vivo* assays for cutaneous toxicity is not only to assess potential acute local irritation but also to evaluate acute, subchronic and chronic systemic toxic effects. During cutaneous toxicity tests, animals are monitored for skin reactions/dermal effects, clinical, gross or microscopic pathological findings depending on the duration of a toxic compound administration and the observation period.

The albino rabbit has been until recently the animal model of choice because of the high permeability and sensitivity to toxic agents exhibited by its skin, which sometimes led to over-prediction showing little relevance to human irritation (Auletta, 2004). Currently, albino rats and occasionally guinea pigs are considered preferable species to assess local irritation. Traditionally, guinea pigs and mice have been used also to perform sensitization tests (EPA, 2003). On the other hand, long-term cutaneous toxicity studies usually require the use of a rodent (albino rat and mouse) as well as a non-rodent model. Minipigs have been proved to be a reliable non-rodent species because their skin demonstrates many physiological similarities with those of humans and pigs (Auletta, 2004). Other non-rodent models are dog and non-human primates, commonly used to test the metabolism of toxic agents and the safety of newly designed recombinant pharmaceutical products (Vail *et al.*, 1998; deBlois and Horlick, 2001).

## Genotoxicity testing

The aim of genotoxicity testing is to detect gene damage induced by the test compound, by measuring chromosome aberration and breakage, point mutation, and other DNA and chromosomal effects *in vivo*. The host-mediated assay is based on the inoculation of a microorganism into a rodent such as mouse, rat or hamster and subsequent assessment of the point mutations found in the microorganisms, after certain treatment of the rodents with the potential mutagen (Gabridge and Legator, 1969; Dhillon *et al.*, 1995; Loomis and Hayes, 1996). On the other hand, to identify chromosome breakage, male rodents are treated with the test compounds and, after mating with untreated females, foetal mortality and survival are recorded (OECD, 1984; EPA, 1998c).

The mouse spot test is capable of detecting somatic gene mutations and is based on *in utero* exposure to the tested chemical. This *in vivo* mutation test works by monitoring the appearance of coloured spots in the coat of the animal that may appear due to altered or lost specific wild-type allele in a pigment precursor cell (EPA, 1998h).

A popular *in vivo* test to identify genetic risks is the rodent bone marrow micronucleus test, although it is not the most sensitive test and it does not improve predictivity of rodent genotoxicity when combined with *in vitro* tests (OECD, 1997a; Zeiger, 1998). However, this *in vivo* test is widely used because it is relatively easy to perform. Another *in vivo* test that has been validated and recommended by Organisation for Economic Cooperation and Development (OECD, 1997b) is the rat liver unscheduled DNA synthesis (UDS) test. It is worth mentioning that none of the above approaches are suggested to be used individually to predict genotoxicity. Negative or positive results should always be confirmed first by *in vitro* or non-mammalian mutagenicity tests.

## Carcinogenicity tests

*In vivo* assays of carcinogenicity examine the possibility that a tested agent might cause tumours and other chemically related effects in one or more animal species. Currently available is the rodent carcinogenicity test which combines the use of F344 rats and B6C3F1 mice (OECD, 1981a, b). This test runs for up to 2 years and involves the use of three different concentrations, five different administration routes and both sexes. The results are based on clinical chemistry, gross and histopathological analysis of more than 40 tissues and organs in order to determine the site, the number and type of tumours (OECD, 1981a, b; Chhabra *et al.*, 1990).

One serious disadvantage of using mice in carcinogenicity testing is their tendency to present high incidence of spontaneous liver or lung tumours in some strains, leading occasionally to inconclusive results (Gad, 1998). Furthermore, because of the long duration of carcinogenicity tests and therefore the old age of laboratory animals, it means that the natural occurrence of tumours increases, making it difficult to distinguish between a real treatment and a background carcinogenic effect. In addition, the most

common mechanisms of tumour development in rodents may not be relevant to human carcinogenicity, posing limitations to any attempt to use *in vivo* data for carcinogen risk assessment.

Tumorigenic tests using dogs are limited because of the high cost and the long duration of the studies (up to 7 years). Furthermore, dogs demonstrate high susceptibility to aromatic amines and therefore care should be taken when using these animals for the evaluation of potential carcinogenic compounds of this category (Loomis and Hayes, 1996).

## Neurotoxicity

The neurotoxic potential of chemical compounds can be assessed by determining relevant effects on the autonomic or central nervous system (CNS), not only in adult but also in developing animals. Clinical signs including changes in behaviour (e.g. movement, motor co-ordination or reflexes, paralysis, tremor, learning and memory), neurochemical (e.g. activity of enzymes associated with neuropathies, cell signalling pathways, synthesis, release and uptake of neurotransmitters), neurophysiological (e.g. electroencephalography, nerve conduction velocity) and neuroanatomical effects are commonly explored.

Rodents are the most frequently used animal models and there is a considerable number of available guidelines to examine the neurotoxicity of xenobiotics on them (OECD, 1997c; EPA, 1998d, e). However, an important issue related to the selection of animal model in neurotoxicity testing is the delayed onset of effects manifested by toxic agents. For example, in the case of assessment of the potential of OPs to produce delayed neuropathy induced by organophosphate (OPIDN), as rodents are not the most sensitive models, the use of the hen model is recommended (OECD, 1995a, b). The two protocols used in the hen model involve either acute or repeated dosing for up to 28 days and determination of the enzymes acetylcholinesterase (AChE) and neuropathy target esterase (NTE), clinical observation and histopathology of the CNS (OECD, 1995a, b).

Furthermore, the need to address the specific risks of the developing nervous system has led to the application of more specific developmental neurotoxicity testing guidelines in rats (EPA, 1998f; OECD, 2003). Although rodents are the animal model of choice, the main problem using them in developmental studies is that the period of enhanced brain growth takes place during the first 10 days after birth, whereas in humans, dogs, guinea and mini pigs, this period is completed prenatally. In conclusion, for predicting developmental neurotoxicity risk in humans, the rat is the recognized model. However, dogs, guinea and mini pigs are also widely used to test pharmaceutical compounds that target children, due to the limitations of the rodent model (Kaufmann, 2003).

## Immunotoxicity

Immunotoxicity tests are designed to detect adverse effects of xenobiotics on the immune system including all the relevant cells, organs and mechanisms of immune response, whether or not there is a measurable disturbance in host resistance. Toxic substances can directly or indirectly cause immunotoxicity, either by inhibiting the enzymatic activity of esterases and serine hydrolases in the immune system or by chronic alteration in metabolism of organs in the immune and nervous system, respectively. Some of the approaches, tests and endpoints used to assess *in vivo* immunotoxicity are presented in Table 4.4 (OECD, 1995c; EPA, 1998g; Barlow *et al.*, 2002; Galloway and Handy, 2003).

Laboratory rodents, especially mice, are used in toxicological tests because basic immunological studies were mainly conducted in this species. The immune elements

**TABLE 4.4 Immunotoxicity endpoints, methods and approaches used for *in vivo* toxicity testing**

| Tests | Methods | Endpoints |
|---|---|---|
| Non-functional tests of immunotoxic response | Immunopathology | Lymphoid organ weight<br>Histopathology of lympoid tissues, including bone marrow |
| | Routine haematology | Immunocyte viability/differential count<br>Immunoglobulin levels |
| Functional tests of immunotoxic response | Measurement of humoral immunity | Antibody response<br>Antibody plaque forming |
| | Measurement of non-specific immunity | Neutrophil and monocyte numbers<br>Phagocytic activity of macrophages |
| | Measurement of cell mediated immunity | Mitogen-induced cell proliferation<br>Natural killer cell activity<br>Mixed lymphocyte reaction<br>Delayed hypersensitivity |
| | Host resistance assays | Mortality and organ histopathology due to bacterial, virus and parasitic infection |

and interactions in rodents and humans are very similar and if the toxicokinetic properties of tested chemicals are close, then the use of mice or rats is recommended (IPCS, 1996; EPA, 1998g).

Chlorpyrifos, an OP with distinctive toxic effects on neurodevelopment, was reported to affect intracellular signalling cascades involved in differentiation and proliferation of T lymphocytes (Blakely *et al.*, 1999) and has recently been implicated as an endocrine disrupting chemical, thus possibly affecting endocrine-mediated immunity. In most experimental studies, cholinergic doses of OPs were shown to reduce immune activity whereas non-cholinergic ones resulted in an increased response, although there is still research to be done to this direction (Pruett, 1992; Galloway and Handy, 2003).

## Transgenic animals in toxicity testing

The recent advances in genetic engineering techniques led to the development of a number of transgenic *in vivo* models that can be used in toxicity testing, mainly in carcinogenicity and mutagenicity, as well as for the study of xenobiotic metabolism. These *in vivo* systems permit the study of toxicological effects of tested compounds on foreign genes of human or other origin that have been genetically transferred to produce transgenic animals, by transgenesis or targeted gene modification. From the point of view of toxicity testing, it is considered that such models will lead faster to results that are more representative of human response to xenobiotics. In addition, transgenic mice can bring significant animal welfare benefits, because they are able to reduce the group size needed in experiments and replace testing to other species, including non-human primates. Finally, these models are also excellent experimental systems and able to address and answer specific mechanistic questions in toxicology more efficiently (Valancius-Mangel and Doetschman, 1999). However, the use of "humanized" animal models in toxicological studies is an expensive process because it involves higher costs for their development and breeding. There is also concern that the human genome, once transferred to laboratory animals may express the same proteins, but it does not guarantee that the protein will have similar function with that found in humans. In addition, there is limited background information on transgenic animals, making the interpretation of data in toxicological studies difficult (Valancius-Mangel and Doetschman, 1999).

The main transgenic genotoxicity systems used in toxicology to follow up *in vitro* genotoxicity positives are the Muta Mouse and Big Blue rat/mouse models, in which the genome is "tagged" with the markers lacZ (β-galactosidase) and lacI, respectively (Gossen *et al.*, 1994; Winegar *et al.*, 1994; Wahnschaffe *et al.*, 2005a, b). Aflatoxin B1, a mycotoxin commonly found in human food and animal feed, has been tested in transgenic C57BL/6N mice and caused lacI mutation in animals' kidney and liver, proving the genotoxicity effect of the mycotoxin and demonstrating the usefulness of transgenic mice in toxicology because not only can this approach quantify mutant frequencies but it also identifies specific types of mutational events (Autrup *et al.*, 1996).

Several models are available to assess the carcinogenicity of a test compound: these include (a) inactivated tumour suppressor gene and DNA repair gene models such as p53+/− and XPA−/− knockout mice, and (b) oncogene activation models like the TgAC and the ras H2 transgenic mice (Valancius-Mangel and Doetschman, 1999).

Other important transgenic system with toxicological application that have been designed to express/lack one or more of the proteins involved in metabolism of xenobiotics and their use has led to a better understanding of metabolic activation and transformation. Most of these transgenic models express human cytochrome P450 (CYP1A2, CYP1B1) or are knock out mice (CYP2E1/CYP1A2 null mice) (Valancius-Mangel and Doetschman, 1999).

## Limitations and implications of animal use in toxicological testing

The closer a test is to the phylogenic species of concern, the more relevant the information obtained and the higher its predictivity. There are basic similarities in biology and xenobiotic metabolism between humans and the different mammalian species used in toxicity testing, making *in vivo* testing the most reliable approach in chemical safety assessment (Knudsen, 1999). However, because of species and strain variability in uptake, metabolism and elimination of xenobiotics, no single type of animal is an ideal model for predicting human or domestic animal toxicity. Consequently, multiple *in vivo* models are usually required to determine toxicity of agents and to contribute to more precise risk assessment, which may have ethical implications.

Currently there is a trend in using well-established inbred (immortal clones of genetically identical individuals) strains of rodents, in order to increase the sensitivity of animal experimentation in toxicological testing by maximizing the reproducibility of the results. While this approach can reduce animal numbers, the results may not be representative of a heterogeneous population; using a heterologous group of one species with low inbreeding may therefore be a more realistic approach reflecting the impact of a toxic compound on a broader spectrum of genes.

In the last three decades there has been an increased sensitivity to ethical issues involved in use of animals for toxicity testing. The tactics of mass demonstrations by animal protection and animal extremist groups has created an atmosphere where many institutions, organizations or companies and their employees are reluctant to

support or carry out any animal testing, before making sure that an alternative test does not exist. In addition, there are economic arguments against the use of animals in toxicity testing. Approaches using animals are undoubtedly more time consuming and more costly to run, compared to *in vitro* models. There are certain standards that should be maintained from laboratories undertaking toxicity testing experiments, such as Good Laboratory Practices (GLP), not only from an animal welfare prospective but also from experimental reproducibility approach, something that adds to the cost.

Furthermore, the quantity and quality of animal feed should also be considered because several studies demonstrated that the nutritional status can interfere with the reproducibility of the results obtained from animal models used in toxicity testing (Kacew, 2001). Other factors contributing to data quality are the health status of the animal stock (specific pathogen free, germfree or with ordinary microflora) and the laboratory experience in handling and caring for the stock. In conclusion, toxicological animal testing, although a valuable tool to predict human and domestic/farm animal toxicity of chemicals, is labour intensive, time and space consuming and raises important animal welfare concerns and uncertainties. For these reasons, there has been increasing interest in the development of *in vitro* alternatives that can help to reduce the use of animals in toxicity testing.

# *IN VITRO* MODELS OF TOXICITY TESTING

## Introduction

*In vitro* cellular models are relatively inexpensive and easy to maintain and manipulate compared to animal models. *In vitro* methods allow the study of direct cellular effects of toxins on specific cell or tissue types in a controlled environment.

However, the main disadvantage of *in vitro* systems over animal models is the lack of systemic effects such as an appropriate balance and supply of growth factors and a system of xenobiotic metabolism and elimination of toxins. The former can be at least partially addressed by adding appropriate growth factors and the latter can be addressed by the use of metabolic activation systems such as the introduction of nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) activated microsomes or a hepatic cell line in a cell culture well insert fitted with a filter (Figure 4.1). The toxin is introduced into the insert and a mixture of metabolized and non-metabolized toxin (but not the microsomes or the hepatic cells) diffuses through the filter into the growth medium containing the target cells. Another

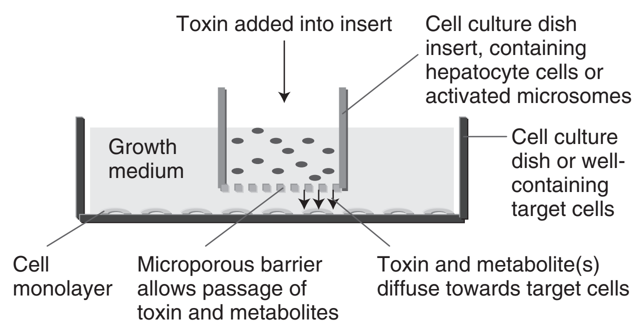


**FIGURE 4.1** Metabolic activation in cell culture systems.

disadvantage is that many cellular systems lack the complexity of cell:cell interactions in tissue, although this can be addressed to varying degrees using co-culture systems or organ culture, which are discussed later.

## Types of cell culture system used in toxicity testing

Before discussing the assays used in the *in vitro* systems it would be useful to discuss the principles, strengths and weaknesses of the main types of *in vitro* system. All cell cultures need to be prepared and maintained under sterile conditions in order to reduce microbial contamination. Cells are maintained under defined conditions of humidity, pH and temperature. They are grown in a specific growth medium, which may have a number of supplements, such as antibiotics, glutamine, serum, etc., which are different for each cell line. Growth conditions should be optimized prior to experimental work. Excellent reviews on practical aspects of cell culture can be found in the following sources (Cohen and Wilkin, 1996; Shaw, 1996; Masters, 2000; Davis, 2002; Gardner *et al*., 2004).

There are a number of types of cell culture available for *in vitro* testing that offer various degrees of complexity and relatedness to the *in vivo* situation. In order of increasing complexity and genetic similarity to the tissue of origin, these include permanent cell lines, primary cultures and organotypic cultures (Figure 4.2) (for reviews see, Noraberg, 2004; Spielmann, 2005; Sundstrom *et al*., 2005).

Permanent cell lines are mitotic and can be finite, established or clonal in nature. They have the advantage that they are relatively easy and inexpensive to maintain compared to animals and they are amenable to cryopreservation under liquid nitrogen. However, if maintained through high numbers of divisions, there is an increasing likelihood of genetic drift that might affect phenotypic properties of relevance to toxicity testing.

Finite cell lines are normally derived from primary cultures (see below) and can survive for 40–50 divisions before finally dying (e.g. fibroblasts). Established cell lines are effectively immortal, having been transformed with a virus, a mutagen or spontaneously. These are generally tumour

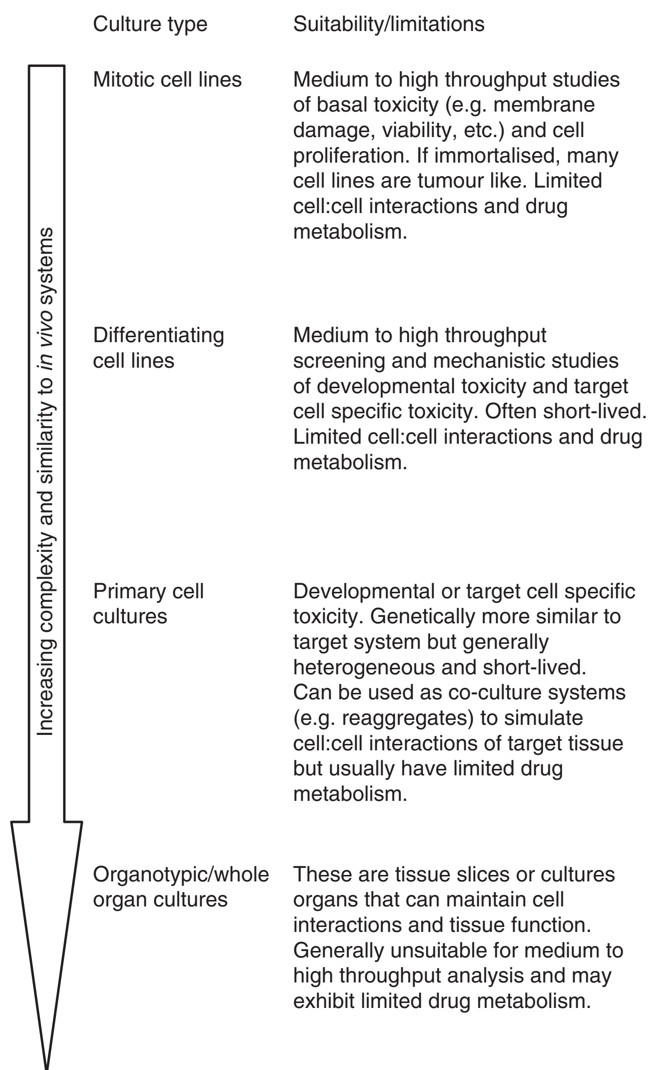


**FIGURE 4.2** Organization of a tiered system for *in vitro* toxicity testing.

like in nature; some widely used examples include mouse 3T3 fibroblasts, HeLa cells, Chinese hamster ovary (CHO) cells. However, several cell lines can be induced to differentiate, making them potentially useful models of specific stages of development, for example the use of nerve growth factor or retinoic acid to induce a neuronal phenotype in cultures of rat PC12 pheochromocytoma and human SH-SY5Y neuroblastoma cells, respectively (Fujita *et al*., 1989; Presgraves *et al*., 2004).

Clonal cell lines are derived from the mitotic division of a single cell seeded in a sterile microtitre plate by limiting dilution as used in the cloning of hybridoma cell lines. Thus, cell lines can be cloned to exhibit a specific trait (e.g. high levels of specific receptors, drug resistance, etc.). While a homogeneous response to toxin treatment might then be expected from such a cell line, there is the risk of losing other features of a more heterogeneous population. In some cases, clones of transfected cell lines provide useful tools for mechanistic studies of cell differentiation and/or toxicity. For example, some cell cultures have been transfected with the certain cytochrome P450 transgenes to make them metabolically more competent during long-term studies (Tzanakakis *et al*., 2002).

Primary cultures are derived by a combination of mechanical and enzymic disruption of the tissue of interest, which releases a collection of cells that resembles the tissue of origin closely both genetically and in terms of cell heterogeneity. It is then possible to enrich in specific cell types by using either selective culture media and/or cell growth inhibitors, immunomagnetic beads or fluorescence activated cell sorting (FACS). If a monoculture is desirable (i.e. predominantly one specific cell type), the resultant culture would then need to be screened for cell specific markers to determine purity/enrichment of the preparation, as a pure culture is rarely achieved. Typically, this would take the form of monitoring cultures for the expression of a unique morphological or molecular trait of the desired cell type to determine the level of enrichment. For example, expression of glial fibrillary protein (GFAP) and aster-like morphology would be good markers for astrocytic glial cells. Cells may be cultured as monolayers or, if a system simulating cell:cell interactions of the tissue of origin is required, cells may be cultured in suspension with mild agitation or using rolling cell culture. Under these circumstances, cells form clusters, spheroids or reaggregates that may continue to grow/proliferate over a period of several days or weeks, making them amenable to studies of long-term effects. For example, brain reaggregates prepared in this way have been maintained for up to several weeks and used in studies of pesticide toxicity (Sales *et al*., 2000). However, many primary cultures tend to be very short lived.

Also, as for established cell lines, such cell cultures do not necessarily exhibit identical cell:cell interaction patterns as those in the tissue of origin and cell types that divide more rapidly could become more predominant than they would be *in vivo*. This deficiency may be addressed by using organotypic cultures. In this case, tissue slices (typically 200 μm thickness) are cut from fresh tissue on a microtome, then subsequently rinsed and cultured in growth medium with agitation, as discussed above. Such slices maintain the complexity of cell:cell interactions and extracellular matrix composition of the original tissue and, in some cases can survive up to several weeks (Sundstrom *et al*., 2005). Nevertheless, even this kind of cellular system lacks the systemic interaction with the immune and circulatory systems that would occur *in vivo* and is not, therefore, a complete substitute for *in vivo* testing. However, using cell cultures as part of a tiered system of increasing complexity from *in vitro* to *in vivo* measurements would improve throughput, decrease costs and allow drastic reduction in the use of live animals in screening compounds for potential toxic effects. Some examples of *in vitro* toxicity testing systems in current use are indicated in Table 4.5.

**TABLE 4.5 Examples of cell culture systems used to model specific types of toxicity**

| Model | Description and comments |
|---|---|
| Neurotoxicity | Differentiating neural cell lines (e.g. human SH-SY5Y and rat PC12 neuroblastoma). Primary cultures, whole rat brain reaggregates and organotypic brain slice cultures (Sales *et al*., 2000). |
| Hepatic toxicity | Human hepatoma HepG2 cell line and subclones expressing CYP1A1, cell lines engineered to express single human or animal P450, primary hepatocyte cultures, longer term collagen sandwich cultures, liver slices and isolated perfused liver (Worth and Balls, 2002). |
| Developmental toxicity | Whole rat embryo cultures, rat limb bud reaggregates cultures and mouse embryonic stem cell lines (Liebsch and Spielmann, 2002). Standard operating procedures (SOPs) available on the ECVAM–INVITTOX databases. |
| Dermal toxicity | Keratinocyte and fibroblast cell lines. Excised rat skin models and human EPISKIN™ and EPIDERM™ skin models (Fentem *et al*., 2001). |
| Immunotoxicity | Antibody production and activation/proliferation of lymphocytes (Karol, 1998). |
| Genotoxicity | Mammalian cell gene mutation and chromosome aberration tests (EPA, 1998i, j). |

## Endpoint determination for *in vitro* testing systems

A good *in vitro* testing system should be sensitive but at the same time yield low levels of false positive and false negative results. It should have endpoint measurements that (i) show dose response relationships for a given toxin, (ii) reflect and are predictive of the *in vivo* pattern of toxicity for a given group of agents, (iii) are objective and reproducible, and (iv) have internal controls. It is also useful if the testing system involves rapid assays of toxicity, allowing medium to high throughput analysis and simultaneous testing of multiple compounds and/or doses. The testing system should also be relatively inexpensive and involve technology and skills that are easily transferable to other laboratory personnel. Importantly, for a testing system to be deemed reliable for the prediction of acute *in vivo* human systemic toxicity it should have been validated through a rigorous international multi-centre validation programme. For example, during the period 1989–1996, 97 international laboratories tested the same reference chemicals (which represent different classes of chemicals with varied human toxicity) in their own *in vitro* systems. The outcomes of these studies are available on an Internet database (URL http://www.cctoxconsulting.a.se/meic.htm) and various aspects of the overall study have been published in eight articles in Alternatives to Laboratory Animals. Over the past 10 years or so, recommendations governing validation of *in vitro* alternatives, including systems for measuring chronic effects, have been published and are regularly reviewed by international organizations such as ECVAM (European Centre for Validation of Alternative Methods), ICCVAM (The Interagency Coordinating Committee on the Validation of Alternative Methods) and OECD. The interested reader is referred to the following articles for more recent publications on validated systems for toxicity testing (Liebsch and Spielmann, 2002; Bhogal *et al*., 2005).

The best way to achieve something approaching an ideal testing system is to include a battery of endpoint measurements in order to minimize the occurrence of false negatives and false positive results. Endpoint determination should give an objective assessment of a cytostatic, cytotoxic or other functional effect. It should also be quantitative or reproducibly qualitative. The selection of endpoints chosen should enable the categorization of toxins in terms of their toxicity relative to other agents of the same or other groups, giving reproducible results in different laboratory settings.

Endpoints can take a variety of forms, including measurements of cell viability, metabolic activity, morphology, changes in protein and gene expression and/or altered subcellular distribution of markers of interest. Some of the main endpoints are summarized below and the reader is referred to other references for further background information (Masters, 2000) and the INVITTOX web site (http://embryo.ib.amwaw.edu.pl/invittox/) for further information and technical details about specific protocols used in toxicity testing *in vitro*.

### *Cell viability*

The traditional method of determining cell viability is to determine the cell density (cells/ml) in a haemocytometer chamber. While this is a very useful method for determining cell number for seeding in cell culture experiments, it does not distinguish between viable and non-viable cells following exposure to toxin. However, the proportion of non-viable cells can be determined in parallel by assessing the percentage of counted cells that take up the dye Trypan blue, which is excluded from viable cells. Though effective, this approach is not suited to high throughput, as measurement can be time consuming and is therefore best suited to small numbers of samples or treatments.

An alternative approach is to use dye uptake assays. For example, neutral red accumulates on the lysosomes of viable cells, after which it can be extracted from cells with an organic solvent and determined spectrophotometrically. A variation on this is to prelabel cells with the dye prior to treatment with toxin; reduced levels of absorbance compared to untreated control cells would be indicative of dye release due to membrane damage.

Appropriately equipped laboratories might choose to use a dual fluorescence assay to determine the proportion of live and dead cells. In one such system, fluorescein diacetate is taken up by viable cells and cleaved by esterases to release fluorescein producing green fluorescence in the cytoplasm, whereas propidium iodide only crosses the membrane of damaged cells and stains the nucleus (DNA) producing red fluorescence (Coder, 1997). Specimens are observed using a fluorescence microscope and quantification can be achieved by image analysis or by FACS analysis.

### Membrane leakage

Leakage of macromolecule through the plasma membrane into the culture medium is an effective means of detecting early or late stages of membrane damage. Cells can be incubated in the presence of $^{3}$H-thymidine, which is incorporated into DNA during cell proliferation. Subsequent loss of $^{3}$H-labelled DNA would be indicative of cell lysis. An alternative approach is to label cells in the presence of $^{51}$Cr which binds to many cellular proteins. Loss of labelled proteins from treated cells would be indicative of membrane leakage/cell lysis. In this sense the $^{51}$Cr release assay is more sensitive than that for $^{3}$H-labelled DNA as the former will detect signs of damage to the membrane at a much earlier stage than the latter.

An attractive alternative to radioisotopic methods is the release of lactate dehydrogenase (LDH) into cell culture medium, which is measured spectrophotometrically. The assay shows sensitivity comparable to the $^{51}$Cr release assay and is amenable to microtitre plate format, which would facilitate medium to high throughput analysis.

### Cell growth and proliferation

Cell growth or proliferation can be measured in a number of ways.

#### *Cloning efficiency*

In the absence of sophisticated equipment, perhaps the simplest method is that of cloning efficiency. In this case, cells would be plated at a density of 100–200 per culture vessel and assessed microscopically for their ability to produce viable colonies after a given period of incubation in the presence and absence of toxin. This is one of the most sensitive methods used for testing the effects of toxins on the growth of mitotic cells.

#### *Mitogenicity*

This can be determined in a number of ways. For example, the mitotic index of cell cultures can be determined by counting the percentage of mitotic figures following staining of DNA with haematoxylin. Alternatively, the proportion of cells in S-phase can be determined by immunohistochemical staining of cells incubated with the thymidine analogue 5-bromo, 2-deoxyuridine.

#### *DNA synthesis*

Another approach is the measurement of cell growth in toxin-exposed cells is via the incorporation of $^{3}$H-thymidine into DNA (Flaskos *et al.*, 1994). This method gives a good quantitative assessment of the effects of toxins on cell proliferation, as the radioisotope is incorporated into cells during S-phase of the cell cycle. High throughput analysis requires the use of a cell harvester to lyse cells and transfer radiolabelled DNA onto filters prior to detection by scintillation counting. Alternatively, cell proliferation can be measured by the incorporation of 5-bromo, 2-deoxyuridine incorporated into S-phase cultured cells, and subsequent quantification by enzyme-linked immunoabsorbent assay (ELISA) (Lanier *et al.*, 1989). Total DNA content can also be determined by detecting fluorescence after incubation of cells with DNA binding dyes such as Hoechst 33258, using a spectrofluorimetric microplate reader or by FACS analysis (Downs and Wilfinger, 1983).

#### *Protein synthesis*

Cell growth can also be measured by total protein content or by protein synthesis. Protein content can be estimated by a number of dye binding assays in microtitre plate format, such as the bicinchoninic acid (BCA) assay (Tuszynski and Murphy, 1990). More rapid analysis can be achieved by fixing and staining cells directly with Coomassie brilliant blue. After washing, the dye is solubilized and the absorbance of protein bound dye measured spectrophotometrically. As a measure of total protein synthesis, cells can be incubated in the presence of radiolabelled amino acids such as $^{35}$S-methionine or $^{14}$C-leucine and TCA precipitable protein assessed for radioactive content by scintillation counting. If an assessment of specific protein changes is required, protein extracts can be separated by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) and proteins stained with Coomassie blue or another protein staining dye. Changes in radiolabelled protein synthesis, which could be masked by the pre-existing protein pool, can be detected by autoradiography following exposure of dried gels onto X-ray film.

### Cell morphology

Changes in cell morphology can be measured microscopically and are very useful in studies of mechanisms of toxicity. However, medium to high throughput analysis of toxicity would require the use of image analysis software to produce more consistent data. Specialist techniques such as Allen video-enhanced contrast differential interference contrast (AVEC-DIC) microscopy facilitate analysis of effects on living cells. Furthermore, high throughput assays could be

developed that measure the underlying molecular changes determined from follow-up studies.

Morphological changes can take various forms, as indicated below.

*Cell volume*

An increase or decrease in cell volume could indicate osmotic changes or may represent the early stages of cell death by necrosis or apoptosis. Cell death or viability changes would then need to be made to confirm the type of cell death occurring, as indicated earlier.

*Cell shape*

Changes in shape may occur following exposure to a toxin. These could include rounding up, flattening, spreading or process outgrowth in cell culture monolayers. Such changes would give an initial indication of altered cell attachment, migration, proliferation or differentiation, indicating potential targets for follow-up molecular studies. For example, OP-induced changes in axon outgrowth in cultured neurons indicated possible changes in proteins associated with axon growth and maintenance, which were then targeted in molecular studies (Hargreaves *et al.*, 2006).

*Membrane integrity*

Changes in membrane integrity may be indicated by surface blebbing, which can occur as a result of cellular stress (e.g. oxidative stress) or during the early stages of apoptosis. These parameters could be measured further by biochemical methods to confirm the underlying molecular events associated with these morphological changes (e.g. free radical generation, lipid peroxidation, caspase activation, etc.).

*Growth patterns*

Growth patterns may change as a result of exposure to toxin. Thus, the proportion of cells growing in colonies or singly would indicate changes in cell:cell interactions and potential changes in cell adhesion proteins, which could be targeted in subsequent molecular studies.

**Metabolic assay**

*ATP levels*

ATP is an essential requirement for many energy dependent processes and its levels can be affected by a variety of toxins. It can be used as a marker for cell viability as it is present in all metabolically active cells and its levels decline rapidly when cells undergo apoptosis or necrosis (Kangas *et al.*, 1984). A number of reagents and kits suitable for high throughput screening are available. Typically, the amount of ATP in cell lysates is determined by the light released from firefly luciferase-catalysed oxidation of D-luciferin in the presence of ATP and oxygen. ATP and its major metabolites (ADP and AMP) in cell culture extracts can also be determined using an HPLC method (Yang and Gupta, 2003).

*Mitochondrial activity*

The activity of succinate dehydrogenase can be assayed by the reduction of the yellow methyl tetrazolium dye to its blue formazan reaction product. The reduced dye forms crystals in metabolically active cells and, after removal of growth medium, can be solubilized in an organic solvent such as dimethyl sulphfoxide and quantified spectrophotometrically in microtitre plate format, e.g. in assays of *in vitro* toxicity of OPs (Hargreaves *et al.*, 2006).

Although metabolic assays such as those indicated above are not direct measurements of cell viability, a significant fall in either measurement is normally taken to indicate a fall in cell viability or cell number.

**Calcium homeostasis**

Many toxins have a direct or indirect effect on mechanisms involved in the control of $Ca^{2+}$ homeostasis. Such effects could be related to a breakdown in plasma membrane integrity, which would also be detected by other methods discussed previously (e.g. leakage of LDH), or it may be the result of a selective effect on $Ca^{2+}$ channels or $Ca^{2+}$ ATPases that regulate $Ca^{2+}$ movements across cellular membranes. Changes in $Ca^{2+}$ flux can be measured using a variety of $Ca^{2+}$ binding dyes that fluoresce when bound to $Ca^{2+}$ (e.g. Quin 2 and Fura 2). Detection requires spectrofluorimetric analysis which is potentially applicable to high throughput analysis of average change for a given cell population. However, analysis of changes in $Ca^{2+}$ flux in individual cells requires a system that has an integrated microscope and image analysis software package.

**Cell or tissue specific markers of toxicity**

This approach is useful for targeting key proteins or enzyme activities involved in the normal function(s) of the target cells or tissues. It is particularly useful in sublethal/ chronic studies of exposure to toxin, in order to determine whether a potentially reversible functional deficit occurs. The principal approaches used at the protein level are those of antibody-based detection and enzyme assays.

*Antibody-based detection*

This involves mainly immunoblotting techniques such as Western blotting and dot blotting coupled with densitometric analysis of antibody reactivity with target antigens. In Western blotting the cell extracts are normally denatured, separated by SDS-PAGE and transferred electrophoretically onto nitrocellulose membrane filters (Towbin *et al.*, 1979) thus creating a replica of the gel pattern that is more accessible to antibody probes. By contrast, in dotblotting an

extract is applied direct in a circular dot onto the membrane filter, without chromatographic separation of proteins. Alternatively, experiments could be carried out on microtitre plates and the levels of antigens of interest determined in fixed cell monolayers by ELISA. Changes in the cellular or tissue distribution of antigens of interest can also be determined by immunohistochemical staining methods using enzyme conjugated or fluorophore-conjugated antibody detection systems in a light microscope or fluorescence microscope, respectively. In the case of fluorescence microscopy quantification of antigen levels or distribution can be done with the aid of image analysis software. These approaches have been used effectively in mechanistic studies of OP toxicity (Hargreaves *et al*., 2006).

*Enzyme assays*

Many cell and tissues can be monitored by the activity of enzymes that are known targets of the xenobiotics in question. For example, as discussed earlier, AChE activity is a good indicator of the acute neurotoxic effects of cholinesterase inhibitors such as OPs and carbamates *in vitro*, whereas NTE is a marker of the delayed neurodegenerative capability of OPs (Ehrich *et al*., 1997). There are also enzymes that provide useful markers of specific cell types, such as tyrosine hydroxylase for dopaminergic neurons (Kuhn and Geddes, 2000), 2′,3′-cyclic nucleotide 3′-phosphohydrolase for glial cells (McMorris, 1977) and alanine aminotransferase for hepatocytes (Amacher *et al*., 1998). Where possible, changes in expression of the enzyme should also be taken into account when interpreting specific activity data.

*Proteomic analysis*

This approach can be useful in mechanistic studies of toxicity as it helps to identify novel protein markers of toxicity. Techniques of this type include the use of two-dimensional PAGE, chromatographic or protein chip fractionation of proteins from cell/tissue lysates or subcellular in order to identify changes in specific protein levels following toxin exposure. Proteins of interest are then normally digested with trypsin and the tryptic fragments are initially identified by "peptide mass fingerprinting" in a matrix-assisted laser desorption ionization–time of flight (MALDI–TOF) mass spectrometer, followed by further confirmation using, e.g. tandem mass spectrometry (MS–MS) (Steen and Mann, 2004). Identification is facilitated by the use of freely available Internet-based gene and protein sequence databases such as MASCOT, SwissProt, etc.

*Genomic analysis*

Changes in the levels of specific proteins detected by proteome analysis could reflect changes in the regulation of protein turnover (i.e. how rapidly it is degraded once synthesized) or changes in gene expression. Furthermore, depending on the cell or tissue fractionation procedures adopted, changes in low abundance proteins that could be functionally important may not necessarily be detected by proteomic methods. Changes in gene expression following exposure to toxin can be determined by a number of methods including reverse transcriptase polymerase chain reaction (RT-PCR) and DNA microarray analysis (Koizimo and Yamada, 2003).

RT-PCR is a targeted approach involving the production of complementary DNA (cDNA), by treatment of cell or tissue RNA with RT. The resultant cDNA is then incubated with primers (probes) that recognize specific genes or DNA sequences of interest, followed by amplification of such sequences by the Taq polymerase reaction. Changes in the level of amplified target sequence would indicate corresponding changes in the expression of the target gene to which the primers are matched. This approach is only semi-quantitative although accuracy can be improved by continuous measurement of amplicon levels using real time RT-PCR. Levels of amplicon are normally compared to changes in household genes such as glyceraldehyde dehydrogenase, as an internal control.

A less targeted approach that, nevertheless, could help to identify novel changes in gene expression is that of DNA microarray analysis. In this technique, up to several thousand genes are immobilized on a template and probed with labelled RNA from control and treated cells or tissues. Image analysis software is then used to determine up- or down-regulation of genes due to toxin exposure. Controls usually involve averaged changes for a series of household genes. The researcher should be aware that a change in levels of gene expression does not necessarily indicate a corresponding change in protein levels or vice versa, as there are multiple levels of control. A multi-disciplinary approach is recommended to get an accurate overall picture of the chain of events following exposure to toxin.

## GENERAL SUMMARY AND CONCLUSIONS

A major issue facing toxicological science today is how to convert experimental data from *in vivo* and *in vitro* models into knowledge about molecular mechanisms of toxicity and safe levels of exposure to the agents tested. A number of animal models that have been used for many years for screening purposes, have been gradually refined with increasing emphasis on improved experimental design, animal welfare and reduced animal use. More recent developments in the area of *in vitro* toxicity testing have contributed significantly to the gradual reduction in animal use for toxicity screening and have helped to improve knowledge relating to mechanisms of cell- or

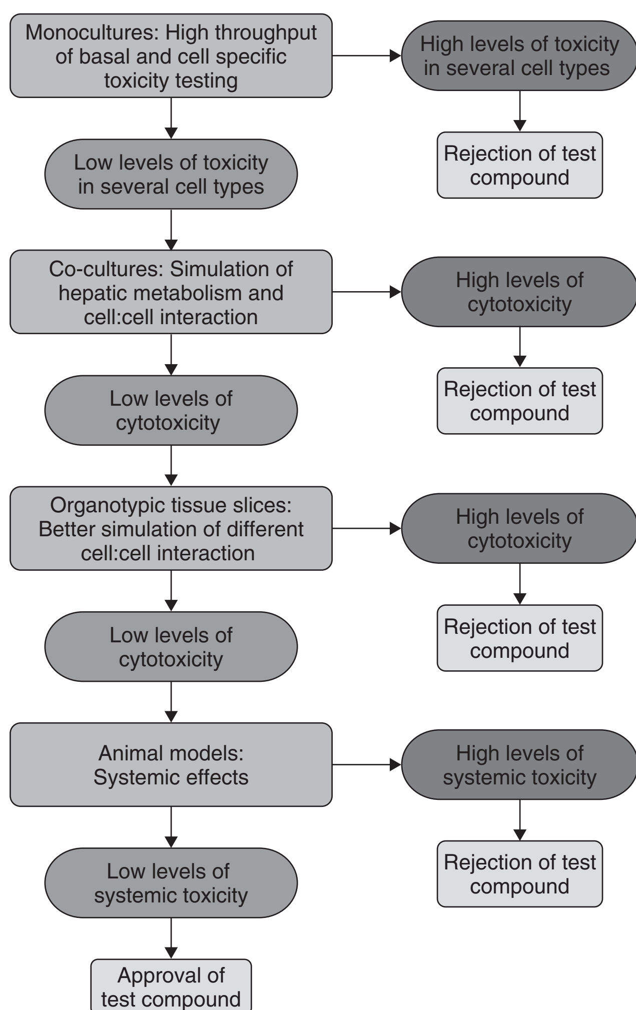


**FIGURE 4.3** Flow chart showing principles of multi-tiered approach to toxicity testing.

organ-specific toxicity. Veterinarians have played an important role in the development of legislation and guidelines relating to the use and replacement of laboratory animals in toxicity assessment.

The generally accepted approach to screening compounds for toxicity is to use a multi-tiered system starting with simple monocultures of specific cell types, followed by co-cultures that simulate metabolic effects and/or cell:cell interactions in the whole organism, before carrying out final testing on animals (Figure 4.3). In this way, it is possible to eliminate compounds that exhibit high levels of basal toxicity in several cell types and reduce unnecessary suffering in animals. In both *in vitro* and *in vivo* testing it is important to use a battery of appropriate endpoint measurements of both basal toxicity and cell/tissue specific toxicity at each level, in order to increase the reliability and predictive potential of the data produced.

*In vitro* toxicology has made significant advances in the last few decades, by improving and finding solutions to its limitations. For example, cell culture systems are now being designed to be metabolically competent and viable for several weeks, making them appropriate not only for short but for long-term toxicity testing (Bhogal *et al.*, 2005). Extending this approach to a wider range of cell culture systems will improve the prospect of using *in vitro* methods for studying a wider range of chronic toxicological phenomena. However, further development and validation of *in vitro* models is required if they are to be adopted on a much wider scale in order to secure further reduction, replacement and refinement of animal models. This will require further improvements in cell culture technology and growth conditions, such as the increased use of hollow fibre systems to facilitate continuous replenishment of media and supplements (Nussler *et al.*, 2001). The increased use of microsomal activation systems and/or established cell lines transfected with genes for mixed function oxidases is another approach that will help to increase the metabolic competence of *in vitro* models. Another promising *in vitro* approach involves the use of adult or embryonic stem cells of human or rodent origin. These cells can be either continuously cultured without being differentiated or they can generate a wide variety of functional mammalian cell types. In addition to its application in developmental toxicity testing, stem cell technology has been successfully used to study mechanisms of toxicity and to screen new pharmaceutical compounds for teratogenicity, genotoxicity, hepatotoxicity and cardiotoxicity (Davila *et al.*, 1998; 2004).

The development of medium to high throughput methods in proteomics, genomics and metabolomics, together with the revolution in bioinformatics, will lead to the accumulation of vast toxicological information, helping to elucidate mechanisms of toxicity in both *in vitro* and *in vivo* models (Bhogal *et al.*, 2005). These approaches will also help in the development and establishment of new biomarkers of effect or exposure.

In conclusion, although it is a desirable goal to replace all animal testing, animal-based toxicological testing is likely to continue for the foreseeable future. Further improvements in animal models will make them more reliable indicators of human toxicity, while the development of improved *in vitro* systems will eventually minimize the use of animals for toxicity studies. The veterinarian's role will therefore be to continue monitoring and improving the welfare of animals used in toxicity testing and to advise on issues relating to the development of *in vitro* alternatives.

# REFERENCES

Amacher DM, Fasulo LM, Charuel C, Comby P, Beaumont K (1998) *In vitro* toxicity of zamifenacin (UK-76,654) and metabolites in primary hepatocyte cultures. *Xenobiotica* **28**: 895–908.

Arnold DL, Grice HC, Krewski DR (1990) *Handbook of In Vivo Toxicity Testing*. Academic Press, San Diego.

Auletta CS (2004) Current *in vivo* assays for cutaneous toxicity: local and systemic toxicity testing. *Pharmacol Toxicol* **95**: 201–8.

Autrup H, Jorgensen EC, Jensen O (1996) Aflatoxin B1 induced lacI mutation in liver and kidney of transgenic mice C57BL/6N: effect of phorone. *Mutagenesis* **11**: 69–73.

Barlow SM, Greig JB, Bridges JW, Carere A, Carpy AJM, Galli CL, Kleiner J, Knudsen I, Koeter HBWM, Levey LS, Madsen C, Mayer S, Narbonne J–F, Pfannkuch F, Prodanchuk MG, Smith MR, Steinberg P (2002) Hazard identification by methods of animal-based toxicology. *Food Chem Toxicol* **40**: 145–91.

Bhogal N, Grindon C, Combes R, Balls M (2005) Toxicity testing: creating a revolution based on new technologies. *TRENDS Biotechnol* **23**: 299–307.

Blakely BR, Yole MJ, Brousseau P, Boermans H, Fournier M (1999) Effects of chlorpyrifos on immune function in rats. *Vet Hum Toxicol* **41**: 140–4.

Buse E, Habermann G, Ostrburg I, Korte R, Weinbauer GF (2003) Reproductive/developmental toxicity and immunotoxicity assessment in the nonhuman primate model. *Toxicology* **185**: 221–7.

Chhabra RS, Huff JE, Schwetz BS, Selkirk J (1990) An overview of prechronic and chronic toxicity/carcinogenicity experimental designs and criteria used by the National Toxicology Programme. *Environ Health Prospect* **86**: 313–21.

Coder DM (1997) Assessment of cell viability. In *Current Protocols in Cytometry,* Robinson JP, Darzynkiewicz Z, Dobrucki J, Hyun W, Nolan J, Orfao A, Rabinovitch P (eds). John Wiley and Sons Inc., Somerset NJ, pp. 9.2.1–14.

Cohen J, Wilkin GP (1996) *Neural Cell Culture. A Practical Approach*. Oxford University Press, Oxford.

Combes RD, Gaunt I, Balls M (2004) A scientific and animal welfare assessment of the OECD health effects test guidelines for the safety testing under the European Union REACH system. *Altern Lab Anim* **32**: 163–208.

Davis J (2002) *Basic Cell Culture. A Practical Approach*. Oxford University Press, Oxford.

Davila JC, Rodriguez RJ, Melchert RB, Acosta Jr D (1998) Predictive value of *in vitro* model systems in toxicology. *Ann Rev Pharmacol Toxicol* **38**: 63–96.

Davila JC, Cezar GG, Thiede M, Strom S, Miki T, Trosko J (2004) Use and application of stem cells in toxicology. *Toxicol Sci* **79**: 214–23.

deBlois D, Horlick RA (2001) Endotoxin sensitization to kinin B(1) receptor agonist in non-human primate model: haemodynamic and pro-inflammatory effects. *Br J Pharmacol* **132**: 327–35.

Dhillon VS, Singh J, Singh H, Kler RS (1995) *In vitro* and *in vivo* genotoxicity of hormonal drugs. VI. Fluoxymesterone. *Mutat Res* **342**: 103–11.

Downs TR, Wilfinger WW (1983) Fluorometric quantification of DNA in cells and tissue. *Anal Biochem* **131**: 538–47.

Ehrich M, Correll L, Veronesi B (1997) Acetylcholinesterase and neuropathy target esterase inhibitions in neuroblastoma cells to distinguish organophosphorus compounds causing acute and delayed neurotoxicity. *Fundam Appl Toxicol* **38**: 55–63.

EPA (Environmental Protection Agency) (1998a) Health effects test guidelines OPPTS 870.7200 Companion animal safety. EPA 712-C-98-349.

EPA (Environmental Protection Agency) (1998b) Health effects test guidelines OPPTS 870.3700 Prenatal developmental toxicity study. EPA 712-C-98-207.

EPA (Environmental Protection Agency) (1998c) Health effects test guidelines OPPTS 870.5450 Rodent dominant lethal assay. EPA 712-C-98-227.

EPA (Environmental Protection Agency) (1998d) Guidelines for neurotoxicity risk assessment. EPA FRL-6011-3. NTIS PB98-117831.

EPA (Environmental Protection Agency) (1998e) Health effects test guidelines OPPTS 870.6200 Neurotoxicity screening battery. EPA 712-C-98-238.

EPA (Environmental Protection Agency) (1998f) Health effects test guidelines OPPTS 870.6300 Developmental neurotoxicity study. EPA 712-C-98-239.

EPA (Environmental Protection Agency) (1998g) Health effects test guidelines OPPTS 870.7800 Immunotoxicity. EPA 712-C-98-351.

EPA (Environmental Protection Agency) (1998h) Health effects test guidelines OPPTS 870.5200 Mouse visible specific locus test. EPA 712-C-98-217.

EPA (Environmental Protection Agency) (1998i) Health effects test guidelines OPPTS 870.5300 *In vitro* mammalian cell gene mutation test. EPA 712-C-98-221.

EPA (Environmental Protection Agency) (1998j) Health effects test guidelines OPPTS 870.5300 *In vitro* mammalian chromosome aberration test. EPA 712-C-98-223.

EPA (Environmental Protection Agency) (2000) Health effects test guidelines OPPTS 870.3550 Reproduction/developmental toxicity screening test. EPA 712-C-00-367.

EPA (Environmental Protection Agency) (2003) Health effects test guidelines OPPTS 870.2600 Skin sensitization. EPA 712-C-03-197.

FDA (Food and Drug Administration) (1982) Toxicological principles for the safety assessment of direct food additives and color additives used in food: guidelines for long-term toxicity studies in the dog. Appendix II, 42–52.

Fentem JH, Briggs D, Chesne C, Elliott GR, Harbell JW, Heylings JR, Portes P, Roguet R, van de Sandt JJ, Botham PA (2001) A prevalidation study on *in vitro* tests for acute skin irritation. 2. Results and evaluation by the Management Team. *Toxicol In vitro* **15**: 57–93.

Flaskos J, McLean WG, Hargreaves AJ (1994) The toxicity of organophosphate compounds towards cultured PC12 cells. *Toxicol Lett* **70**: 71–6.

Foote RH, Carney EW (2000) The rabbit as a model for reproductive and developmental toxicity studies. *Reprod Toxicol* **14**: 477–93.

Fujita K, Lazarovici P, Guroff G (1989) Regulation of the differentiation of PC12 pheochromocytoma cells. *Environ. Health Perspect* **80**: 127–42.

Gabridge MG, Legator MS (1969) A host-mediated microbial assay for the detection of mutagenic compounds. *Proc Soc Exp Biol Med* **130**: 831.

Gad SC (1998) Toxicity testing, carcinogenesis. In *Encyclopedia of Toxicology*, Wexler P (ed.), vol. 3. Academic Press, San Diego, pp. 289–93.

Gad SC, Gad SC (2006) *Animal Models in Toxicology*. Marcel Dekker Inc., New York.

Galloway T, Handy R (2003) Immunotoxicity of organophosphorus pesticides. *Ecotoxicol* **12**: 345–63.

Gardner DK, Lane M, Watson AJ (2004) *A Laboratory Guide to the Mammalian Embryo. A Practical Approach*. Oxford University Press, Oxford.

Gossen JA, de Leeuw WJ, Vijg J (1994) LacZ transgenic mouse models: their application in genetic toxicology. *Mutat Res* **307**: 451–9.

Hargreaves AJ, Fowler MJ, Sachana M, Flaskos J, Bountouri M, Coutts IC, Glynn P, Harris W, McLean WG (2006) Inhibition of neurite outgrowth in differentiating mouse N2a neuroblastoma cells by phenyl saligenin phosphate: effects on MAP kinase (ERK 1/2) activation, neurofilament heavy chain phosphorylation and neuropathy target esterase. *Biochem Pharmacol* **71**: 1240–7.

Hurtt ME, Cappon GD, Browning A (2003) Proposal for a tiered approach to developmental toxicity testing for veterinary pharmaceutical products for food-producing animals. *Food Chem Toxicol* **41**: 611–19.

IPCS (International Programme on Chemical Safety) (1996) Principles and methods for assessing direct immunotoxicity associated with exposure to chemicals. Environmental Health Criteria, vol. 180. International Programme on Chemical Safety. World Health Organization. Geneva.

Kacew S (2001) Confounding factors in toxicity testing. *Toxicology* **160**: 87–96.

Kangas L, Gronroos M, Nieminen AL (1984) Bioluminescence of cellular ATP: a new method for evaluating cytotoxic agents *in vitro*. *Med Biol* **62**: 338–43.

Karol MH (1998) Target organs and systems: methodologies to assess immune system function. *Environ Health Perspect* **106**: 533–40.

Kaufmann W (2003) Current status of developmental neurotoxicity: an industry prospective. *Toxicol Lett* **140–1**: 161–9.

Knudsen I (1999) Temporal equivalence between test species and humans: general toxicity issues. *Regul Toxicol Pharmacol* **30**: 42–7.

Koizimo S, Yamada H (2003) DNA microarray analysis of altered gene expression in cadmium-exposed human cells. *J Occup Health* **45**: 331–4.

Kuhn DM, Geddes TJ (2000) Molecular footprints of neurotoxic amphetamine action. *Ann NY Acad Sci* **914**: 92–103.

Lanier TL, Berger EK, Eacho PI (1989) Comparison of 5-bromo-2-deoxyuridine and [$^3$H]thymidine for studies of hepatocellular proliferation in rodents. *Carcinogenesis* **10**: 1341–3.

Liebsch M, Spielmann H (2002) Currently available *in vitro* methods used in the regulatory toxicology. *Toxicol Lett* **127**: 127–34.

Loomis TA, Hayes AW (1996) Toxicologic testing methods. In *Loomis's Essentials of Toxicology*, Loomis TA, Hayes AW (eds). Academic Press, San Diego, pp. 205–48.

Masters J (2000) *Animal Cell Culture. A Practical Approach*. Oxford University Press, Oxford.

McMorris AF (1977) Norepinephrine induces glial-specific enzyme activity in cultured glioma cells. *Proc Natl Acad Sci USA* **74**: 4501–4.

Nebbia C (2001) Biotransformation enzymes as determinants of xenobiotic toxicity in domestic animals. *Vet J* **161**: 238–52.

Noraberg J (2004) Organotypic brain slice cultures an efficient and reliable method for neurotoxicological screening and mechanistic studies. *Altern Lab Animals* **32**: 329–37.

Nussler AK, Wang A, Neuhaus P, Fischer J, Yuan J, Liu L, Zeilinger K, Gerlach J, Arnold PJ, Albrecht W (2001) The suitability of hepatocyte culture models to study various aspects of drug metabolism. *ALTEX* **18**, 91–101.

OECD (Organisation for Economic Cooperation and Development) (1981a) Carcinogenicity studies. OECD guidance 451 adopted 12-05-1981.

OECD (Organisation for Economic Cooperation and Development) (1981b) Combined chronic toxicity/Carcinogenicity studies. OECD guidance 453 adopted 12-05-1981.

OECD (Organisation for Economic Cooperation and Development) (1984) Genetic toxicology: rodent dominant lethal test. OECD guidance 478 adopted 4-04-1984.

OECD (Organisation for Economic Cooperation and Development) (1987) Acute dermal toxicity. OECD guidance 402 adopted 24-02-1987.

OECD (Organisation for Economic Cooperation and Development) (1995a) Delayed neurotoxicity of organophosphorus substances. Following acute exposure. OECD guidance 418 adopted 27-07-1995.

OECD (Organisation for Economic Cooperation and Development) (1995b) Delayed neurotoxicity of organophosphorus substances: 28 day repeated dose study. OECD guidance 419 adopted 27-07-1995.

OECD (Organisation for Economic Cooperation and Development) (1995c) Repeated dose 28 day toxicity study in rodents. OECD updated guidance 407 adopted 27-07-1995.

OECD (Organisation for Economic Cooperation and Development) (1997a) Mammalian bone marrow chromosomal aberration test. OECD guidance 475 adopted 21-07-1997.

OECD (Organisation for Economic Cooperation and Development) (1997b) Unscheduled DNA synthesis (UDS) test with mammalian liver cells *in vivo*. OECD guidance 486 adopted 21-07-1997.

OECD (Organisation for Economic Cooperation and Development) (1997c) Neurotoxicity study in rodents. OECD guidance 424 adopted 21-07-1997.

OECD (Organisation for Economic Cooperation and Development) (2001a) Two generation reproduction toxicity study. OECD guidance 416 adopted 22-01-2001.

OECD (Organisation for Economic Cooperation and Development) (2001b) Prenatal developmental toxicity study. OECD guidance 414 adopted 22-01-2001.

OECD (Organisation for Economic Cooperation and Development) (2003) Developmental neurotoxicity study. OECD draft new guidelines 426.

Presgraves SP, Ahmed T, Borwege S, Joyce JN (2004) Terminally differentiated SH-SY5Y cells provide a model system for studying neuroprotective effects of dopamine agonists. *Neurotox Res* **5**: 579–98.

Pruett SB (1992) Immunotoxicity of organophosphorus compounds. In *Organophosphates, Chemistry, Fate and Effects*, Chambers JE, Levi PE (eds). Academic Press, New York, pp. 123–49.

Russell WM, Burch RL (1959) *The Principles of Humane Experimental Technique*. Methuen, London.

Sales KM, Kingston ST, Atterwill CK, Purcell WM (2000) Avian whole-brain spheroid cultures: applications in pesticide toxicity. *Pest Manag Sci* **56**: 825–7.

Shaw AJ (1996) *Epithelial Cell Culture. A Practical Approach*. Oxford University Press, Oxford.

Spielmann H (2005) Predicting the risk of developmental toxicity from *in vitro* assays. *Toxicol Appl Pharmacol* **207**: S375–80.

Steen H, Mann M (2004) The ABC's (and XYZ's) of peptide sequencing. *Nat Rev Mol Cell Biol* **5**: 699–711.

Sundstrom L, Morrison III B, Bradley M, Pringle A (2005) Organotypic cultures as tools for functional screening in the CNS. *Drug Discov Today* **10**: 993–1000.

Towbin S, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* **76**: 4350–4.

Tuszynski GP, Murphy A (1990) Spectrophotometric quantitation of anchorage-dependent cell numbers using the bicinchoninic acid protein assay reagent. *Anal Biochem* **184**: 189–91.

Tzanakakis ES, Waxman DJ, Hansen LK, Remmel RP, Hu WS (2002) Long-term enhancement of cytochrome P4502B1/2 expression in rat hepatocyte spheroids through adenovirus-mediated gene transfer. *Cell Biol Toxicol* **18**: 13–27.

Vail DM, Chun R, Thamm DH, Garrett LD, Cooley AJ, Obradovich JE (1998) Efficacy of pyridoxine to ameliorate the cutaneous toxicity associated with doxorubicin containing pegylated (Stealth) liposomes: a randomized, double-blind clinical trial using a canine model. *Clin Cancer Res* **4**: 1567–1571.

Valancius-Mangel V, Doetschman T (1999) Potential uses of transgenic and gene-targeted animals in toxicologic research. In *Molecular Biology of the Toxic Response* Puga A, Wallace KB (eds). Taylor and Francis, Philadelphia, pp. 27–51.

Wahnschaffe U, Bitsch A, Kielhorn J, Mangelsdorf I (2005a) Mutagenicity testing with transgenic mice. Part I: Comparison with the mouse bone marrow micronucleus test. *J Carcinog* **4**: 3.

Wahnschaffe U, Bitsch A, Kielhorn J, Mangelsdorf I (2005b) Mutagenicity testing with transgenic mice. Part II: Comparison with the mouse spot test. *J Carcinog* **4**: 4.

Winegar RA, Lutze LH, Hamer JD, O'Loughlin KG, Mirsalis JC (1994) Radiation-induced point mutations, deletions and micronuclei in lacI transgenic mice. *Mutat Res* **307**: 479–87.

Worth A, Balls M (2002) Alternative (non-animal) methods for chemicals testing: current status and future prospects. *Altern Lab Anim* **30**(Suppl. 1): 1–125.

Yang MS, Gupta RC (2003) Determination of energy charge potential in the C6 glioma and the Hepg-2 cell culture. *Toxicol Mechan Methods* **13**: 97–101.

Zeiger E (1998) Identification of rodent carcinogens and noncarcinogens using genetic toxicity tests: premises, promises and performance. *Regul Toxicol Pharmacol* **28**: 85–95.

C H A P T E R

# 5

# Epidemiology of animal poisonings

*Sharon M. Gwaltney-Brant*

## INTRODUCTION

Although animals are exposed to potentially toxic agents on a daily basis, actual poisoning cases are uncommon when compared to other conditions of veterinary concern (infectious disease, trauma, metabolic disease, neoplasia, etc.). Clients will often present their animal with a suspicion of poisoning, only for the veterinarian to determine that the animal is suffering from an unrelated ailment. When evaluating information regarding suspected poisoning cases, it is important to consider the full exposure and patient history before determining whether a particular exposure is related to a clinical syndrome, as temporal coincidence does not necessarily equal causality.

The fundamental rule of toxicology as stated by Paracelsus, considered to be the "Father" of toxicology, is "the dose makes the poison." Obviously the dose required to induce toxicosis will depend on a variety of factors, including the agent in question, species of animal exposed and route of exposure. Based on information from poison control centers, the majority of animal exposures to potentially toxic agents result in no signs developing (Hornfeldt and Murphy, 1992; Forrester and Stanley, 2004). However, animal poisonings *do* occasionally occur from exposures to natural or man-made hazards. Knowledge of the most common features of animal poisonings can aid in instituting measure that may help in minimizing exposures of animals to toxicants.

## DEMOGRAPHICS

Demographic information on animal poisonings has largely come from human or animal poison control centers that animal owners have contacted regarding potential exposures to toxic agents (Haliburton and Buck, 1983; Hornfeldt and Borys, 1985; Hornfeldt and Murphy, 1992, 1997, 1998; Forrester and Stanley, 2004) or from surveys of veterinary emergency centers or teaching hospitals (Osweiler, 1975; Cope *et al*., 2006). In all these reports, dogs and cats are the species that owners most frequently seek assistance with potential poisonings, accounting for 95–98% of all reported animal cases (Hornfeldt and Murphy, 1998; Xavier and Kogika, 2002; Forrester and Stanley, 2004; Giuliano Albo and Nebbia, 2004). Approximately 2–5% of reported cases involved other species of domesticated animals, exotic animals and wildlife. These percentages have changed considerably since the 1883, when dogs and cats accounted for 44% of calls to an animal poison control center, with production animals (bovine, porcine, ovine) and equines making up 35% of calls (Trammel *et al*., 1985).

Forrester and Stanley (2004) reported that exposures occurred more commonly in the summer, and this is consistent with data from the ASPCA Animal Poison Control Center's AnTox™ database (Figure 5.1), although a peak in December, associated with the holiday season, is also present in the APCC data (ASPCA Animal Poison Control Center, unpublished data, 2005). The majority (>90%) of animal poisonings are accidental and acute in nature and occur near or at the animal owner's home (Hornfeldt and Murphy, 1992, 1998; Khan *et al*., 1999). Malicious intent comprises less than 1% of all exposures to potentially toxic agents. The majority (70–95%) of exposures is due to acute ingestion, followed by acute dermal exposures (Hornfeldt and Murphy, 1992; Forrester and Stanley, 2004). Inhalation, envenomation, bites, ocular and parenteral routes of exposure account for less than 1% and chronic exposures comprise approximately 1% of all exposures. In 97% of exposures, a single agent is involved (Hornfeldt and Murphy, 1992, 1998).

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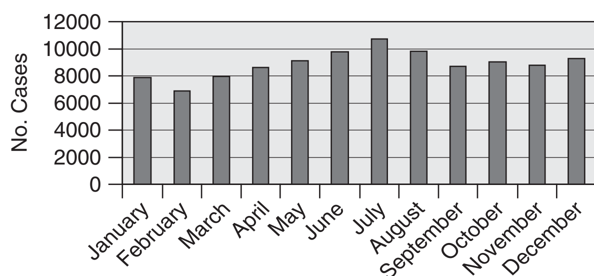


**FIGURE 5.1** Monthly distribution of cases managed by ASPCA Animal Poison Control Center in 2005 (unpublished data).

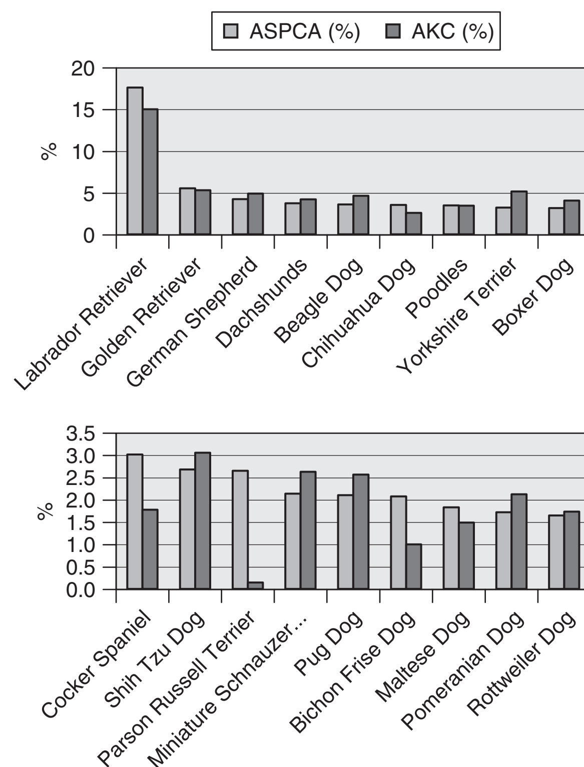


**FIGURE 5.2** Comparison of top twenty purebred dog breeds in relation to frequency of exposures to potentially toxic agents reported to the ASPCA Animal Poison Control Center with the relative popularity of the breed based on Registration Statistics from the American Kennel Club in 2006 (ASPCA Animal Poison Control Center, unpublished data, 2005).

## DOGS

Perhaps at least partly because of their inquisitive natures and willingness to investigate everything with their mouths, dogs far outrank other species when it comes to owners seeking aid for potential poisonings, making up 70–80% of all animal cases (Hornfeldt and Murphy, 1992, 1998; Xavier and Kogika, 2002; Forrester and Stanley, 2004).

There is a tendency in the veterinary community to consider certain breeds, such as Labrador retrievers, to be more prone to exposure to potentially toxic agents. However, to verify such an impression, one must take into account the relative popularity of a particular breed. In other words: Are there more poisoning cases with Labrador retrievers because they really are more inclined to misadventure, or is it just that there are more Labradors? A recent survey of the APCC database in 2005 (ASPCA Animal Poison Control Center, unpublished data, 2005) evaluated over 68,000 exposures of non-mixed breed dogs and compared the relative breed incidence to the 2005 Registration Statistics reported by the American Kennel Club, the oldest and largest purebred dog registry in the United States (American Kennel Club (AKC), 2006). The results of this comparison are displayed in Table 5.1 and the top 20 breeds are compared graphically in Figure 5.2. Labradors account for approximately 15% of AKC registered breeds, with almost three times more registered than the next most popular breed, the golden retriever. Labradors accounted for 17.5% of canine exposures in APCC cases, which was also three times more than the next most popular breed (the golden retriever) and which was not significantly different from the AKC statistics. In analyzing the data in Table 5.1, a few breeds do appear to be over-represented in regards to exposures to potentially toxic agents. For instance, the Bichon Frise ranks 26th in AKC registrations yet ranks 15th in the APCC data, occurring in APCC cases (2.1%) at twice the incidence of AKC (1%); Welsh springer spaniels make up 0.03% of AKC registered dogs, but account for 0.18% of APCC dogs. However, care must be taken when evaluating the data from certain breeds (such as greyhounds, border collies and Parson Russell terriers), as many individuals of these breeds are registered with their own independent breed registry rather than the AKC; this may be one reason all three of these dog breeds appear over-represented in APCC data.

Previous reports of poisoning cases in dogs have indicated no gender predisposition, and most reports indicate that adults are most commonly involved, with an average age of approximately 4 years (±3.6 years) (Khan *et al*., 1999; Cope *et al*., 2006). Given the wide age ranges in these studies (0.15–15 years) and the relatively low average age, it would appear that young adult dogs are most inclined to be exposed to potentially toxic agents.

## CATS

Due, perhaps, to their more discriminating habits and appetites, cats account for only 11–20% of reported animal exposures to potential toxicants, which is three times less frequent than dogs (Hornfeldt and Murphy, 1992, 1998;

**TABLE 5.1 Comparison of purebred dog breeds in relation to frequency of exposures to potentially toxic agents reported to the ASPCA Animal Poison Control Center with the relative popularity of the breed based on registration statistics from the American Kennel Club**

| Breed | ASPCA (%) | AKC (%) | ASPCA rank | AKC rank |
|---|---|---|---|---|
| Labrador Retriever | 17.589 | 14.972 | 1 | 1 |
| Golden Retriever | 5.518 | 5.268 | 2 | 2 |
| German Shepherd Dog | 4.206 | 4.889 | 3 | 4 |
| Dachshunds | 3.711 | 4.188 | 4 | 6 |
| Beagle Dog | 3.565 | 4.626 | 5 | 5 |
| Chihuahua Dog | 3.535 | 2.560 | 6 | 11 |
| Poodles | 3.453 | 3.436 | 7 | 8 |
| Yorkshire Terrier | 3.193 | 5.130 | 8 | 2 |
| Boxer Dog | 3.150 | 4.047 | 9 | 7 |
| Cocker Spaniel | 3.012 | 1.775 | 10 | 15 |
| Shih Tzu Dog | 2.677 | 3.050 | 11 | 9 |
| Parson Russell Terrier | 2.647 | 0.145 | 12 | 72 |
| Miniature Schnauzer Dog | 2.131 | 2.622 | 13 | 10 |
| Pug Dog | 2.099 | 2.560 | 14 | 12 |
| Bichon Frise Dog | 2.073 | 0.997 | 15 | 26 |
| Maltese Dog | 1.829 | 1.485 | 16 | 19 |
| Pomeranian Dog | 1.718 | 2.119 | 17 | 14 |
| Rottweiler Dog | 1.647 | 1.728 | 18 | 16 |
| Siberian Husky Dog | 1.566 | 1.026 | 19 | 25 |
| Border Collie Dog | 1.539 | 0.258 | 20 | 55 |
| Australian Shepherd Dog | 1.331 | 0.676 | 21 | 34 |
| Miniature Pinscher Dog | 1.312 | 1.244 | 22 | 22 |
| Shetland Sheepdog | 1.120 | 1.551 | 23 | 18 |
| Boston Terrier | 1.074 | 1.722 | 24 | 17 |
| West Highland White Terrier | 0.980 | 0.825 | 25 | 32 |
| Weimaraner Dog | 0.844 | 0.936 | 26 | 29 |
| Basset Hound | 0.839 | 0.965 | 27 | 27 |
| Lhasa Apso Dog | 0.795 | 0.426 | 28 | 39 |
| Doberman Pinscher Dog | 0.782 | 1.267 | 29 | 21 |
| German Shorthaired Pointer | 0.670 | 1.441 | 30 | 20 |
| Cavalier King Charles Spaniel | 0.667 | 0.834 | 31 | 31 |
| English Springer Spaniel | 0.664 | 0.950 | 32 | 28 |
| Pembroke Welsh Corgi Dog | 0.626 | 1.156 | 33 | 23 |
| Brittany Dog | 0.619 | 0.853 | 34 | 30 |
| Australian Cattle Dog | 0.614 | 0.156 | 35 | 70 |
| Dalmatian Dog | 0.578 | 0.113 | 36 | 77 |
| Cairn Terrier | 0.569 | 0.406 | 37 | 41 |
| Great Dane Dog | 0.558 | 1.047 | 38 | 24 |
| Soft Coated Wheaten Terrier | 0.530 | 0.219 | 39 | 59 |
| Chow Chow Dog | 0.517 | 0.182 | 40 | 64 |
| Bulldog | 0.601 | 2.232 | 41 | 13 |
| Greyhound | 0.485 | 0.015 | 42 | 134 |
| Pekingese Dog | 0.464 | 0.374 | 43 | 48 |
| Vizsla Dog | 0.379 | 0.387 | 44 | 44 |
| Newfoundland Dog | 0.377 | 0.379 | 45 | 46 |
| Portuguese Water Dog | 0.377 | 0.153 | 46 | 71 |
| Papillon Dog | 0.371 | 0.652 | 47 | 35 |
| Rhodesian Ridgeback Dog | 0.360 | 0.263 | 48 | 54 |
| Shiba Inu Dog | 0.333 | 0.174 | 49 | 66 |
| American Eskimo Dog | 0.319 | 0.045 | 50 | 108 |
| Airedale Terrier | 0.303 | 0.290 | 51 | 52 |
| Collies | 0.434 | 0.554 | 52 | 36 |
| Bernese Mountain Dog | 0.282 | 0.378 | 53 | 47 |
| Havanese Dog | 0.274 | 0.390 | 54 | 43 |
| Akita Dog | 0.272 | 0.308 | 55 | 51 |
| Mastiff Dog | 0.266 | 0.738 | 56 | 33 |
| Chinese Shar pei Dog | 0.264 | 0.385 | 57 | 45 |
| Chesapeake Bay Retriever | 0.256 | 0.362 | 58 | 49 |
| Scottish Terrier | 0.252 | 0.412 | 59 | 40 |
| Wirehaired Fox Terrier | 0.249 | 0.124 | 60 | 76 |
| English Setter Dog | 0.246 | 0.071 | 61 | 97 |
| American Staffordshire Terrier | 0.245 | 0.182 | 62 | 63 |
| Italian Greyhound | 0.229 | 0.252 | 63 | 56 |
| Toy Fox Terrier | 0.207 | 0.082 | 64 | 88 |
| Silky Terrier | 0.208 | 0.175 | 65 | 65 |
| French Bulldog | 0.205 | 0.457 | 66 | 38 |
| Samoyed Dog | 0.204 | 0.133 | 67 | 75 |
| Alaskan Malamute Dog | 0.195 | 0.223 | 68 | 58 |
| Whippet Dog | 0.185 | 0.190 | 69 | 61 |
| Welsh Springer Spaniel | 0.179 | 0.028 | 70 | 124 |
| Pointer | 0.178 | 0.042 | 71 | 110 |
| Bullmastiff Dog | 0.172 | 0.398 | 72 | 42 |
| Great Pyrenees Dog | 0.166 | 0.242 | 73 | 57 |
| Tibetan Terrier | 0.165 | 0.076 | 74 | 93 |
| English Cocker Spaniel | 0.163 | 0.143 | 75 | 74 |
| Saint Bernard Dog | 0.160 | 0.473 | 76 | 37 |
| Schipperke Dog | 0.156 | 0.108 | 77 | 80 |
| Bloodhound | 0.150 | 0.338 | 78 | 50 |
| Old English Sheepdog | 0.144 | 0.159 | 79 | 69 |
| Irish Setter Dog | 0.132 | 0.165 | 80 | 67 |
| Welsh Terrier | 0.128 | 0.079 | 81 | 90 |
| Standard Schnauzer Dog | 0.127 | 0.064 | 82 | 99 |
| Border Terrier | 0.124 | 0.103 | 83 | 82 |
| Chinese Crested Dog | 0.122 | 0.281 | 84 | 53 |
| Keeshond Dog | 0.116 | 0.077 | 85 | 91 |
| Japanese Chin Dog | 0.108 | 0.161 | 86 | 68 |
| Brussels Griffon Dog | 0.106 | 0.196 | 87 | 60 |
| Flat Coated Retriever | 0.100 | 0.064 | 88 | 98 |
| Basenji Dog | 0.096 | 0.107 | 89 | 81 |
| Staffordshire Bull Terrier | 0.092 | 0.093 | 90 | 84 |
| Cardigan Welsh Corgi Dog | 0.090 | 0.111 | 91 | 79 |
| Irish Terrier | 0.084 | 0.034 | 92 | 117 |
| Bull Terrier | 0.080 | 0.189 | 93 | 63 |
| Belgian Malinois Dog | 0.079 | 0.085 | 94 | 87 |
| Norwegian Elkhound | 0.071 | 0.073 | 95 | 96 |
| Bearded Collie Dog | 0.070 | 0.053 | 96 | 101 |
| Giant Schnauzer Dog | 0.066 | 0.112 | 97 | 78 |
| Belgian Sheepdog | 0.064 | 0.040 | 98 | 111 |
| Norwich Terrier | 0.063 | 0.080 | 99 | 89 |
| American Foxhound | 0.055 | 0.005 | 100 | 151 |
| Bouvier Des Flandres Dog | 0.054 | 0.092 | 101 | 85 |
| Nova Scotia Duck Tolling Retriever | 0.054 | 0.038 | 102 | 113 |
| Gordon Setter Dog | 0.054 | 0.088 | 103 | 86 |
| Black and Tan Coonhound | 0.052 | 0.017 | 104 | 131 |

(*Continued*)

**TABLE 5.1 (Continued)**

| Breed | ASPCA (%) | AKC (%) | ASPCA rank | AKC rank |
|---|---|---|---|---|
| Smooth Fox Terrier | 0.052 | 0.052 | 105 | 103 |
| King Charles English Toy Spaniel | 0.051 | 0.028 | 106 | 123 |
| Neapolitan Mastiff Dog | 0.049 | 0.034 | 107 | 116 |
| Norfolk Terrier | 0.047 | 0.040 | 108 | 112 |
| Greater Swiss Mountain Dog | 0.045 | 0.076 | 109 | 92 |
| Irish Wolfhound | 0.044 | 0.096 | 110 | 83 |
| Manchester Terrier | 0.044 | 0.050 | 111 | 104 |
| German Wirehaired Pointer | 0.035 | 0.144 | 112 | 73 |
| Petit Basset Griffon Vendeen Dog | 0.035 | 0.031 | 113 | 118 |
| Belgian Tervuren Dog | 0.032 | 0.055 | 114 | 100 |
| Australian Terrier | 0.031 | 0.045 | 115 | 107 |
| Afghan Hound | 0.028 | 0.073 | 116 | 95 |
| Tibetan Spaniel | 0.028 | 0.052 | 117 | 102 |
| Pharaoh Hound | 0.023 | 0.012 | 118 | 140 |
| Italian Spinoni Dog | 0.023 | 0.036 | 119 | 115 |
| Bedlington Terrier | 0.022 | 0.030 | 120 | 120 |
| Lakeland Terrier | 0.022 | 0.019 | 121 | 129 |
| Borzoi Dog | 0.020 | 0.076 | 122 | 94 |
| Field Spaniel | 0.020 | 0.015 | 123 | 135 |
| Clumber Spaniel | 0.019 | 0.029 | 124 | 122 |
| Irish Water Spaniel | 0.019 | 0.014 | 125 | 136 |
| Kerry Blue Terrier | 0.017 | 0.048 | 126 | 105 |
| Finnish Spitz Dog | 0.016 | 0.007 | 127 | 148 |
| Miniature Bull Terrier | 0.015 | 0.023 | 128 | 126 |
| Wirehaired Pointing Griffon Dog | 0.013 | 0.045 | 129 | 109 |
| Saluki Dog | 0.013 | 0.031 | 130 | 119 |
| Lowchen Dog | 0.013 | 0.012 | 131 | 139 |
| Briard Dog | 0.012 | 0.030 | 132 | 121 |
| Puli Dog | 0.010 | 0.017 | 133 | 132 |
| Affenpinscher Dog | 0.009 | 0.026 | 134 | 125 |
| Polish Sheepdog | 0.009 | 0.013 | 135 | 138 |
| Ibizan Hound | 0.009 | 0.017 | 136 | 133 |
| German Pinscher Dog | 0.009 | 0.011 | 137 | 142 |
| Curly Coated Retriever | 0.009 | 0.018 | 138 | 130 |
| Anatolian Shepherd Dog | 0.009 | 0.037 | 139 | 114 |
| American Water Spaniel | 0.007 | 0.020 | 140 | 128 |
| Komondor Dog | 0.006 | 0.008 | 141 | 145 |
| English Foxhound | 0.006 | 0.002 | 142 | 154 |
| Sealyham Terrier | 0.004 | 0.008 | 143 | 146 |
| Harrier Dog | 0.004 | 0.005 | 144 | 153 |
| Dandie Dinmont Terrier | 0.004 | 0.006 | 145 | 149 |
| Sussex Spaniel | 0.003 | 0.008 | 146 | 143 |
| Scottish Deerhound | 0.003 | 0.021 | 147 | 127 |
| Kuvasz Dog | 0.003 | 0.013 | 148 | 137 |
| Glen of Imaal Terrier | 0.003 | 0.005 | 149 | 150 |
| Canaan Dog | 0.003 | 0.008 | 150 | 147 |
| Black Russian Terrier | 0.001 | 0.012 | 151 | 141 |

ASPCA Animal Poison Control Center, unpublished data (2005) and American Kennel Club (2006).

Xavier and Kogika, 2002; Forrester and Stanley, 2004). In 2005, 72% of cats exposed to potential toxicants were identified as domestic shorthairs, 9% as domestic long-hairs and 5% as domestic mediumhairs (ASPCA Animal Poison Control Center, unpublished data, 2005). Excluding these types of cats due to the generic nature of their classification, the top 10 purebred cats were Siamese (28%), Persian (15%), Main coon (11%), Himalayan (9%), ragdoll (5%), Bengal (4%), Russian blue (3%), Abyssinian (3%), Manx (2%) and Burmese (2%). Of these, the Persian, Maine coon, Siamese, Abyssinian and ragdoll are listed in the 10 most popular breeds registered by the Cat Fancier's Association in 2005. As with dogs, no gender or age difference's in incidence of exposure have been noted. Cats may, due to their grooming habits, be more susceptible to toxicants that come into contact with their fur; this is especially problematic with agents to which cats are exquisitely sensitive (e.g. ethylene glycol).

## OTHER SPECIES

Demographic information on potential poisonings in animal species other than dogs and cats is largely lacking. Production animals are generally kept in large groups, meaning that when a toxicosis occurs, there is potential for a multiple animals of varying age and genders to be exposed. Most production animals are kept in some form of confinement, which limits the potential for exposure to toxic agents. However, mistakes in management, such as feed mixing errors or improper ventilation, may result in acute or chronic toxicosis in large numbers of animals.

Wildlife is almost continuously exposed to toxic agents in the environment, but specific demographic information is not available in most cases. Seasonality of poisoning incidences in wildlife would vary with the agent involved. For instance, avian botulism in waterfowl tends to be seasonal, with most cases occurring between the months of July and September (Locke and Friend, 1989).

## AGENTS INVOLVED

There is an unlimited number of agents by which exposed animals may become poisoned, and for the most part, which specific agents are involved in animal poisonings will be dependent upon what is available in the animals' environment, the potential or inclination for the animal to be exposed to the agent, the amount of agent to which

**TABLE 5.2 Exposures of animals to various toxic agents reported by veterinary clinics and poison control centers, 1975–2006**

| Agent | 1975 | 1978–1981 | 1990 | 1993 | 2006 |
|---|---|---|---|---|---|
| Chocolate | NR | NR | NR | NR | 26% |
| Glycols | 1% | NR | 10% | NR | 4% |
| Metals | 2% | 7% | 1% | NR | 0% |
| Miscellaneous | 47% | 56% | 17% | 34% | 3% |
| Pesticides++ | 23% | 20% | 21% | 21% | 13% |
| Pharmaceuticals | NR | 10% | 25% | 24% | 22% |
| Plants | 1% | 12% | 12% | 10% | 5% |
| Rodenticides | 27% | 7% | 14% | 7% | 26% |

NR: not reported; ++: combined insecticide and herbicide exposures. Osweiler (1975), Haliburton and Buck (1983), Hornfeldt and Murphy (1992, 1998), and Cope *et al*. (2006).

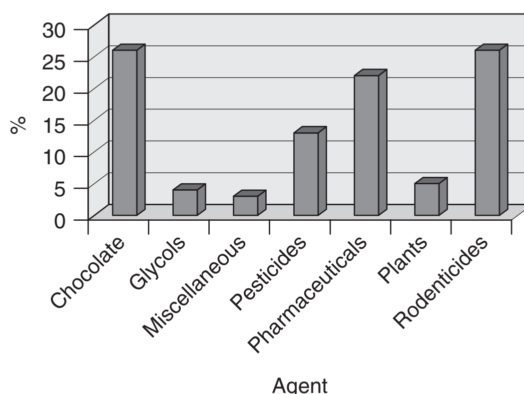


**FIGURE 5.3** Most common agents involved in canine exposures presenting to a veterinary emergency center in 2006 (Cope *et al*., 2006).

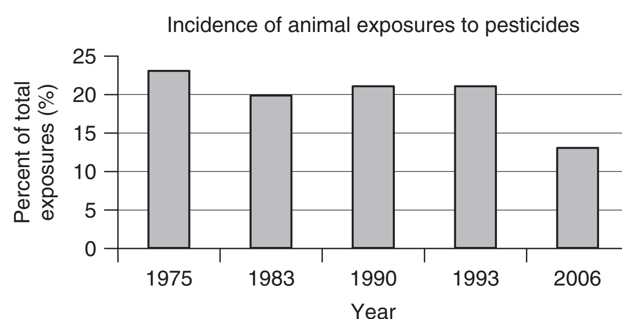


**FIGURE 5.4** Comparison of incidence of animal exposures to pesticides reported to human poison control centers and veterinary clinics (Osweiler, 1975; Haliburton and Buck, 1983; Trammel *et al*., 1985; Hornfeldt and Murphy, 1992).

the animal is exposed, and the individual sensitivity of the animal to the effects of the agent. The potential for exposure to specific agents may be uniform throughout the year or may be seasonal, depending on the agent and the species involved. For instance, exposures to lawn care products, such as herbicides and insecticides, would be expected to be more common during the seasons when these products are most in use (i.e. spring and summer).

Table 5.2 summarizes the most common agents involved in animal exposures reported to veterinary emergency referral centers and human or animal poison control centers in the United States. These exposures predominantly involve companion animals, particularly dogs and cats, and are consistent with the types of animal exposures reported by poison control centers in other countries (Xavier and Kogika, 2002; Giuliano Albo and Nebbia, 2004). Rodenticides, pharmaceuticals and chocolate make up the majority of agents in the most recent report summarized in Figure 5.3 (Cope *et al*., 2006). Rodenticides and chocolate made up approximately one quarter of all exposures, followed by pharmaceutical agents, which accounted for 22% of exposures. Pharmaceuticals exposures have increased since first reports in 1983 (Beasley and Trammel, 1994), likely due to the increased use of these agents in veterinary and human medicine over the past 20 years. The most common pharmaceutical agents associated with animal exposures are analgesics (primarily non-steroidal antiinflammatory drugs) and central nervous system drugs (sedatives, antipsychotics, stimulants, etc.) (Hornfeldt and Murphy, 1992, 1998). None of the reported data from poison control centers included information specifically on chocolate cases, but a veterinary emergency hospital reported that exposure of dogs to chocolate accounts for one in every four presentations for potential toxicosis (Cope *et al*., 2006). Pesticides have historically been responsible for large numbers of exposures and toxicoses in domestic animals (Beasley and Trammel, 1994), but the incidence appears to be declining (Figure 5.4). This may be due in part to the development of newer herbicides and insecticides that have a much higher margin of safety in mammals than those used in the past, as well as perhaps better public awareness of the potential hazards of these agents to pets.

Less information is available regarding exposure of non-canine and non-feline animals to potential toxicants. For livestock, plant poisoning causes tremendous economic losses to producers, estimated to exceed $350 million per year (Galey, 1996). Losses from poisonous plants are due to deaths as well as loss of productivity. Other agents reported to be responsible for significant livestock loss include mycotoxins, organophosphate and carbamate insecticides, nitrate and lead, although lead poisoning appears to be on the decline in livestock. For waterfowl, avian botulism is a concern, resulting in the loss of thousands to millions of birds each year (Locke and Friend, 1989). Lead toxicosis was once a significant cause of loss of waterfowl in the United States due to ingestion of lead shot left by hunters, but the incidence of lead toxicosis has decreased following an enforced ban on the use of lead shot on waterfowl (Gwaltney-Brant, 2004). However, lead intoxication still occurs in raptors that ingest upland prey that have shot embedded in their tissues. Wild animals ingesting the tissues of animals that have been euthanized with

barbiturates are at risk of toxicosis (Hayes, 1988), and toxicosis has been reported in a variety of species feeding on animals intentionally poisoned with pesticides (Stroud, 1998; Wobeser *et al.*, 2004). Intentional poisoning of wildlife with pesticides is most commonly accomplished using carbofuran and aldicarb, while accidental or secondary poisoning of wildlife by pesticides most often involves strychnine, famphur, fenthion and avitrol (Stroud, 1998). Oil spills near sea shores have resulted in significant loss of life of animals living in or around these areas, and industrial pollution from agricultural or industrial effluents have similarly cause deaths in a variety of wildlife species.

## SIGNS AND OUTCOMES

Based on poison control center statistics, the majority (57–63%) of animal exposures to potential toxicants result in no signs for the patient, either due to insufficient level of exposure or successful decontamination by animal caretakers (Figure 5.5). Mild signs developed in 25–27% of animal exposures to suspected toxicants, moderate signs developed in 7–8%, major signs developed in approximately 1% and death occurred in 2–3% of cases where outcomes were known (Hornfeldt and Murphy, 1992, 1998). Mild signs are those in which some clinical signs developed but are expected to be mild and self-limiting and not in need of treatment (e.g. hypersalivation, mild vomiting, etc.). Moderate signs are those in which signs that develop are more pronounced, prolonged or of a systemic nature and merit some form of treatment, although the signs would not be expected to be life-threatening (e.g. protracted vomiting, severe diarrhea, mild to moderate hypotension). No residual effects would be expected following recovery. Major signs are those in which life threatening conditions exist or there is potential for significant residual disability or disfigurement following recovery from the acute episode (e.g. seizures, renal injury, etc.).

Agents most frequently reported to be associated with mild to moderate signs in animals are pharmaceuticals (19%), pesticides (insecticides and herbicides, 14%), plants (10%), cleaning products (8%) and mushrooms (3%) (Hornfeldt and Murphy, 1998). Agents associated with major signs included pesticides (45%), pharmaceuticals (25%), plants (15%), rodenticides (9%) and cleaning products (8%). Deaths were most commonly associated with exposures to pesticides (24%), pharmaceuticals (17%), rodenticides (16%), plants (9%), automotive products (predominantly ethylene glycol, 9%) and cleaning products (8%). Organophosphate insecticides, ethylene glycol and long acting anticoagulant rodenticides were the top three agents responsible for deaths in animals.

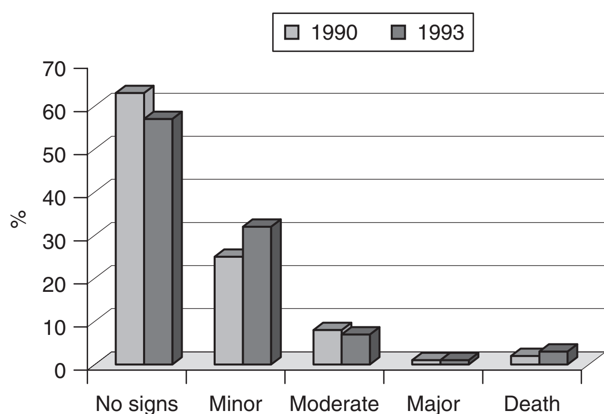


**FIGURE 5.5** Degree of illness in animals following exposure to potentially toxic agents (Hornfeldt and Murphy, 1992, 1998).

## CONCLUSIONS

Poisonings are a serious cause of morbidity and mortality in animals, particularly in dogs and cats. Based on information obtained by veterinary clinics and human and animal poison control centers the incidence of animal poisoning does not appear to be waning, although the agents to which animals are exposed does change with time. While significant pesticide exposures may be on the decline due to the development of newer and less toxic pesticides, exposures to other agents, such as chocolate and pharmaceutical agents may be on the rise. Knowing what agents have the potential to be involved in serious toxicoses should allow veterinarians to better educate their clients on means of preventing animal poisonings through the appropriate use of household products and the removal of potential hazards from the animals' environments.

## REFERENCES

American Kennel Club (2006) Dog Registration Statistics, 2004–2005 (http://www.akc.org/reg/dogreg_stats.cfm).

Beasley VR Trammel HL (1994) Incidence of poisonings in small animals. In *Current Veterinary Therapy X*, Kirk RW (ed.). Saunders, Philadelphia, PA, pp. 97–113.

Cat Fancier's Association (2005) Top 10 breeds. (http://www.cfa.org/ezine/features.html#top10).

Cope RB, White KS, More E, Holmes K, Nair A, Chauvin P, Oncken A (2006) Exposure-to-treatment interval and clinical severity in canine poisoning: a retrospective analysis at a Portland Veterinary Emergency Center. *J Vet Pharmacol Therap* **29**: 233–6.

Forrester MB, Stanley SK (2004) Patterns of animal poisonings reported to the Texas Poison Center Network: 1998–2002. *Vet Hum Toxicol* **46**: 96–9.

Galey FD (1996) Disorders caused by toxicants. In *Large Animal Internal Medicine*, 2nd edn, Smith BP (ed.). Mosby, St. Louis, MO, pp. 1974–91.

Giuliano Albo A, Nebbia C (2004) Incidence of poisonings in domestic carnivores in Italy. *Vet Res Commun* **1**: 83–8.

Gwaltney-Brant SM (2004) Lead. In *Small Animal Toxicology 2nd edn*, Peterson ME, Talcott PA (eds). Saunders Elsevier, St. Louis, MO, pp. 204–10.

Haliburton JC, Buck WB (1983) Animal poison control center: summary of telephone inquiries during the first three years of service. *J Am Vet Med Assoc* **182**: 514–15.

Hayes B (1988) Deaths caused by barbiturate poisoning in bald eagles and other wildlife. *Can Vet J* **29**: 173–4.

Hornfeldt CS, Borys DJ (1985) Review of veterinary cases received by the Henepin Poison Center in 1984. *Vet Hum Toxicol* **27**: 525–8.

Hornfeldt CS, Murphy MJ (1992) 1990 Report of the American Association of Poison Control Centers: poisonings in animals. *J Am Vet Med Assoc* **200**: 1077–80.

Hornfeldt CS, Murphy MJ (1997) Poisonings in animals: the 1993–1994 report of the American Association of Poison Control Centers. *Vet Hum Toxicol* **39**: 361–5.

Hornfeldt CS, Murphy MJ (1998) American Association of Poison Control Centers report on poisonings of animals, 1993–1994. *J Am Vet Med Assoc* **212**: 358–61.

Khan SA, Schell MM, Trammel HL, Hansen SK, Knight MW (1999) Ethylene glycol exposures managed by the ASPCA National Animal Poison Control Center from July 1995 to December 1997. *Vet Hum Toxicol* **41**: 403–6.

Locke LN, Friend M (1989) Avian botulism: geographic expansion of a historic disease. *US Fish, Wildl Leaflet* **13.2.4**: 1–6.

Osweiler GD (1975) Sources and incidence of small animal poisoning. *Vet Clin Small Anim* **5**: 589–604.

Stroud RK (1998) Wildlife forensics and the veterinary practitioner. *Sem Avian Exotic Pet Med* **7**: 182–92.

Trammel HL, Buck WB, Beasley VR (1985) National Animal Poison Control Center: seven years of service. *29th Annual Proceedings of the American Assciation of Veterinary Laboratory Diagnosticians*, 183–91.

Wobeser G, Bollinger T, Leighton FA, Blakley B, Mineau P (2004) Secondary poisoning of eagles following intentional poisoning of coyotes with anticholinesterase pesticides in western Canada. *J Wildl Dis* **40**: 163–72.

Xavier FG, Kogika MM, de Sousa Spinosa H (2002) Common causes of poisoning in dogs and cats in a Brazilian veterinary teaching hospital from 1998 to 2000. *Vet Hum Toxicol* **44**: 115–16.

C H A P T E R

# 6

# Chemicals of terrorism

*Tina Wismer*

## INTRODUCTION

Chemical agents may be chosen by terrorists as they can be dispersed over large areas and can eventually penetrate even the most well-defended positions. These agents can be deployed against specific targets, and depending on the agent used, the effects can be immediate or delayed. Chemical agents can cause incapacitation, disorientation, or death. Many of the more commonly used agents can be produced inexpensively and easily stored.

There are four basic types of military agents that can be used for chemical terrorism: choking agents (chlorine, phosgene), blister agents (mustard, Lewisite, phosgene oxime), blood agents (cyanide, hydrogen cyanide), and nerve agents (tabun, sarin, soman, VX). Chemical warfare agents can be delivered by a variety of methods: bomb, spray tanks, rockets, missiles, land mines, and artillery projectiles (USACHPPM, 2001a). Few indicators of a chemical attack may be evident at first. The initial observation of unusual signs and symptoms that correlate with nerve, vesicant, blood, or pulmonary agent exposures should raise immediate suspicion of poisoning.

The history of chemical warfare dates back to the beginning of the last century. The first chemical warfare agent of modern times was chlorine. It was used by the German army at Ypres in 1915 against the Allies. Experiencing some success, the Germans then began to mix chlorine with phosgene, or deployed phosgene alone as a weapon. Phosgene, arsenicals, blister agents, and mustard gas were estimated to be responsible for approximately 1.3 million casualties during the war, including at least 90,000 fatalities (Raffle *et al.*, 1994; Bingham *et al.*, 2001; HSDB, 2005). Phosgene accounted for 80% of all gas deaths.

Chemical warfare agents do not need to be lethal to be disruptive. Although the mortality rate was not as high as with phosgene, mustard gas was the number one cause of casualties in World War I (Raffle *et al.*, 1994). Mustard gas caused severe injuries, and due to its persistence, remained a hazard and barrier to troop movements.

Chemical weapons have been used in a few recent civil wars. Phosgene was used by Egyptian bombers against Yemeni royalist forces in the Yemeni Civil War in the 1980s (Evison *et al.*, 2002). The Iraqi military used both mustard gas and HCN against the Kurds leading to as many as 3000 deaths (Somani and Babu, 1989). Iraq also used "Yellow rain" (mixture of mycotoxins, mustard gas, and nerve agents) against Iran in 1984. The fatality rate was 20% (Drasch *et al.*, 1987).

With the changing political climate around the world, several terrorist groups have used chemical warfare to bring attention to themselves and further their cause. Aum Shinrikyo (Supreme Truth) has used several chemical attacks recently to promote their agenda. In June 1994, they attempted to assassinate three Japanese judges by releasing sarin gas in the community where they lived. Aum Shinrikyo had modified a truck with a special device to release the sarin gas. Seven people, but not the judges, died and 280 were injured. Emboldened by this partial success, on March 20, 1995, Aum Shinrikyo carried out a sarin gas attack on the Tokyo subway. The terrorists used sharpened umbrellas to puncture lunch boxes and bags filled with dilute sarin. Eleven people died and more than 5500 were injured (Kaplan and Marshall, 1996). On April 20, 1995, Aum Shinrikyo changed their chemical agent and released phosgene gas in a Yokohama train station, causing injuries to 300 people.

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Chemical agents also have their problems as agents of terror. They are most effective in confined spaces. Chemical warfare agents dissipate quickly and degrade or adhere to surfaces. Attacks need to be well coordinated for dispersal of the agent and escape from the area of deployment.

Both livestock and pet animals can be exposed to chemical agents during a terrorist attack. Treatment of animals is secondary to treating human casualties. Humane euthanasia may be the only recourse due to financial or logistical concerns.

## CHLORINE GAS

### Background

Chlorine (Agent CL, $Cl_2$) has been used as a choking or pulmonary agent for military purposes under the name bertholite (Budavari, 2000). It is a greenish-yellow diatomic gas with an irritating, pungent, or suffocating odor (Lewis, 1997; Budavari, 2000; NIOSH, 2003). Chlorine gas is heavier than air and will settle in low areas.

### Pharmacokinetics/toxicokinetics

The initial effects of chlorine gas exposure can appear very rapidly, depending upon the concentration. Due to chlorine's water solubility and chemical reactivity, it can have a greater effect on the lower respiratory tract as a large percentage bypasses the upper airways. Respiratory, dermal, and ocular irritation starts immediately and acute lung injury peaks in 12–24 h.

### Mechanism of action

Chlorine gas is a strong irritant, or in concentrated amounts, may be corrosive to mucous membranes when inhaled or ingested. When chlorine combines with tissue water it produces hydrochloric acid, and reactive oxygen species. These free radicals are potent oxidizers, causing tissue damage. The damage to the respiratory epithelium leads to alveolar capillary congestion which is followed by high fibrinogen edematous fluid (Noe, 1963). Hypoxemia results from development of atelectasis, emphysema, and membrane formation. Death usually occurs within 48 h from cardiac arrest secondary to hypoxia (Decker, 1988).

### Toxicity

Inhalation is the main route of chlorine gas exposure. Chlorine gas is very irritating on contact and can be caustic to the eyes, skin, nose, throat, and mucous membranes. Ocular exposure can result in severe or permanent eye injury. Dermal exposure may cause erythema, pain, and irritation. Both liquid chlorine and high concentrations of gaseous chlorine can cause dermal burns (Raffle *et al.*, 1994). Signs of exposure to chlorine gas include rhinorrhea, ataxia, syncope, muscle weakness, dermatitis, dyspnea, tachypnea, pneumonia, bronchospasm, and acute lung injury. High concentrations may cause laryngospasm, cardiovascular collapse, tachycardia, and respiratory arrest (Noe, 1963). Respiratory symptoms may be immediate or delayed up to several hours depending on the concentration (Bingham *et al.*, 2001). Hypoxia is common and death may be rapid. See Table 6.1 for severity of signs expected after inhalation at certain parts per million (ppm) concentrations of chlorine gas.

Symptoms generally disappear within 6 h after mild exposures, but may continue for more than 24 h with severe exposures. Moderate or severe exposures can result in chronic respiratory dysfunction (Decker, 1988; Schwartz *et al.*, 1990).

Chlorine is both teratogenic and carcinogenic. Chlorine at 100 ppm when given to pregnant rats in their water caused both biochemical and metabolic effects in the newborns (RTECS, 2006). Carcinogenicity has only been

**TABLE 6.1 Different effects of exposure levels of chlorine gas**

| Values in ppm | Effect |
|---|---|
| 0.2–3.5 | Odor detection (some tolerance develops) |
| 1–3 | Mild mucous membrane irritation that can be tolerated for up to 1 h |
| 3 | Extremely irritating to the eyes and respiratory tract |
| 5 | Severe irritation of eyes, nose, and respiratory tract; intolerable after a few minutes |
| 14–21 | Immediate irritation of the throat; dangerous if exposed for 30–60 min |
| 15 | Irritation of the throat |
| 30 | Moderate irritation of the upper respiratory tract; immediate chest pain, vomiting, dyspnea, cough |
| 35–50 | Lethal in 60–90 min |
| 40–60 | Toxic pneumonitis and acute lung injury; dangerous for even short periods |
| 430 | Lethal over 30 min |
| 1000 | Fatal within a few minutes |

Lewis (2000), HSDB (2005), and Bingham *et al.* (2001).

demonstrated in chronic exposures (Morris *et al.*, 1992; RTECS, 2006).

## Treatment

Move animals into fresh air and monitor for respiratory distress. If coughing or dyspnea develops, evaluate for hypoxia, acidosis, respiratory tract irritation, bronchitis, or pneumonitis. Oxygen supplementation along with intubation and ventilation may be needed. Beta adrenergic agonists can help if bronchospasm develops (Guloglu *et al.*, 2002).

Flush eyes with copious amounts of room temperature 0.9% saline or water for at least 15 min. Fluorescein staining should be performed to check for corneal defects (Grant and Schuman, 1993). Animals should be bathed with copious amounts of soap and water. Chlorine blood concentrations are not clinically useful as it converts directly to hydrochloric acid in the lungs and in the tissues.

Animal models have suggested that corticosteroids can hasten recovery from severe chlorine gas poisoning (Traub *et al.*, 2002); however, administration of steroids to exposed humans has not been shown to provide any significant change (Chester *et al.*, 1977). Pigs exposed to chlorine gas responded best to a combination of aerosolized terbutaline and budesonide than to either therapy alone (Wang *et al.*, 2004). Another study in sheep exposed to chlorine gas and then nebulized with 4% sodium bicarbonate had decreased mortality and had improved arterial blood gas values (Chisholm *et al.*, 1989).

## Concluding remarks

Rescuers should wear self-contained breathing apparatus (SCBA) and have protective clothing when entering contaminated areas. Chlorine dissipates quickly in warm climates and does not leave an environmental residue (Munro *et al.*, 1999). The potential for secondary contamination is low, as the gas is not carried on contaminated clothing.

# PHOSGENE

## Background

Phosgene (Agent CG, carbonyl chloride, C—$Cl_2$—O) is classified as a choking agent. It is a colorless, noncombustible, and highly toxic gas. At room temperature phosgene is easily liquefied (NIOSH, 2003; Proctor and Hughes, 2004; ACGIH, 2005; CHRIS, 2005) and at high concentrations, the gas has an odor described as strong, suffocating, and pungent. Lower concentrations are referred to as smelling like green corn or "haylike" (Raffle *et al.*, 1994; Budavari, 2000; Pohanish, 2002). Phosgene will sink in water (Budavari, 2000; Bingham *et al.*, 2001).

## Pharmacokinetics/toxicokinetics

Dyspnea develops 2–6 h post exposure in most patients, but may be delayed up to 15 h (Borak and Diller, 2001). With high concentrations (>200 ppm), phosgene can cross the blood air barrier in the lung and cause hemolysis and coagulopathies (Sciuto *et al.*, 2001).

## Mechanism of action

Phosgene is a lower respiratory tract irritant. Due to its low water solubility and low irritancy of the upper respiratory system, phosgene is able to penetrate deeply into the lungs (Franch and Hatch, 1986). Phosgene gas contacts water in the lungs, where it is hydrolyzed into hydrochloric acid causing cellular injury (Murdoch, 1993). Phosgene also acylates sulfhydryl, amine, and hydroxyl groups (Borak and Diller, 2001). This results in protein and lipid denaturation, changes in membrane structure, and disruption of enzymes. Phosgene increases pulmonary vascular permeability, leading to increased fluid accumulation in the lung interstitium and alveolae. This fluid accumulation results in gas diffusion abnormalities and pulmonary edema (Diller, 1985; Ghio *et al.*, 1991). Phosgene also decreases energy metabolism and disrupts the glutathione redox cycle. Animals exposed to phosgene have elevated levels of leukotrienes and neutrophil chemotactic agents. Neutrophils congregate in the lung releasing cytokines and other reactive mediators which contribute to pulmonary injury (Ghio *et al.*, 1991; Sciuto *et al.*, 1995). Bronchiolar epithelium is damaged, resulting in local emphysema and partial atelectasis. Death is due to anoxia secondary to pulmonary edema.

## Toxicity

Most exposures to phosgene are from inhalation. The odor of phosgene gas is not sufficient to warn individuals of toxic levels and with high concentrations, olfactory fatigue can occur (Borak and Diller, 2001; ACGIH, 2005). The degree of pulmonary injury relates to the concentration and length of exposure (Bingham *et al.*, 2001) and initial symptoms are not considered to be a good indicator of prognosis (Diller, 1985).

Exposure to concentrations less than 3 ppm may not be immediately accompanied by symptoms, but delayed effects usually occur within 24 h of exposure. Concentrations as low as 3–5 ppm can cause immediate conjunctivitis, rhinitis, pharyngitis, bronchitis, lacrimation, blepharospasm, and

upper respiratory tract irritation and extended (170 min) exposure was fatal (Diller, 1985; Wells, 1985; Proctor and Hughes, 2004). A dose of 50 ppm for 5 min may cause pulmonary edema and rapid death (Chemstar, 1996; Borak and Diller, 2001; RTECS, 2006).

A lag time of 1–6 h before the onset of respiratory distress and pulmonary edema is common with acute, high-dose exposures (>50 ppm/min). Signs can be delayed for up to 24 h (most common) or 72 h with exposures to lower concentrations (Pohanish, 2002; Proctor and Hughes, 2004). Thoracic radiographs can show evidence of pulmonary edema within 1–2 h of high-dose exposure, 4–6 h after moderate exposure, and approximately 8–24 h after low-dose exposure (Diller, 1985).

Progressive dyspnea, productive cough, cyanosis, and hemoptysis are common initial signs following exposure (Wells, 1985; Borak and Diller, 2001). Hypoxemia and hypoventilation are common secondary to respiratory distress (Wells, 1985). Animals may develop secondary gastrointestinal (GI), hepatic, renal, or brain injury, due to lack of oxygenation. In a dog model, severe phosgene poisoning caused initial bradycardia followed by tachycardia and progressive hypotension (Patt *et al.*, 1946). Cardiac failure may occur secondary to severe pulmonary edema (Borak and Diller, 2001; Proctor and Hughes, 2004).

Direct contact with the liquefied material can cause dermal burns and severe eye irritation, corneal opacification, and frostbite (Proctor and Hughes, 2004). Corneal opacification has also been produced in cats exposed to highly concentrated phosgene gas (Grant and Schumann, 1993).

Prognosis is directly related to the extent of pulmonary injury. If the animal survives 24–48 h, the prognosis improves, as pulmonary edema begins to resolve after 2–3 days. Survivors may have suppressed natural killer cell activity and are more susceptible to infectious agents. Secondary infections may become evident 3–5 days after exposure. They may also have persistent exertional dyspnea, reduced exercise capacity, and abnormal pulmonary function tests (Borak and Diller, 2001).

## Treatment

If inhalation exposure occurs, remove animals to fresh air (higher ground) and monitor for respiratory distress. Exposed skin should be washed with soap and water. Exposed eyes should be flushed for 15 min with tepid water. Asymptomatic animals should be monitored for 12–24 h for development of pulmonary edema (Borak and Diller, 2001). Symptomatic animals should receive 100% oxygen. If arterial blood gases or $pO_2$ continues to fall, intubation and ventilation is recommended. Plasma phosgene levels are not clinically useful and there is no specific antidotal agent. Management of patients with pulmonary edema from phosgene is the same as for an ARDS (acute respiratory distress syndrome) patient. Diuretics are not indicated and can worsen volume depletion. Mechanical ventilation with oxygen and PEEP (positive end-expiratory pressure) is the mainstay of treatment.

Intravenous (IV) fluids can help with cardiovascular support, but monitor closely for signs of overhydration. Colloids are preferred as they will remain in the vascular space for a longer period of time. Most arrhythmias will resolve with adequate oxygenation.

Nebulized beta adrenergic agonists are recommended if bronchospasm occurs. In a rabbit inhalation study, animals exposed to toxic levels of phosgene were dosed with IV aminophylline and subcutaneous terbutaline. If given within 10-min post exposure, it prevented non-cardiogenic pulmonary edema (Kennedy *et al.*, 1989). *N*-acetylcysteine (Mucomyst®) administered intratracheally to rabbits 45–60 min after inhalational exposure to phosgene (1500 ppm/min) decreased pulmonary edema, production of leukotrienes, lipid peroxidation, and maintained normal glutathione levels as compared to rabbits exposed to phosgene only (Sciuto *et al.*, 1995). Ibuprofen was shown to protect against acute lung injury from phosgene in rats and rabbits (Guo *et al.*, 1990). Supplemental oxygen and sodium bicarbonate were beneficial in dog experiments (Mautone *et al.*, 1985). Aerosolized surfactant improved lung compliance in a dog model, but the effects of the surfactant on the development or resolution of pulmonary edema were not determined (Mautone *et al.*, 1985). Animal experiments also suggest that corticosteroids, prostaglandin E1, and atropine may be helpful in treating phosgene-induced pulmonary edema (Chemstar, 1996).

## Concluding remarks

Phosgene is heavier than air and will pool in low lying areas. This heavy vapor density, 3.4 times that of air, made phosgene practical for trench warfare. Phosgene is considered to be non-persistent in the environment. Air concentrations are reduced by atmospheric water, such as rain or fog (Borak and Diller, 2001). Rescuers should wear proper protective clothing when treating exposed patients. Fortunately, the potential for secondary contamination of rescue personnel is low, as the gas does not persist in fabric or leather. Phosgene is used extensively in industry as a chemical precursor and this widespread availability makes it an attractive agent for terrorist use.

# MUSTARD GAS

## Background

Mustard gas (Agent H, $C_4$—$H_8$—$C_{12}$—S) is a vesicant agent used in chemical warfare. It produces corrosion and

necrosis of the skin, eyes, and respiratory tract. It is an organic lipophilic sulfide which is a bifunctional alkylating agent (Borak and Sidell, 1992; Lewis, 1997). Mustard agent is a clear oily liquid in its pure state. Due to impurities, however, it is normally amber to black, or yellow to brown in color (Munro *et al*., 1999; USACHPPM, 2001b; HSDB, 2005). The liquid becomes aerosolized when dispersed by spraying or by explosive blast from a shell or bomb (USACHPPM, 2001a). Mustard gas has an odor resembling garlic, mustard, or horseradish. Systemic poisoning occurs more easily in warm climates than in temperate ones. Mustard gas is still considered a major threat by the US Military, as it is easily manufactured and is both incapacitating and lethal.

## Pharmacokinetics/toxicokinetics

Mustard gas is toxic by all routes of exposure (oral, inhaled, dermal, and ocular) (EPA, 1985b; Sidell *et al*., 1997; Lewis, 2000; Pohanish, 2002). Skin penetration of both the liquid and vapor is rapid, and mustard causes both localized cellular and systemic damage (NATO, 1973). Mustard penetrates down hair follicles and sweat glands within minutes. Dermal absorption of mustard varies by species. About 20% of a dermal dose is absorbed through human skin, while up to 75% was absorbed through the skin in rats (Smith, 1999; Hambrook *et al*., 1992). Ocular absorption also happens within minutes. The latent period for absorption is inversely related to the dose, temperature, and humidity (NATO, 1973). Lesions develop within 2–3 h with high-level exposures and 8–10 h after milder exposures (Requena *et al*., 1988).

The chemical reaction with biological tissue occurs rapidly, but symptoms are typically delayed by several hours (Grant and Schuman, 1993; Sidell *et al*., 1997). Dog studies show that equilibrium between blood and tissues was achieved within 5 min after inhalation (IARC, 1975). Once inside the body, mustard accumulates (in descending order) in fat, skin with subcutaneous fatty tissue, brain, kidney, muscle, liver, cerebrospinal fluid, spleen, and lung (Drasch *et al*., 1987; Somani and Babu, 1989). There are no measurable levels of mustard in the liquid from a skin blister (Drasch *et al*., 1987). Urine is the major route of excretion in rabbits, mice, and rats. After IV administration in rats and mice, the majority is excreted in 72–96 h (Maisonneuve *et al*., 1993; Dacre and Goldman, 1996). Only about 6% is eliminated in the feces (IARC, 1975). Bone marrow damage is not evident for 3–5-days post exposure. Leukopenia usually occurs at day 7–10 following exposures (Garigan, 1996).

## Mechanism of action

The mechanism of action for cellular damage by mustard is unknown, but four theories have been proposed: alkylation of deoxyribonucleic acid, oxidative stress upon cell components, depletion of glutathione, and an inflammatory response (Smith *et al*., 1995). Mustard gas is a bifunctional alkylating agent. It forms covalent cross-links between the double strands of DNA which inhibits DNA synthesis. Mustard-induced blistering appears to be correlated to DNA damage in the basal cells (Andreassi, 1991; Cowan *et al*., 1998). Mustard penetrates the skin, and damages the cells separating the epidermis from the dermis. Mustard disrupts the hemidesmosomes forming a blister between the two layers (Sidell *et al*., 1997). Skin biopsies from mustard gas blisters revealed a separation of the basal cells from one another and the development of multinucleated cells (Bismuth *et al*., 1995).

Mustard also causes oxidative stress on intracellular molecules. Mustard rearranges in the aqueous of the cytosol to form an electrophilic ethylene episulfonium intermediate (Smith, 1999). The episulfonium ion reacts with sulfhydryl groups leading to increased intracellular calcium. The increased calcium level breaks down the microfilaments needed to maintain cell integrity and induces apoptosis by activation endonucleases, proteases, and phospholipases (Smith *et al*., 1995).

Mustard gas may also exert its toxicity via depletion of glutathione. Without glutathione, reactive oxygen species react with membrane phospholipids causing loss of membrane function, fluidity, and integrity (Smith *et al*., 1995). Inflammation and sulfur mustard-increased proteolytic activity are also implicated in contributing to mustard pathology (Cowan *et al*., 1998).

## Toxicity

Mustard gas is a radiomimetic as it produces lesions similar to radiation (Andreassi, 1991; Sidell *et al*., 1997). Tissues with high cell turnover are the most affected. Being a vesicant, mustard gas can produce erythema, severe pruritus, blistering, ulceration, and necrosis of exposed skin (Borak and Sidell, 1992; ITI, 1995; Dacre and Goldman, 1996; Budavari, 2000; Pohanish, 2002). With dermal exposure, the skin initially appears pale and then becomes erythematous within a few hours of exposure (Requena *et al*., 1988). Blistering usually starts on the 2nd day and progresses for several more days. Erythema disappears in 3–7 days, while the ulcers take 6–8 weeks to heal (Garigan, 1996; Sidell *et al*., 1997). Brown or black hyperpigmentation usually occurs after resolution of the burns, especially in areas with thinner skin (Requena *et al*., 1988). Skin involvement is most severe at warm and moist sites (genitalia, perineal regions, groin, skin folds, and axillae). This is due to the high number of sweat glands in these areas. The smallest reported blister-causing dose on the skin is 0.02 mg of mustard (Smith *et al*., 1995).

The eyes are very sensitive to the effects of mustard gas (NATO, 1973; Borak and Sidell, 1992; Dacre and

TABLE 6.2 $LD_{50}$ for mustard gas

| Species | Route | $LD_{50}$ (mg/kg) |
|---|---|---|
| Human | Oral | 0.7 |
| | Dermal | 100 |
| Mouse | Dermal | 92 |
| | SQ | 20 |
| Rat | Oral | 17 |
| | Dermal | 5 |
| | SQ | 1.5 |

Lewis (2000) and RTECS (2006).

Goldman, 1996). Pain, lacrimation, corneal ulceration, along with photophobia, swelling, blepharospasm, and blindness can be seen (Garigan, 1996). Conjunctivitis appears early, developing 4–6 h after exposure. Eye lesions have been reported at a *Ct* (concentration × time) of 10 mg-min/m$^3$ (Sidell *et al.*, 1997). Porcelain-white areas in the episcleral tissues adjacent to the cornea and formation of large, tortuous, sausage-shaped varicose veins are pathognomonic signs of mustard gas poisoning (Grant and Schuman, 1993). Chronic conjunctivitis and keratopathy have been reported in people (Blodi, 1971).

The main non-dermal toxic effects are on the respiratory tract. Irritation or ulceration of the respiratory tract can occur (Vogt *et al.*, 1984; Borak and Sidell, 1992; ITI, 1995; Dacre and Goldman, 1996; Budavari, 2000). Cough, dyspnea, and pulmonary edema may occur up to 24 h after inhalation; 1 ppm in air is a lethal concentration for dogs and 0.650 ppm mustard results in a 33% mortality rate in rabbits (OHM/TADS, 2005). See Table 6.2 for $LD_{50}$ information in various species by multiple routes of exposure.

Mild pulmonary exposures produce rhinorrhea, sneezing, epistaxis, and cough within 12–24 h of exposure. Large exposures can cause pulmonary damage. In severe cases, arterial blood gases indicate hypoxia and respiratory acidosis. Seizures appear to occur only following extremely acute, high doses (Sidell *et al.*, 1997).

Leukopenia, thrombocytopenia, pancytopenia, and anemia have been reported due to depressed myelopoiesis from destruction of precursor cells in the bone marrow (Vogt *et al.*, 1984; Borak and Sidell, 1992; Dacre and Goldman, 1996). Bone marrow aplasia can be seen in severe cases. Secondary to bone marrow damage, overwhelming infection can result in death (Sidell *et al.*, 1997).

Mustard gas is a possible human and animal teratogen. It has been linked to an increased incidence of cleft lip and cleft palate in Iranian children born during the Iran–Iraq war (Taher, 1992). Abnormalities of the musculoskeletal system were observed in the offspring of rats orally dosed with mustard gas, but such effects may only occur at doses high enough to be toxic to the dams (Dacre and Goldman, 1996; RTECS, 2006). Other rat and rabbit studies showed no correlation (Hackett *et al.*, 1987). Mustard gas is considered both carcinogenic and neoplastic. Mustard gas has caused carcinomas in the skin, appendages, lungs, thorax, and blood (leukemia) of rats and mice via both inhalation and IV exposure (RTECS, 2006).

## Treatment

Move animal into fresh air. Do not induce emesis. Activated charcoal administration after oral ingestion is controversial. It appears to have some beneficial effects if administered within 1 h of ingestion. Sodium thiosulfate has been used as a "mustard scavenger" and giving 2% sodium thiosulfate solution orally may help in cases with ingestion exposures (Borak and Sidell, 1992; Dacre and Goldman, 1996). If signs or symptoms of esophageal burns are present, consider endoscopy to determine the extent of injury. Perforation and stricture formation could result.

Flush eyes with tepid water for at least 15 min. After flushing, instill 2.5% sodium thiosulfate to help neutralize the mustard. Time to decontamination is very important with ocular exposures. Mustard droplets disappear from the eye very quickly, and late flushing of the eye generally provides no benefit (Sidell *et al.*, 1997). Topical antibiotics and mydriatics should be used if corneal lesions are present (Sidell *et al.*, 1997). Corneal transplants have been performed on some human patients with good results and may be considered for some valuable animals (Blodi, 1971).

Bathe animals with copious amounts of soap and water. Dermal decontamination needs to be implemented quickly, as once mustard has reacted with the skin, it cannot be easily removed (Sidell *et al.*, 1997). A 2.5% sodium thiosulfate solution has been used to neutralize dermal mustard exposures (Garigan, 1996). Dilute (0.5%) hypochlorite solutions may also be used for skin decontamination (Borak and Sidell, 1992). Monitor for dermal effects, which are delayed and progressive. Dermal lesions behave like a chemical burn or radiomimetic effect. Topical silver sulfadiazine should be applied to all affected areas. In a mouse model, topically applied dexamethasone and diclofenac reduced inflammatory parameters when applied within 4 h (Dachir *et al.*, 2004). Healing can take weeks to months and infection is common (Borak and Sidell, 1992). Removal and debridement of closed blisters is controversial. Blisters should be left intact until they rupture spontaneously or unless they are extremely large or inhibit motion (Roberts, 1988). Tetanus toxoid should be given to all equine and ovine patients. Tetanus prophylaxis of other species should be determined on a case-to-case basis.

Monitor for coughing and respiratory distress. If respiratory abnormalities occur, monitor arterial blood gases and/or pulse oximetry, and thoracic radiographs. Thoracic radiographs may show an infiltrate within the first 2 days (Smith, 1999). Nebulization of 2.5% sodium thiosulfate

may help neutralize the mustard gas. *N*-acetylcysteine (Mucomyst®) is also a potential mustard gas antagonist (Garigan, 1996). A loading dose of 140 mg/kg should be given, followed by 40 mg/kg every 4 h for a total of 17 doses (Garigan, 1996). Administer oxygen and inhaled beta agonists if needed. Intubation and ventilation may be needed. Combinations of parenteral dexamethasone, promethazine, vitamin E, and heparin have shown protective effects against mustard gas poisoning in laboratory animals. (Vojvodic *et al*., 1985; Requena *et al*., 1988).

Monitor complete blood count (CBC) with platelets for 2 weeks after exposure. Leukopenia develops at 7–10 days following a severe acute exposure (Garigan, 1996). Prophylactic antibiotics are not recommended, but a broad spectrum antibiotic should be started if leukopenia develops (Sidell *et al.*, 1997). Antibiotic choice should be based on culture and sensitivity if possible.

Mustard can be detected in air, urine, and body tissues using different methods. Mustard gas or its thiodiglycol metabolite can be detected in urine up to a week after acute exposure using gas chromatography-mass spectrometry (GC-MS) (Vycudilik, 1985). Other than for confirming the diagnosis, measuring mustard gas levels is not likely to be of value in the management of the patient.

## Concluding remarks

Due to its low volatility, mustard is persistent in the environment. It persists for shorter periods of time in a hot climate, but reaches higher vapor concentrations more rapidly. In temperate areas mustard may persist for more than 1 week, but in desert conditions, persistence is reduced to about 1 day. Mustard will bind to vegetation for days to weeks (USACHPPM, 2001b). Detection tubes can be used to detect airborne levels of mustard gas (IARC, 1975).

The potential for secondary contamination is high. Rescue personnel must wear protective clothing, eye protection, and a respirator (HSDB, 2005). Mustard gas penetrates wood, leather, rubber, and paints. Medical personnel treating mustard-exposed patients have developed toxicity.

# LEWISITE

## Background

Lewisite ($C_2$—$H_2$—As—$Cl_3$) is a substituted arsine. Lewisite was first synthesized in 1918 by a research team headed by US Army Captain W.L. Lewis (Sidell *et al.*, 1997). It is an oily vesicant (blister-causing) liquid with potential terrorist use. Lewisite smells like geraniums. Pure Lewisite is colorless, but impurities and age cause the color to darken (amber to black or violet to brown, to olive-green) (HSDB, 2005). Lewisite remains a liquid at low temperatures and is persistent in colder climates. It hydrolyzes rapidly, making it difficult to maintain a biologically active concentration on a humid day (Sidell *et al.*, 1997; AAR, 2000; Lewis, 2000; HSDB, 2005). The synthesis of Lewisite involves adding arsenic trichloride to acetylene, using aluminum chloride as a catalyst. This results in a mixture of about 20% Lewisite, other arsine compounds, and an explosive component. Lewisite can be dispersed in air as a very fine droplet spray over a large distance (Grant and Schuman, 1993; Lewis, 2000).

## Pharmacokinetics/toxicokinetics

Lewisite can cause systemic signs when ingested, inhaled, or when absorbed dermally or ocularly (Sidell *et al.*, 1997; HSDB, 2005). Inhalation of vapor causes immediate pain and if high enough concentrations are inhaled, death can be seen within 10 min (DeRosa *et al.*, 2002). Dermal absorption occurs within 3–5 min, especially following liquid exposures (Sidell *et al.*, 1997). The volume of distribution of arsenic after Lewisite administration, is several liters/kilogram, indicating extensive tissue distribution (HSDB, 2005). A distribution study using rabbits revealed that the liver, lungs, and kidneys had the highest concentration of arsenic after Lewisite administration (>7 times blood concentration) (HSDB, 2005). Arsenic can cross the placenta and is passed into the milk, and nursing animals may be at risk (Barlow and Sullivan, 1982).

Animal studies showed that excretion of Lewisite oxidation products into the bile caused focal necrosis of the liver and necrosis of biliary vessel mucosa with peribiliary hemorrhages (Munro *et al.*, 1999). The same study in rabbits found that arsenic was eliminated with a half-life in blood of 55–75 h (HSDB, 2005). The excretion of oxidized Lewisite products into the bile by the liver may result in injury to the intestinal mucosa (Munro *et al.*, 1999).

## Mechanism of action

Besides being a vesicant, Lewisite is an arsenical compound which causes systemic effects. Lewisite directly effects enzyme systems. The exact mechanism of action is unknown, but it inhibits a variety of enzymes (pyruvic oxidase, alcohol dehydrogenase, succinic oxidase, hexokinase, and succinic dehydrogenase) (DeRosa *et al.*, 2002). Lewisite binds with thiol groups on these enzymes, resulting in decreased ATP production. Ocular injuries following Lewisite exposure are due in part to the liberation of hydrochloric acid. Deep penetration of Lewisite into the cornea and aqueous humor causes rapid necrosis (Goldfrank *et al.*, 1998).

Lewisite causes increased capillary permeability. Systemic absorption and increased permeability can cause a significant loss of blood plasma and is called "Lewisite shock" since it is similar to that of shock observed in severe burns. The leakage of fluid into the extravascular space results in hypotension (Sidell *et al.*, 1997). The exact mechanism of increased capillary permeability is not known. Theories include a capillary dilating material released from skin or tissue, or alternatively, enhanced permeability from an interference with the metabolism of capillary endothelial cells (Goldman and Dacre, 1989). Lung capillaries appear to be the most affected due to absorption via the respiratory tract and first pass through the lungs following dermal exposure. Pulmonary edema or ARDS can develop (Sidell *et al.*, 1997).

## Toxicity

Due to its method of dispersal (bursting charge of an explosive), the main routes of absorption are dermal and respiratory. Lewisite first acts as a vesicant, then as a pulmonary irritant, and finally as a systemic poison. Lewisite is similar to mustard gas in that it damages the skin, eyes, and airways; however, it differs in that its clinical effects appear within seconds of exposure and it is about 10 times more volatile than mustard gas (Budavari, 2000). Exposure to Lewisite is very painful, in contrast to mustard. See Table 6.3 for $LD_{50}$ of various species by different routes of exposure.

Dermal contact results in immediate pain. Both the vapor and liquid Lewisite can penetrate skin. Reddening of the skin becomes evident within 15–30 min after exposure (EPA, 1985a; Sidell *et al.*, 1997; Pohanish, 2002). Evidence of tissue destruction (grayish epithelium) will be present within minutes of skin contact (Goldman and Dacre, 1989; Sidell *et al.*, 1997). Severe blisters develop within 12 h after exposure (EPA, 1985a; Goldman and Dacre, 1989; Sidell *et al.*, 1997). The blisters may rupture, usually about 48 h after occurrence, with copious amounts of fluid seeping from the site (Goldman and Dacre, 1989). With dermal exposure, as little as 0.5 ml may cause severe systemic effects and 2 ml may be lethal. Severe edema can be seen due to the Lewisite's increased capillary permeability. Dermal burns are generally deeper than those with mustard gas. Healing occurs much faster than with sulfur mustard-induced lesions and is generally complete within 4 weeks (Goldman and Dacre, 1989; Sidell *et al.*, 1997).

**TABLE 6.3 $LD_{50}$ for Lewisite**

| Species | Route | $LD_{50}$ (mg/kg) |
|---|---|---|
| Mouse | Dermal | 12 |
| Rat | Dermal | 15 |
| | Subcutaneous | 1 |
| | Oral | 50 |
| Human | Dermal | 30 |

RTECS (2006), Sidell *et al.* (1997), and DeRosa *et al.* (2002).

Ocular contact causes immediate pain, lacrimation, and blepharospasm. Permanent blindness may occur if eye exposure occurs for more than 1 min without rapid decontamination (EPA, 1985a; Pohanish 2002). A small droplet (0.001 ml) can cause perforation and loss of vision (Sidell *et al.*, 1997).

Inhalation of the Lewisite vapor may result in irritation to nasal passages and profuse nasal discharge and violent sneezing (HSDB, 2005). Inhalation of 6 ppm can be lethal (USACHPPM, 2001c). Following inhalation of vapor, coughing and hemoptysis commonly occur (Sidell *et al.*, 1997; HSDB, 2005). Lesions following Lewisite inhalation are similar to the lesions produced by mustard gas exposures (Sidell *et al.*, 1997). Dogs that inhaled lethal doses died of necrotizing pseudomembranous laryngotracheobronchitis (Goldman and Dacre, 1989). A thick membrane was noted in the nostrils, larynx, and trachea with purulent bronchitis. Edema, hemorrhage, and emphysema were seen in the lungs (Goldman and Dacre, 1989). Death can be seen within 10 min with high concentrations (EPA, 1985a).

Unlike arsenic or mustard gas, Lewisite does *not* cause damage to the bone marrow or immunosuppression (Sidell *et al.*, 1997). Even though Lewisite is a substituted arsine, it also does not appear to directly cause hemolysis of the red blood cells (RBC) (HSDB, 2005). Hypovolemia, secondary to fluid loss, can be severe enough to cause renal dysfunction (Sidell *et al.*, 1997). Arrhythmias may occur as a result of hypovolemia rather than a direct toxic effect of Lewisite on the myocardium (Sidell *et al.*, 1997). Lewisite was fetotoxic to rats and rabbits, but not teratogenic (Goldman and Dacre, 1989; RTECS, 2006). It is a suspected carcinogen due to its arsenic content (Goldman and Dacre, 1989). Lewisite blood levels are not clinically useful, but an arsenic blood level below 7 $\mu$g/100 ml is considered normal.

## Treatment

Move animals to fresh air and monitor for coughing and respiratory distress. Monitor blood gases and $SpO_2$ in patients with significant exposures. If coughing or difficulty breathing develops, administer oxygen and assist ventilation as needed. Bronchospasm should be treated with inhaled beta agonists and possibly corticosteroids. Monitor electrolytes and packed cell volume (PCV) as animals can become hemoconcentrated. Crystalloids should be given with caution not to overhydrate the patient (Goldfrank *et al.*, 1998). Consider urinary alkalinization and maintain good urine output. Monitor for liver and kidney failure and secondary infection.

Emesis is not recommended due to the irritant and vesicant nature of Lewisite. Dilute oral ingestions with milk or water. Activated charcoal is of unknown benefit in Lewisite ingestion and as severe irritation or vesication (blistering) of the esophagus or GI tract is likely to occur it is not recommended. Endoscopy may be used to determine the extent of injury. Perforation and stricture formation may occur after ingestion.

Flush eyes with copious amounts of tepid water for at least 15 min. A 5% BAL (dimercaprol, British Anti-Lewisite) compounded ophthalmic ointment applied within 2 min may prevent a significant reaction. Treatment at 30 min will lessen the ocular reaction but does not prevent permanent damage (Goldfrank *et al.*, 1998).

Animals should be rinsed with copious amounts of water. A 5% solution of sodium hypochlorite (diluted liquid household bleach) should be used as soon as possible on contaminated skin. Topical application of a 5% BAL ointment within 15 min of an exposure has been reported to be effective in diminishing the blistering effects of Lewisite (Smith, 1999). Wash BAL ointment off after 5 min. The ointment may cause stinging, itching, or urticaria. Burns should be managed as discussed previously under mustard gas. Pain control is very important.

Chelation is indicated if there is coughing, dyspnea, pulmonary edema, or skin burns larger than palm size (Goldfrank *et al.*, 1998). BAL is an effective arsenic chelator, but requires painful deep intramuscular injections and has numerous side effects (hypertension, tachycardia, vomiting, lacrimation, sweating). BAL will increase the clearance rate of arsenic, but it is contraindicated in animals with liver damage (Goldfrank *et al.*, 1998). 2,3-Dimercaptosuccinic acid (DMSA, Succimer®) appears to be a very effective arsenic chelator in animals (Graziano *et al.*, 1978). DMSA is an oral agent and is relatively nontoxic. It may be used following BAL. 2,3-Dimercapto-1-propanesulfonic acid (DMPS) is related to DMSA and is used for heavy metal poisoning, especially in Europe. It has been effective in protecting rabbits from the lethal effects of Lewisite (Aposhian *et al.*, 1982).

Urine arsenic levels may be tested using several methods. Urinary arsenic levels of less than 100 μg are considered normal (Proctor and Hughes, 2004). Concentrations between 700 and 1000 μg/l (0.7–1.0 mg/l) indicate a potentially harmful exposure (Proctor and Hughes, 2004).

### Concluding remarks

Lewisite can remain in the environment for about 1 day. It reacts with water to yield a solid arsenoxide that also has vesicant properties. When in contact with strong alkalis, Lewisite is decomposed to less harmful substances. The potential for secondary contamination is high and material spilled on clothing may be transferred to rescuers or medical personnel. Lewisite is considered a terrorist threat as it is easy to produce and has a quick onset of signs. Carcasses should be buried deeply (away from water supplies), rendered, or incinerated to insure safety of the food supply.

## PHOSGENE OXIME

### Background

Phosgene oxime (Agent CX, "Nettle Rush," CH—$Cl_2$—NO) is a halogenated oxime used as a blistering agent in chemical warfare. Other halogenated oximes include diiodoformoxime, dibromoformoxime, and monochloroformoxime, but phosgene oxime is the most irritant of the group and the only one considered a terrorist warfare threat. Phosgene oxime can be found as a liquid or as a colorless, low melting point crystalline solid, readily soluble in water. The solid form can produce enough vapor to cause symptoms (Sidell *et al.*, 1997). Phosgene oxime has an unpleasant, peppery, and irritating odor. Phosgene oxime is not a true vesicant as it does not cause skin blisters but it does have a rapid dermal corrosive effect. It can be dispersed as a liquid or vapor causing almost immediate tissue damage upon contact (Sidell *et al.*, 1997).

### Pharmacokinetics/toxicokinetics

Absorption in both dermal and inhalational exposures is complete and rapid (within seconds) (Sidell *et al.*, 1997). Dermal lesions form within seconds. As phosgene is soluble in water, it will dissolve in sweat and move to other non-exposed areas of the body (DeRosa *et al.*, 2002). Pulmonary edema can be evident on thoracic radiographs within 2 h of high-dose exposure, 4–6 h of moderate exposure, and approximately 8–24 h after low-dose exposure (Sidell *et al.*, 1997).

### Mechanism of action

The exact mechanism of action is unknown, but it has been proposed that phosgene oxime reacts with SH and $NH_2$ groups (US Army, 1996; Sidell *et al.*, 1997). Phosgene oxime exerts its greatest effects in the first capillary bed it encounters (Sidell *et al.*, 1997).

### Toxicity

Both liquid and vaporous phosgene oxime cause intense, immediate pain, and local tissue destruction on contact

with skin, eyes, and mucuous membranes (Sidell *et al.*, 1997). Damage to the eyes, skin, and airways is similar to that caused by mustard gas. Following dermal contact with either the liquid or vapor, grayish tissue damage may be seen within several minutes. The damaged areas are erythematous and extremely painful. Within 1 h, the area becomes edematous. Browning of the skin and blistering occurs the next day. In about 3 weeks, desquamation, necrosis, crust formation, and purulent exudate occur (US Army, 1996; Sidell *et al.*, 1997). Pain can last for several days. Skin irritation begins at 0.2 mg/min/$m^3$ (12 s) for humans and is intolerable 3 mg/min/$m^3$ (1 min) (USACHPPM, 2001d).

Phosgene oxime is very irritating to the eyes. Very low concentrations can cause lacrimation, inflammation, and temporary blindness and high concentrations can cause permanent corneal lesions and blindness (US Army, 1996; Sidell *et al.*, 1997; USACHPPM, 2001d). Inhalation or oral absorption may cause respiratory tract irritation, dyspnea, and pulmonary edema. The non-cardiogenic pulmonary edema may occur after a several hour delay. Death is due to respiratory arrest.

## Treatment

There is no antidote for phosgene oxime exposure. Move animals into fresh air. Emesis is not recommended after oral ingestion because of the irritant and corrosive effects of phosgene oxime. Immediately dilute oral ingestions with milk or water. Activated charcoal is also not recommended after ingestion, since the primary toxicity is expected to be a local corrosive injury rather than systemic effects from absorption. Charcoal may also obscure endoscopy findings and induce emesis.

Flush eyes with tepid water until pH returns to neutrality and remains so for 30 min after irrigation is discontinued (Brodovsky *et al*., 2000). Decontamination after ocular exposure is critical since phosgene oxime is absorbed within seconds. Corneal ulcers should be treated with mydriatic cycloplegics to prevent synechia development (Grant and Schuman, 1993; Brodovsky *et al*., 2000). For more severe corneal lesions, topical steroids, citrate, ascorbate, and tetracycline or doxycycline may be used to aid in re-epithelialization (Grant and Schuman, 1993).

The skin should be flushed with large volumes of water and mild soap. As phosgene oxime reacts so quickly with tissue, decontamination is not expected to be entirely effective after pain has been produced. Chloramine and phenol towelettes are ineffective for dermal decontamination. Isotonic sodium bicarbonate or 0.5% hypochlorite may remove phosgene oxime that has not yet reacted with tissue. Ulcerated skin lesions should be treated just like a thermal burn. Topical silver sulfadiazine is recommended (Roberts, 1988). Healing of dermal lesions can take from 1 month to over a year (US Army, 1996; Sidell *et al*., 1997). Both sheep and horses should receive tetanus prophylaxis. Other species should be vaccinated at the veterinarian's discretion. Large amounts of opioid analgesics may be needed to help control pain.

Monitor arterial blood gases, pulse oximetry, and thoracic radiographs in patients following significant exposures. Non-cardiogenic pulmonary edema may take 12–24 h to develop. If dyspnea develops, administer 100% humidified oxygen, perform endotracheal intubation, and provide assisted ventilation as required. Beta adrenergic agonists may help if bronchospasm develops. Administer IV fluids but ensure that the animal does not become over hydrated (Hoffman, 2002).

## Concluding remarks

Phosgene oxime is considered non-persistent in the environment. It hydrolyzes rapidly in aqueous alkaline solutions. The potential for secondary contamination is high. Veterinary personnel should wear aprons, rubber gloves, and masks when treating un-decontaminated patients to avoid self-contamination. Phosgene oxime is of interest to terrorists as it penetrates garments and rubber much more quickly than other chemical warfare agents. Phosgene oxime can also be mixed with other chemical warfare agents (e.g. VX). The phosgene oxime will cause skin damage which will increase the dermal absorption of the second agent (Sidell *et al*., 1997).

# CYANIDE AND HYDROGEN CYANIDE

## Background

Cyanogen and cyanogen halides (cyanogen bromide, cyanogen chloride, and cyanogen iodide) have been used historically as military chemical warfare agents (Barr, 1985; ACGIH, 2005). Today cyanide is most likely to be used for a terrorist weapons in the form of hydrogen cyanide or cyanogen chloride.

Hydrogen cyanide (Agent AC, HCN, prussic acid) is a colorless gas with a faint bitter almond-like odor (ACGIH, 2005). Hydrocyanic acid is the liquefied form of hydrogen cyanide (Lewis, 1997). Cyanogen chloride (Agent CK, ClCN) is either a colorless irritant gas or liquid with a pungent odor (ACGIH, 2005). It was developed to be slightly heavier than air and to have greater environmental persistence. It can release hydrogen chloride and hydrogen cyanide when it contacts water, acids, or by thermal decomposition. The water-soluble salt forms (calcium cyanide, sodium cyanide, and potassium cyanide) will form HCN gas when mixed with a strong acid.

Animals with cyanide poisoning may have an odor of bitter almonds in their gastric or ruminal contents or expired breath. The ability to smell the bitter almond-like odor of cyanide is genetically determined, and 20–60% of the population cannot detect its presence (Hall and Rumack, 1986). Cyanide and related compounds are classified as blood agents.

## Pharmacokinetics/toxicokinetics

Cyanide and hydrogen cyanide can be absorbed by inhalation, ingestion, ocularly, and through intact skin (Ballantyne, 1983; Hall and Rumack, 1986). Cyanide rapidly diffuses into tissues and irreversibly binds to its target sites. Dermal absorption of significant amounts of hydrogen cyanide gas has not been reported. There have been no reports of systemic poisoning in humans ocularly exposed to cyanide; however, rabbits have died following ocular exposure to NaCN, KCN, and HCN (Ballantyne, 1983).

Cyanide is distributed to all organs and tissues. The concentration of cyanide in red cells is greater than that in plasma by a factor of 2 or 3 (HSDB, 2005). Cyanide accumulates in neural tissue. It preferentially accumulates in the hypothalamus, with levels about 40% higher compared to the hippocampus, cerebellum, and cortex (Borowitz *et al*., 1994). In acute cyanide intoxication, there are no specific pathological changes.

Cyanide is metabolized by rhodanase in the liver to thiocyanate (Hall and Rumack, 1986). This reaction complexes cyanide with endogenous sulfur or sulfur supplied from the sodium thiosulfate antidote. Once thiocyanate is formed it is excreted mainly in the urine. Half-life for the metabolism of cyanide to thiocyanate is 20 min to 1 h (Feldstein and Klendshoj, 1954). In animals, the dose of cyanide that produces signs is very close to the lethal dose and death can occur within seconds to minutes.

## Mechanism of action

Cyanide causes its toxicity by forming a stable complex with ferric iron ($Fe^{3+}$) in cytochrome oxidase enzymes. Since oxygen is unable to re-oxidize the reduced cytochrome a3, this inhibits cellular respiration, oxygen utilization, and ATP production, resulting in deprivation of oxygen to the body at the cellular level (Way *et al*., 1988). In the brain, cyanide decreases oxidative metabolism, increases glycolysis, and inhibits brain glutamic acid decarboxylase, thereby decreasing gamma aminobutyric acid (GABA) (Bingham *et al*., 2001). The corpus callosum, hippocampus, corpora striata, and substantia nigra are commonly damaged in cyanide poisoning (Grandas *et al*., 1989).

Early in cyanide toxicosis, central nervous system (CNS), respiratory, and myocardial depression also contribute to decreased oxygenation of the blood and decreased cardiac output (Hall and Rumack, 1986). There is also evidence of lipid peroxidation by measurement of elevated levels of conjugated dienes in mouse brain and kidneys at 15 and 30 min after cyanide exposure (Ardelt *et al*., 1994). Cyanide salts are irritating upon ingestion and can cause corrosion of the oral, esophageal, or gastric mucosa (HSDB, 2005).

## Toxicity

Signs following acute cyanide exposure include syncope, or CNS stimulation, dizziness, dyspnea, seizures, paralysis, apnea, and coma (Vogel *et al*., 1981; Hall and Rumack, 1986). Tachypnea and hyperpnea are followed rapidly by respiratory depression. Signs of severe hypoxia without cyanosis can suggest the diagnosis. Signs in birds are similar to those in mammals. Chickens will pant, and have rapid eye blinking, excess salivation, and lethargy (Wiemeyer *et al*., 1986). Mydriasis is common in severe poisonings and blindness may occur from cyanide-induced damage to optic nerves and retina (Vogel *et al*., 1981; Grant and Schuman, 1993). Nausea, vomiting, and abdominal pain may occur, especially after ingestion of cyanide salts (Vogel *et al*., 1981; Hall and Rumack, 1986; Singh *et al*., 1989). Metabolic acidosis and lactic acidosis are frequent metabolic derrangements associated with cyanide poisoning. Blood gases show a decreased arterial–venous oxygen saturation difference due to the cellular inability to extract oxygen (Paulet, 1955; Graham *et al*., 1977).

The blood, both arterial and venous, becomes cherry red from accumulated oxyhemoglobin (Lewis, 2000; Bingham *et al*., 2001). The skin may also be a bright pink color from the high concentration of oxyhemoglobin in the venous return (HSDB, 2005). On funduscopic examination, retinal arteries and veins will appear equally red. The arterialization of venous blood gases occurs early in the process of cyanide poisoning (Hall and Rumack, 1986). Tachycardia and hypertension may be seen in the initial phases of cyanide poisoning followed by bradycardia and hypotension in the late phases (Vogel *et al*., 1981; Hall and Rumack, 1986). Cyanide exposure can produce death within minutes. See Table 6.4 for $LD_{50}$ of HCN by various routes.

Cyanide can be measured in blood. No symptoms are expected at concentrations less than 0.2 mg/l, tachycardia can be seen at 0.5–1.0 mg/l, obtundation at 1.0–2.5 mg/l, coma and respiratory depression at levels greater than 2.5 mg/l, and death with blood levels greater than 3 mg/l (Graham *et al*., 1977). No adverse reproductive studies were found for cyanide or hydrogen cyanide, but in

**TABLE 6.4 $LD_{50}$ for hydrogen cyanide**

| Species | Route | $LD_{50}$ (μg/kg) |
|---|---|---|
| Mouse | IM | 2700 |
| | IP | 2990 |
| | Oral | 3700 |
| Rat | SQ | 3700 |

ACGIH (2005), Bingham *et al.* (2001), Budavari (2000), HSDB (2005), ITI (1995), Lewis (2000), OHM/TADS (2005), and RTECS (2006).

laboratory animals, cyanide compounds did cause resorptions, malformations, and teratogenic effects in offspring (Willhite, 1983).

## Treatment

Move animals to fresh air. Emesis is not recommended due to the rapid progression of the clinical signs and potential for seizures, coma, or apnea. Activated charcoal may be beneficial if administered immediately after ingestion as the absorption of cyanide is rapid. Flush eyes for at least 15–20 min with tepid water. Wash all contaminated animals thoroughly with soap and water.

Monitor arterial blood gases and serum electrolytes (Vogel *et al.*, 1981; Hall and Rumack, 1986). Administer 100% humidified oxygen with assisted ventilation if needed to maintain an elevated $pO_2$. Hyperbaric oxygen therapy is approved for cyanide poisoning by the Undersea Medical Society (Myers and Schnitzer, 1984). It has been suggested to improve clinical outcome, but experimental animal studies have been questionable (Way *et al.*, 1972). For severe acidosis ($pH < 7.1$) administer sodium bicarbonate, but acidosis may be difficult to correct prior to administration of antidotes in cyanide toxicosis. Control seizures with benzodiazepines or barbiturates.

Cyanide toxicosis usually occurs and progresses so rapidly that treatment is rarely administered soon enough to be effective. Antidotal agents should be used if the animal is in respiratory distress or a coma. Treatment for cyanide poisoning includes several steps. Sodium nitrite should be given IV over 15–20 min, as soon as vascular access is established. Quick administration can cause hypotension. Sodium nitrite reacts with hemoglobin in the RBC forming methemoglobin. The methemoglobin will combine with free cyanide to form cyanomethemoglobin. If possible, monitor methemoglobin levels during nitrite administration. The goal is to maintain methemoglobin levels below 30% (Hall and Rumack, 1986).

Follow sodium nitrite with IV administration of sodium thiosulfate. Sodium thiosulfate supplies sulfur for the rhodanase reaction (Hall and Rumack, 1987). Thiocyanate is formed and excreted in the urine. Oxygen, combined with traditional nitrite/thiosulfate therapy, provides better results than thiosulfate alone (Way *et al.*, 1972). It is believed that oxygen may reverse the cyanide–cytochrome oxidase complex and aid in the conversion to thiocyanate following thiosulfate administration (Graham *et al.*, 1977).

Hydroxocobalamin, a vitamin B12 precursor, is a cobalt containing chelator. It is the chelator of choice in Europe and Australia. Hydroxocobalamin is not approved for use in the United States. Hydroxocobalamin reverses cyanide toxicosis by combining with cyanide to form cyanocobalamin (vitamin B12) (Hall and Rumack, 1987). It has been shown to be effective in treating cyanide-poisoned laboratory animals and has the advantage of producing neither methemoglobinemia nor hypotension, as sodium nitrite does. Dicobalt–EDTA (Kelocyanor®) is also highly effective in chelating cyanide. It is used clinically in Europe, Israel, and Australia, but it is not available in the United States (Hillman *et al.*, 1974). Another methemoglobin-inducing agent used in some European countries is 4-dimethylaminophenol hydrochloride (4-DMAP). It has a more rapid onset of methemoglobin production than sodium nitrite. Methemoglobin peaks at 5 min after 4-DMAP versus 30 min after sodium nitrite. 4-DMAP is coadministered with thiosulfate. Excessive methemoglobin production can be a major complication and hemolysis may occur with therapeutic doses (van Dijk *et al.*, 1986; Weger, 1990).

Animal studies to identify alternate cyanide antidotes have tested stroma-free methemoglobin solutions, alpha-ketoglutaric acid, chlorpromazine, hydroxylamine, phenoxybenzamine, centrophenoxine, naloxone hydrochloride, etomidate, para-aminopropiophenone, and calcium-ion-channel blockers (Ashton *et al.*, 1980; Amery *et al.*, 1981; Dubinsky *et al.*, 1984; Leung *et al.*, 1984; Ten Eyck *et al.*, 1985; Johnson *et al.*, 1986; Bright and Marrs, 1987; Yamamoto, 1990; Budavari, 2000). These antidotes have shown some promise in the laboratory setting, but have not been tried during actual poisoning situations.

Blood cyanide levels can be useful in confirming the diagnosis. However, unless the results are available within a reasonable time, it is not clinically useful. Cyanide can be measured by several methods but most take several hours to complete. Biological specimens can be tested using spectroscopy, GC, gas–liquid chromatography, paper chromatography, ion specific electrode, fluorimetric, microdistillation, and paper strip (Groff *et al.*, 1985; Fligner *et al.*, 1992; HSDB, 2005). Cyanide and thiocyanate levels can be measured in timed urine collections to provide information on cyanide clearance, but it is rarely done clinically. Handling of samples is very important due to the volatile nature of HCN. The loss of cyanide from specimens can be minimized by rapid analysis after death, collection of blood from a closed source, storage in a fluoride–oxalate tube with minimal dead space, and frozen at −20°C (Bright *et al.*, 1990).

## Concluding remarks

Because HCN is lighter than air, it has a long half-life in air. However, HCN is rapidly dispersed and diluted to non-toxic concentrations. Cyanide does not concentrate in soil or plant material, but can mix with water. Water converts HCN gas to HCN liquid. Treat contaminated water with ozone, hydrogen peroxide, or calcium or sodium hypochlorite bleach. The potential for secondary contamination of rescue personnel is high. Boots, gloves, googles, full protective clothes, and a self-contained positive pressure breathing apparatus are needed (AAR, 2000).

As a chemical warfare agent, cyanide is not easy to disseminate; however, it is widely available which increases the chances of its use in terrorist activities (Burklow *et al.*, 2003). Cyanide works much better as a terrorist weapon in an enclosed space.

# MILITARY NERVE AGENTS

## Background

Military nerve agents are probably the most poisonous of the known chemical warfare agents and are sufficiently toxic that even a brief exposure may be fatal. They were originally synthesized by the Germans during World War II in search of alternatives to the embargoes against insecticidal nicotine. Military nerve agents are rapidly acting, anticholinesterase organophosphate (OP) compounds. Military nerve agents are more potent than OP insecticides and military nerve agents contain a C—P bond that is unique and very resistant to hydrolysis, except in highly alkaline solutions. At ambient temperatures, nerve agents are viscous liquids, not gases.

Military nerve agents are generally divided into "G" and "V" agents. The "G" agents (tabun, sarin, soman) were developed during World War II and are called "G" agents because they were first synthesized in Germany. The "G" agents are very volatile and present a vapor hazard. The vapors are denser than air, thus they stay close to the ground (Garigan, 1996). Tabun (Agent GA, $C_5$—$H_{11}$—$N_2$—$O_2$—P) was first of the "G" agents to be synthesized in 1936, followed by sarin (GB, $C_4$—$H_{10}$—F—$O_2$—P) in 1938 and soman (GD, $C_7$—$H_{16}$—F—$O_2$—P) in 1944 (Sidell *et al.*, 1997).

Tabun is the easiest of the "G" agents to manufacture. It is a fruity-smelling (like bitter almonds) combustible colorless to brownish liquid. Contact with bleaching powder generates cyanogen chloride (EPA, 1985c). It may also undergo hydrolysis in the presence of acids or water, releasing hydrogen cyanide (Munro *et al.*, 1999; Budavari, 2000; HSDB, 2005). Sarin is a colorless liquid with almost no odor in its pure state (Budavari, 2000). Soman is a colorless liquid with a fruity or camphor odor (Budavari, 2000). Soman can release hydrogen fluoride when in contact with acids (EPA, 1985c).

The "V" agents ("V" for venomous) were developed in 1954 in the United Kingdom and are more stable than the "G" agents (Sidell *et al.*, 1997). "V" agents, such as VX, contain a sulfur group and are alkylphosphonothiolates, they are more toxic and persistent on surfaces than G-series agents. VX ($C_{11}$—$H_{26}$—N—$O_2$—P—S) is a nonvolatile, amber colored, odorless liquid (Sidell *et al.*, 1997).

## Pharmacokinetics/toxicokinetics

Nerve agents can be absorbed following ocular exposure, oral ingestion, inhalation, and dermal contact (HSDB, 2005; RTECS, 2006). These nerve agents are absorbed without producing any irritation or other sensation on the part of the exposed person or animal (HSDB, 2005). Inhalation of military nerve agents will have initial effects on the airways within seconds. Inhalation of a large amount of the vapor will result in sudden loss of consciousness, apnea, flaccid paralysis, and seizures within seconds to 2–3 min (Sidell *et al.*, 1997). Peak effects are seen within 20–30 min and death is usually due to respiratory failure (Berkenstadt *et al.*, 1991). Dermal exposures to nerve agents have a slower onset of action. Following exposure to a large drop or more will result in clinical effects within 30 min but with small drops, a delay of up to 18 h can be seen (Sidell *et al.*, 1997). With an ingestion, initial symptoms begin in 20–30 min and are usually GI. There is no taste to solutions containing nerve gas agents (Grob, 1956).

Distribution in the body is slightly different for each of the nerve agents. Distribution of sarin is to the brain, liver, kidney, and plasma of mice (Little *et al.*, 1986). Radiolabeled soman was evenly distributed throughout the mouse brain after IV administration, with higher levels in the hypothalamus (Wolthuis *et al.*, 1986). Tabun was also found in high concentrations in the hypothalamus after IV administration in mice (Hoskins *et al.*, 1986). An unusual feature of soman toxicity is its apparent storage in body "depots" and release over time. This results in eventual death in animals who survive the initial dose of soman (Wolthuis *et al.*, 1986).

The military nerve agents differ from other OPs in the rapidity of "aging" of the OP–enzyme complex. "Aging" is thought to be due to the loss of an alkyl group, whereby the inhibitor–enzyme complex becomes resistant to reactivation (Young *et al.*, 1999). The half-life ($T_{1/2}$) of aging: soman within minutes, sarin about 5 h, tabun and VX both greater than 40 h (Garigan, 1996).

The nerve agents are hydrolyzed by plasma and tissue enzymes to their corresponding phosphoric and phosphonic acids. Oxidative enzymes are also involved in metabolism (HSDB, 2005). Sarin is hydrolyzed in the body to isopropyl-methylphosphonic acid (IMPA). IMPA in mice

studies was generally present at 20-fold higher concentrations than sarin in most tissues, exceeding sarin by 4 times in the brain (Little *et al.*, 1986). In mice studies, the majority of administered radioactive sarin was detoxified and excreted by the kidneys (Little *et al.*, 1986). Mouse studies reveal that approximately 50% of injected soman is converted to free pinacolyl-methylphosphonic acid within 1 min, and the half-life of this metabolite is less than 1 h (Reynolds *et al.*, 1985). Soman is mainly eliminated via enzymatic hydrolysis, in competition with binding to target acetylcholinesterase (AChE) (HSDB, 2005).

**TABLE 6.5** $LD_{50}$ (mg/kg) for various nerve agents

| Species | Route | Sarin | Soman | Tabun | VX |
|---|---|---|---|---|---|
| Mouse | IP | | | | 0.050 |
| | Dermal | 1.08 | 7.8 | 1 | |
| | IM | | 0.089 | | |
| Rat | SQ | | | 0.162 | 0.012 |
| | Oral | 0.55 | | 3.7 | |
| | Dermal | | | 18 | |
| | IM | | 0.062 | | |
| Human | Dermal | 28 | 5 | 14 | 0.14 |

RTECS (2006) and Sidell *et al.* (1997).

## Mechanism of action

OPs competitively inhibit AChE by binding irreversibility to its esteric site (phosphorylation). Inhibition of the AChE enzyme results in accumulation of acetylcholine and excessive stimulation at muscarinic, nicotinic, and CNS cholinergic sites. Increased acetylcholine at autonomic neuroeffector junctions results in increased smooth muscle contractions and secretions, but its effect at skeletal muscle junctions is partly stimulatory (fasciculations) and partly inhibitory (muscle weakness, paralysis) (Garigan, 1996). The effects on the sino-atrial node of the heart is inhibitory, causing bradycardia (Namba *et al.*, 1971). Acetylcholine accumulation in the CNS can cause ataxia, seizures, and coma. These high levels induce massive neuronal deaths in various brain areas, particularly in limbic and cortical structures. Death from nerve agents is due to paralysis of the diaphragm, airway obstruction from increased bronchial secretions, or depression of the CNS respiratory center (Garigan, 1996).

VX is also thought to possibly react directly with receptors of other neurotransmitters, such as norepinephrine, dopamine, and GABA. VX appears to have CNS effects that are unrelated to AChE activity and these agents may produce prolonged effects following convulsive doses (Young *et al.*, 1999).

## Toxicity

Symptoms of acute exposure to OPs may include muscarinic, nicotinic, and CNS signs. The muscarinic effects include sweating, hypersalivation, increased bronchial secretions, miosis, bradycardia, hypotension, vomiting, and diarrhea, bronchoconstriction, and urinary and fecal incontinence. The nicotinic effects include fasciculations and weakness of muscles (including the diaphragm), tachycardia, hypertension, and mydriasis. The CNS effects of nerve agents include restlessness, anxiety, headaches, seizures, and coma (Garigan, 1996). Effects after inhalation begin within seconds to minutes post exposure (Sidell *et al.*, 1997; HSDB, 2005). Death can occur within minutes from inhibition of AChE function.

The "G" nerve gases do not readily penetrate intact skin, but toxicity significantly increases if the skin becomes permeable. Dermal toxicity of VX is high, even through intact skin as the liquid does not evaporate quickly (Berkenstadt *et al.*, 1991; Sidell *et al.*, 1997). With dermal exposures, a very small drop on the skin may cause sweating and fasciculations at the site, starting within 18 h of exposure. A larger drop may cause loss of consciousness, seizures, apnea, and flaccid paralysis, with effects beginning within 30 min. Liquid tabun in the eye can result in death nearly as quickly as an inhalational dose (EPA, 1985c).

On a weight basis, toxicity in descending order is VX > soman > sarin > tabun. As used, sarin is the most potent of the "G" nerve agents and VX is about 3 times more potent a respiratory agent as sarin (HSDB, 2005). VX is 300 times more lethal than tabun on skin (Sidell *et al.*, 1997). See Table 6.5 for $LD_{50}$ of the various nerve agents.

Plasma cholinesterase values usually recover in a few days or weeks, due to the irreversible nature of OP inhibition. RBC AChE recovers more slowly (several days to 4 months) depending on the severity of the depression (Grob, 1956). Delayed neurotoxicity has not been reported in humans following nerve agent exposure. However, delayed peripheral neurotoxicity has been reported in animal studies. Soman, at a dose of 1.5 mg/kg, produced severe delayed neuropathy in the atropinized hen assay (Willems *et al.*, 1984). No prenatal mortality or fetal toxicity was noted in soman-poisoned rats or rabbits, even at doses producing significant maternal toxicity, but other nerve gases showed post-implantation mortality and fetotoxicity (HSDB, 2005; RTECS, 2006).

## Treatment

Remove animal from the toxic environment. Administer oxygen if needed. Intubation and ventilation may be necessary if signs progress. Flush eyes with copious amounts of tepid 0.9% saline or water for at least 15 min. Wash all exposed animals 3 times with soap and water. The use of a dilute bleach solution (1:10 with water), ethanol, or tincture of green soap may be more efficacious (Cancio, 1993).

Military personnel carry skin decontaminating kits and towelettes impregnated with an alkaline chloramine and phenol mixture which can break down nerve agents (M291 Skin Decontaminating Kit, Rohm and Haas). The kit has been tested for safety and efficacy against mustard vesicant agents and OP nerve agents. A topical skin protectant (IB1) has also been tested as a pretreatment for exposure to sulfur mustard and VX (Kadar *et al*., 2003).

Emesis is not recommended in oral ingestion due to the rapid development of signs. Activated charcoal might provide benefits even after a topical exposure. Control seizures with diazepam, methocarbamol, or barbiturates as needed before proceeding with other treatments.

Atropine sulfate is reversal agent. It is used for the treatment of muscarinic effects of nerve agent poisoning, and will not reverse nicotinic effects (muscular weakness, diaphragmatic weakness, etc.). Atropine does not affect the AChE–insecticide bond, but blocks the effects of accumulated acetylcholine at the synapse. Atropinization should be continued until the nerve agent is metabolized (Midtling *et al*., 1985). Effects of overdosing with atropine include hyperthermia, tachycardia, inspiratory stridor, irritability, and dilated and unresponsive pupils (Meerstadt, 1982).

Pralidoxime chloride (2-PAM) can be used to treat the nicotinic signs. Pralidoxime is probably most effective when administered in the first 1–3 h. Pralidoxime is not as effective in the treatment of soman poisoning, due to the quick "aging" (within minutes) of the compound (Sidell *et al*., 1997). Since VX-inhibited cholinesterase ages slowly, administration of 2-PAM chloride is effective in reactivating the enzyme for up to 48 h after exposure (Sidell and Groff, 1974).

In Belgium, Israel, The Netherlands, Scandinavia, Portugal, and West Germany, obidoxime dichloride (Toxogonin, LUH6) is the favored oxime. It may be a less toxic and more efficacious alternative to pralidoxime in poisonings from OPs containing a dimethoxy or diethoxy moiety (De Kort *et al*., 1988). HI-6 is an alternative oxime that has excellent AChE regenerating action with VX and very good action with sarin (GB). It has a good response to soman, but provides poor to no response following tabun exposures (Hoffman, 1999). HI-6 is given in conjuction with atropine and diazepam (Kusic *et al*., 1991).

With human exposures to nerve agents, autoinjectors (AtroPen®, Mark I®, Combopen MC®) are available for use. Most available autoinjectors combine atropine and pralidoxime. Autoinjectors are not used in veterinary medicine as they are not adaptable for different sized patients.

The "G" agents have been detected in urine and water using capillary GC and GC-MS (Okudera *et al*., 1997; Kientz, 1998). Immunoassay has been used to detect VX in biological samples (Ci *et al*., 1995). Nerve agent detection is not as clinically relevant as the measuring of AChE. AChE activity can be used as a diagnostic indicator or a screening test. The test can be run on plasma, serum, or whole blood. AChE activity that is less than 50% of normal is generally associated with severe symptoms (Midtling *et al*., 1985). Blood AChE does not always correlate well with clinical signs and poisoning has been diagnosed in patients with "normal" values.

## Concluding remarks

The "G" agents evaporate and disperse over several hours, they are non-persistent in the environment (Garigan, 1996). In contrast, VX is an oily liquid that can remain in the environment for weeks or longer after being dispersed (Garigan, 1996; Sidell *et al*., 1997; Munro *et al*., 1999; Budavari, 2000). Environmental persistence is estimated to be 0.5–1 day for tabun, 1–2 days for soman, 5 days for sarin, and several weeks for VX. Environmental cleanup of OP spills depend on changing the pH to promote hydrolysis to inactive phosphate diester compounds (EPA, 1978). Contaminated soil can be treated with either alkaline substances (sodium carbonate, sodium bicarbonate, calcium hydroxide, and calcium carbonate) or chlorine-active compounds (sodium hypochlorite or calcium hypochlorite) (EPA, 1975).

Veterinarians and staff can be dermally exposed to contaminated animals. Rubber gowns, aprons, and gloves along with respiratory protection must be worn. Once the animals are bathed, the risk of secondary contamination is low. Leather absorbs OPs and is extremely difficult to decontaminate. Leather collars, muzzles, and other items should be disposed of by incineration.

The production of nerve agents is beyond the capabilities of most terrorist groups. Production requires a significant background in chemistry and outlay of capital. The production of sarin by the Aum Shinrikyo cult was estimated to take 1 year to make, involved 80 persons led by a PhD level scientist, and cost about $30 million (Leitenberg, 1999).

## REFERENCES

AAR (2000) *Emergency Handling of Hazardous Materials in Surface Transportation*. Bureau of Explosives, Association of American Railroads, Washington, DC.

ACGIH (2005) Threshold Limit Values (TLVs(R)) for Chemical Substances and Physical Agents and Biological Exposure Indices (BEIs(R)). *American Conference of Governmental Industrial Hygienists*, Cincinnati, OH.

Amery WK, Wauquier A, van Neuten JM (1981) The anti-migrainous pharmacology of flunarizine (R14950), a calcium antagonist. *Drug Exp Clin Res* **7**: 1–10.

Andreassi L (1991) Chemical warfare and the skin. *Int J Dermatol* **30**: 252–3.

Aposhian HV, Mershon MM, Brinkley FB (1982) Anti-Lewisite activity and stability of meso-dimercaptosuccinic acid and 2,3-dimercapto-1-propanesulfonic acid. *Life Sci* **31**: 2149–56.

Ardelt BK, Borowitz JL, Maduh EU (1994) Cyanide-induced lipid peroxidation in different organs: subcellular distribution and hydroperoxide generation in neuronal cells. *Toxicology* **89**: 127–37.

Ashton D, van Reempts J, Wauquier A (1980) Behavioral, electroencephalographic and histological study of the protective effect of etomidate against histotoxic dysoxia produced by cyanide. *Arch Int Pharmacodyn Ther* **254**: 196–213.

Ballantyne B (1983) Acute systemic toxicity of cyanides by topical application to the eye. *J Toxicol Cut Ocular Toxicol* **2**: 119–29.

Barlow SM, Sullivan FM (1982) Arsenic and its compounds. In *Reproductive Hazards of Industrial Chemicals*. Academic Press, London, UK, pp. 62–82.

Barr SJ (1985) Chemical warfare agents. *Topic Emerg Med* **7**: 62–70.

Berkenstadt H, Marganitt B, Atsmon J (1991) Combined chemical and conventional injuries – pathophysiological, diagnostic and therapeutic aspects. *Isr J Med Sci* **27**: 623–6.

Bingham E, Chorssen B, Powell CH (2001) *Patty's Toxicology*, 5th edn, vol. 3. John Wiley & Sons, New York.

Bismuth C, Blanchet-Bardon C, Baud FJ (1995) Delayed admission of five soldiers intoxicated with mustard gas. *Ann Emerg Med* **26**: 715.

Blodi FC (1971) Mustard gas keratopathy. *Int Opthalmol Clin* **11(3)**: 1–13.

Borak J, Diller WF (2001) Phosgene exposure: mechanisms of injury and treatment strategies. *J Occupat Environ Med* **43(2)**: 110–19.

Borak J, Sidell FR (1992) Agents of chemical warfare: sulfur mustard. *Ann Emerg Med* **213**: 303–7.

Borowitz JL, Rathinavelu A, Kanthasamy A (1994) Accumulation of labeled cyanide in neuronal tissue. *Toxicol Appl Pharmacol* **129**: 80–5.

Bright JE, Inns RH, Tuckwell NJ (1990) The effect of storage upon cyanide in blood samples. *Hum Exp Toxicol* **9**: 125–9.

Bright JE, Marrs TC (1987) Effects of *p*-aminopropiophenone (PAPP), a cyanide antidote, on cyanide given by intravenous infusion. *Hum Toxicol* **6**: 133–7.

Brodovsky SC, McCarty AC, Snibson G (2000) Management of alkali burns an 11-year retrospective review. *Ophthalmology* **107**: 1829–35.

Budavari S (2000) *The Merck Index*, 12th edn. on CD-ROM. Version 12:3a. Chapman & Hall/CRCnetBASE, Whitehouse Station, NJ.

Burklow TR, Yu CE, Madsen JM (2003) Industrial chemicals: terrorist weapons of opportunity. *Pediatr Ann* **32(4)**: 230–4.

Cancio LC (1993) Chemical casualty decontamination by medical platoons in the 82nd airborne division. *Mil Med* **158**: 1–5.

Chemstar (1996) *Phosgene Pulmonary Exposure Information*, 2nd edn. Chemical Manufacturers Association, Phosgene Panel, Arlington, VA.

Chester EH, Kaimal J, Payne CB (1977) Pulmonary injury following exposure to chlorine gas. Possible beneficial effects of steroid treatment. *Chest* **72**: 247–50.

Chisholm CD, Singletary EM, Okerberg CV (1989) Inhaled sodium bicarbonate therapy for chlorine inhalation injuries (Abstract). *Ann Emerg Med* **18**: 466.

CHRIS (2005) *CHRIS Hazardous Chemical Data*. US Department of Transportation, US Coast Guard, Washington, DC. (Internet Version). Edition expires in 2005. Thomson MICROMEDEX, Greenwood Village, CO.

Ci YX, Zhou YX, Guo ZQ, Rong KT, Chang WB (1995) Production, characterization and application of monoclonal antibodies against the organophosphorus nerve agent VX. *Arch Toxicol* **69(8)**: 565-7.

Cowan FM, Broomfield CA, Smith WJ (1998) Sulfur mustard exposure enhances Fe receptor expression on human epidermal keratinocytes in cell culture: implications for toxicity and medical countermeasures. *Cell Biol Toxicol* **14**: 261–6.

Dachir S, Fishbeine E, Meshulam Y, Sahar R, Chapman S, Amir A, Kadar T (2004) Amelioration of sulfur mustard skin injury following a topical treatment with a mixture of a steroid and a NSAID. *J Appl Toxicol* **24**: 107–13.

Dacre JC, Goldman M (1996) Toxicology and pharmacology of the chemical warfare agent sulfur mustard. *Pharmacol Rev* **48**: 289–326.

Decker WJ (1988) Reactive airways dysfunction syndrome following a single acute exposure to chlorine gas (Abstract). *Vet Hum Toxicol* **30**: 344.

De Kort WL, Kiestra SH, Sangster B (1988). The use of atropine and oximes in organophosphate intoxications: a modified approach. *Clin Toxicol* **26**: 199–208.

DeRosa CT, Holler JS, *Allred M, et al. (2002) Managing hazardous* materials incidents. *Agency for Toxic Substances and Disease Registry* (www.atsdr.cdc.gov).

Diller WF (1985) Pathogenesis of phosgene poisoning. *Toxicol Ind Health* **1(2)**: 7–15.

Drasch G, Kretschmer E, Kauert G (1987) Concentrations of mustard gas (bis(2-chloroethyl)sulfide) in the tissues of a victim of a vesicant exposure. *J Forens Sci* **32**: 1788–93.

Dubinsky B, Sierchio JN, Temple DE (1984) Flunarizine and verapamil: effects on central nervous system and peripheral consequences of cytotoxic hypoxia in rats. *Life Sci* **34**: 1299–306.

EPA (1975) *Guidelines for the Disposal of Small Quantities of Unused Pesticides (EPA-670/2-75-057)*. US Environmental Protection Agency, Washington, DC, pp. 315–30.

EPA (1978) *Identification and Description of Chemical Deactivation/Detoxification Methods for the Safe Disposal of Selected Pesticides (SW-156c)*. US Environmental Protection Agency, Washington, DC, pp. 44–88.

EPA (1985a) *EPA Chemical Profile on Lewisite*. US Environmental Protection Agency, Washington, DC.

EPA (1985b) *EPA Chemical Profile on Mustard Gas*. US Environmental Protection Agency, Washington, DC.

EPA (1985c) *EPA Chemical Profile on Sarin; Tabun*. US Environmental Protection Agency, Washington, DC.

Evison D, Hinsley D, Rice P (2002) Chemical weapons. *Br Med J* **324**(7333): 332–5.

Feldstein M, Klendshoj NC (1954) The determination of cyanide in biologic fluids by microdiffusion analysis. *J Lab Clin Med* **44**(1): 166–70.

Fligner CL, Luthi R, Linkaityte-Weiss E (1992) Paper strip screening method for detection of cyanide in blood using CYANTESMO test paper. *Am J Forens Med Pathol* **13**: 81–4.

Franch S, Hatch GE (1986) Pulmonary biochemical effects of inhaled phosgene in rats. *J Toxicol Environ Health* **19(3)**: 413–23.

Garigan T (1996) *Medical Treatment of Chemical Warfare Casualties*. Uniformed Services Academy of Family Physicians, Okinawa, Japan. http:www.usafp.org/op_med/fieldclinical/chemcascare.html (accessed May 15, 2006).

Ghio AJ, Kennedy TP, Hatch GE, Tepper JS (1991) Reduction of neutrophil influx diminishes lung injury and mortality following phosgene inhalation. *J Appl Physiol* **71**(2): 657–65.

Goldfrank LR, Flomenbaum NE, Lewis NA (1998) *Goldfrank's Toxicologic Emergencies*, 6th edn. Appleton & Lange, Stamford, CN.

Goldman M, Dacre JC (1989) Lewisite: its chemistry, toxicology, and biological effects. *Rev Environ Contam Toxicol* **110**: 75–115.

Graham DL, Laman D, Theodore J (1977) Acute cyanide poisoning complicated by lactic acidosis and pulmonary edema. *Arch Intern Med* **137**: 1051–5.

Grandas F, Artieda J, Obeso JA (1989) Clinical and CT scan findings in a case of cyanide intoxication. *Mov Disord* **4**: 188–93.

Grant WM, Schuman JS (1993) *Toxicology of the Eye*, 4th edn. Charles C. Thomas, Springfield, IL.

Graziano JH, Cuccia D, Friedheim E (1978) The pharmacology of 2,3-dimercaptosuccinic acid and its potential use in arsenic poisoning. *J Pharmacol Exp Ther* **207**: 1051–5.

Grob D (1956) The manifestations and treatment of poisoning due to nerve gas and other organic phosphate anticholinesterase compounds. *Arch Intern Med* **98**: 221–39.

Groff WA, Stemler FW, Kaminskis A (1985) Plasma free cyanide and blood total cyanide: a rapid completely automated microdistillation assay. *Clin Toxicol* **23**: 133–63.

Guloglu C, Kara IH, Erten PG (2002) Acute accidental exposure to chlorine gas in the Southeast of Turkey: a study of 106 cases. *Environ Res* **88**: 89–93.

Guo YL, Kennedy TP, Michael JR, Sciuto AM, Adkinson Jr NF, Gurtner GH (1990) Mechanism of phosgene-induced lung toxicity: role of arachidonate mediators. *J Appl Physiol* **69**: 1615–22.

Hackett PL, Rommereim RL, Burton FG, Buschbom RL, Sasser LB (1987) *Teratology Studies on Lewisite and Sulfur Mustard Agents: Effects of Sulfur Mustard in Rats and Rabbits*. US Army Medical Research and Development Command, Fort Detrick, Frederick, MD.

Hall AH, Rumack BH (1986) Clinical toxicology of cyanide. *Ann Emerg Med* **15**: 1067–74.

Hall AH, Rumack BH (1987) Hydroxycobalamin/sodium thiosulfate as a cyanide antidote. *J Emerg Med* **5**: 115–21.

Hambrook JL, Harrison JM, Howells DJ (1992) Biological fate of sulphur mustard (1,1′-thiobis(2-chloroethane)): urinary and faecal excretion of 35S by rat after injection or cutaneous application of 35S-labelled sulphur mustard. *Xenobiotica* **22**: 65–75.

Hillman B, Bardhan KD, Bain JTB (1974) The use of dicobalt edetate (Kelocyanor) in cyanide poisoning. *Postgrad Med J* **50**: 171–4.

Hoffman RS (1999) Soman poisoning and autoinjectors and reactivators. *Proceedings, NACCT Meeting*, La Jolla, CA.

Hoffman RS (2002) Respiratory principles. In Goldfrank's Toxicologic Emergencies, 7th edn, Goldfrank LR, Flomenbaum NE, Lewin NA (eds). McGraw-Hill, New York.

Hoskins B, Fernando JC, Dulaney MD (1986) Relationship between the neurotoxicities of soman, sarin and tabun, and acetylcholinesterase inhibition. *Toxicol Lett* **30**: 121–9.

HSDB, Hazardous Substances Data Bank (2005) National Library of Medicine, Bethesda, MD (Internet Version). Edition expires in 2005. Thomson MICROMEDEX, Greenwood Village, CO.

IARC (1975) *IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Man*, vol. 9. World Health Organization, Geneva, Switzerland, pp. 181–92.

ITI (1995) *Toxic and Hazardous Industrial Chemicals Safety Manual*. The International Technical Information Institute, Tokyo, Japan.

Johnson JD, Meisenheimer TL, Isom GE (1986) Cyanide-induced neurotoxicity: role of neuronal calcium. *Toxicol Appl Pharmacol* **84**: 464–9.

Kadar T, Fishbine E, Meshulam J, Sahar R, Amir A, Barness I (2003) A topical skin protectant against chemical warfare agents. *Isr Med Assoc J* **5**(10): 717–9.

Kaplan DE, Marshall A (1996) *The Cult at the End of the World: The Terrifying Story of the Aum Doomsday Cult, from the Subways of Tokyo to the Nuclear Arsenals of Russia*. Crown Publishing, New York.

Kennedy TP, Michael JR, Hoidal JR, Hasty D, Sciuto AM, Hopkins C, Lazar R, Bysani GK, Tolley E, Gurtner GH (1989) Dibutyryl cAMP, aminophylline, and beta-adrenergic agonists protect against pulmonary edema caused by phosgene. *J Appl Physiol* **67**(6): 2542–52.

Kientz CE (1998) Chromatography and mass spectrometry of chemical warfare agents, toxins and related compounds: state of the art and future prospects (review). *J Chromatogr* **814**: 1–23.

Kusic R, Jovanovic D, Randjelovic S (1991) HI-6 in man: efficacy of the oxime in poisoning by organophosphorus insecticides. *Hum Exp Toxicol* **10**: 113–18.

Leitenberg M (1999) Aum Shinrikyo's efforts to produce biological weapons: a case study in the serial propagation of misinformation. *Terror Polit Viol* **11**(4): 149–58.

Leung P, Sylvester DM, Chiou F (1984) Stereospecific effect of naloxone hydrochloride on cyanide intoxication. *Toxicol Appl Pharmacol* **83**: 525–30.

Lewis RJ (1997) *Hawley's Condensed Chemical Dictionary*, 13th edn. John Wiley & Sons, Inc, New York.

Lewis RJ (2000) *Sax's Dangerous Properties of Industrial Materials*, 10th edn. Van Nostrand Reinhold Company, New York.

Little PJ, Reynolds ML, Bowman ER (1986) Tissue disposition of (3H)sarin and its metabolites in mice. *Toxicol Appl Pharmacol* **83**: 412–19.

Maisonneuve A, Callebat I, Debordes L (1993) Biological fate of sulphur mustard in rat: toxicokinetics and disposition. *Xenobiotica* **23**: 771–80.

Mautone AJ, Katz Z, Scarpelli EM (1985). Acute responses to phosgene inhalation and selected corrective measures (including surfactant). *Toxicol Ind Health* **1**(2): 37–57.

Meerstadt PWD (1982) Atropine poisoning in early infancy due to Eumydrin drops. *Br Med J* **285**: 196–7.

Midtling JE, Barnett PG, Coye MJ (1985) Clinical management of field worker organophosphate poisoning. *West J Med* **142**: 514–18.

Morris RD, Audet AM, Angelillo IF (1992) Chlorination, chlorination by-products, and cancer: a meta-analysis. *Am J Public Health* **82**: 955–63.

Munro NB, Talmage SS, Griffin GD (1999). The sources, fate, and toxicity of chemical warfare agent degradation products. *Environ Health Perspect* **107**: 933–74.

Murdoch CM (1993) Toxicity of gases. In *Occupational Toxicology*, Stacey NH (ed.). Taylor and Francis, London, pp. 233–49.

Myers RAM, Schnitzer BM (1984) Hyperbaric oxygen use: update 1984. *Postgrad Med J* **76**: 83–95.

Namba T, Nolte CT, Jackrel J (1971) Poisoning due to organophosphate insecticides. Acute and chronic manifestations. *Am J Med* **50**: 475–92.

NATO (1973) *NATO Handbook on the Medical Aspects of NBC Defensive Operations*, AMedP-6, Part III. North Atlantic Treaty Organization, Brussels, Belgium, pp. 1, 3, 7, 10.

NIOSH (2003) *NIOSH Pocket Guide to Chemical Hazards*. NIOSH, Cincinnati, OH.

Noe JT (1963) Therapy for chlorine gas inhalation. *Ind Med Surg* **32**: 411–14.

OHM/TADS. *Oil and Hazardous Materials Technical Assistance Data System*. US Environmental Protection Agency. Washington, DC (CD Rom Version). Edition expires in 2005. Thomson MICROMEDEX, Greenwood Village, CO.

Okudera H, Morita H, Iwashita T (1997) Unexpected nerve gas exposure in the city of Matsumoto: report of rescue activity in the first sarin gas terrorism. *Am J Emerg Med* **15**: 527–8.

Patt HM, Tobias JM, Swift MN, Postel S, Gerard RW (1946) Hemodynamics in pulmonary irritant poisoning. *Am J Physiol* **147**: 329–39.

Paulet G (1955) Valeur et mecanisme d'action de l'oxygenotherapie dans le traitement de l'intoxication cyanhydrique. *Arch Internat de Physiologie et de Biochimie* **63**: 340–60.

Pohanish RP (2002) *Sittig's Handbook of Toxic and Hazardous Chemicals and Carcinogens*, 4th edn. William Andrew Publishing/Noyes, Park Ridge, NJ.

Proctor NH, Hughes JP (2004) *Proctor and Hughes' Chemical Hazards of the Workplace*, 5th edn. John Wiley & Sons, New York.

Raffle PAD, Adams PH, Baxter PJ (1994) *Hunter's Diseases of Occupations*. Little, Brown & Co, Boston, MA.

Requena L, Requena C, Sanchez M (1988) Chemical warfare. Cutaneous lesions from mustard gas. *J Am Acad Dermatol* **19**: 529–36.

Reynolds ML, Little PJ, Thomas BF (1985) Relationship between the biodisposition of (3H)soman and its pharmacological effects in mice. *Toxicol Appl Pharmacol* **80**: 409–20.

Roberts JR (1988) Minor burns (Part II). *Emerg Med Ambul Part Care News* **10**: 4–5.

RTECS. Registry of Toxic Effects of Chemical Substances. National Institute for Occupational Safety and Health. Cincinnati, OH (CD

Rom Version). Edition expires in 2006. Thomson MICROMEDEX, Greenwood Village, CO.

Schwartz DA, Smith DD, Lakshminarayan S (1990) The pulmonary sequelae associated with accidental inhalation of chlorine gas. *Chest* **97**: 820–5.

Sciuto AM, Strickland PT, Kennedy TP, Gurtner GH (1995) Protective effects of *N*-acetylcysteine treatment after phosgene exposure in rabbits. *Am J Respir Crit Care Med* **151**: 768–72.

Sciuto AM, Moran TS, Narula A, Forester JS (2001) Disruption of gas exchange in mice after exposure to the chemical agent phosgene. *Mil Med* **116**(9): 809–14.

Sidell FR, Groff WA (1974) The reactivatibility of cholinesterase inhibited by VX and sarin in man. *Toxicol Appl Pharmacol* **27**(2): 241–52.

Sidell FR, Takafuji ET, Franz DR (1997) *Textbook of Military Medicine: Medical Aspects of Chemical and Biological Warfare*. TMM Publications, Washington, DC.

Singh BM, Coles N, Lewis RA (1989) The metabolic effects of fatal cyanide poisoning. *Postgrad Med J* **65**: 923–5.

Smith KJ (1999) The prevention and treatment of cutaneous injury secondary to chemical warfare agents. Application of these finding to other dermatologic conditions and wound healing. *Dermatol Clin* **17**(1): 41–60.

Smith KJ, Hurst CG, Moeller RB (1995) Sulfur mustard: its continuing threat as a chemical warfare agent, the cutaneous lesions induced, progress in understanding its mechanism of action, its long-term health effects, and new developments for protection and therapy. *J Am Acad Dermatol* **32**: 765–76.

Somani SM, Babu SR (1989) Toxicokinetics of sulfur mustard. *Int J Clin Pharmacol Ther Toxicol* **27**(9): 419–35.

Taher AA (1992) Cleft lip and palate in Tehran. *Cleft Palate Craniofac J* **29**(1): 15–16.

Ten Eyck RP, Schaerdel AD, Ottinger WE (1985) Stroma-free methemoglobin solution: an effective antidote for acute cyanide poisoning. *Am J Emerg Med* **3**: 519–23.

Traub SJ, Hoffman RS, Nelson LS (2002) Case report and literature review of chlorine gas toxicity. *Vet Hum Toxicol* **44**(4): 235–9.

USACHPPM (2001a) Detailed Facts about Sulfur Mustard Agents H and HD. US Army Center for Health and Promotion and Preventive Medicine, Aberdeen Proving Ground, MD. http://chppm-www.apgea.army.mil/dts/docs/dethhd.pdf (accessed May 15, 2006).

USACHPPM (2001b) General Facts about Sulfur Mustard Agents H and HD. US Army Center for Health and Promotion and Preventive Medicine, Aberdeen Proving Ground, MD. http://chppm-www.apgea.army.mil/dts/docs/genhhd.pdf (accessed May 15, 2006).

USACHPPM (2001c) Detailed Facts about Blister Agent Lewisite 218-14-1096. US Army Center for Health and Promotion and Preventive Medicine. Aberdeen Proving Ground, MD. http://chppm-www.apgea.army.mil/dts/docs/detlew.pdf (accessed May 15, 2006).

USACHPPM (2001d) Detailed Facts about Blister Agent Phosgene Oxime (CX). US Army Center for Health Promotion and Preventative Medicine. Aberdeen Proving Ground, MD. http://chppm-www.apgea.army.mil/dts/docs/detcx.pdf (accessed May 15, 2006).

US Army (1996) NATO Handbook on the Medical Aspects of NBC Defensive Operations FM 8-9. NATO Information Service, Brussels. http://www.fas.org/nuke/guide/usa/doctrine/dod/fm8-9/toc.htm (accessed May 15, 2006).

van Dijk A, Douze JMC, van Heijst ANP (1986) Clinical evaluation of the cyanide antagonist 4-DMAP. (Abstract). *II World Congress of the World Federation of Associations of Clinical Toxicology and Poison Control Centers*, Brussels, Belgium.

Vogel SN, Sultan TR, Ten Eyck RP (1981) Cyanide poisoning. *Clin Toxicol* **18**: 367–83.

Vogt Jr RF, Dannenberg Jr AM, Schofield BH (1984) Pathogenesis of skin lesions caused by sulfur mustard. *Fundam Appl Toxicol* **4**: S71–83.

Vojvodic V, Milosavljevic Z, Boskovic B (1985) The protective effect of different drugs in rats poisoned by sulfur and nitrogen mustards. *Fundam Appl Toxicol* **5**: S160–8.

Vycudilik W (1985) Detection of mustard gas bis(2-chloroethyl)-sulfide in urine. *Forens Sci Int* **28**: 131–6.

Wang J, Zhang L, Walther SM (2004) Administration of aerosolized terbutaline and budesonide reduces chlorine gas-induced acute lung injury. *J Trauma* **56**: 850–62.

Way JL, End E, Sheehy MH (1972) Effect of oxygen on cyanide intoxication. IV. Hyperbaric oxygen. *Toxicol Appl Pharmacol* **22**: 415–21.

Way JL, Leung P, Cannon E, Morgan R, Tamulinas C, Leong-Way J, Baxter L, Nagi A, Chui C (1988) The mechanism of cyanide intoxication and its antagonism. *CIBA Found Symp* **140**: 232–43.

Weger NP (1990) Treatment of cyanide poisoning with 4-dimethylaminophenol (DMAP): experimental and clinical overview. *Middle East J Anesth* **10**: 389–412.

Wells BA (1985) Phosgene: a practitioner's viewpoint. *Toxicol Ind Health* **1**(2): 81–92.

Wiemeyer SN, Hill EF, Carpenter JW, Krynitsky AJ (1986) Acute oral toxicity of sodium cyanide in birds. *J Wildl Dis* **22**: 538–46.

Willems JL, Nicaise M, De Bisschop HC (1984) Delayed neuropathy by the organophosphorous nerve agents soman and tabun. *Arch Toxicol* **55**: 76–7.

Willhite CC (1983) Developmental toxicology of acetonitrile in the Syrian golden hamster. *Teratology* **27**: 313–25.

Wolthuis OL, Vanwersch RA, Van Helden HP (1986) Residual behavioral incapacitation after therapy of soman intoxication: the effect of a soman simulator. *Neurobehav Toxicology Teratol* **8**: 127–30.

Yamamoto HA (1990) Protection against cyanide-induced convulsions with alpha-ketoglutarate. *Toxicology* **61**: 221–8.

Young RA, Opresko DM, Watson AP (1999) Deriving toxicity values for organophosphate nerve agents: a position paper in support of the procedures and rationale for deriving oral RfDs for chemical warfare nerve agents. *Hum Ecol Risk Assess* **5**: 589–634.

C H A P T E R

# 7

# Regulatory considerations in veterinary toxicology

*Lynn O. Post*

## INTRODUCTION

This chapter is organized into six sections: Section I: Food and Drug Administration and the Center for Veterinary Medicine; Section II: Environmental Protection Agency; Section III: US Department of Agriculture; Section IV: Drug Enforcement Administration; Section V: Occupational Safety and Health Administration; and Section VI: Statues, Regulations, and Guidelines. Sections I through V describe the federal agencies that are important to animal health, including processes, organizational structure and mission, and selected programs. Testing methods and pharmacovigilance for the Center for Veterinary Medicine, the Environmental Protection Agency, and the US Department of Agriculture are described in Sections I through III. Pharmacovigilance is the emerging area of monitoring for unintended harmful effects of marketed animal health products.

This chapter is an overview to a complex and dynamic area that is under the constant influence of competing interests (consumer groups, industrial groups, and professional organizations), new legislation, and legal precedents, as well as a new generation of animal health products and issues. Appropriate Internet addresses are included in the list of references for the reader to consult more complete and/or updated information from Internet home pages of the respective federal agencies and other reliable organizations.

## SECTION I. FOOD AND DRUG ADMINISTRATION AND THE CENTER FOR VETERINARY MEDICINE

### Creation and organization of the FDA and CVM

Animal drugs and medical devices are regulated by the Food and Drug Administration's (FDA) Center for Veterinary Medicine (CVM) under the Federal Food, Drug, and Cosmetic Act (FFDCA). The FFDCA and FDA are widely recognized as the preeminent regulatory legislation and agency in the nation. The first Federal Food and Drug Act was passed in 1906. It prohibited interstate shipment of adulterated or misbranded food, drink, and drugs, and was administered by the US Department of Agriculture's (USDA) Bureau of Chemistry. In 1938 landmark broader legislation was passed requiring manufacturers to provide evidence of product safety before distributing new drugs. This legislation also provided authority to conduct manufacturing site inspections, establish tolerances for unavoidable contaminants and use court injunctions as enforcement tools. In 1940, FDA was transferred from USDA, ultimately becoming part of the Department of Health, Education and Welfare (DHEW), which became the Department of Health and Human Services (DHHS). During the early 1950s, a veterinary branch was established within the FDA's Bureau of Drugs. Subsequently, two major amendments to the

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FFDCA were passed. The Food Additive Amendments of 1958 expanded FDA's authority over animal food additives and drug residues in animal-derived foods. The Kefauver–Harris Drug Amendments of 1962 brought major changes to the FFDCA, requiring manufacturers to test new drugs for effectiveness before marketing them, and to report adverse drug events promptly to FDA. These requirements were also applied through a modified process to products already on the market. Recognizing the importance of animal health products the FDA established the Bureau of Veterinary Medicine (all Bureaus became Centers in 1984) in 1965. However, animal drugs were still regulated under three different sections of the FFDCA, either as new drugs, antibiotics, or if labeled for food-animals, as food additives. Because this situation produced a regulatory scheme for animal drugs that was confusing and more complex than for human drugs, the Animal Drugs Amendments of 1968 were passed consolidating the various sections of the FFDCA that applied to animal drugs in one section (512) of the FFDCA. More complete discussions of the history of the FFDCA and CVM are available (Teske, 1995; FDA Center for Veterinary Medicine, 1999). More recent animal health product-related amendments to the FFDCA are given in Table 7.1 (FDA Center for Veterinary Medicine, 2000).

**TABLE 7.1 Recent major animal health product-related amendments to the FFDCA**

| Amendment | Summary of changes |
|---|---|
| Generic Animal Drug and Patent Term Restoration Act (GADPTRA) | Enacted 1988.<br>Provides for approval of generic copies of animal drug products that have been previously approved and shown to be safe and effective.<br>Provided for prescription status for animal drugs. |
| Animal Drug Use Clarification Act of 1994 (AMDUCA) | Allows veterinarians to prescribe extra-label uses of approved animal drugs and approved human drugs for animals under certain conditions.<br>Allows FDA to restrict extra-label use in certain circumstances.<br>Allows FDA to establish a safe level for a residue for such extra-label use by regulation or order and require the development of analytical methods for residue detection. |
| Animal Drug Availability Act of 1996 (ADAA) | Amended the definition of substantial evidence of effectiveness.<br>Created a new category of drugs – "Veterinary Feed Directive Drugs".<br>Permitted a range of doses to appear on animal drug product labeling, rather than one optimum dose.<br>Provided for Licensing of Feed Mills and eliminated the requirement for Medicated Feed Applications. |

FDA is an agency within the DHHS. The senior official within FDA is the FDA Commissioner. The agency is organized by product responsibility into Centers: Center for Veterinary Medicine (CVM); Center for Drugs Evaluation and Research (CDER); Center for Biologics Evaluation and Research (CBER); Center for Devices and Radiological Health (CDRH); and the Center for Food Safety and Nutrition (CFSAN). The FDA is also organized geographically into Regional Offices and component Districts where field staff are assigned.

## Approving and monitoring animal drugs

Under the FFDCA, the CVM is responsible for ensuring that animal drugs and medicated feeds are safe and effective for their intended uses and that food from treated animals is safe for human consumption. To accomplish its mission, the CVM is organized into pre-market review, post-market monitoring, and research components. In addition, FDA field staff conduct inspections of FDA-regulated animal health industries and products. Prior to a product being marketed it must have an approved New Animal Drug Application (NADA). In order for an NADA to be approved there must be substantial evidence that the new animal drug is effective and safe for its intended uses under the conditions of use prescribed, recommended, or suggested in the proposed labeling. In addition, methods, facilities, and controls used for the manufacturing, processing, and packaging of the drug must be shown to be adequate to preserve its identity, strength, quality, and purity (Good Manufacturing Practices, 21 CFR 210 and 211). For food-animal products safety includes safety of drug residues in animal-derived food. Tolerances for residues of new animal drugs in food may be found in 21 CFR 556. Safety would also include an assessment of the effects of the proposed animal drug on the environment and on human health. Following approval products are monitored to ensure safety and effectiveness and maintained under actual conditions of use.

## Testing methods for animal drugs

FDA has developed extensive detailed guidelines for the conduct of studies required to obtain approval to market products. This section provides a brief description of the animal safety and efficacy studies and human safety studies that may be performed. They are provided to familiarize the student with the general scope of information required for product approval, not to provide guidance in designing studies for product approval. The specific pertinent agency guideline should be consulted prior to designing a study protocol.

### CVM *target animal safety and product efficacy testing requirements*

*Introduction*

A veterinary drug sponsor must show that a drug is safe to use as described in the proposed labeling (21 CFR 514.1(b)(1) and 512(d) of the FFDCA; FDA Center for Veterinary Medicine, 1989). The information needed to determine a products safety in the target species depends on several factors: proposed use, type of product, chemistry, intended species, breed, and class of animal (calves versus mature ruminants), claims and previous use history. Existing data collected from previous studies are used to develop and refine protocols for toxicity studies. While the CVM target animal safety guideline is intended to provide general directions for acquiring essential information, all the data requirements for a standard target animal safety study are not required for every product, and special circumstances may require the collection of additional data that is not specified for the standard study.

*Good laboratory practices and general study requirements*

Laboratory studies in animals are conducted according to Good Laboratory Practice (GLP) regulations (21 CFR Section 58). Target animal safety studies are also subject to GLP regulations. The GLP regulations ensure that methods and procedures for collecting, processing, and reporting data are standardized and verified for the entire study. GLPs ensure an adequate level of accuracy and quality control. Many GLP requirements are inherent in any well-designed scientific study. On the other hand, adherence to GLP standards does not guarantee the scientific validity of the study.

Since animal husbandry requirements often differ for laboratory animals and domestic animals, domestic animals utilized in clinical studies are not maintained under GLP conditions. Appropriate diagnostic tests, vaccinations, prophylactic, and therapeutic treatments are completed prior to the treatment phase of the study. During this initial phase a complete physical examination is performed and baseline data are collected by a qualified person. The test product formulation must be identical to the proposed marketed product. The route of administration should be the same as the proposed labeling, however, the toxicity or nature of the product may require a different formulation. For example, due to feed refusal, a drench of a product may be required instead of delivery of the drug through a medicated feed.

Data are collected at predetermined intervals during the trial. Clinical observations are recorded at specified intervals, usually twice a day, 7 days a week during the entire study or modified according to the study protocol. During the study appropriate clinical pathology procedures are conducted in all test groups, usually only half of the animals selected at random from each group at predetermined intervals. Histopathologic examinations may be required from all or a portion of the animals. Tissues from the highest treatment and control groups must be examined. If microscopic lesions are observed, corresponding tissues from the next lower treatment group are examined until a no observable effect level (NOEL) is established.

*Drug tolerance test*

Under controlled conditions, the target animal response to a toxic dose is characterized by clinical signs. Clinical signs include changes in behavior and appearance and gross lesions in animals that die during the study. Clinical pathology and histology data reveal physiological functions that are most readily affected by the drug. The duration of the toxicity studies may range form acute to chronic.

Target animal safety studies require systemic testing for new products or new chemical entities and additional animal species. They are not required for drugs administered locally, such as otic, ophthalmic, intra-mammary, and intra-articular preparations. This does not apply to generalized dermal or topical drugs that may act systemically. The market formulation of a drug must be administered according to the proposed conditions of use. If volume or palatability becomes a limiting factor for administration, gavage, intravenous, multiple sites, or incremental doses may be used. A vehicle control group must be included in the experimental design.

Usually, up to ten times (10×) the maximum proposed clinical dose is administered. The toxic dose (10×) may be reduced or increased in order to manifest toxic signs without causing death. The main purpose of the tolerance test is to characterize toxic signs which can be used to develop toxicity study protocols at lower doses (1×, 3×, and 5×). The toxicity studies are used to design the clinical efficacy study protocol.

*Toxicity study*

The objective of toxicity studies in target animal species are (1) to demonstrate safety of the drug product under condition of use, (2) to demonstrate signs and effects associated with toxicity, and (3) to demonstrate a margin of safety at 5× or below (FDA Center for Veterinary Medicine, 1989). If the drug has a narrow margin of safety with an intended use in debilitated animals, safety studies in diseased animals may be necessary. This may require more animals per treatment group because of greater variability. Special studies may be requested by FDA, such as lameness, reproductive, dermal irritation, specific routes of administration, tissue disposition, and combination drugs.

### *FDA-required toxicological testing in food-producing animals*

*General considerations*

Studies must be conducted according to GLPs. For compounds used in food-producing animals, FDA is concerned with intermittent and chronic exposure of people to relatively low concentrations of residues. FDA tailors the type of toxicological testing needed to show safety for a specific compound. Factors, which are considered, include the proposed use, the potential human exposure of the parent compound and/or its residues, as deduced by structure–activity relationships. Some compounds need only a minimum of testing; others may need very extensive studies in a number of diverse biological systems.

The purpose of the toxicology studies is to define the biological effect(s) of the sponsored compound and its quantitative limits. FDA generally asks that the sponsor (owner of the NADA), at a minimum, to determine a dose of the compound that produces an adverse biological effect in test animals and a dose that does not produce any significant toxicological or pharmacological effect (NOEL). The spacing of the doses should provide an assessment of the dose–response relationship (FDA Center for Veterinary Medicine, 2005).

*Testing requirements*

FDA recommends the following studies as the minimum necessary for each sponsored compound:

- A battery of genetic toxicity tests.
- A 90-day feeding study in both a rodent species (usually the rat) and a non-rodent mammalian species (usually the dog).
- A two-generation reproduction study with a teratology component in rats. Two litters should be produced in each generation. If toxicity occurs at a lower dose, with a higher incidence, or with greater intensity in the second generation as compared to the first, then the study should include a third generation.

FDA may require the following additional studies, which should be conducted using an approved protocol:

- Chronic bioassays for oncogenicity in each of two rodent species when indicated by positive results in genetic toxicity tests.
- One-year feeding studies in a rodent species (usually the rat) and in a non-rodent species (usually the dog) are needed, when human residue exposure exceeds 25-μg/kg body weight/day. This daily intake is equivalent to 1 part per million in total solid diet of 1500 g, normally consumed by a 60-kg human subject. The FDA will calculate permissible exposure, when evidence indicates that the residue bioaccumulates in the tissues of target animals.
- A teratology study in a second species when the compound is structurally related to a known teratogen, when the compound has hormonal activity that may affect the fetus, or when the compound shows adverse effects in the reproduction/teratology study indicating that the compound may be a teratogen.
- Other specialized testing if necessary to define the biological effect of the compound. Examples of specialized studies include testing for neurotoxicity, for immunotoxicity, for hormonal activity, for toxicity following *in utero* exposure, or for toxicity from a "biomass" product.

If the testing shows that the sponsored compound is a carcinogen, FDA will apply the "no-residue" requirement of Section 409(c)(3)(A), 512(d)(1)(H), or 706(b)(5)(B) of the Act as operationally defined in 21 CFR subpart E of Part 500. FDA will calculate the concentration of residue giving no significant risk of cancer from the tumor data using a statistical extrapolation procedure. In the absence of information establishing the mechanism of carcinogenesis for a particular chemical, FDA will use a non-threshold, linear-at-low-dose extrapolation procedure that determines the upper limit of the risk (Gaylor and Kodell, 1980; Farmer *et al.*, 1982). In the extrapolation FDA will use the upper 95% confidence limit on the tumor data and a permitted maximum lifetime risk to the test animal of 1 in 1 million.

*Acceptable daily intake and safety factors*

For other toxicological endpoints, FDA will calculate the acceptable daily intake (ADI) from the results of the most sensitive study in the most sensitive species. From that study the ADI is the highest dose showing NOEL divided by the appropriate safety factor (Table 7.2).

*Sex steroids*

Although not all sex steroids are demonstrated carcinogens, the evidence supports FDA's conclusion that all endogenous sex steroids and synthetic compounds with similar biological activity should be regarded as suspect

**TABLE 7.2 CVM: human food safety studies and safety factors**

| Type of study | Safety factor |
|---|---|
| Chronic | 100 |
| Reproduction/teratology<br>(100 for a clear indication of maternal toxicity, 1000 for other effects) | 100 or 1000 |
| 90 days | 1000 |

carcinogens, and that the endogenous sex steroids are not genotoxic agents. If these compounds produce an oncogenic response in experimental animals, the mechanism of action is not related to a direct chemical interaction with DNA. Specifically, tumor development is a consequence of hyper-proliferation of endocrine-sensitive tissue resulting from persistent over-stimulation of the hormonal system.

The safety of endogenous sex steroids and their simple ester derivatives can be assured without additional animal study data. Large quantities of these compounds are produced by *de novo* synthesis in people and food-producing animals. The FDA has concluded that no physiological effect will occur in individuals ingesting animal tissues that contain endogenous steroids equal to 1% or less of that produced daily in the population with the lowest production. In the case of estradiol and progesterone, prepubertal boys synthesize the least; in the case of testosterone, prepubertal girls synthesize the least. The daily production values and the calculated increase permitted above the amount naturally present in untreated target animals are listed in Table 7.3. The product is considered safe within the meaning of the Act, if data acceptable to the FDA demonstrate that under the proposed conditions of use, the residue concentration of the endogenous sex steroid in treated food-producing animals does not exceed the permitted increase at the time of slaughter.

*Synthetic sex steroids*

In addition to the standard requirements, FDA recommends the following additional tests:

- A 180-day study in rhesus monkeys or another suitable subhuman primate is required to assess the effect of the sponsored compound on various parameters including ovulation, menstrual cycle, and circulating levels of gonadotropins, and sex steroids. The study should establish a dose that gives a no observed hormonal response.
- FDA will also normally use a safety factor of 100 for the study in subhuman primates. If a carcinogenic response is observed in a non-endocrine-sensitive tissue, FDA will determine the dose that will satisfy the "no-residue" requirement of the act using the tumor data from that tissue and a statistical extrapolation procedure.

**TABLE 7.3 CVM: human food safety and endogenous sex steroids**

| Sex steroids | Daily production (μg) | Permitted increased exposure (μg) |
|---|---|---|
| Estradiol | 6 | 0.06 |
| Progesterone | 150 | 1.50 |
| Testosterone | 32 | 0.32 |

## Animal efficacy studies

Efficacy studies are clinical studies. The purpose of an efficacy study is to evaluate the response of the test article under actual conditions of use. Clinical studies to establish efficacy are also important to the overall safety assessment of the product. GLP regulations do not apply because clinical studies are conducted in a clinical environment under actual conditions of use, rather than in a rigorously controlled laboratory setting. For example, veterinary practitioners may enroll their client's dogs in a non-steroidal anti-inflammatory study and food-animal producers may participate in a production drug study. In efficacy studies, special care must be exercised in handling animals to avoid undue stress which may alter the response to the drug, greatly affecting the study results. If there is a need to collect samples for laboratory analysis that is unrelated to the collection of efficacy data, all animals are sampled at specified times in order to minimize bias.

The design of the efficacy trial is flexible and depends on the species, breed, drug, class of production animal, and data endpoints. In some instances, such as for production drugs, many animals are required per treatment group for the efficacy trial because of variability due to environmental conditions and in the test animals. For typical therapeutic products, the number of subjects required for an animal drug clinical trial is about 5–10% of the number required for an equivalent human drug clinical trial. Additionally, while toxicity is not the objective of these studies, animals are observed periodically (usually daily) for clinical signs and abnormalities. These observations give valuable information for designing additional safety study protocols or reveal information that would appear in the safety information on the product label. Some animals may be necropsied at the end of the study if animals exhibit signs of toxicity. On the other hand, the drug may also be discontinued in an animal exhibiting toxic signs.

Even though the efficacy studies are non-GLP, the test methods and procedures for administering the test article are verified and monitored throughout the study. This ensures that the drug is being delivered to the animals according to the protocol. For example, an oral drug may administer with or without food, or the mixing procedure for dispersing a production drug in feed must be periodically validated.

After all requirements have been met, FDA approval of the product allows it to be legally marketed and promoted as a new animal drug. The label must contain the product claim(s), pharmacology, side effects, precautions, and warnings. After approval, the drug is monitored through periodic and special industry drug experience reports (DERs) to CVM. These reports include adverse experiences and may result in label changes related to product safety.

### *Regulatory requirements for reporting animal adverse drug events*

#### *Introduction*

CVM does not evaluate complaints for pesticides and animal vaccines. These complaints should be sent to the Environmental Protection Agency (EPA) or the USDA. The CVM will accept safety information for animal feeds, animal devices, human drugs used in animals, and approved animal drugs. Mandatory reporting of safety complaints by the company are only required for approved animal drugs to include medicated feeds. Animal medicated feeds may be mixed incorrectly or given to the wrong species, such as monensin intended for cattle is fed to horses. Alternately, animals feeds may be contaminated with natural or man-made toxins, such as aflatoxin and polychlorinated biphenyls (PCBs), with the potential for serious consequences in humans and the target species.

Although, there are no regulatory requirements for the firm to report post-approval safety information for animal devices, CVM will take regulatory action when necessary. Pre-market approval is not required: the FDA does not require formal pre-market approval for devices used in veterinary medicine (510(k) of the FFDCA). Firms that manufacture radiation emitting devices need to register their products under the radiological health regulations, administered by the Center for Devices Radiological Health. FDA does have regulatory oversight over veterinary devices and can take appropriate regulatory action if a veterinary device is misbranded, mislabeled, or adulterated. It is the responsibility of the manufacturer and/or distributor of these articles to assure that these animal devices are safe, effective, and properly labeled.

The Center for Drug Evaluation and Research does not require the pharmaceutical company to report safety complaints from extra-label use of human drugs in animals. CVM will process and evaluate the complaints concerning the extra-label use of human drugs in animals, such as methimazole use for hyperthyroid cats.

#### *Pharmacovigilance*

Pharmacovigilance consists of the means and methods for monitoring and ensuring the safety and efficacy of marketed medicinal products. (*Note*: For the purpose of this section, the term "pharmaco-" is used to encompass not only drugs, but all types of medicinal products.) Pharmacovigilance is made necessary by the limitations of pre-approval or pre-registration studies for animal medicinal products. Economic and practical considerations limit the number of animals included in safety and efficacy studies. Further, many of the animals utilized during the testing phase consist of experimental animals that are homogenous in age, breed, and genetics. More variation is obtained when the drug is tested in clinical studies, but these types of studies generally have few animals. Thus, while premarketing studies may be adequate in demonstrating efficacy and common adverse drug reactions, they generally have limited power to detect less frequently occurring adverse events that may occur when the product is finally utilized under actual conditions of use in the general veterinary community.

Veterinary pharmacovigilance within the federal government is typically based on the receipt of spontaneous reports involving complaints of product performance. Spontaneous refers to reports that are voluntarily submitted by veterinarians, animal producers, and animal owners. The reports may be submitted by mail, phone, fax, or e-mail. The reports may be originally submitted to either the drug company involved or directly to CVM. Product complaints might involve a suspected animal injury, failure of the product to perform (or ineffectiveness), or a product defect.

The key to understanding the spontaneous AER (adverse event report) reporting process is in recognizing and appreciating the word "suspected". Any complaint must start with the establishment of an actual AER. The pharmacoepidemiologist understands that any one spontaneously reported AER might be misrepresented, or perhaps not existed at all. A certain level of bias is automatically introduced when the reporter associates the AER with the administration of a specific drug. Lacking evidence that unquestionably documents and supports the AER occurrence, information criteria must be established for accepting an AER into a pharmacovigilance system. This information should include an identifiable reporter, an identifiable animal, an identifiable product, and an adequately described adverse event.

In the case of the existence of an actual AER, a reporter lodges a complaint in the form of an AER. The AER is the mechanism through which all relevant aspects of the AE are described. The main goal of the pharmacovigilance program receiving an AER should be to ensure that all needed data elements are accurately and fully described. In many cases, follow-up information may be needed to augment the original AER. Even if the most accurate information is obtained for an AER, the possibility of one or more alternative non-drug-related explanations for the AE occurrence cannot be discounted.

This type of system establishes some level of assurance that the drug under question was "associated" with the reported AER. Strength of association is often determined by utilizing objective-based guidelines termed algorithms. The main strength of the spontaneous reporting system is in detecting the occurrence of drug-related adverse effects that are uncommon and cannot be detected by premarketing studies, which generally have low statistical power. Once a trend or suspected adverse reaction is detected, other methods may be used to investigate the suspected AER and determine the most appropriate course

of action. For instance, the drug company may be requested to provide more information or to conduct studies investigating the specific issue, or expert opinions may be sought from relevant veterinary specialists. Recommended actions might include labeling changes, the recall of specific batches of product, or even the removal of the product from the market.

#### *FDA/CVM adverse drug event reporting system for approved animal drugs*

The regulations that specifically address the spontaneous reporting obligations of the drug sponsors of FDA-approved animal drugs are contained in "Records and reports concerning experience with new animal drugs for which an approved application is in effect" (21 CFR 514.80). Reporting of AERs is mandatory for the pharmaceutical industry. It is voluntary for veterinarians and consumers.

For industry reporting, the regulations categorize AERs into three categories: significant product defect reports that should be submitted to an FDA District Office within 3 days (21 CFR 514.80(b)(1)); AERs involving unexpected animal injury and unexpected product ineffectiveness that should be submitted within 15 working days to CVM (21 CFR 514.80(b)(2)); and the remaining types of AERs and product defects which should be submitted at periodic intervals to CVM (21 CFR 514.80(a)(4)). All categories of complaints are required to be submitted by the drug sponsor on the OMB-approved Form FDA 1932 ("Veterinary Adverse Drug Reaction, Lack of Effectiveness, and Product Defect Report").

Significant product defects are those involving either label mix-ups or a significant departure of the product form approved specifications, where the product defect may result in immediate harm to animals. Corrective action is accomplished through the FDA Field Office responsible for the manufacturing site.

AERs requiring 15-day submissions are serious and unexpected (21 CFR 514.3 and 514.80):

- fatal or life threatening;
- require professional intervention;
- causes an abortion, or stillbirth, or infertility, or permanent disability disfigurement;
- unexpected AERs are not on the current label or may be pathophysiologically related to an AER on the label.

Unexpected refers to either information that is not contained either on product labeling or as part of the approved FDA application. Regulations further require reporting of unexpected adverse events that are associated with either clinical use, studies, investigations, or tests, whether or not determined to be attributable to the suspected drug. A key phrase is whether or not determined to be attributable to the suspected drug. CVM expects the drug sponsor to submit all AERs rather than only those reports the firm believes are associated with the AER. CVM considers selective report submission (filtering) to introduce a bias that confounds the evaluation of submitted AERs.

All AERS submitted to CVM are evaluated by a staff of veterinarians assigned to this task. All relevant information is extracted from the AERs and entered into the appropriate fields of a relational database, using standard terminology. A six-step algorithm, evaluation process is utilized to assign a score that represents the strength of the veterinarian's opinion that the use of the drug was associated with the adverse clinical sign.

The database is utilized by product manager, veterinarians assigned to monitor the safety and efficacy of specific products. In the event that a potential problem is identified, the Monitored Adverse Reaction Committee (MARC) is convened to assist in evaluating information related to the potential problem. The MARC also recommends appropriate regulatory action, in the event the problem is considered significant. AER information is released to the public by means of summary report publication on the CVM website. Additional requests for AER information can be made through the Freedom-of-Information officer.

AER submission is required only from companies marketing FDA-approved animal drugs. There are no requirements for submitting AERs for human drugs used in animals or unapproved products labeled for animals. CVM will accept reports directly from consumers and veterinarians regarding extra-label use of human drugs and unapproved animal drug products. CVM has limited ability to monitor the performance of these drugs in animals.

Veterinarians are encouraged to report AERs directly to the manufacturer/sponsor. The manufacturer should record the information and send a report of their investigation to CVM. Another reporting option is to call the CVM AER hotline (1-888-FDA-VETS) to report AERs. An FDA veterinarian will return the phone call. The reporter can also either call or write the CVM to obtain a pre-postage-paid reporting form.

AER reporting to CVM has significantly increased over the last decade. During the early 1990s, the CVM received approximately 1000 AERs each year. CVM received about 33,000 AER reports for the year 2005. Reasons for the increase are numerous, but include the new types of drugs approved for use in companion animals, label information provided for contacting drug companies, and the interest of the public in reporting perceived product problems.

#### ***The future of global pharmacovigilance reporting***

The USDA and FDA are participants in the construction of a global AER platform. The reporting format for AERs will be standardized but not the causality assessment, evaluation of risk for each AER, and the pharmacoepidemiologic approach that may include a mathematical analyses.

Standardized evaluation of AERs is impractical because conditions of use for a drug vary from country to country. Spontaneous reporting does not readily lend itself to classical epidemiologic analyses because the numerator (number of animals) is under-reported and the denominator (number of doses) is unknown. Therefore, it is extremely difficult to calculate a true incidence.

Global harmonization of reporting requirements for AERs is no small task. It must be kept in mind that, unlike human AERs, veterinary medicine deals with multiple species which adds more complexity to a complicated process. In addition, reporting requirements differ substantially from country to country because missions, goals, resources, administrative procedures, and AER sources vary considerably. The variations reflect the fundamental differences in the worldwide agencies and the laws they administer. The International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Medicinal Products (VICH, 2005) is a trilateral (EU–Japan–USA with two observer countries: Australia and Canada) program aimed at harmonizing technical requirements for animal medicinal product registration to include pre-approval review and post-approval monitoring (International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Medicinal Products). In 1996, at the inception of VICH, one goal was the harmonization of pharmacovigilance reporting requirements: definitions, data elements, electronic submission protocols, and medical dictionaries.

Successful efforts of VICH will lead to the timely transmission of AER information between all parties and organizations involved in animal pharmacovigilance. Electronic reporting standards would also available to consumers and veterinarians who could report directly to the regulatory agency rather than the company. The standardized AER information will be disseminated worldwide for examination and analysis by all interested parties.

# SECTION II. ENVIRONMENTAL PROTECTION AGENCY

## Creation of the EPA

During the 1960s activists became increasingly effective in focusing attention on environmental and public health issues. Rachael Carson's book, Silent Spring (1962), provided a worldwide view of the effects of indiscriminate use of pesticides. By 1969, the word "ecology" had become part of American culture. Under criticism that his environmental committees were largely ceremonial bodies, President Nixon appointed a White House Committee in December of 1969 to consider whether there should be a separate environmental agency. About the same time, Congress sent the National Environmental Policy Act (NEPA) to the president and he signed NEPA on January 1, 1970.

It was against the background of environmental activism that Reorganization Plan No. 3 was sent to Congress by the Nixon Administration. Under Reorganization Plan No. 3, the EPA was formed in 1970 to establish and enforce environmental protection standards; to conduct environmental research; to provide assistance to other agencies in combating environmental pollution; and to assist in developing and recommending new policies for environmental protection. The regulatory activities for environmental protection were consolidated from 15 components of five executive departments and independent agencies into a single agency, the EPA. Certain pesticide regulatory functions were transferred from FDA and USDA to EPA. The enactment of major new environmental laws and important amendments since 1970 have expanded and refined the role of the EPA (Table 7.4).

The EPA is organized into major Offices headed by assistant EPA administrators which develop and implement EPA's policies and programs. For instance the Office of Prevention, Pesticides and Toxic Substances (OPPTS) develops national strategies for toxic substance control and promotes pollution prevention and the public's right to know about chemical risks. A component Office, the Office of Pesticide Programs (OPP), regulates the use of all pesticides in the United States and establishes maximum levels for pesticide residues in food. The EPA also has geographically organized regions. Each EPA Regional Office is responsible, within selected states, for the execution of the Agency's programs, considering regional needs and the implementation of federal environmental laws (USEPA, 2000b).

## Pesticides and the Federal Insecticide, Fungicide, and Rodenticide Act

The Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) has a major impact on the practice of veterinary medicine because it provides EPA the authority to regulate pesticide use in animals. Under FIFRA the term pesticide includes products, such as insecticides, fungicides, rodenticides, insect repellants, weed killers, antimicrobials, and swimming pool chemicals. Pesticides are intended to prevent, destroy, repel, or reduce pests of any sort. If a pesticide can be used with "reasonable certainty of no harm", it is granted a "registration" that permits its sale and use according to requirements set by EPA to protect human health and the environment. During its evaluation of a proposed pesticide the EPA considers the toxicity of the pesticide, the quantity and frequency of pesticide application, and the amount of pesticide that remains on the food by the time it is marketed.

**TABLE 7.4 Major statutes that form the basis for the programs of the EPA**

| Statute or law | Description |
|---|---|
| Federal Food, Drug, and Cosmetic Act, Delaney Clause (1958) | FFDCA prohibited any man-made chemical in food that caused cancer in any animal, including man. |
| National Environmental Policy Act (1969) | NEPA is the basic national charter for protection of the environment. |
| Clean Air Act (1970) | CAA is the comprehensive federal law that sets standards regulates air emissions from area, stationary, and mobile sources. |
| Occupational Safety and Health Act (1970) | OSHA ensures worker and workplace safety from hazards, such as exposure to toxic chemicals, excessive noise levels, mechanical dangers, heat or cold stress, or unsanitary conditions. |
| Federal Insecticide, Fungicide, and Rodenticide Act (1972) | FIFRA provided federal control of pesticide distribution, sale, and use and required users (farmers, utility companies, and others) to register when purchasing pesticides. |
| Endangered Species Act (1973) | ESA provides a program for the conservation of threatened and endangered plants and animals and the habitats in which they are found. |
| Safe Drinking Water Act (1974) | SDWA was established to protect the quality of drinking water in the United States. |
| Resource Conservation and Recovery Act (1976) | RCRA gave EPA the authority to control hazardous waste during the generation, transportation, treatment, storage, and disposal of hazardous waste. |
| Toxic Substances Control Act (1976) | TSCA gave EPA the ability to track about 75,000 industrial chemicals produced or imported into the United States. |
| Clean Water Act (1977) | CWA gave EPA the authority to set effluent standards on an industry basis (technology based) and continued the requirements to set water quality standards for all contaminants in surface waters. |
| Comprehensive Environmental Response Compensation and Liability Act (1980) | CERCLA created a tax on the chemical and petroleum industries and provided broad federal authority to cleanup abandoned hazardous waste sites (Superfund). |
| Emergency Planning and Community Right-to-Know Act (1986) | EPCRA was enacted by Congress as the national legislation on community safety. |
| Superfund Amendments and Reauthorization Act (1986) | SARA amended CERCLA and reflected EPA's experience in administering the complex Superfund program. |
| Pollution Prevention Act (1990) | PPA encouraged industry to control toxic emissions by using cost-effective changes in production. |
| Oil Pollution Act (1990) | OPA streamlined and strengthened EPA's ability to prevent and respond to catastrophic oil spills through a tax on oil. |
| Food Quality Protection Act (1996) | FQPA amended FIFRA and FFDCA. |

The FIFRA was enacted in 1947 as a consumer protection statute to regulate the manufacture, sale, distribution, and use of pesticides. The USDA administered FIFRA. Pesticide registration was required before marketing in interstate commerce, and the pesticide product had to bear a label with the manufacturers name and address, name brand and trademark of the product, net contents, ingredient list, warning statements to prevent injury, and directions for use. Subsequent amendments added more product classes including nematicides, plant regulators, defoliants, and desiccants (1959); and a requirement that all pesticide labels contain a Federal Registration Number and safety information words, such as "warning", "danger", "caution", and "keep out of reach of children"(1964).

In 1970, authority for FIFRA was transferred from the USDA to the newly created EPA. Another amendment in 1972 provided the EPA with authority to regulate pesticides to prevent unreasonable adverse effects on the environment. The 1972 amendment changes included requirements to follow the label, heavy fines for violations, classification of pesticides as "restricted use" or "general use", state certification, manufacturer registration and inspection by EPA, and scientific evidence that the product is effective and safe for humans, crops, and animals. Subsequent clarifying amendments provided for significant changes to improve the registration process including generic standards for the active ingredient rather than for each formulated product, re-registration of all products registered prior to 1975, conditional registration where efficacy data may be waived, state enforcement authority and defined the phrase: "to use any registered pesticide in a manner inconsistent with its labeling".

In 1988, the EPA was required to re-register existing pesticides that were originally registered before current scientific and regulatory standards were formally established. The Food Quality Protection Act (FQPA) of 1996 is the most recent significant amendment to FIFRA.

## Pesticide residues in foods

When pesticides are used in producing food, they may remain in small amounts called residues. To protect the public from potentially harmful effects caused by pesticide residues on food, the EPA regulates the amount of each pesticide that may remain on or in each food. EPA regulates pesticides under two major federal statutes. Under the FIFRA, EPA registers pesticides for use in the United States and prescribes labeling and other regulatory requirements to prevent unreasonable adverse effects on health or the environment. Under the FFDCA, EPA establishes tolerances (maximum legally permissible levels) for pesticide residues in food. Tolerances are established as part of the pre-market registration process. Exceeding the tolerance initiates enforcement actions where the commodity may be subject to seizure. Tolerance limits apply to both domestic and imported food. Some foods do not require tolerances because the exposure and toxicity data show that the food is safe when the pesticide is used according to the directions on the label.

Several other government agencies enforce EPA's pesticide tolerances in food. The FDA tests food produced in the United States and imported food. State agencies also test food produced in the United States. The USDA tests milk, meat, and eggs. The USDA and FDA have programs designed to develop statistically valid information on pesticide residues in foods. This information is provided to EPA for use in risk assessments for pesticides. When the USDA detects violations of tolerances in their data collection program, they notify FDA.

EPA has responded to both large and small environmental disasters during the past 30 years. Less visible but just as important is EPA's present effort to reassess all the pesticide and other ingredient tolerances and exemptions that were in effect as of 1996 when the FQPA of 1996 was passed. It amended both the FIFRA and the FFDCA. These amendments changed the way EPA regulates pesticide residues. Examples of FQPA changes include a single residue safety standard of "a reasonable certainty of no harm"; requirement for an explicit determination that pesticide tolerances are safe for children (includes an additional safety factor); limits consideration of benefits when setting tolerances to non-threshold (such as carcinogenic) effects; requirement to review existing pesticide tolerances within 10 years; authorization to require new data, including potential endocrine effects; imposition of civil penalties; and expedites review of safer pesticides.

## EPA companion animal safety testing

Companion animal safety studies for EPA registered pesticides apply only to dogs and cats because of widespread use of these external products for pests (USEPA, 1998). The companion animal safety study is required for pesticide registration. Companion animal safety studies for pesticide formulations for the treatment of external pests are intended to demonstrate that an adequate margin of safety exists with overuse or misuse (40 CFR Section 792 and 40 CFR 160, GLP Standards). Data from companion animal safety studies for pesticides serve as a basis for product labeling. The study can be compared to an acute dermal toxicity study, and is limited because an NOEL is not required. External pesticide products include collars, sprays, dips, shampoos, and spot treatments.

The design of a companion animal safety study should reflect the product label: method of administration, species and age group, and frequency of application. The criteria for use of the product should be used in the study. A control group should receive a concurrent vehicle at the 5× level. The vehicle should contain the inert ingredients at the maximum levels that would appear in the 5× formulation. Negative (untreated) controls may occasionally be employed to determine whether adverse effects are due to the inert ingredients in a formulation. The test formulation is applied to several groups of (six/sex) experimental animals at the label dose and multiples of this dose (3× and 5×). For exaggerated doses (3× and 5×), products specifically prepared for this type of study that contain higher concentrations of the active ingredient are preferred. If the drug cannot be formulated at the 3× or 5× concentration, due to volume constraints, multiple treatments at frequent intervals may be necessary. If the high-dose level (at least 5×) produces no evidence of drug-related toxicity, a full study (three-dose levels) may not be necessary. Multiple pesticides formulated into a single product may also be evaluated. Depending on the severity of clinical signs of toxicity, products with less than 5× margin of safety may be considered for registration. The route of administration of these product studies should be topical. The skin or hair should not be prepared in any manner unless such directions appear on the label. If the product label recommends several treatments, multiple treatments at frequent intervals are included in the study design based on label claims and instructions for use.

The species recommended for treatment on the product label is included in the study. Studies are preformed on representative classes of healthy dogs and cats by size, weight, sex, and age based on label claims. For instance, if only adults over 6 months of age are the label population of animals to receive treatment, only adults are enrolled in the study. If the product is registered for puppies and kittens, the label should state a minimum age for this group and this class should be included the study.

An equal number of animals per sex are used at each dosage level. Animals are appropriately examined and prepared during the acclimation period.

Clinical observations are conducted at hourly intervals for at least 4 h after the last treatment, and twice daily for the duration of the study. If adverse reactions are observed, the observation period on the day of treatment is extended to a time at which no toxic signs are observed. Observations should include all systems. Special attention is directed to observations of central nervous system signs of seizures, tremors, and salivation; and gastrointestinal signs of vomiting and diarrhea. Observations and measurements are reported for a minimum of 14-day post-treatment and longer if appropriate. Various samples and measurements are collected throughout the study. Individual body weights and food consumption are measured during the acclimation and periodically during the study. Animals that die or are euthanized in a moribund state are subjected to a gross necropsy and abnormal tissues are examined histopathologically to determine the cause of death. Routine post-study necropsy is not required. Clinical pathology samples including red cell cholinesterases, when appropriate, are assessed prior to treatment, 24-h post-treatment, and on day 7 of treatment.

## Chronic toxicity testing: pesticides in animal-derived food

### *Introduction*

The USEPA requires a variety of toxicity testing prior to registration of pesticides. The 870 Series Final Guidelines describe study protocols that meet testing requirements of the FIFRA and the Toxic Substances Control Act (TSCA): Reproduction and Fertility (870.3800) and Combined Chronic Toxicity/Carcinogenicity (870.4300) (USEPA, 1998, 2000a).

### *Reproduction toxicity testing*

The two-generation reproduction study provides information on the effects of a test substance on the male and female reproductive systems. The study should be conducted in accordance with the GLP Standards stipulated in 40 CFR Part 160 (FIFRA) and 40 CFR Part 792 (TSCA). The test substance is administered to parental (P) animals prior to and during their mating, during the resultant pregnancies, and through the weaning of their F1 offspring. The substance is then administered to selected offspring during their growth into adulthood, mating, and production and weaning of a subsequent generation. The rat is the most commonly used species for testing. Each control group should contain a sufficient number of mating pairs to yield approximately 20 pregnant females. Each test group should contain a similar number of mating pairs. At least three-dose levels and a concurrent control should be used. The dose levels should be spaced to produce a gradation of toxic effects. The highest dose should be chosen to induce some reproductive and/or systemic toxicity but not death or severe suffering. The intermediate-dose levels should produce minimal observable toxic effects. The lowest-dose level should not produce any evidence of either systemic or reproductive toxicity. The highest dose tested should not exceed 1000 mg/kg/day (or 20,000 ppm in the diet), unless potential human exposure data indicate the need for higher doses. A concurrent control group should be used. The test substance is usually administered by the oral route (diet, drinking water, or gavage). The animals should be dosed with the test substance on a daily basis. Daily dosing of the parental males and females should begin when they are 5–9-week old, and that of the offspring should begin at weaning. Daily dosing should begin at least 10 weeks before the mating period and continue until termination.

The endpoints measured should provide data regarding the performance of the male and female reproductive systems including gonadal function, the estrous cycle, mating behavior, conception, gestation, parturition, lactation, and weaning, and on the growth and development of the offspring. The study may also provide information about the effects of the test substance on neonatal morbidity, mortality, target organs in the offspring, and preliminary data on prenatal and postnatal developmental toxicity and serve as a guide for subsequent tests. Additionally, since the study design includes *in utero* as well as postnatal exposure, it provides the opportunity to examine the susceptibility of the mature/neonatal animal. For further information on functional deficiencies and developmental effects, additional study segments can be incorporated into the protocol, utilizing the guidelines for developmental toxicity or developmental neurotoxicity (USEPA, 1996).

### *Combined chronic toxicity/carcinogenicity testing*

The objective of the combined chronic toxicity/carcinogenicity study is to determine the effects of a substance in a mammalian, species following prolonged and repeated exposure. If chronic/carcinogenic effects are detected in the first mammalian species, usually the rat, further long-term studies may be conducted in another species, usually the dog. The design and conduct should allow for the detection of neoplastic effects and a determination of the carcinogenic potential as well as general toxicity, including neurological, physiological, biochemical, and hematological effects and exposure-related morphological (pathology) effects.

Preliminary studies providing data on acute, subchronic, and metabolic responses should be conducted. The rate of exposure is generally oral, dermal, or inhalation. For the

combined chronic toxicity/carcinogenicity study, the rat is the species of choice for oral and inhalation studies, whereas the mouse is species of choice for the dermal route. The choice of the route of administration depends on the physical and chemical characteristics of the test substance and the product exposure in humans. The duration of studies should be at least 18 months for mice and hamsters and 24 months for rats. The following general requirements apply to all combined chronic toxicity/carcinogenicity studies regardless of the route of administration. Testing should be started with young healthy animals as soon as possible after weaning and acclimatization, but no later than 8 weeks of age. At commencement of the study, the weight variation of animals used should be within 20% of the mean weight for each sex. At least 100 rodents (50 males and 50 females) should be allotted randomly to each dose level and concurrent control group. At least 20 additional rodents (10 males and 10 females) should be used for satellite dose groups and the satellite control group. The purpose of the satellite group is to allow for the evaluation of chronic toxicity after 12 months of exposure to the test substance. The number of animals in any group should not fall below 50% during the course of the study at 15 months in mice and 18 months in rats, so that a meaningful and valid interpretation of negative results can be achieved. Survival in any group should not fall below 25% at 18 months in mice and 24 months in rats.

#### *Organization for Economic Cooperation and Development*

The Organization for Economic Cooperation and Development (OECD) carried forward recommendations of an international expert group and questioned the value of clinical pathology findings, including hematology, clinical chemistry, beyond 12 months in chronic, and combined chronic/carcinogenicity studies (Weingand *et al.*, 1996). As a result, hematology and clinical chemistry measures beyond 12-month period have been eliminated. The observational and functional tests on nervous system and behavior have also been revised per OECD. Recently, the requirement for the 6-month long-term studies in the dog has been modified. Long-term studies (6 months or longer) in the dog will not be conducted unless warranted by findings in long-term rat studies.

## EPA adverse effects information reporting

At EPA, regulatory responsibilities for pesticides are centralized at the OPP. Regulatory authority is derived from Section 6(a)(2) of the FIFRA. The regulations that specifically address reporting of adverse effects of pesticides are contained in Reporting Requirements for Risk/Benefit Information (40 CFR Section 159). Reporting of AERs is mandatory for industry.

Pesticides are broadly defined to include insecticides, herbicides, fungicides, and various other substances that are not animal drugs used to control pests. A pesticide is also any substance or mixture of substances intended for use as a plant regulator, defoliant, or desiccant. The EPA collects information on pesticide use, whether intended or unintended. Adverse effects information may include information on human or animal injury, plant injury, or environmental contamination. All adverse effects information is accepted regardless of the registered label use of the product.

Specific industry reporting requirements for adverse effects information depends on the category of the information. Reports of human death should be submitted within 15 calendar days as an individual report. A variety of other information, including suspected product defects, human epidemiology and exposure studies, and pest resistance reports should be submitted within 30 calendar days as individual reports. Reports involving major and moderate human injury, major injury to plants and wildlife, water contamination, and public health product ineffectiveness should be submitted after a 1-month accumulation as individual reports.

Animal reports receive less priority than human reports. Reports involving any type of domestic animal injury, and some minor types of human, plant, and wildlife injury, should be reported within 2 months, after a 3-month accumulation of reports. The information may be submitted in an aggregate format. An aggregate report for a specific time period includes a count of domestic injuries within categories of assessed seriousness. Information concerning the specific details of each individual AER is not included in an aggregate. The EPA, however, may request additional information for any AERs that involved the death of an animal.

The EPA/OPP gathers adverse effects information from other sources. Information may result from complaints made directly to the EPA. This AER information is entered into the EPA Incident Database. Information may also be obtained through the National Pesticide Communications Network, based at Oregon State University, and other sources. Much of this information may be entered into the EPA Incident Data System. Some aggregate reports may also be entered into the system.

Various product divisions within OPP review the AER information. A complete review of product-related information is performed when it is being considered for re-registration. A Special Review may be set in motion when EPA has reason to believe that the use of a pesticide may result in unreasonable adverse effects, including acute toxicity to domestic animals. Subsequent regulatory actions may range from removal of the product from the market to changes in permitted uses.

Since the EPA/OPP regulates all pesticides, jurisdictional AER issues resulting from extra-label use are not a

consideration. Further, the mission of OPP is to monitor the safety of pesticides in humans and animals regardless of the intended use. Evaluation of domestic animal AERs involving pesticides labeled for therapeutic use on animals is given the same attention as a pesticide labeled for use on plants. Intended use is more likely to affect what regulatory actions may be subsequently taken to improve the overall safety of product use.

# SECTION III. US DEPARTMENT OF AGRICULTURE

## Regulatory authority and mission

The authority for regulating veterinary biologics in the United States is provided in the Virus–Serum–Toxin Act (VSTA), enacted in 1913 and amended in 1985 (USDA APHIS Center for Veterinary Biologics, 2005a). It requires, with some exceptions, that all veterinary biologics be licensed. The Licensing and Policy Development (LPD) unit in the Center for Veterinary Biologics (CVB), Veterinary Services, Animal and Plant Health Inspection Service (APHIS), and US Department of Agriculture (USDA) enforces the VSTA. The Act authorizes the Secretary of USDA to prescribe regulations governing the preparation and marketing of veterinary biologics shipped into, within, or from the United States. The VSTA makes it unlawful to sell worthless, contaminated, dangerous, or harmful veterinary biologics or to ship veterinary biologics in or from the United States unless they are prepared in a licensed establishment in compliance with USDA regulations. It requires the issuance of a permit by USDA prior to the importation of a veterinary biological product and gives the department the authority to test veterinary biological products prior to importation. In case of violation, the Act permits USDA to remove or suspend establishment and/or product licenses and permits. It also gives authority for detention, seizure, and condemnation of products and injunctions against products or establishments.

## Definition and functions

The regulations in 9 CFR 101.2(w) define veterinary biological products to be all viruses, serums, toxins, and analogous products of natural or synthetic origin that are intended for use in the diagnosis, treatment, or prevention of diseases of animals. The products include diagnostics, antitoxins, vaccines, live or killed microorganisms, and their antigenic or immunizing components. The LPD reviews license applications for production facilities and biological products and for importation. It reviews production methods, labels, and supporting data involved in the licensing and permit process and issues licenses and permits. An exemption from this licensing provision is given for products prepared by (1) a person solely for administration to that person's own animals; (2) a veterinarian for use in his or her own licensed practice under a veterinarian–client–patient relationship; and (3) a person operating a State licensed facility solely for distribution of product within the State of production in a State that has the State regulatory USDA-approved control program for veterinary biologics. The VSTA provides for the issuance of conditional or special licenses for products in order to meet an emergency condition.

A proposed rule to amend the VSTA (9 CFR Parts 101 and 116) was published in the Federal Register on August 17, 2005 (Vol. 70, No. 158, p. 48325) (USDA APHIS Guidance Documents, 2005b). The proposed rule requires veterinary biologics licensees to record specific information concerning adverse events associated with the use of biological products that they produce or distribute and to compile and submit those records in a summary report to the APHIS every 12 months for products licensed for 1 year or more; for newly licensed products. A summary report would have to be submitted at 6-month intervals during the first year of the product license and at 12-month intervals thereafter. The VSTA did not explicitly require licensees to maintain records of adverse events associated with the use of veterinary biologics, nor do the regulations in Part 116.5 provide specific guidance in determining whether an adverse event should be considered an indication that raises questions regarding the purity, safety, potency, efficacy, preparation, testing, or distribution of biologics.

Adverse event and AER are defined as follows:

- As any observation in animals, whether or not the cause of the event is known, that is unfavorable and unintended and that occurs after any use (on or off label) of a biological product.
- For products administered to animals, this would include events related to a suspected lack of expected efficacy.
- AER: as a communication concerning the occurrence of an adverse event from an identifiable first-hand reporter that includes at least the following information: an identifiable reporter; an identifiable animal; an identifiable biological product; and one or more adverse events.

### *Animal immunobiologic vigilance program*

Regulation of animal immunobiologics is centralized within the APHIS and CVB. Regulatory authority is derived from the VSTA. Animal immunobiologics modulate the immune system for the prevention, treatment, or diagnosis of disease in animals. Animal immunobiologics typically include

vaccines, toxoids, and serum derivatives, which may be used in diagnostic test kits.

Immunobiologic vigilance (or vaccinovigilance) may in some respects be more complicated than monitoring the safety of animal drugs, or even therapeutic animal pesticides. Vaccines are generally utilized for the prevention of disease. The real or perceived failure of a vaccine to perform may be dependent on a number of factors and difficult to assess. Suspected vaccine failure in individuals is particularly difficult to assess. Further, assessing AERs involving adverse events that are manifested some time after vaccine administration can be complicated. In addition to the "active ingredient", vaccines can include other ingredients, such as preservatives, stabilizers, and adjuvants, which can affect the product performance. Vaccinovigilance is a particularly challenging discipline that may require substantially more information from formal studies in order to verify suspected problems.

Until the proposed rule is finalized to amend the VSTA, USDA currently has no regulations that require the immunobiologic industry to routinely submit AERs to CVB. Since the immunobiologic industry does not submit AERS, the CVB has limited ability to monitor AERs involving animal safety. USDA may at times request companies to voluntarily submit AERs that are related to a particular safety issue. Although, the USDA does not presently mandate specific AER record keeping practices, the USDA does have the ability to conduct investigations at the immunobiologic manufacturing site.

At present, modest levels of CVB resources are allocated to direct vaccinovigilance activities. However, CVB can direct resources, as needed, to safety issues. While regulatory actions may be more likely to result in the removal of the registered product from the market, other actions may include manufacturing changes or label revisions.

## Animal care and welfare requirements

Researchers who conduct studies funded by the federal government are subject to a number of requirements imposed by various federal agencies. The Animal Welfare Act (AWA) is administered by the USDA APHIS Animal Care Program (USDA APHIS Animal Care Program Publications, 2000). It applies specifically to dogs, cats, and a number of other listed species as well as to any other warm-blooded animals designated by the Secretary. It requires covered facilities to register and comply with AWA regulations. Examples of types of requirements established to implement AWA include living space, lighting, heating, ventilation, drainage, transportation, feeding and watering, and veterinary care. It establishes the requirement for an Institutional Animal Care and Use Committee, which must include a veterinarian and an external member. This committee reviews the program and inspects the facility periodically and reviews protocols for research conducted within the facility. It does not apply to animal agriculture or non-research horses. It does apply to dealers and exhibitors. Federal agencies such as National Institutes of Health (NIH) may impose additional requirements.

As a practical approach to ensuring that their programs and facilities meet these requirements, many organizations participate in the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International's accreditation process (Association for Assessment and Accreditation of Laboratory Animal Care, 2005). AAALAC was founded in 1965 to address laboratory animal welfare issues. AAALAC International is a non-regulatory, non-profit corporation. Its mission is to promote high standards for animal care, use, and welfare and enhance life sciences research and education through its accreditation process.

# SECTION IV. DRUG ENFORCEMENT ADMINISTRATION

## Mission and public health impact

Illegal drug use is a widespread problem in the United States. In 1998, an estimated 13.6 million Americans used an illicit drug at least once during the 30 days prior to being interviewed. During the same period, nearly 1 in 10 youths aged 12–17 years was an illicit drug user, and 130,000 were current users of Heroin. The Drug Enforcement Administration (DEA) was established on July 1, 1973. Its mission is to enforce the controlled substances laws and regulations of the United States and to bring to the criminal justice system, or any other competent jurisdiction, those organizations and principal members of organizations who are involved in the growing, manufacture, or distribution of controlled substances in the United States.

## Illicit veterinary drugs of public health concern

Approved veterinary scheduled drugs, which are illicitly used in humans, include boldenone (Equipoise), ketamine, stanozolol (Winstrol), and trenbolone (Finajet). Veterinary products containing anabolic steroids that are exclusively intended for administration through implants to cattle or other non-human species and which have been approved by the CVM, US FDA are excluded from all schedules (Drug Enforcement Agency: 21 CFR Parts 1300–1316, List of Scheduling Actions and Controlled Substances, 1999b; Drugs of Abuse, 1997).

Ketamine hydrochloride, known as "special k" and "k", is a general anesthetic for human and veterinary use.

Ketamine produces effects similar to pentachlorophenol (PCP) with the visual effects of lysergic acid diethylamide (LSD). Ketamine sold on the streets comes from diverted legitimate supplies, primarily veterinary clinics. Its appearance is similar to that of pharmaceutical grade cocaine, and it is snorted, placed in alcoholic beverages, or smoked in combination with marijuana. The incidence of ketamine abuse is increasing. Ketamine was placed in Schedule III of the Controlled Substances Act (CSA) in August 1999 (Drug Enforcement Agency: 21 CFR Parts 1300–1316, Schedules of Controlled Substances, 1999a).

Concerns over a growing illicit market and prevalence of abuse combined with the possibility of harmful long-term effects of steroid use led Congress in 1991 to place anabolic steroids into Schedule III of the CSA. The CSA defines anabolic steroids as any drug or hormonal substance chemically and pharmacologically related to testosterone (other than estrogens, progestins, and corticosteroids) that promotes muscle growth. Most illicit anabolic steroids are sold at gyms, competitions, and through mail operations. For the most part, these substances are smuggled into this country. Those commonly encountered on the illicit market include boldenone (Equipoise), ethlestrenol (Maxibolin), fluoxymesterone (Halotestin), methandriol, methandrostenolone (Dianabol), methyltestosterone, nandrolone (Durabolin, DecaDurabolin), oxandrolone (Anavar), oxymetholone (Anadrol), stanozolol (Winstrol), testosterone, and trenbolone (Finajet). Physical side effects include elevated blood pressure and cholesterol levels, severe acne, premature balding, reduced sexual function, and testicular atrophy. In males, abnormal breast development (gynecomastia) can occur. In females, anabolic steroids have a masculinizing effect, resulting in more body hair, a deeper voice, smaller breasts, and fewer menstrual cycles. Several of these effects are irreversible. In adolescents, abuse of these agents may prematurely stop the lengthening of bones, resulting in stunted growth (Drug Enforcement Agency: 21 CFR Parts 1300–1316, List of Scheduling Actions and Controlled Substances, 1999b).

## DEA regulatory requirements for veterinarians

Federal DEA regulations are contained in 21CFR 1300 and 1316 (US Department of Justice, 2005). Clinicians who are authorized to prescribe controlled substance may do so for legitimate medical purpose in the context of a valid veterinary-client–patient relationship. Under these regulations the prescribing practitioner is held responsible in case the prescription does not conform to the regulations for records and reports (21 CFR 1304), submitting proper ordering forms (21 CFR 1305), and for meeting the requirements for prescriptions (21 CFR 1306 (1)). Requirements for prescribing Schedule II drugs are much more stringent than those are for Schedules III, IV, and V. For example, an oral order or refilling of the prescription for Schedule II drugs is prohibited under the CSA. DEA has divisional offices in various regions, including in Atlanta, Boston, Chicago, Dallas, Denver, Detroit, El Paso, Houston, and Los Angeles. In addition, each division has several district offices.

# SECTION V. OCCUPATIONAL SAFETY AND HEALTH ADMINISTRATION

## Organization and mission

Harmful chemical compounds in the form of vapors, fumes, mists, gases, liquids, and solids are encountered in the workplace by inhalation, absorption (through direct skin contact), or to a lesser extent by ingestion. Airborne chemical hazards have great occupational health significance. The degree of worker risk from exposure to any given substance depends on the nature and potency of the chemicals toxic effects and the duration and intensity of exposure (Occupational Safety and Health Administration, Informational Booklet on Industrial Hygiene, 2005c).

In 1970 Congress passed the Occupational Safety and Health Act to assure so far as possible every worker in the nation safe and healthful working conditions (Occupational Safety and Health Administration, The Occupational Safety and Health Act of 1970, 2005a). The Act requires employers to maintain a safe workplace and created the Occupational Safety and Health Administration (OSHA). This agency's mandate includes regulatory responsibility for establishing mandatory occupational exposure standards, as well as related research, training and education and enforcement. OSHA is part of the Department of Labor and is headed by the Assistant Secretary of Labor for Occupational Safety and Health. Major OSHA programs are administered through Directorates such as the Directorate for Safety Standards Programs which provides workplace standards and regulations to ensure safe working conditions.

*Offices and District Offices*. Under the Act many states have OSHA-approved occupational safety and health programs which function *in lieu* of OSHA programs or standards. OSHA has broad workplace safety responsibilities which pertain to physical hazards such as electromagnetic radiation, temperature, noise and vibration; ergonomic hazards such as repeated motions or heavy lifting; and biological hazards including bacterial and viral pathogens, in addition to the chemical hazards that are the primary subject of this section. The Act also established the National Institute for Occupational Safety and Health (NIOSH) within the (DHHS). NIOSH is authorized to develop and establish recommended occupational safety and health standards by conducting research and

experimental programs for the development of new and improved occupational safety and health standards.

### Exposure limits

OSHA limits occupational exposure to hazardous chemicals by establishing exposure limits and directing changes in employer work processes or equipment, or when that is not feasible requiring personal protective equipment. Unlike drugs, biologics, pesticides, or food additives, industry does not need to provide evidence of safety, or obtain an approval prior to manufacturing and marketing new products which may entail occupational exposure to hazardous chemicals. Thus, exposure limits may be adopted after the chemical has entered the occupational environment. The Act specifies that for regulating toxic chemicals OSHA must adopt standards which most adequately assure, to the extent feasible, on the basis of the best available evidence, that no employee will suffer material impairment of health or physical capability. Thus, some consideration must be given to the issue of technological achievability for industry in promulgating standards.

OSHA standards are called permissible exposure limits (PELs). They are legal standards that may not be exceeded. PELs have been controversial and susceptible to successful legal challenges. Thus, many chemicals do not have PELs. In the absence of OSHA PEL's employers utilize recommended occupational exposure limits established by acknowledged sources in order to meet OSHA's requirements for worker safety. NIOSH has recommended exposure limits (RELs). The American Conference of Governmental Industrial Hygienists Threshold Limit Values (TLVs) for Chemical Substances and Physical Agents in the Work Environment are very widely utilized (National Oceanographic and Atmospheric Administration, 1998). TLVs refer to airborne concentrations of substances and represent conditions under which it is believed that nearly all workers may be repeatedly exposed day after day without adverse health effects. There are three categories of occupational exposure limits:

1 *Time-weighted average (TWA)*: The TWA concentration for a conventional 8-h workday and 40-h workweek, to which it is believed that nearly all workers may be repeatedly exposed, day after day, without adverse effect.
2 *Short-term exposure limit (STEL)*: The concentration to which it is believed that workers can be exposed for a short period of time without suffering from irritation, tissue damage, or narcosis, it is not a separate exposure limit but supplemental to the TWA and limited to a 15-min TWA.
3 *Ceiling*: The concentration that should not be exceeded during any part of the working exposure (The American Conference of Governmental Industrial Hygienists, 2005).

The OSHA Hazard Communication Standard (29 CFR 1910.1200), also called the Right-to-Know Law, ensures that the hazards of all chemicals are evaluated and the information concerning their hazards is transmitted to employers and employees. It requires chemical manufacturers and importers to obtain or develop a material safety data sheet (MSDS) for each hazardous chemical they produce or import. Distributors are responsible to insure that their customers are provided a copy of these MSDSs. An MSDS must contain information describing the physical and chemical properties, health hazards, routes of exposure, precautions for safe handling and use, emergency procedures, and control measures. The MSDS has become a well-established document for disseminating health and safety information about chemical products (Occupational Safety and Health Administration, Technical Center Brochure, 2005b). The NIOSH Pocket Guide to Chemical Hazards is another useful document for health and safety information about hazardous chemicals (National Institute for Occupational Safety and Health, 1997).

## SECTION VI. STATUTES, REGULATIONS, AND GUIDELINES

The three federal agencies (FDA, EPA, and USDA), which are responsible for regulating animal health products all maintain surveillance programs for monitoring products after they are marketed. The laws, which provide regulatory authority, and thus the regulatory goals, regulations, and approach by these three agencies are different. By extension, the pharmacovigilance programs utilized for monitoring the safety and effectiveness of marketed animal medicinal products are different. Thus, they have some common and some different statues, regulations, and guidelines.

### Notice and comment rulemaking procedures and access to agency records

Statutes are legislation passed by congress and signed by the president. They are the laws that provide the authority for regulatory agencies to operate. Regulations are basic tools for achieving a regulatory agency's goals, such as consumer or environmental protection. Regulations inform the affected industries and the public of statutory requirements and the agencies procedures. Regulations interpret the law and spell out the details needed to implement the general

provisions in the statutes. Regulations also describe the product approval process for many individual products or set forth required standards of product composition or performance.

Change is constant and agencies must issue new regulations and amend or revoke old ones. All regulations must be authorized by statute, and the rulemaking procedures must conform to requirements of the Administrative Procedures Act which applies to all executive branch departments and agencies of the federal government. The administrative process for establishing regulations used is referred to as "Notice and Comment Rulemaking".

New and amended regulations and other notices are disseminated by publication in the Federal Register which is issued daily by the Federal Government (Federal Register). Regulations are codified in the Code of Federal Regulations (CFR) which is updated annually. Preparing and issuing a regulation is a complex and lengthy process because agencies must be sure any new rule is needed and well conceived. Stakeholders such as affected industry, and consumers generally must be given an opportunity to participate through the notice and comment rulemaking process. Ordinarily, this is done by publishing in the Federal Register a notice of proposed rulemaking and inviting comments, within a specified time frame, typically 60 or 90 days. In appropriate cases public hearings are also held. The written and public hearing comments then must be reasonably responded to before issuing a final regulation. After reviewing all comments an agency publishes the final rule in the Federal Register and announces the effective date of the regulation. The regulation then becomes part of the CFR.

Guidelines establish practices of general applicability and do not include decisions or advice on specific situations. Guidelines are not legal requirements, but may be relied on with the assurance that it is an acceptable procedure or standard. A guideline represents the formal position of the agency on a matter and obligates the agency to follow it until it is amended or revoked. The guideline development process is similar to the regulation publication process. Draft guideline availability and request for comment is typically announced in the Federal Register and comments are considered when developing final guidelines.

## Freedom of information and public access to regulatory agency records

The Freedom of Information Act (FOIA) went into effect in 1967. It provides access to information in government files and requires that each government agency publish descriptions of its operations and procedures. Each agency must also make available opinions, orders, and statements of public policy that affect the public. Any person can obtain information through an FOIA request. Certain types of information are not available under FOIA. For example, national security-related, trade secrets and commercial information and personnel and medical files are exempt. Agencies are given a certain period of time to process requests, typically within 20 days of receipt. The key to obtaining the desired information is to make the request sufficiently detailed and specific to allow identification of the record(s). Information that would be important to veterinarians would include adverse reaction information for animal drugs, regulatory letters written by the agency, Compliance Policy Guidelines (to direct FDA field staff in enforcement activities), and Guidelines implementing regulations. Fortunately most of this information is now available on federal agency Internet home pages. For instance, Warning Letters to manufacturers for violations of Good Manufacturing Practices, and livestock producers or veterinarians involved in causing violative drug residues may be viewed on FDA's FOIA site. Summary information on reports of adverse drug reactions is available on the FDA CVM's home page.

# REFERENCES

Association for Assessment and Accreditation of Laboratory Animal Care (2005) http://www.aaalac.org/.

Code of Federal Regulations. http://www.gpoaccess.gov/cfr/index.html.

Drug Enforcement Agency, Washington, DC (1997) Drugs of Abuse, Superintendent of Documents, Mail Stop SSOP, 20402-9328.

Drug Enforcement Agency, Washington, DC (1999a) Schedules of Controlled Substances: Placement of Ketamine into Schedule III, 64FR (133), 37673–5.

Drug Enforcement Agency, Washington, DC (1999b) List of Scheduling Actions and Controlled Substances, Office of Diversion Control, Drug and Chemical Evaluation Section, 20537.

Farmer JH, Kodell RL, Gaylor, DW (1982) Estimation and extrapolation of tumor probabilities from a mouse bioassay with survival/sacrifice components. *Risk Anal* **2**: 27–34.

FDA Center for Veterinary Medicine, Rockville, MD (1989) Target Animal Safety Guidelines for New Animal Drugs. http://www.fda.gov/cvm/Guidance/Guideline33.htm.

FDA Center for Veterinary Medicine, Rockville, MD (1999) A Brief History of CVM. http://www.fda.gov/cvm/aboutbeg.htm.

FDA Center for Veterinary Medicine, Rockville, MD (2000) Specific Subject Index. http://www.fda.gov/cvm/index.html.

FDA Center for Veterinary Medicine, Rockville, MD (2005) General Principles for Evaluating the Safety of Compounds Used in Food-Producing Animals Guidelines. http://www.fda.gov/cvm/Guidance/GFI003.htm.

Federal Register. http://www.gpoaccess.gov/fr/index.html.

Gaylor DW, Kodell RL (1980) Linear interpolation algorithm for low dose risk assessment of toxic substances. *J Environ Pathol Toxicol* **4**: 305–12.

International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Medicinal Products, VICH (2005) http://vich.eudra.org/.

National Institute for Occupational Safety and Health, Cincinnati, OH (1997). NIOSH Pocket Guide to Chemical Hazards. http://www.cdc.gov/niosh/npg/.

National Oceanographic and Atmospheric Administration, Washington, DC (1998) Occupational Exposure Limits. http://www.noaa.gov.

Occupational Safety and Health Administration, Washington, DC (2005a) The Occupational Safety and Health Act of 1970 and Amendments. http://www.osha.gov/pls/oshaweb/owadisp.show_document?p_table=OSHACT&p_id=2743.

Occupational Safety and Health Administration, Washington, DC (2005b) Salt Lake Technical Center Brochure, Technical Assistance. http://www.osha.gov/SLTC/index.html.

Occupational Safety and Health Administration, Washington, DC (2005c) OSHA 3143 Informational Booklet on Industrial Hygiene. http://www.osha-slc.gov/Publications/OSHA3143/OSHA3143.htm.

Teske RH (1995) *Veterinary Pharmacology and Therapeutics*, 7th edn, Adams RH (ed.). Iowa State University Press, Ames, IA, pp. 1131–6.

The American Conference of Governmental Industrial Hygienists, Cincinnati, OH (2005) Threshold Limit Values (TLVs) for Chemical Substances and Physical Agents and Biological Exposure Indices. http://www.acgih.org.

US Department of Justice (2005) DEA. http://www.usdoj.gov/dea/.

USDA (2000) APHIS Animal Care Program Publications. http://www.aphis.usda.gov/ac/publications.html.

USDA (2005a) APHIS, Center for Veterinary Biologics. http://www.aphis.usda.gov/vs/cvb/index.htm.

USDA (2005b) APHIS, Guidance Documents. http://www.aphis.usda.gov/vs/cvb/regsandguidance.htm.

USEPA, Washington, DC (1996) Reproductive toxicity risk assessment guidelines. Federal Register 61 FR 56274-56322.

USEPA, Washington, DC (1998) Health Effects Test Guidelines, OPPTS 870.7200, Companion Animal Safety, Prevention, Pesticides and Toxic Substances. http://www.epa.gov/opptsfrs/publications/OPPTS_Harmonized/870_Health_Effects_Test_Guidelines/index.html.

USEPA, Washington, DC (2000a) Subpart E – Specific Organ/Tissue Toxicity: Reproduction and Fertility Effects, 40CFR 798.4700. US Government Printing Office, Washington, DC.

USEPA, Washington, DC (2000b) Programs. http://www.epa.gov/epahome/programs.htm.

Weingand K, Brown G, Hall R, Davies D, Gossett K, Neptun D, Waner T, Matsuzawa T, Salemink P, Froelke W, Provost JP, Negro GD, Batchelor J, Nomura M, Groetsch H, Boink A, Kimball J, Woodman D, York M, Fabianson-Johnson E, Lupart M, Melloni E (1996) Harmonization of animal clinical pathology testing in toxicity and safety studies. *Fundam Appl Toxicol* **29**: 198–201.

CHAPTER

# 8

# Toxicology and the law

*Michael J. Murphy*

## INTRODUCTION

Much of the law of admissibility of scientific expert testimony arises from toxic tort cases. Consequently, the discipline of toxicology has strongly influenced the legal standards by which scientific expert testimony is admitted in a legal venue.[1]

This chapter uses a hypothetical example of legal issues that may arise in an animal toxicosis case to illustrate application of the law to a feed case. It is intended to introduce toxicologists, and other scientists, to a broad overview of some of the legal issues that may arise when a medical case become a "legal case." This chapter is not, and is not intended to be, a comprehensive review of the law. Neither it is intended to provide an outline of steps to take for a particular case in a particular jurisdiction.[2]

This chapter primarily relies on the Federal Rules of Evidence, and limited case law, to identify issues[3] that may arise when the findings of a medical diagnosis are used in the courtroom. It focuses on the admissibility of expert testimony in a legal proceeding. However, the reasons for conducting toxicology analyses, jurisdiction, standard of proof, and evidence, are briefly summarized to provide a foundation for the role of the expert testimony. The chapter concludes with some questions to consider when analyzing a toxicology case.

[1]The particular county, or geographical area, in which a court with jurisdiction may hear and determine a case. Black's Law Dictionary.

[2]It is the power of the court to decide a matter in controversy and presupposes the existence of a duly constituted court with control over the subject matter and the parties.

[3]A single, certain, and material point, deduced by the allegations and pleadings of the parties, which is affirmed on the one side and denied on the other.

## REASONS FOR CONDUCTING TOXICOLOGY ANALYSES

Toxicology has been defined as the scientific *study of adverse effects of chemicals on living organisms* (Eaton and Klaassen, 2001). A toxicologist is one trained to examine the nature of those effects and assess the probability of their occurrence (Eaton and Klaassen, 2001). Exposure, dose response, and variability of that response are fundamental principles in the science of toxicology (Eaton and Klaassen, 2001). A showing that the toxicologist offered as an expert witness has applied these fundamental principles to the facts of the case at hand will likely be useful in the determination of whether the toxicologist's testimony will or will not be admissible as evidence. The factors that the judge uses to make this determination are discussed in the expert witness section at the end of this chapter.

A toxicologist relies on analytical toxicology analyses in many toxicology cases. In most instances, it is known at the time the sample is submitted for analytical chemistry analysis that the results of that analysis will be used for legal purposes. Racing chemistry laboratories are one example in the veterinary profession where it is known from the outset that the results of the analytical chemistry testing performed will be used in a legal enforcement setting. In some instances however, the purpose to which the results will be applied is not known at the time of sample submission, or, more commonly, that purpose changes after the testing is completed.

### *When the reason for testing the sample is known*

Samples are analyzed for the presence of chemical toxins for many reasons and in many settings. Analyses may take place in academic, governmental, or private laboratories. Analyses performed in these laboratories may each

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serve a different purpose, such as research, enforcement actions, preclinical testing, or clinical diagnoses.

Toxicology analyses in academic settings are primarily conducted to support one's research or scholarly efforts. One example is the development of new analytical methods. Another example is the characterization of adverse effects of a particular dose of a particular chemical in a given species. Similarly, researchers may observe the clinical signs and adverse effects of animals exposed to chemicals, then compile these effects in retrospective or prospective case reports in peer-reviewed literature. Such literature may be useful to support a general causation[4] argument in a legal case. However, the medical case itself or even the analyses performed on that medical case may not be admissible in court because of insufficient documentation supporting authenticity. Other laboratories may provide such documentation.

Veterinary diagnostic laboratories normally have some government support to assist in the clinical diagnosis of diseased animals. Such analyses are normally aimed at assisting in the determination of whether an animal may or may not have been exposed to a particular chemical, and if so, whether the animal has or has not experienced a toxicosis as a result of that exposure. These chemical analyses may give qualitative, semi-quantitative, or quantitative results.

Many contemporary analytical chemistry procedures, performed in each of the above laboratory types, allow detection of the presence of chemicals in biological samples that represent only "background" or "normal" exposure to that chemical. One example is micronutrients, such as vitamins A and E, copper, iron, magnesium, selenium, and zinc. Similarly, lead may be detected at "background" concentrations in blood, liver, or kidney by many analytical toxicology methods available today. Consequently, the detection of the chemical is not "diagnostic" for toxicosis due to that chemical, but merely indicates exposure. Information beyond the concentration of a chemical in tissues is often required to reach a toxicosis diagnosis. The distinction between "exposure to" and "toxicosis from" a chemical is fundamental in a legal venue.

Samples are received from many sources. Most diagnostic laboratories accept samples from veterinarians, animal owners, animal industries, and others. The circumstances of the collection and handling of the sample prior to submission in the diagnostic laboratory are rarely known with certainty. Similarly, routine protocols in a diagnostic laboratory may be different than those in an enforcement laboratory, particularly with respect to sample tracking, documentation, and disposal.

Some government and some private laboratories focus on performing chemical analyses in support of legal enforcement actions. Examples of such laboratories include racing chemistry laboratories, and State or Federal Departments of Agriculture or Health. Many of these laboratories have inspectors who insure proper sample collection and transport to the laboratory. In addition, sample tracking, control, retention, and disposal are generally better documented in laboratories devoted to enforcement action, than those devoted to research or routine diagnostic testing. The analytical methods used in an enforcement setting have often been validated by AOACI,[5] or a similar entity. This validation often demonstrates that the method has been shown to be reliable in multiple laboratories for quantifying the concentration of an analyte or a group of analytes in a specific matrix.

The analytical methods useful in a diagnostic setting may be different from those in an enforcement or research setting. The analytical methods used to support a diagnostic case are often influenced by cost and turn-around-time. On the other hand, those in an enforcement setting may be more influenced by reliability of the results reported. These factors directly and indirectly influence the collection of the original sample, tracking of the sample in the laboratory, sample retention, document retention, and sample disposal protocols. Protocols appropriate for the development of a method in a research laboratory may not be appropriate for use in an enforcement setting. These factors should be taken into account and samples sent to the appropriate laboratory when the reason for the analysis is known. The reason is not always known.

#### *When the reason for testing the sample is not known or changes after testing*

Problems are more likely to occur if the purpose for which the sample is tested is not known at the time of testing, or changes after the testing is completed. These problems may give rise to angst on the part of the toxicologist, counsel, and others. This angst may occur if sample collection, tracking, analysis, and interpretation have not been documented in a way that supports proof of reliability in a legal venue. Questions of authenticity and reliability are more likely to arise in analyses performed in a research, routine clinic, or diagnostic laboratory setting than those collected and performed by enforcement personnel. Medical samples submitted and tested for one purpose may not be reliable if used for another purpose. In short, the lack of sample tracking or chain of custody documentation may seriously erode the admissibility of laboratory results. A diagnosis may not be supported without laboratory results and a legal case may not succeed without the diagnosis. Put more bluntly, insufficient sample collection, retention, and chain of custody documentation can be a

[4] The fact of being the cause of something produced or of happening. The act by which an effect is produced.

[5] Association of Official Analytical Chemists International.

huge problem for a plaintiff or prosecutor in a legal venue. A hypothetical case may help illustrate the application of a number of the following legal issues.

### *Hypothetical case*

A hypothetical individual employee of a feed additive manufacturer intentionally adds a foreign chemical to a feed premix during a step in the manufacturing process. The feed premix is distributed to many states. Many local mills purchase the premix and incorporate it into the feed in appropriate amounts. The mill then delivers that feed to its customers – animal owners. These animal owners offer that finished feed to their animals in appropriate amounts. These animals ingest the feed, then develop adverse effects. An adverse effect in one of the animals is death. An animal that died after ingesting this feed is submitted to a local diagnostic laboratory by the animal owner. The owner of the animal has not made any association between the new feed and the death of the animal, so the feed is not delivered when the animal is, and the new feed delivery is not part of the history provided to the diagnostic laboratory. The laboratory does a routine necropsy and case work up. A feed additive is mentioned to the animal owner as one of the possible differential diagnostic causes for the lesions seen at necropsy. The owner then takes a grab sample of feed out of the total mixed ration mixer and has her teenager drive it down to the diagnostic laboratory. The chemical is detected in the feed at concentrations sufficiently high to cause the lesions observed in the dead animal.

Various types of legal claims[6] could arise from this case. The results of diagnostic testing may be offered as fact by the animal owner, feed manufacturer, State and Federal agencies, or some combination thereof, against one or more parties. An insurance claim and government enforcement action are discussed very briefly before continuing with issues that arise in civil or criminal litigation.[7]

The results of a routine diagnostic case may now be offered as fact in an insurance claim. The animal owner will claim that the feed provided by the distributor caused the toxicosis experienced by the owner's animals. The animal owner may file a claim against the distributor of the feed for the harm done to her animals. The distributor, or the distributor's insurance company, may settle the claim with the animal owner if all agree. If all do not agree, a civil suit may be filed. The issues that arise in the civil suit are discussed in the next section.

[6] A cause of action. To demand as one's own or as one's right, to assert, to urge, to insist. Means by or through which claimant obtains possession or enjoyment or privilege or thing.

[7] A lawsuit. Legal action, including all proceedings therein. Contest in a court of law for the purpose of enforcing a right or seeking a remedy.

The dispute[8] between the animal owner and the feed distributor involves private parties. The case may also give rise to an enforcement action. These actions may be on the part of one or more agencies in one or more States, the Federal government, or any combination of State and Federal government. One or more agencies of the State in which the animal was harmed may have statutory authority to protect the health of animals, humans, or both. These agencies may include the State Department of Agriculture, the State Department of Health, the State Department of Natural Resources, or the State Board of Animal Health. A state agency may choose to take enforcement action against the feed distributor, premix manufacturer, or both in order to protect the health of animals or humans in its state.

The state agency may collect its own samples, and perform its own analyses prior to taking any enforcement action. The agency may determine that the concentration of chemical in the feed is greater than a published tolerance or action level for that chemical in that feed. This agency action may end with a fine, recall, or other administrative action, if both sides agree.

If both sides do not agree, the feed distributor and premix manufacturer may "appeal" the agency's decision. This appeal may go to an administrative law judge, or a similar official within, or outside, the agency, depending on the administrative structure of that particular agency. If the feed manufacturer or premix manufacturer agrees with the ruling of the administrative law judge, the dispute is finished. If all do not agree, the distributor or manufacturer of the feed may choose to appeal the decision of the administrative law judge to a district civil court. This puts the dispute in a venue similar to a civil suit as discussed below.

Agencies are given deference at the district court level, making this an increasingly difficult, but not insurmountable, argument to win. The specialty of Administrative Law is devoted to the details of administrative procedure that arises in such actions. Administrative Law is beyond the scope of this chapter.

Federal agencies may also have an interest in the animal case. The Federal Food and Drug Administration (FDA) may have an interest in the case because the feed additive impacted interstate commerce when it crossed state lines and impacted the health of the public when it caused toxicosis of a food animal. The FDA has authority to protect the public health. It has authority to take administrative action against the manufacturer of the feed similar to that discussed for the state agencies above.

Although agency decisions are normally given deference in the courtroom, regulations developed by agencies may not always address the issue of causation. Agency

[8] A conflict or controversy, a conflict of claims or rights, an assertion of a right, claim, of demand on one side, met by contrary claims or allegations on the other.

regulations are often promulgated to protect populations rather than individuals.[9] The effect on an individual referred to as specific causation is discussed below. The distinction between enforcement actions taken by agencies to benefit the public as a whole and legally admissible specific causation in an individual is not always appreciated.

This distinction may be illustrated by a hypothetical example. A bulk tank load of milk is tested in the receiving bay and found to be positive for the presence of beta-lactam antibiotics using a test kit marketed and validated for this purpose. The entire load of milk is dumped and the producer whose individual sample is positive is charged for the load of milk. Paying for the tanker load of milk in this situation is a contractual agreement between the milk producer and the bulk milk buyer.

The test kit is approved and validated for use in administrative action. That action is to assist in the public policy of preventing exposure of humans to beta-lactam antibiotics to prevent allergic reactions and to reduce the likelihood of developing strains of bacteria that are pathogenic to humans and resistant to beta-lactam antibiotics. The test kit is sufficient to support the administrative action of preventing these antibiotics from entering the human food supply.

It may not be sufficient, however, for litigation aimed at prosecuting one for the source of the beta-lactam. Further analytical chemistry analysis may be required to distinguish, penicillic acid, from penicillin G from, from ticarcillin, as an extreme example. This distinction may be important because of the implication raised as to the potential source of the antibiotic in the bulk tank – feed, versus a treated cow, versus "spiking" the bulk tank. In short, a test that is entirely acceptable for agency action may not be acceptable for a specific type of litigation. This concept is expanded upon in the section on specific causation below. See Dr. Post's chapter on Regulatory Veterinary Medicine for a further discussion of agency issues.

The results of diagnostic testing may rarely be offered as fact in a criminal case. Animal poisoning cases have rarely risen to the level of criminal action. The addition of the chemical to the feed with the intention of causing a toxicosis may be a crime based on animal cruelty or chemical terrorism statutes. The executive branch of government would then prosecute the case. The state – often through its Attorney General's office – may choose to prosecute under an animal cruelty statute. Similarly, the Department of Justice may file criminal prosecution charges if the act is considered to be one of chemical terrorism. In these instances the state or federal government is a direct party in the case.

This discussion of legal venue is not exhaustive. The purpose of this section is merely to indicate how a routine clinical or diagnostic case could become involved in a variety of legal venues including, insurance claims, administrative action, civil and criminal litigation at the state and federal levels.

In summary, those samples used for the purpose known at the time of submission to a laboratory can, with some reasonable care, be handled, analyzed, interpreted, and stored in a manner consistent with that purpose. Angst may arise on the part of the animal owner, veterinarian, laboratory personnel, counsel, and others, in those instances where the application of results of testing is used in a venue not anticipated at the time of sample submission, particularly in those laboratories not accustomed to litigation work. This angst may rise when the documentation of laboratory results is found not to be sufficiently authentic or reliable to support a legal case. The importance of authenticity and reliability is discussed in the Evidence section below, immediately after a brief discussion of jurisdiction and standard of "proof."

## JURISDICTION – WHAT ARE THE RULES IN THIS FIGHT AND WHO DECIDES THE WINNER?

Ok, the parties have met, discussed, and agreed to disagree on reaching a settlement[10] on the insurance claim. They decide to take the case "to court." The owner of the animals will normally be the plaintiff,[11] and the distributor of the feed will normally be the defendant,[12] in this legal case. The manufacturer of the premix is also likely to be named as a defendant. The animal owner may be a natural person or may have formed a legal entity[13] such as a family farm corporation. The feed manufacturer and premix manufacturer may be businesses organized as sole proprietorships, partnerships, corporations, or other legal entities.

A question arises as to "which court" will decide the dispute. The state and federal governments already have authority to rule on disputes between their citizens. This authority ultimately arises from the respective constitutions.

[9] See e.g. *Troy Corp*, *AFL-CIO,* and *Simpson.*

[10] Payment or satisfaction. In legal parlance, it implies meeting of the minds or parties to transaction or controversy, an adjustment of differences or accounts, a coming to an agreement.

[11] A person who brings an action; the party who complains or sues in a civil action and is so named on the record. A person who seeks remedial relief.

[12] The person defending or denying; the party against whom relief or recovery is sought in an action or suit to the accused in a criminal case.

[13] A real being. An organization or being that possesses separate existence for tax purposes.

State constitutions most commonly delegate authority to the state judicial system to enforce the laws passed by the state legislature. Many state judicial systems have a three tiered structure composed of district courts, courts of appeals, and a Supreme Court, each with increasing authority. Similarly, the federal constitution authorizes the federal judicial system which has district courts, courts of appeals, and the Supreme Court.

Most civil suits that are filed in the United States do not actually go to trial. A settlement is reached in the vast majority of civil cases. This settlement may be reached by the parties on their own, after mediation or arbitration, or even after litigation has begun, but before a judgment is entered.

Mediation and arbitration are two forms of "alternative dispute resolution" (ADR). An outcome reached after ADR may or may not be disputed later in court depending on the final agreement of the parties in the ADR process. Judges often encourage ADR even when civil litigation is filed. Authority may be given by contract. Disputing parties may have agreed to not litigate at all but rather to use ADR. ADR is increasingly used to settle disputes between companies because it can be faster and cheaper than litigation, and because both parties have more control over the outcome of the dispute. Two parties who agree to submit themselves to ADR must grant authority to the mediator or arbitrator at the outset of the deliberation by way of contract. This authority prevails for as long as the contract between the parties is in force. Authority already exists for the government.

Parties with an ongoing relationship may have made a contractual agreement to argue any disputes that arise under that contract according to the laws of a particular state. For example, the manufacturer of the feed additive and the manufacturer of the final feed may have agreed to argue any dispute they may have under that contract according to the laws of Minnesota. One reason for this may be that both parties agree that the cost of arguing jurisdiction for a distributor with outlets in many states, and a manufacturer with plants in many states, is not in the economic best interest of either company.

The parties will need to decide on the state in which the dispute – lawsuit – will be litigated if they have not already made this decision by prior contract. Individuals, and particularly companies, may be citizens of more than one jurisdiction; consequently parties may need to decide which judicial system they choose to "sue out" the case in. This decision may influence the outcome of the dispute because of differences in laws on a particular issue between states. Similarly, federal law on a particular point may be different than that of state law on the same point, so one party may prefer to argue in federal court and the other in state court. The choice of venue may then become a significant part of the dispute that must be decided before the merits of the case can be argued. This jurisdictional decision will normally be made by the judge in the venue where the case was originally filed.

Each state has rules governing the procedures to be followed in conducting a civil trial; and procedures to be followed in conducting a criminal trial. Although these procedural rules may have many similarities between states, they are not uniform across all states. The Federal rules are more consistent nationwide.

The federal judicial system also has rules governing Civil Procedure and those governing Criminal Procedure. These rules are the same across states, although they may be interpreted differently in different federal judicial districts. In short, administrative rules guide administrative actions, state rules guide state civil or state criminal actions, and federal rules guide federal civil and criminal actions.

In our hypothetical example, the manufacturer of the premix and the manufacturer of the feed may have agreed to argue any disputes that arise under their business contract according to the laws of Minnesota. The animal owner who chooses to sue the manufacturer of the feed may choose to sue in state court in the state in which the owner fed the feed to their animal, or perhaps in the federal court representing the same geographic area. One argument that may be used to argue that the case belongs in the federal court system is that the feed crossed state lines and therefore impacted interstate commerce. Other elements that may be required to establish federal jurisdiction are beyond the scope of this chapter.

A judgment will be entered by the judge if the dispute goes all the way through trial. Thus the animal owner may receive compensation at the insurance claim, settlement, or judgment level of this dispute if the animal owner prevails.

The point of this section is primarily to indicate that the procedures by which the dispute is handled may be different between states for an insurance claim, arbitration, mediation, or litigation. These distinctions between state court systems are beyond the scope of this chapter. On the other hand, the Federal Judicial system has procedures that are generally applied across the country. They are more similar nationwide, so they are described below to indicate some procedural issues that may arise in toxicology cases. Although many disputes involving animal toxicoses are not tried in federal courts, the Federal procedural rules provided below illustrate issues that are likely to be similar across the country.

## STANDARD OF PROOF – HOW SURE DO I NEED TO BE?

One party needs to persuade the other party once a dispute develops. This need is normally referred to as the

burden of persuasion in a legal setting.[14–16] The burden of persuasion is colloquially referred to as a "burden of proof" – a phrase that toxicologists may find familiar. This burden of persuasion is different in civil cases than it is in criminal cases. Both legal burdens may be different than that of a routine medical diagnosis. The level of medical certainty needed to treat is often presented by quoting the Hippocratic Oath "first do no harm." The degree of medical certainty required to treat a disease may only be that needed to give appropriate general and supportive treatment, but not that which would be required to reach an etiological diagnosis. Reaching an etiological diagnosis to any degree of medical certainty is not always done in clinical practice.

Similarly, routine cases in a diagnostic laboratory may not reach an etiological diagnosis to any degree of medical certainty either. In many instances reaching an etiological diagnosis is limited by time, cost, and the needs of the animal owner and veterinarian. This need may be merely to have guidance for providing supportive treatment. Other limits on the ability to provide an etiological diagnosis may be the available samples, the condition of the samples, the willingness of the owner to spend the money required to reach such a diagnosis, and other factors.

This distinction between a medical diagnosis and a "legal" diagnosis is appreciated by the court system. This distinction is summarized in *Wynacht*:

> There is a fundamental distinction between Dr. Z ...'s ability to render a medical diagnosis based on clinical experience and her ability to render an opinion on causation of W..'s injuries. Beckman apparently does not dispute, and the Court does not question, that Dr. Z is an experienced physician, qualified to diagnose medical conditions and treat patients. The ability to diagnose medical conditions is not remotely the same, however, as the ability to deduce, delineate, and describe, in a scientifically reliable manner, the causes of these medical conditions. Wynacht v. Beckman Instruments, Inc. 113 F. Supp. 2d 1205, 1209 (E.D. Tenn., 2000).

In short, the diagnosis reached in a routine medical clinic is not always sufficient to meet a legal burden of persuasion. Similarly, the diagnosis reached in a routine veterinary clinic may not meet this burden either.

The legal burden of persuasion is different in civil and criminal cases. The burden in civil cases is usually either a preponderance of the evidence or clear and convincing evidence. A preponderance of the evidence is also referred to as a "more likely than not" standard. Juries are sometimes instructed that this is a 51% persuasion standard. In a civil case, the plaintiff has the burden of persuading the finder of fact – judge or jury – that his or her claim is valid with a preponderance of the evidence. Similarly, the defense has the burden of persuading the finder of fact that their defense is valid with a preponderance of the evidence.

The burden of persuasion in criminal cases is much higher. Here, the burden of persuasion is "beyond a reasonable doubt." The prosecutor has the burden of persuading the fact finder – judge or jury – beyond a reasonable doubt, that the crime was committed by the defendant. Conversely, the defendant raises "reasonable doubt" that the prosecution has met this burden. The merits of both civil and criminal cases are argued based on evidence. Some evidence will require interpretation. Interpretation of facts is the role of the expert. The role and types of evidence follow.

## EVIDENCE

Legal cases are decided based on an application of the law to the facts of a particular case. A dispute about facts themselves or interpretation of those facts arises in many legal cases. Disputes of fact are decided by the trier of fact. The trier of fact is the judge in a bench trial. The trier of

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[14] Article III. Presumptions in Civil Actions and Proceedings.
Rule 301. Presumptions in General in Civil Actions and Proceedings

In all civil actions and proceedings not otherwise provided for by Act of Congress or by these rules, a presumption imposes on the party against whom it is directed the burden of going forward with evidence to rebut or meet the presumption, but does not shift to such party the burden of proof in the sense of the risk of non-persuasion, which remains throughout the trial upon the party on whom it was originally cast.

[15] Rule 302. Applicability of State Law in Civil Actions and Proceedings

In civil actions and proceedings, the effect of a presumption respecting a fact which is an element of a claim or defense as to which State law supplies the rule of decision is determined in accordance with State law.

[16] Rule 1101. Applicability of Rules:

(a) Courts and judges: These rules apply to the United States district courts, the District Court of Guam, the District Court of the Virgin Islands, the District Court for the Northern Mariana Islands, the United States Courts of Appeals, the United States Claims Court, 1 and to United States bankruptcy judges and United States magistrate judges, in the actions, cases, and proceedings and to the extent hereinafter set forth. The terms "judge" and "court" in these rules include United States bankruptcy judges and United States magistrate judges.

(b) Proceedings generally: These rules apply generally to civil actions and proceedings, including admiralty and maritime cases, to criminal cases and proceedings, to contempt proceedings except those in which the court may act summarily, and to proceedings and cases under title 11, United States Code.

fact is the jury in a jury trial. The trier of fact learns of these facts largely through evidence.

All facts are not admissible as evidence. The judge must determine which facts are admissible as evidence and which are not as a preliminary matter in the case. Specifically, the judge must determine "the qualifications of a person to be a witness", and "the admissibility of evidence."[Rule 104 (a)] The weight and credibility of the evidence is not determined at this preliminary stage. Rather "evidence relevant to weight or credibility" is normally "introduce[d] before the jury."[Rule 104 (e)]

The major factor used to determine the admissibility of evidence is relevance. "Relevant evidence" and "material fact" are two phrases that are often used synonymously. "Relevant evidence" is "evidence having any tendency to make the existence of any fact that is of consequence to the determination of the action more probable or less probable than it would be without the evidence."[Rule 401] "[A]ll relevant evidence is admissible" with few exceptions.[Rule 401] "Evidence which is not relevant is not admissible."[Rule 402] For example, testimony that the sky is blue may not be admitted as relevant, because the color of the sky has no tendency to indicate that the feed did or did not cause the toxicosis in the animals.

The judge decides which facts are admissible as evidence and which are not. The factors that influence the judge's decision on admissibility vary with the type of fact. Documents, specimens and testimony are three sources of fact that a party may desire to enter into evidence in toxicology cases.

### Documents

Documents require authentication before they can be considered as evidence. Document authentication is of increasing interest in many laboratories revisiting record retention procedures. Authentication of documents is covered by Rule 901 of the Federal Rules of Evidence.[17] Subsections of this rule specify the procedure for authenticating public[18] or ancient[19] documents. A laboratory will likely have additional influence on its record keeping. For example, veterinary diagnostic laboratories are adopting ISO standards, contract research laboratories adhere to Good Laboratory Practice standards,[20] many state agencies have state specific data practices requirements. While the specific requirements may vary, the general purpose is to authenticate the accuracy of the final report.

The case report and the documents supporting that case report may be required to authenticate that report in a legal proceeding. Authentication of case documents is one reason that many laboratories have established record retention procedures. A document and its underlying support is retained for 7 years in some diagnostic laboratories. The specimens themselves are also important in these proceedings.

### Specimens

Specimens are often relevant in toxicology cases. Toxicologists are well aware of the importance of accurate sample identification to establish the relevance of the results of testing a particular sample. Laboratories involved in racing chemistry, regulatory enforcement, and GLP studies often have significant sample tracking documents to establish that the result of a test can be tracked to the original specimen. These sample tracking or chain of custody documents are often needed to establish that the sample tested does, in fact, relate to the case report. The absence of sample tracking or such chain of custody data may eviscerate a legal claim. The federal judicial system is aware of this as well. It considers as relevant evidence "Comparison by the trier of fact or by expert witnesses with specimens which have been authenticated."[Rule 901 (3)] Split sample testing in racing chemistry is an example of comparison by an expert witness with authenticated samples. Such split sample testing may be desirable in toxicology cases that reach an insurance claim, civil or criminal case level.

Toxicologists are aware of the potential for different results when different analytical methods are used. This awareness has given rise over the years to recognition of the need to "validate" tests and test results. The federal judicial system is also aware of this need. It requires "[e]vidence describing a process or system used to produce a result and showing that the process or system produces an accurate result."[Rule 901 (9)] AOACI is an example of an entity that validates an analytical method for a particular matrix. The plaintiff has the burden of persuading the judge that the analytical results go with the correct sample, and the results of that sample's testing are on the correct report. Authenticating sample results for such proceedings are one reason for standard operating procedures, quality assurance samples, method validation, split sample testing, and other such procedures.

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[17] Rule 901. "The requirement of authentication or identification as a condition precedent to admissibility is satisfied by evidence sufficient to support a finding that the matter in question is what its proponent claims."

[18] Rule 901 (7). "Evidence that a writing authorized by law to be recorded or filed and in fact recorded or filed in a public office, or a purported public record, report, statement, or data compilation, in any form, is from the public office where items of this nature are kept."

[19] Rule 901 (8). "Evidence that a document or data compilation, in any form, (A) is in such condition as to create no suspicion concerning its authenticity, (B) was in a place where it, if authentic, would likely be, and © has been in existence 20 years or more at the time it is offered."

[20] 21 CFR 58.

Analytical toxicologists know the limits of interpretation of the results of a given test. However, some who see these reports may not be aware of these limits. So toxicologists may take care in the wording of interpretive comments made.

*Testimony*

Testimony is the third means of introducing evidence. Testimony is often gathered during the discovery portion of a legal case by taking the deposition of witnesses. Testimony may also be given orally at trial. Witnesses are under oath both at the deposition and at trial. Testimony given in a deposition may be entered into evidence at trial. This is sometimes done because the witness is not available on the trial date. Testimony may be given in a deposition, then again at trial by the same person on the same subject.

Testimony is given by both "lay" witnesses and expert witnesses. Admission of testimony from a lay witness is evaluated differently than that of an expert witness. A lay witness must have personal knowledge of the facts to which she or he is testifying. Specifically, "[a] witness may not testify to a matter unless evidence is introduced sufficient to support a finding that the witness has personal knowledge of the matter. Evidence to prove personal knowledge may, but need not, consist of the witness' own testimony. …"[Rule 602]

Lay witnesses must testify to their personal knowledge. This personal knowledge is their recollection of the event. For example, the animal owner in our hypothetical example may testify that she did purchase the feed, that she fed it to her animals, that she observed certain clinical signs in her animals, and that she delivered a dead animal to the veterinary diagnostic laboratory.

The animal owner may be cross-examined to help verify details of the events. The animal owner's recollection may be refreshed by referring to a written document pursuant to Rule 612.[21]

Examples of a writing to which the animal owner may refer are a receipt and a case report from the diagnostic facility or veterinary clinic. The receipt could be used to refresh the memory of the animal owner as to precisely when and where the feed was purchased. The case report could be used to refresh the memory of the animal owner as to when the animal died, when the diagnostic testing was performed, and the results of the testing. An expert witness may be required, however, to interpret the laboratory report.

## ADMISSIBILITY OF EXPERT TESTIMONY

The lay witness is not the only source of testimony. Lay testimony is a source of facts. Some of these facts must be interpreted, which is the role of the expert. Expert testimony is the other main form of introducing testimonial evidence. Expert testimony is commonly used at trial and has become the topic of a number of evidentiary rules. Federal Rules of Evidence 701 through 706 follow in their entirety.[22–27]

Expert testimony must not only be relevant as with a lay witness, but it must also be *reliable*. Discussion of this reliability factor constitutes most of the remainder of this

[21] Except as otherwise provided in criminal proceedings by section 3500 of title 18, United States Code, if a witness uses a writing to refresh memory for the purpose of testifying, either:

(1) while testifying, or

(2) before testifying, if the court in its discretion determines it is necessary in the interests of justice, an adverse party is entitled to have the writing produced at the hearing, to inspect it, to cross-examine the witness thereon, and to introduce in evidence those portions which relate to the testimony of the witness. … If a writing is not produced or delivered pursuant to order under this rule, the court shall make any order justice requires, except that in criminal cases when the prosecution elects not to comply, the order shall be one striking the testimony or, if the court in its discretion determines that the interests of justice so require, declaring a mistrial. Rule 612. As amended March 2, 1987, eff. October 1, 1987.

[22] Rule 701. Opinion Testimony by Lay Witnesses

If the witness is not testifying as an expert, the witness' testimony in the form of opinions or inferences is limited to those opinions or inferences which are (a) rationally based on the perception of the witness, and (b) helpful to a clear understanding of the witness' testimony or the determination of a fact in issue, and (c) not based on scientific, technical, or other specialized knowledge within the scope of Rule 702. (As amended March 2, 1987, eff. October 1, 1987; April 17, 2000, eff. December 1, 2000.)

[23] Rule 702. Testimony by Experts

If scientific, technical, or other specialized knowledge will assist the trier of fact to understand the evidence or to determine a fact in issue, a witness qualified as an expert by knowledge, skill, experience, training, or education, may testify thereto in the form of an opinion or otherwise, if (1) the testimony is based upon sufficient facts or data, (2) the testimony is the product of reliable principles and methods, and (3) the witness has applied the principles and methods reliably to the facts of the case. Rule 702. (As amended April 17, 2000, eff. December 1, 2000.)

[24] Rule 703. Bases of Opinion Testimony by Experts

The facts or data in the particular case upon which an expert bases an opinion or inference may be those perceived by or made known to the expert at or before the hearing. If of a type reasonably relied upon by experts in the particular field in forming opinions or inferences upon the subject, the facts or data need not be admissible in evidence in order for the opinion or inference to be admitted. Facts or data that are otherwise

chapter. In virtually all legal cases, the judge makes preliminary rulings that determine whether the expert may testify at all, and if so, upon which opinions. Reliability is the primary factor in the judge's determination of admissibility of expert testimony. Two approaches to determine the reliability of expert testimony have evolved, namely the "*Frye* test" and the "*Daubert* test."

### *Frye*

The *Frye* test is the original standard of reliability for expert testimony, and it remains in several state court systems, including Minnesota (see Table 8.1). The *Frye* test is often summarized as the "general acceptance" standard. A brief history of *Frye* may illustrate its use in a legal setting.

In 1923, a defendant was convicted of second degree murder (*Frye*, 1923). The defendant appealed the trial court's ruling because the court[28] did not allow the defendant to offer expert testimony. The expert testimony would have used a precursor to the "lie detector test" known as the "systolic blood pressure deception test" ("SBPD test"). The defendant's expert claimed that the SBPD test could prove whether or not a person being examined by the SBPD was attempting to deceive the examiner or conceal his guilt with regard to the crime. The judge did not allow this testimony, and the defendant was convicted. The ruling was appealed.

**TABLE 8.1 Test of admissibility of expert testimony in some states.[1]**

| *Frye* test | *Daubert* (all three cases) | *Daubert* and *Kumho* only |
|---|---|---|
| Alabama | Arkansas | Kentucky |
| Arizona | Delaware | New Hampshire |
| California | Louisiana | North Carolina |
| Florida | Massachusetts | Ohio |
| Illinois | Mississippi | Rhode Island |
| Kansas | Nebraska | South Dakota |
| Maryland | Oklahoma | |
| Michigan | Texas | |
| Minnesota | Wyoming | |
| Missouri | | |
| New Jersey | | |
| New York | | |
| North Dakota | | |
| Pennsylvania | | |
| Washington | | |

[1] Modified from Bernstein, David E, Jackson, Jeffrey D. Jackson, The Daubert Trilogy in the States, 44 Jurimetrics J 351 (2004).

The court of appeals held that when "admitting expert testimony deduced from a well-recognized scientific principle or discovery, the thing from which the deduction is made must be sufficiently established to have gained general acceptance in the particular field in which it belongs" (*Frye*

inadmissible shall not be disclosed to the jury by the proponent of the opinion or inference unless the court determines that their probative value in assisting the jury to evaluate the expert's opinion substantially outweighs their prejudicial effect.Rule 703. (As amended March 2, 1987, eff. October 1, 1987; April 17, 2000, eff. December 1, 2000.)

[25] Rule 704. Opinion on Ultimate Issue:

(a) Except as provided in subdivision (b), testimony in the form of an opinion or inference otherwise admissible is not objectionable because it embraces an ultimate issue to be decided by the trier of fact.

(b) No expert witness testifying with respect to the mental state or condition of a defendant in a criminal case may state an opinion or inference as to whether the defendant did or did not have the mental state or condition constituting an element of the crime charged or of a defense thereto. Such ultimate issues are matters for the trier of fact alone. (As amended October 12, 1984.)

[26] Rule 705. Disclosure of Facts or Data Underlying Expert Opinion

The expert may testify in terms of opinion or inference and give reasons therefore without first testifying to the underlying facts or data, unless the court requires otherwise. The expert may in any event be required to disclose the underlying facts or data on cross-examination. (As amended March 2, 1987, eff. October 1, 1987; April 22, 1993, eff. December 1, 1993.)

[27] Rule 706. Court Appointed Experts

(a) Appointment: The court may on its own motion or on the motion of any party enter an order to show cause why expert witnesses should not be appointed, and may request the parties to submit nominations. The court may appoint any expert witnesses agreed upon by the parties, and may appoint expert witnesses of its own selection. An expert witness shall not be appointed by the court unless the witness consents to act. A witness so appointed shall be informed of the witness' duties by the court in writing, a copy of which shall be filed with the clerk, or at a conference in which the parties shall have opportunity to participate. A witness so appointed shall advise the parties of the witness' findings, if any; the witness' deposition may be taken by any party; and the witness may be called to testify by the court or any party. The witness shall be subject to cross-examination by each party, including a party calling the witness.

(b) Compensation: Expert witnesses so appointed are entitled to reasonable compensation in whatever sum the court may allow. The compensation thus fixed is payable from funds which may be provided by law in criminal cases and civil actions and proceedings involving just compensation under the fifth amendment. In other civil actions and proceedings the compensation shall be paid by the parties in such proportion and at such time as the court directs, and thereafter charged in like manner as other costs.

(c) Disclosure of appointment: In the exercise of its discretion, the court may authorize disclosure to the jury of the fact that the court appointed the expert witness.

(d) Parties' experts of own selection: Nothing in this rule limits the parties in calling expert witnesses of their own selection.
(As amended March 2, 1987, eff. October 1, 1987.)

[28] The judge is referred to as "the court" in legal writing.

at 1013). This holding is commonly abbreviated as the "general acceptance" test. The trial court decision held, because the defendant had insufficient proof that the SBPD test had gained "general acceptance." Consequently, the defendant's expert was not allowed to testify, so this expert testimony was not entered into evidence, and this evidence was then not available to support an argument to overturn the defendant's conviction.

*Frye* was a federal criminal case, but the holding was later adopted in federal civil cases. Over time, *Frye* was adopted by most state courts for both civil and criminal proceedings. Minnesota reaffirmed its use of *Frye* in *Goeb* v. *Tharaldson*. Minnesota Supreme Court cases on the subject are briefly summarized to illustrate differences with the *Daubert* test below.

Minnesota adopted *Frye* in 1952 in the case of *State* v. *Kolander*. In 1980, Minnesota added an analysis to decide that testimony developed through hypnosis was inadmissible (*State* v. *Mack* at 768–69). Consequently, the Minnesota test for admissibility of novel scientific evidence has evolved into a two-prong *Frye–Mack* standard. The *Frye* prong first requires that a novel scientific technique be generally accepted in the relevant scientific community, then the *Mack* prong requires that the particular evidence derived from that test have a foundation that is scientifically reliable (Goeb at 814, see also State v. Anderson, State v. Jobe, State v. Moore).

The Minnesota Supreme Court reconsidered the *Frye–Mack* two-prong test after *Daubert* passed the United States Supreme Court.[29] It rejected *Daubert* and sustained *Frye–Mack*, stating:

> Having reviewed the cases and the commentary surrounding this issue, we reaffirm our adherence to the Frye–Mack standard and reject Daubert. Therefore, when novel scientific evidence is offered, the district court must determine whether it is generally accepted in the relevant scientific community. See Moore, 458 N.W.2d at 97–8; Schwartz, 447 N.W.2d at 424–26. In addition, the particular scientific evidence in each case must be shown to have foundational reliability. See Moore, 458 N.W.2d at 98; Schwartz, 447 N.W.2d at 426–8. Foundational reliability "requires the 'proponent of a * * * test [to] establish that the test itself is reliable and that its administration in the particular instance conformed to the procedure necessary to ensure reliability.' " Moore, 458 N.W.2d at 98 (alteration in original) (quoting State v. Dille, 258 N.W.2d 565, 567 (Minn., 1977)). Finally, as with all testimony by experts, the evidence must satisfy the requirements of Minn. R. Evid. 402 and 702 – be relevant, be given by a witness qualified as an expert, and be helpful to the trier of fact. (See State v. Nystrom, 596 N.W.2d 256, 259 (Minn., 1999).)

The Minnesota Supreme Court has held that the Frye–Mack standard for admission "facilitates more objective and uniform rulings" and reduces the "undesired element of subjectivity * * * [in] evidentiary rulings" *Schwartz*, at 424 that may occur with *Daubert*. The Minnesota approach requires that an appellate court review a novel scientific or non-scientific technique for reliability before an expert may offer it (*State of Minnesota* v. *DeShay*) including DNA testing by PCR-STR (*State of Minnesota*, v. *Nose*). With very rare exceptions, a layperson may not testify in the form of opinions or inferences (*Ray* v. *Miller Meester*).

However, *Frye* is no longer the standard in federal cases. The *Frye* test was superseded in federal courts by the enactment of the Federal Rules of Evidence (*Daubert* at 589). The *Frye* test has also been replaced by the *Daubert* test in many state court systems (see Table 8.1). The remainder of this section is devoted to *Daubert* since it has been adopted by the federal courts and many state court systems to determine whether expert testimony is, or is not, admitted into evidence today.

### *Daubert*

*Daubert* is one of three United States Supreme Court cases addressing the issue of admissibility of expert testimony. In addition, the Federal Court system has published a Reference Manual on Scientific Evidence to assist judges in applying these rulings. This Reference Manual contains a section devoted to toxicology and a separate section devoted to medical testimony. Much of the material below is summarized from these sources.

Three cases with rulings on the admissibility of expert testimony have reached the United States Supreme Court in recent years. The cases are *Daubert* v. *Merrell Dow Pharmaceuticals*, Inc 509 U.S. 579 (1993), *General Electric Co* v. *Joiner*, 522 U.S. 136 (1997), and *Kumho Tire Co* v. *Carmichael*, 119 S. Ct. 1167 (1999). Both *Daubert* and *General Electric* were toxic tort cases. *General Electric* and *Kumho* are discussed first.

The holdings in *General Electric* and *Kumho* are of legal review and admission of testimony from non-scientists as experts, so they are summarized briefly. The holding in

[29] There have been no developments in Minnesota since Mack and Schwartz to convince us that the Frye–Mack standard is now incompatible with those same rules of evidence in existence at the time of these decisions. Cf. Leahy, 882 P.2d at 328 (reasoning that by applying the Frye standard after the adoption of the evidence code, the California Supreme Court had concluded Frye was compatible with the code); State v. Copeland, 922 P.2d 1304, 1314 (Wash. 1996) (noting that by adopting the rules of evidence and continuing to adhere to Frye, the Washington Supreme Court "signaled that Frye and the evidence rules coexist as the law of th[e] state [of Washington]").

*General Electric* is primarily of legal interest. In *General Electric*, the Supreme Court held that the correct standard for an appellate court to apply in reviewing a district court's evidentiary ruling is an "abuse of discretion" standard. This holding most commonly has the effect of strengthening the decision made by the district court whether it is to admit or to not admit the expert testimony. Of interest to toxicologists and other scientists, is the urging by Justice Breyer in *General Electric* that judges avail themselves of court appointed experts (General Electric at ...). *Kumho Tire* is not directly on point for toxicologists either.

The Supreme Court held in *Kumho Tire* that the phrases "technical" and "other specialized" knowledge in the Federal Rule of Evidence 702, allows testimony from those with "skill" or "experience" based expertise and not just "science" based expertise. The Supreme Court held that an expert in tire failure analysis could testify as an expert even though his expertise was "skill" or "experience" based, rather than "science" based *(Kumho* at 1171). The *Frye–Mack* test in Minnesota offers very few exceptions to the rule that lay witnesses may not give expert testimony. The holdings in *Daubert* are of most direct interest to toxicologists, and other scientists.

Expert testimony is different from lay testimony. One difference is the possibility that "junk science" may be used to confuse, rather than assist, the trier of fact. Consequently an additional test of admissibility is applied to expert testimony. Such testimony must not only be relevant, but it must also be *reliable*. This concept is explained in the *Daubert* case. Justice Breyer, "believe[s] there is an increasingly important need for law to reflect sound science" (Justice Breyer, in the Reference Manual on Scientific Evidence, p. 5). Justice Breyer also writes in the Manual on Scientific Evidence:

> The judge is the evidentiary gatekeeper (*General Electric Co* v. *Joiner*, 522 U.S. 136 (1997); *Daubert* v. *Merrel Dow Pharms Inc*, 509 U.S .579 (1993)). "The judge, without interfering with the jury's role as trier of fact, must determine whether purported scientific evidence is "reliable" and will "assist the trier of fact" thereby keeping from juries testimony that, in Pauli's sense, isn't even good enough to be wrong. Justice Breyer, in Manual on Scientific Evidence, p. 6.
>
> The purpose of Daubert's gatekeeping requirement "is to make certain that an expert whether basing testimony upon professional studies or personal experience, employs in the courtroom the same level of intellectual rigor that characterizes the practice of an expert in the relevant field." *Kumho Tire Co* v. *Carmichael*, 119 S. Ct. 1167 (1999) at 1176. See also, *Rosen* v. *Ciba-geigy Corp*, 78 F.3d 316 (7th Cir) cert denied, 519 U.S. 819 (1996) at 318.

Clearly, the United States Supreme Court in *Daubert* assigned the trial judge a "gatekeeping" responsibility. This responsibility is to make a "preliminary assessment of whether the reasoning or methodology underlying the testimony is scientifically valid and whether that reasoning or methodology properly can be applied to the facts in issue" (*Daubert* at 589). The "gatekeeper" function requires district court judges to screen proffered expert testimony (*Daubert* at 589). This screening is intended to ensure that expert testimony "is not only relevant, but reliable" (*Daubert* at 589). To be relevant, the testimony "requires a valid scientific connection to the pertinent inquiry as a precondition to admissibility" (*Daubert* at 591–2). Much has been written about the reliability portion of this screening.

To meet the reliability standard, the judge must determine whether testimony is "grounded in the methods and procedures of science" (*Daubert* at 590). Consequently, an expert must account for "how and why" he or she reached the expert opinion (Kumho at note 27). The judge's determination is "flexible" (*Daubert* at 594), so the trial judge has broad latitude to determine *how* to test an expert's reliability (Kumho at 1176), and this determination may be different in a criminal case than in a civil one (Kumho at 1176). Nevertheless, guidelines for determining the reliability of expert testimony exist.

Four factors were presented in *Daubert* to assist the judge in determining whether a theory or technique has been derived by the scientific method (*Daubert* at 593–94). These four factors are used to determine the theory's testability (*Carmichael* at 1522), because "[w]hether [a theory or technique] can be (and has been) tested" is the "methodology [that] distinguishes science from other fields of human inquiry" (*Daubert* at 593). In short, the four factors are testability, peer review or publication, the existence of known or potential error rates, and standards controlling the techniques' operation (*Daubert* at 593).

In summary, the trial judge decides whether or not expert testimony is allowed. The means by which the judge makes this decision is flexible, but the judge often tests both the credentials of the expert and the opinion that expert offers. The expert's opinion must meet "the same standard of intellectual rigor" inside the court room as outside of it (*Kumho* at 1176). This showing of intellectual rigor requires that the expert demonstrate both "how and why" the opinion was reached. This may be done by satisfying four factors, namely (1) whether the theory or technique can be tested, (2) whether it is published or peer reviewed, (3) whether it has known or potential error rates, and (4) what the control standards are.

These factors may be applied to both the analytical and interpretive portions of a toxicology case. Both *Daubert* and *General Electric* were toxic tort cases. This may be one reason that a chapter devoted to toxicology appears in the Reference Manual for Scientific Evidence. This Manual

offers some questions to be asked of purported toxicology experts.

Three questions may be asked in the evaluation of a toxicology expert to determine whether the expert's testimony is reliable – and therefore admissible. These questions may be reduced to (1) is the expert qualified, (2) has general causation been established, and (3) has specific causation been established?

### *Is the expert qualified?*

The individual wanting to be allowed to testify as an expert must be "qualified as an expert by knowledge, skill, experience, training, or education, …"[Rule 702] Toxicologists are often argued to be experts based on education, board certification, and other means. Although "… no single academic degree, research specialty, or career path qualifies an individual as an expert in toxicology. … A number of indicia of expertise can be explored, …" (Reference Guide on Toxicology at 415).

1 *Has the person been trained and educated in the discipline of toxicology?*

An advanced degree in toxicology presumptively supports the argument that the person has been trained and educated in the discipline of toxicology. One may go to the credentialing and other means portions of the analysis if the person possesses an advanced degree in toxicology. Not all toxicologists possess such a degree, however.

Many well-qualified toxicologists do not have an advanced degree in toxicology, but do have an advanced degree in related disciplines such as pharmacology, biochemistry, environmental health, industrial hygiene, or other similar fields (Reference Guide on Toxicology at 415). However, not all individuals in possession of these related degrees are toxicologists. So further evidence of training and education is often required of those who do not have an advanced degree in toxicology but do have an advanced degree in a related discipline.

This training and education is often in the form of college level course work or continuing education programs. The number of required courses in toxicology cannot be stated, but one such course is not enough. Specifically, "a single course in toxicology is unlikely to provide sufficient background for developing expertise in the field" (Reference Guide on Toxicology, p. 415). "A physician without particular training or experience in toxicology is unlikely to have sufficient background to evaluate the strengths and weaknesses of toxicological research (see Mary Sue Henifin). "[M]ost physicians have little training in chemical toxicology and lack an understanding of exposure assessment and dose–response relationships"(Mary Sue Henifin). The same is true for veterinarians, and many other health care professionals. So, while the number of college courses, continuing education, or other training programs required to support an argument that one is a toxicologist is not established, one such course is most likely too few. Consequently, an MD or DVM without an advanced degree in toxicology may well be found to not be a toxicologist based on training and education if they have only a course in professional school to support the claim. Certification is another way to support the argument that one is a toxicologist.

2 *Has the person been certified as a toxicologist?*

Some professions have a subspecialty in toxicology with a board certification. Physicians may be certified by the American Board of Medical Toxicology.[30] Veterinarians may be certified by the American Board of Veterinary Toxicology.[31] The American Board of Toxicology (ABT) was established to provide certification of individuals trained and experienced in toxicology without consideration of whether the individual has a professional Medical or Veterinary Medical degree.[32] However, individuals with an MD or DVM degree may also take the ABT certifying examination. A very few individuals are certified by more than one of these boards. Certification by other boards may be relevant for a particular legal case. A person with an advanced degree in toxicology and board certification has strong support for an argument that they are a toxicologist. This may be one reason that the American Association of Veterinary Laboratory Diagnosticians (AAVLD) requires a board certified toxicologist be on staff for an accredited laboratory. A person with an advanced degree but no board certification, or board certification but no advanced degree, may have other criteria to support a finding that they are a toxicologist.

3 *Do other criteria support an argument that the person is a toxicologist?*

Such other criteria may be membership in one or more toxicology organizations, peer-reviewed or other publications, research grants, scientific advisory panels, university appointments, and the like (Mary Sue Henifin p. 418). These criteria are often documented in one's resume or curriculum vitae.

So, a person with a professional degree, plus an advanced degree in toxicology, plus board certification in toxicology, plus membership in one or more toxicology organizations, peer-reviewed toxicology literature, grants, service on scientific advisory panels, and a university appointment has very strong support for an argument that they are an expert in toxicology. On the other hand, a person with a professional degree, no advanced degree in toxicology, no board certification in toxicology,

[30] A list of approved medical subspecialties is available at: http://www.abms.org/approved.asp

[31] www.abvt.org

[32] www.abtox.org

and no other publications, grants, and the like has a very weak argument, if any, that they are an expert in toxicology. The courts recognize the distinction between a professional degree and expertise required in a legal setting. This distinction is aptly described in *Wynacht* v. *Beckman Instruments*.

> There is a fundamental distinction between Dr. Z…'s ability to render a medical diagnosis based on clinical experience and her ability to render an opinion on causation of W…'s injuries. Beckman apparently does not dispute, and the Court does not question, that Dr. Z is an experienced physician, qualified to diagnose medical conditions and treat patients. The ability to diagnose medical conditions is not remotely the same, however, as the ability to deduce, delineate, and describe, in a scientifically reliable manner, the causes of these medical conditions.

Many combinations of education, certification, and other criteria that fall between the two above extremes are possible for toxicology experts. These would, of course, be decided by the judge in a specific case. Once found to be an expert, the toxicologist will most likely be asked to give an opinion on general causation.

## General causation

"[T]he methodology prescribed by both the World Health Organization (WHO) and the National Academy of Sciences (NAS) for determining whether a person has been adversely affected by a toxin" have been described in *Mancuso* v. *Consolidated Edison Co*. as a three step procedure. Scientific validity may be argued in three steps "(1) the validity of the underlying principle, (2) the validity of the technique applying the principle, and (3) the proper application of the technique on a particular occasion." (Paul C. Giannelli)

> First, the level of exposure of plaintiff to the toxin in question must be determined; second, from a review of the scientific literature, it must be established that the toxin is capable of producing plaintiff's illness – called "general causation" – and the dose/response relationship between the toxin and the illness – i.e. the level of exposure which will produce such an illness – must be ascertained; and third, "specific causation" must be established by demonstrating the probability that the toxin caused this particular plaintiff's illness, which involves weighing the possibility of other causes of the illness – a so-called "differential diagnosis." (*Mancuso* at 399)
>
> Toxicologists may arrive at an expert opinion in a variety of ways. "The basis of the toxicologist's expert opinion in a specific case is a thorough review of the research literature and treatises concerning effects of exposure to the chemical at issue. To arrive at an opinion, the expert assesses the strengths and weaknesses of the research studies. The expert also bases an opinion on fundamental concepts of toxicology relevant to understanding the actions of chemicals in biological systems." (Manual on Scientific Evidence at 415)

Two key legal concepts of causation have emerged as courts have attempted to keep junk science out of the courtroom. These concepts are general causation and specific causation. The discussion of specific causation follows in the next section.

"Causation is frequently a crucial issue in toxicology cases. Establishing causation means providing scientific evidence from which an inference of cause and effect may be drawn" (Reference Manual on Scientific Evidence at 32). The Manual goes on to describe the process of arriving at general causation.

> Once the expert has been qualified, he or she is expected to offer an opinion on whether the plaintiff's disease was caused by exposure to a chemical. To do so, the expert relies on the principles of toxicology to provide a scientifically valid methodology for establishing causation and then applies the methodology to the facts of the case.
>
> "An opinion on causation should be premised on three preliminary assessments. First, the expert should analyze whether the disease can be related to chemical exposure by a biologically plausible theory. Second, the expert should examine if the plaintiff was exposed to the chemical in a manner that can lead to absorption into the body. Third, the expert should offer an opinion as to whether the dose to which the plaintiff was exposed is sufficient to cause the disease." (Reference Guide on Toxicology at 419)

Courts define general causation as "the capacity of a product to cause injury" (Siharath). General causation is a scientifically established cause-and-effect relationship. To satisfy this burden, sufficient testing must be done to establish that a disease or condition can arise after exposure to a certain substance. Peer-reviewed literature of epidemiology studies, case reports, *in vitro*, and animal studies may be used to support a general causation argument.

A toxic tort plaintiff must first show that the substance to which he was allegedly exposed is capable of causing his injury – general causation (see e.g. Raynor 7). General causation asks whether exposure to a substance causes harm to anyone (see Navigating Uncertainty).

In short, is the alleged chemical capable of causing the disease observed at any dose or exposure? In our hypothetical above, has the chemical that was added to the feed additive been shown to cause the clinical signs and lesions observed in the species of animal in this case? If not, the expert's testimony may not be allowed in the case. If so, an analysis of specific causation would be required.

## SPECIFIC CAUSATION: THE DIFFERENTIAL DIAGNOSIS

General causation answers the question of whether the chemical in question may cause the disease observed. Specific causation is aimed at answering the question of whether the chemical in question did in fact cause the disease in the specific case at hand. This concept has been stated in a variety of ways. Specific causation is "proof that the product in question caused the injury of which the plaintiff complains" (*Siharath*). It is a tendency to show that the person's alleged exposure, in fact, caused his or her condition (*Siharath* at see also DT Ralston). In other words, it is a showing that the said exposure was the actual cause of the injury (see e.g. Raynor). An analysis of specific causation answers the question of whether exposure to the specific chemical in question did or did not cause the disease experienced by the plaintiff, or in the hypothetical, plaintiff's animals. The specific causation analysis requires consideration of other potential causes of the disease.

Consideration of other causes of the disease is termed differential diagnosis. Differential diagnosis is a common occurrence in the practice of medicine. Differential diagnosis evidence is often crucial to show specific causation (see Lennon). Without some evidence that the substance in question caused the specific injury to the specific plaintiff, courts are likely to grant the defendant summary judgment. Courts generally agree that, whenever there are different causes for the plaintiff's disease, an expert must perform a differential diagnosis before testimony will be admitted (see Gianelli). Courts accept the general validity of the technique of differential diagnosis (US versus Downing). An expert opinion based on a properly performed differential diagnosis analysis is not likely to be inadmissible (*Westberry* at 263). More specifically, "[t]o the extent that a doctor utilizes standard diagnostic techniques in gathering this information, the more likely we are to find that the doctor's methodology is reliable" (In *re* Paoli, at 758).

Put differently, "[a]n expert who opines that exposure to a compound caused a person's disease engages in deductive clinical reasoning. … The opinion is based on an assessment of the individual's exposure, including the amount, the temporal relationship between the exposure and disease, and other disease-causing factors. This information is then compared with scientific data on the relationship between exposure and disease. The certainty of the expert's opinion depends on the strength of the research data demonstrating a relationship between exposure and the disease at the dose in question and the absence of other disease-causing factors (also known as confounding factors)." (Reference guide at 422–3, see also Joseph Sanders).

However, simply stating that a differential diagnosis was performed is not enough. This issue was discussed in *Viterbo*:

> We do not hold, of course, that admissibility of an expert opinion depends upon the expert disproving or discrediting every possible cause other than the one espoused by him. Here, however, Dr. Johnson has admitted that Viterbo's symptoms could have numerous causes and, without support save Viterbo's oral history, simply picks the cause that is most advantageous to Viterbo's claim. Indeed, Dr. Johnson's testimony is no more than Viterbo's testimony dressed up and sanctified as the opinion of an expert. Without more than credentials and a subjective opinion, an expert's testimony that "it is so" is not admissible. (*Viterbo* v. *Dow Chemical*)
>
> This formulation is repeated by Judge Becker in *United States* v. *Downing.*
>
> The process of differential diagnosis is undoubtedly important to the question of "specific causation." If other possible causes of an injury cannot be ruled out, or at least the probability of their contribution to causation minimized, then the "more likely than not" threshold for proving causation may not be met. But, it is also important to recognize that a fundamental assumption underlying this method is that the final, suspected "cause" remaining after this process of elimination must actually be capable of causing the injury. That is, the expert must "rule in" the suspected cause as well as "rule out" other possible causes. And, of course, expert opinion on this issue of "general causation" must be derived from a scientifically valid methodology. (*Cavallo* v. *Star Enterprise*)

The order of proving causation is important. General causation should be proven first, then the specific causation differential diagnosis analysis. In virtually all cases, differential diagnosis does not provide general causation – it can only provide specific causation.[33] This may be part of the reasoning that argues that epidemiological data

[33] See generally, *Raynor* v. *Merrell Pharmaceuticals*, 101 F.3d 129, 138–39 (D.C. Cir. 1996). See also, *Kelly, Grimes, Rutigliano, Hall*, In *re* Breast Implant, National Bank of Commerce and Wynacht.

is rarely determinative in a specific causation analysis, and is most commonly used in the general causation argument.

To show that said exposure was the actual cause of the injury, toxic tort plaintiffs must prove the admissibility of their expert testimony in both general causation and specific causation context by a preponderance of proof (*Daubert* at 593). A successful plaintiff must not only show that, more likely than not, the substance can cause the injury in question, but also that, more likely than not, the plaintiff's specific injury was in fact caused by the substance (*DeLuca*, Ronald J. Allen).

A *Daubert* analysis should be performed. In fact, "a trial court that fails to justify its decision not to use *Daubert* factors risks reversal" (*Black* v. *Food Lion*). *Daubert* factors may be used when assessing the admissibility of clinical medical testimony (*Moore*). This opinion is because reliable opinions are reached using the "methods and procedures of science." Scientific validity is the foundation of "*evidentiary reliability*" (*Daubert* at 590, see also, Bert Black *et al.*).

So the expert in our case must not only rule in the chemical added to the feed additive as the cause of the clinical signs and lesions observed, but must also rule out other diseases that cause these clinical signs or lesions in that species of animal.

## APPLICATION OF DAUBERT IN TOXICOLOGY CASES

The application of these legal rules to specific toxicology cases may be useful. Testimony may be weakened if there is no Daubert inquiry at all (Goebel, 2000).

The differential diagnosis portion of analyzing specific causation is important. Expert testimony from a forensic toxicologist has been properly excluded for insufficient proof to rule in the chemical in question and to rule out other diseases (Wills, 2004). The testimony of a toxicologist was properly excluded because the toxicologist was not a medical doctor and therefore not qualified to offer reliable differential diagnosis analysis (Plourde, 2003) On the other hand, testimony was properly allowed from two marine biologists who each performed a differential diagnosis analysis in a case involving marine animals (Clausen, 2003).

Dose is important in toxicology cases. Testimony of a treating physician, toxicologist, and industrial hygienist were excluded, and the exclusion affirmed because the literature did not support a finding of general causation at the relevant exposure (Amorgianos, 2002). Testimony of a toxicologist was properly excluded because he performed no dose assessment and showed no statistically significant link between the chemical and the type of cancer present (Burleson, 2004).

Reliance on peer-reviewed literature is also important. A toxicologist's testimony was excluded, but the exclusion was reversed, because the toxicologist's testimony was supported by peer-reviewed literature (Bocanegra, 2003).

## SOME QUESTIONS TO CONSIDER WHEN USING LABORATORY RESULTS IN A LEGAL CASE

1 Was the legal aspect of the case known at the time the samples were submitted? If not, is the sample tracking or chain of custody sufficiently well documented to establish each relevant step of the analysis? Can the relevance of the documents be authenticated? Does chain of custody or sample tracking documentation exist?
2 What type of legal venue is the work to be used in? Administrative enforcement, civil litigation, criminal litigation? What standard of persuasion is appropriate for this venue?
3 Can the relevance of the analytical results be authenticated? What documentation exists that the samples were collected from the appropriate animal? What documentation exists to demonstrate proper sample identification throughout the laboratory testing process? What documentation exists to demonstrate that the process used produces an accurate result – what validation processes are in place? Is the interpretation of the test results accurate for the type of analytical test performed?
4 Is the testimony to be as a lay – or fact – witness?
5 Is the testimony to be as an expert witness?
   A Is the expert a toxicologist?
   B Does the scientific literature support an argument of general causation?
   C Was a differential diagnosis performed by the laboratory?
      - Rule-in:
         – Can the expert provide reliable testimony that the sample received by the laboratory is representative of the diseased animals?
         – Can the expert provide reliable testimony that the animals were in fact exposed to a toxic dose of the chemical in question? To what degree of certainty does the analytical methodology demonstrate exposure of the animal to the chemical in question?
         – Can the expert provide reliable testimony that the animals experienced the disease expected from exposure to a toxic dose of the chemical in question? Is reliable testimony of the clinical signs and lesions available?

- Rule-out:
  - Can the expert provide a differential diagnosis list?
  - Can the expert provide reliable testimony that diseases other than the purported one were ruled out? Can the expert explain how and why these other diseases were ruled out? Were scientifically valid methods used to rule out these diseases?

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## REFERENCES

*AFL-CIO* v. *OSHA* 965 F.2d 962, 969–70 (11th Cir. 1992).

*Amorgianos* v. *National R.R. Passenger Corp.*, 303 F.3d 256 (2d Cir. 2002). Testimony of a treating physician, toxicologist, and industrial hygienist were excluded, and the exclusion affirmed because the literature did not support a finding of general causation at the relevant exposure levels when workers were exposed to xylene and developed asymmetric polyneuropathy and other neurological symptoms.

Bernstein DE, Jackson, JD (2004) The Daubert Trilogy in the States, *Jurimetrics J* **44**: 351.

*Black* v. *Food Lion, Inc.*, 171 F.3d 308, 311–12 (5th Cir. 1999) ("In the vast majority of cases, the district court first should decide whether the factors mentioned in *Daubert* are appropriate. Once it considers the *Daubert* factors, the court then can consider whether other factors, not mentioned in *Daubert*, are relevant to the case at hand."); see also *Goebel* v. *Denver and Rio Grande W. R.R. Co.*, 215 F.3d 1083, 1087 (10th Cir. 2000).

*Bocanegra* v. *Vicmar Services, Inc.*, 320 F.3d 581 (5th Cir.), cert. denied, 124 S. Ct. 180 (2003). A toxicologist's testimony was excluded, but the exclusion was reversed, because the toxicologist's testimony was supported by peer-reviewed literature and relied on generally accepted principles.

*Bonner* v. *ISP Techs., Inc.*, 259 F.3d 924 (8th Cir. 2001).

*Burleson* v. *Texas Department of Criminal Justice*, 393 F.3d 577 (5th Cir. 2004). Testimony of a toxicologist excluded and the exclusion was affirmed because he performed no dose assessment and offered no epidemiological studies showing a statistically significant link between thorium dioxide exposure and the type of lung and throat cancer present.

*Carmichael* v. *Samyang Tire Inc.*, 923 F. Supp. 1514, 1522 (S.D. Ala. 1996 at 1520–21).

*Cavallo* v. *Star Enterprise*, 892 F. Supp. 756, 771 (E.D. Va. 1995), aff'd in part, rev'd in part, 100 F.3d 1150 (4th Cir. 1996). See also, *Implant Litig.*, 11 F. Supp. 2d 1217, 1230 (D. Colo. 1998); *Kelley* v. *American Heyer-Schulte Corp.*, 957 F. Supp. 873, 882 (W.D. Tex. 1997); *Rutigliano* v. *Valley Bus. Forms*, 929 F. Supp. 779, 783 (D.N.J. 1996); *Hall* v. *Baxter Healthcare Corp.*, 947 F. Supp. 1387, 1413 (D. Or. 1996).

*Clausen* v. *M/V New Carissa*, 339 F.3d 1049 (9th Cir. 2003). Dueling marine biologist's testimony was allowed and the allowance was affirmed, because each expert performed differential diagnosis analysis in which they first "ruled in" six potential causes then both experts ruled out four of them. They disagreed on the remaining two.

*Daubert* v. *Merrell Dow Pharms., Inc.*, 509 U.S. 579 (1993). at 590 n.9. See also Bert Black *et al.*, Science and the Law in the Wake of Daubert: A New Search for Scientific Knowledge, 72 TEX. L. REV. 715 (1994); Joseph Sanders, Scientific Validity, Admissibility, and Mass Torts after Daubert, 78 MINN. L. REV. 1387 (1994).

*Daubert* I, 509 U.S. at 593 n. 10; see e.g., *Carnegie Mellon Univ.* v. *Hoffmann-LaRoche, Inc.*, 55 F. Supp. 2d 1024, 1030 (N.D. Cal. 1999).

*DeLuca* v. *Merrell Dow Pharms.*, 911 F.2d 941, 958 (3d Cir. 1990).

*Dodge* v. *Cotter Corp.*, 328 F.3d 1212 (10th Cir. 2003).

Eaton DL, Klaassen CD *(2001)* Principles of toxicology. In *Casarett and Doull's Toxicology, The Basic Science of Poisons*, Chapter 2. 6th edn, Klaassen CD (ed.). McGraw Hill, New York, p. 11-34.

Federal Rules of Evidence. December 31, 2004. judiciary.house.gov/media/pdfs/printers/108th/evid2004

*Frye* v. *United States*, 293 F. 1013 (D.C. Cir. 1923).

*General Electric Co.* v. *Joiner*, 522 U.S. 136 (1997).

*Goeb* v. *Tharaldson*, 615 N.W.2d 800, 816 (Minn., 2000).

*Goebel* v. *Denver & Rio Grande Western R.R.*, 215 F.3d 1083 (10th Cir. 2000).

*Goebel* v. *Denver & Rio Grande Western R.R.*, No. 02-1391 (10th Cir. October 9, 2003).

*Grimes* v. *Hoffmann-LaRoche, Inc.*, 907 F. Supp. 33, 38 (D.N.H. 1995).

*Hall* v. *Baxter Healthcare Corp.*, 947 F. Supp. 1387, 1413 (D. Or. 1996). (holding that "[t]estimony regarding specific causation in a given patient is irrelevant unless general causation is established").

In *re* Breast Implant Litigation, 11 F. Supp. 2d 1217, 1230 (D. Colo 1998).

In *re* Paoli, 35 F.3d at 758.

Joseph Sanders, Scientific Validity, Admissibility and Mass torts After Daubert, 78 Minn L Rev. 1387 (1994); Susan R. Poulter, Science and Toxic Torts: Is there a rational Solution to the Problem of Causation ? 7 High Tech L.J. 189 (1992).

*Kelley* v. *American Heyer-Schulte Corp.*, 957 F. Supp. at 882.

*Kumho Tire Co.* v. *Carmichael*, 119 S. Ct. 1167 (1999). See also *Braun* v. *Lorillard Inc.*, 84 F.3d 230, 234 (7th Cir. 1996); *Rosen* v. *Ciba-Geigy Corp.*, 78 F.3d 316, 318 (7th Cir. 1996), and *Black* v. *Food Lion, Inc.*, 171 F.3d 308, 311 (5th Cir. 1999).

*Lennon* v. *Norfolk & W. Ry. Co.*, 123 F. Supp. 2d 1143, 1154 (N.D. Ind. 2000).

*Mancuso* v. *Consolidated Edison Co.*, 56 F. Supp. 2d 391, 403 (S.D.N.Y. 1999), rev'd on other grounds, 216 F.3d 1072 (2d Cir. 2000), at 394–95.

Mary Sue Henifin *et al.*, Reference Guide on Toxicology, II, in Reference Manual on Scientific Evidence.

*Moore* v. *Ashland Chemical, Inc.*, 126 F.3d 679 (5th Cir. 1997), rehearing en banc granted, opinion vacated, 151 F.3d 269 (5th Cir. 1998).

*National Bank of Commerce* v. *Associated Milk Producers, Inc.*, 22 F. Supp. 2d 942, 963 (E.D. Ark. 1998), aff'd, 191 F.3d 858 (8th Cir. 1999).

Paul C. Giannelli (1980) The Admissibility of Novel Scientific Evidence: Frye v. United States, a Half-Century Later, 80 COLUM. L. REV. 1197, 1201.

*Plourde* v. *Gladstone,* No. 02-9136 (2d Cir. June 27, 2003) (unpublished). The testimony of a toxicologist was excluded, and the exclusion affirmed, because the toxicologist was not a medical doctor and was therefore unqualified to offer a reliable differential

diagnosis regarding the development of symptoms after exposure of pesticide following spraying the neighbor's farm.
*Ray,* v. *Miller Meester Advertising*, Inc., Filed June 16, 2003, File No. 9817380.
*Raynor* v. *Merrell Pharms., Inc*., 104 F.3d 1371, 1376 (D.C. Cir. 1997).
*Raynor* v. *Merrell Pharmaceuticals*, 101 F.3d 129, 138-39 (D.C. Cir. 1996).
Reference Manual on Scientific Evidence. Second Edition. Federal Judicial Center. 2000. The manual is available at: www.fjc.gov/public/pdf.nsf/lookup/sciman00.pdf/$file/sciman00.pdf
Ronald J. Allen (1991) The Nature of Judicial Proof, 13 CARDOZO L. REV. 373.
Rule 104.(a)
Rule 104. (e)
Rule 401.
Rule 901. (9)
Rule 402
Rule 602.
Rule 901. (3)
*Rutigliano* v. *Valley Business Forms*, 929 F. Supp. at 783.
*Siharath* v. *Sandoz Pharms. Corp*., 131 F. Supp. 2d 1347, 1352 (N.D. Ga. 2001) (citing Wheat v. Sofamor, S.N.C, 46 F. Supp. 2d 1351, 1357 (N.D. Ga. 1999) (product liability action excluding testimony that failed to establish (1) that Parlodel is capable of causing stroke and (2) that Palodel did in fact cause plaintiffs' strokes)).
*Siharath*, 131 F. Supp. 2d at 1352. See e.g. D. T. Ralston, Toxic Tort Causation – Not Just Chemical Exposure Plus Symptoms, Maely's Daubert Rep., Vol. 4, No. 5, at 15–25 (2000).
*Simpson* v. *Young*, 854 f.2d 1429, 1435 (D.C. Cir, 1988)
*State of Minnesota* v. *DeShay*, June 11, 2002, MN Court of Appeals, C9-01-1128. File No. K200600502
*State* v. *Anderson*, 379 N.W.2d 70, 79 (Minn., 1985) (Graphology "is accorded a low measure of scientific reliability in predicting character or state of mind and is not generally accepted in the scientific fields of psychology and psychiatry.")
*State* v. *Jobe*, 486 N.W.2d 407, 419–20 (Minn., 1992) (Admission of expert testimony based on DNA test results is proper because the principles underlying forensic DNA testing are generally accepted, and the laboratory complied with the appropriate standards and controls, thus rendering the results legally reliable.)
*State* v. *Mack,* 292 N.W.2d 764, 768–69, 772 (Minn., 1980),
*State* v. *Moore*, 458 N.W.2d 90, 97–98 (Minn., 1990) (Admission of expert testimony on blood spatter interpretation was proper where the district court determined that the theory was generally accepted and the theory's application was legally reliable).
*State* v. *Kolander*, 236 Minn. 209, 221–22, 52 N.W.2d 458, 465 (1952).
*State* v. *Moore*, 458 N.W.2d 90, 97–98 (Minn., 1990)
*State* v. *Schwartz*, 447 N.W.2d 422 (Minn., 1989).
*State* v. *Rose*, 667 N.W.2d 386, 397 (Minn., 2003).
*Troy Corp* v. *Browner*, 129 F.3rd 1290 (D.C. Cir 1997).
*United States* v. *Hansen*, 262 F.3d 1217 (11th Cir. 2001), cert. denied, 535 U.S. 1111 (2002).
*United States* v. *Ledesma*, No. 99-8026 (10th Cir. February 14, 2000) (unpublished).
*United States* v. *Downing*, 753 F.2d 1224, 1234 (3d. Cir. 1985).
*Viterbo* v. *Dow Chemical Co.*, 111. 826 F.2d 420 (5th Cir. 1987) at 424.
*Westberry* v. *Gislaved Gummi AB*, 178 F.3d 257, 263 (4th Cir. 1999).
William O. Dillingham, Patrick J. Hagan, Rodrigo E. Salas. Blueprint for General Causation Analysis in Toxic Tort Litigation† Submitted by the authors on behalf of the FDCC Toxic Tort and Environmental Law Section.
*Wills* v. *Amerada Hess Corp*., 379 F.3d 32 (2d Cir. 2004). Testimony from a forensic toxicologist was excluded, and the exclusion was affirmed, because the toxicologist did not offer sufficient support for an oncogene theory that exposure to benzene and polycyclic hydrocarbons had caused squamous cell carcinoma, and because he had not ruled out smoking and alcohol as causes.
*Wynacht* v. *Beckman Instruments, Inc.*, 113 F. Supp. 2d 1205, 1209 (E.D. Tenn., 2000).

## FURTHER READING

Carruth RS, Goldstein BD (2001) Relative risk greater than two in proof of causation in toxic tort litigation. *Jurimetrics* **41**: 195–209.
Giannelli, PC (1980) The Admissibility of novel scientific evidence: Frye v. United States, a Half-Century Later, 80 *Colum L Rev* **1197**: 1205.
Goldstein BD (1993) Invited Paper "Linking scientific and technical expertise to the courts: a scientist's view of barriers and incentives". Presented at the Demonstration Project Planning Conference, Federal Judicial Center, Washington, DC. November 5, 1993.
Goldstein BD (1997) Toxic Substances: Scientific Status, Modern Scientific Evidence: *The Law and Science of Expert Testimony.* Faigman, Kaye, Saks and Sanders (eds), West Publishing Company, St. Paul, MN, pp. 277–99.
Goldstein BD (1997). Basic laws for proving causation of disease, *New Jersey Lawyer*, 6:6, 72, February 10, 1997.
Goldstein BD, Gallo MA (1995). Overview of toxicology. Shepard's *Expert and Scientific Evidence Quarterly*, **3-1**, 45–64.
Goldstein BD, Henifin MS (2000) *Reference Guide on Toxicology*. 2nd edn, *Reference Manual on Scientific Evidence*. Federal Judicial Center, 401–37.
Goldstein BD, Carruth RS (eds) (2002) Toxic Substances: Scientific Status, In *Modern Scientific Evidence: The Law and Science of Expert Testimony,* 2nd edn, Faigman, Kaye, Saks and Sanders (eds). West Publishing Company, St. Paul, MN.

# Part 2

## Organ Toxicity

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CHAPTER

# 9

# Neurotoxicity

*Donna Mensching and Petra A. Volmer*

## INTRODUCTION

Neurotoxicants abound. Some of the most potent, well-known neurotoxicants are "all natural" and all around us. The toxin of the anaerobic bacterium *Clostridium botulinum*, for instance, is popularly considered to be the most toxic natural substance on the planet. Just 1 ng is lethal to a human (Kotsonis *et al*., 2001). Strychnine, a rodenticide commercially available in some geographic regions today, is a toxic alkaloid first isolated from the seeds of *Strychnos nux vomica* and *S. ignatii* hundreds of years ago (Talcott, 2006). The deadly venom of the brilliant Australian blue-ringed octopus (*Hapalochlaena maculosa* and *H. lunulata*) contains tetrodotoxin (TTX) which causes respiratory paralysis and death within minutes of a sting via blockade of sodium channels (Scheumack *et al*., 1978; White, 1995).

Many toxicants of human historical significance affect the nervous system. One of the most notorious toxicoses occurred in Minamata Bay, Japan, in the 1950s and for decades to follow when thousands of people were affected with neurologic deficits due to the ingestion of fish contaminated with mercury. The bay had been contaminated by the Chisso Corporation, which used the mercury in the manufacture of acetaldehyde, a plastic intermediate. Over time, the mercury bioaccumulated in the fish causing neurologic deficits in the people and animals that ingested them. Due to their advanced cerebellar ataxia, resident cats that subsisted on these fish were described as "suicidal" and were often seen falling into the water (Smith and Smith, 1975; Francis, 1994). Mercury toxicosis has invaded popular culture as well. The phrase "mad as a hatter" did not originate with Lewis Carroll's *Alice in Wonderland* (1865) but was popularized years prior. It stemmed from the bizarre behavior of hat manufacturers who used mercury in the curing process of the felt and ultimately suffered neurotoxic effects from the occupational exposure (Francis, 1994).

In addition to the plethora of "all natural" neurotoxicants, a multitude of synthetic pesticides (insecticides, rodenticides, etc.) directed at the nervous system of target species has been developed. One example of historical significance was the delayed neuropathy associated with the intentional contamination of Jamaican ginger alcohol ("Jake") with tri-*ortho* cresyl phosphate (TOCP), an organophosphorus (OP) compound, during the Prohibition years. The TOCP was present in lindol, a substitute solvent added to the Jake in 1929 by Hub Products Corporation president, Harry Gross, to cut costs. The resulting upper motor neuron spasticity and paralysis affected more than 50,000 people and were irreversible (Woolf, 1995).

The efficacy of such compounds, natural or manmade, against non-target species, unfortunately, underscores the importance of a thorough understanding of neurotoxic mechanisms in veterinary medicine. Because of its unique anatomy, specialized functions, and high-metabolic requirements, the nervous system is highly susceptible to toxic effects, often despite adaptations to protect itself (i.e. the blood–brain barrier). The following discussion addresses the basic structural and functional causes of neurotoxicity.

## STRUCTURAL TOXICOSES

Structural changes caused by neurotoxicants can be further divided into those that cause a neuronopathy, an axonopathy, or a myelinopathy.

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## Neuronopathy

A neurotoxicant that results in neuronopathy directly targets the neuronal cell body, resulting in cell death and secondary axonal degeneration. Gliosis, proliferation of astrocytes and/or microglial cells, is a common response to loss of neurons (Anthony *et al.*, 2001). With few exceptions, this type of injury is irreversible. Examples of such toxicants include the aforementioned methyl mercury which preferentially targets the cell bodies of the occipital cortex and the cerebellum via an unproven mechanism. Blindness and motor incoordination are common manifestations of lesions in these areas. Atchison (2005) postulates that the selective toxicity to cerebellar granule cells is due to the stimulation of $M_3$ muscarinic acetylcholinergic receptors and inhibition of $GABA_A$ (gamma aminobutyric acid) receptors. In veterinary medicine, methyl mercury intoxication is most likely seen in animals that subsist on a diet of contaminated fish, as with the "dancing" cats of Minamata Bay.

A second example of a neuronotoxicant is the heavy metal, trimethyltin [$(CH_3)_3Sn$], used as a plasticizer and antifungal agent (Anthony *et al.*, 2001). It causes diffuse injury, but the pyramidal cells of CA1, CA3, and CA4 regions of the hippocampus are most sensitive. Signs experimentally produced in rats include aggression, increased irritability, tremors, seizures, and hyperactivity (Shin *et al.*, 2005). Like methyl mercury, the mechanism of trimethyltin neuronopathy is not well understood.

A third example of a toxicant that can directly target neurons of particular relevance in veterinary medicine is the anthracycline glycoside antineoplastic agent, doxorubicin (Adriamycin®). Its potential for bone marrow suppression and cardiac toxicity with veterinary use, however, is much greater than its neurotoxic potential (Anthony *et al.*, 2001; Plumb, 2005). Although doxorubicin exerts its antineoplastic effect via interference with transcription, the neurons of the dorsal root ganglia and autonomic ganglia are most susceptible to injury. The selectivity of the effect is thought to be due to a lack of blood–tissue barrier within the ganglia (Anthony *et al.*, 2001).

Domoic acid, the neurotoxicant responsible for amnesic shellfish poisoning (ASP) in people and wildlife, is produced by the diatom *Pseudo-nitzschia* spp. Popular belief holds that Alfred Hitchcock's 1963 movie, "The Birds" was inspired by an outbreak of domoic acid poisoning among California seabirds in 1961. The toxin is thought to exert its excitatory and cytotoxic effects on hippocampal cells due to its high affinity for binding to the kainate receptor, which is a glutamate receptor subtype. Cellular excitation ensues, resulting in an influx of $Ca^{2+}$ ions, upregulation of the c-Fos gene, and cell death (Jeffery *et al.*, 2004).

A large animal example of neuronotoxicosis is yellowstar thistle (*Centaurea solstitialis*) ingestion by horses which occurs typically in the dry summer and fall. It results in neurologic signs including involuntary lip and tongue movements, difficulty in prehending food, tremors, writhing, possible circling, persistent slow movements (dystonia), and significant weight loss. The disease is also called nigropallidal encephalomalacia as a result of the areas of the brain that are uniquely affected (the globus pallidus and the pars reticularis of the substantia nigra). These regions and the clinical signs draw a striking similarity to Parkinson's disease in people (Burrows and Tyrl, 2001; Sanders *et al.*, 2001) which has been associated with selective loss of dopaminergic neurons (van den Munckhof *et al.*, 2006). Several guaianolide sesquiterpene lactones have been isolated from *Centaurea* species. *In vitro* ranking of their toxicity in order of most to least reveals repin, subluteolide, janerin, cynaropicrin, acroptilin, and solstitialin. Cynaropicrin and an analog of solstitialin are cytotoxic in primary cultures of rat substantia nigra cells, and thus raise further suspicion for their role in the toxicosis. Aspartic and glutamic acids, two excitatory amino acid neurotransmitters, are also present in *Centaurea* (Burrows and Tyrl, 2001). Their role, if any, in the mechanism of nigropallidal encephalomalacia has yet to be determined.

A final example of a neuronopathic toxicant is currently a common drug of abuse called methylenedioxyamphetamine (MDMA) or "Ecstasy." MDMA selectively targets serotonergic and dopaminergic cells depending on the species exposed, and long-term, irreversible effects may be seen (Gouzoulis-MayFrank and Daumann, 2006). Acute physiologic effects in people include tachycardia, hypertension, euphoria, heightened sexual awareness, urinary urgency, nausea, chills, sweating, and hyperthermia, among others. Signs consistent with serotonin syndrome (hyperactivity, agitation, mental confusion, hyperthermia, tachycardia, and tremors) have been observed in experimentally exposed rats (Easton and Marsden, 2006). Further research is indicated to define species-specific effects, particularly the extent to which serotoninergic or dopaminergic neurons are involved. Veterinary practitioners should be aware of the potential for accidental exposure to small animals.

## Axonopathy

The second class of structural lesions caused by neurotoxicants is axonopathy. With damage to the axon, the neuronal cell body remains intact, but the portion of the axon distal to the lesion degenerates, resulting in a "chemical transection" distal to the lesion that is functionally identical to a physical transection of the axon. This is also known as Wallerian or axonal degeneration. Changes in the Nissl substance, the protein synthetic material comprising of free polyribosomes and rough endoplasmic reticulum, become evident histologically in response to this degeneration. These changes include chromatolysis

(dissolution of the Nissl substance) as well as margination of the Nissl and the nucleus to the periphery of the cell body. Not surprisingly, those neurons with axons of greatest length are most susceptible to axonal damage. To highlight the susceptibility of distal axons, a subclassification of axonopathy affecting these axons has been termed "central peripheral distal axonopathy." This is in contrast to "central peripheral proximal axonopathy" which involves axons proximal to the spinal cord (Anthony *et al.*, 2001). A third subclassification of axonopathy has been termed "dying back axonopathy." The latter represents progressive death of the axon toward the cell body with time and continued injury. In the peripheral nervous system (PNS), the prognosis for at least partial regeneration is good, but this is less true in the central nervous system (CNS). Secondary demyelination is also possible with axonal injury (Mandella, 2002). Both sensory and motor axons can be affected.

Many agents cause axonopathies, yet a few are particularly relevant to veterinary medicine. The first example, commonly used as an antineoplastic drug, is the vinca alkaloid, vincristine, which is derived from the periwinkle plant, *Vinca rosea* or *Catharanthus rosea*. Vincristine exerts its therapeutic effect by binding to tubulin, inhibiting microtubular formation, disrupting the formation of the mitotic spindle, and arresting cell division at metaphase (Burrows and Tyrl, 2001; Roder, 2004a). Neurotoxicity in the form of axonal degeneration can occur as a result of disruption of fast axonal transport (rate of 400 mm/day normally) which relies on functional integrity of the microtubules (Anthony *et al.*, 2001). Cats are more sensitive to the neurotoxic potential of vincristine. With discontinuation of therapy and appropriate supportive care, animals exhibiting signs of peripheral neuropathy may improve over several weeks to months (Roder, 2004a). Colchicine, an antimetabolite derived from the autumn crocus (*Colchicum autumnale*) and the glory lily (*Gloriosa* spp.), also inhibits spindle formation (Burrows and Tyrl, 2001; Roder, 2004a). It is used in veterinary medicine for the treatment of amyloidosis associated with Shar Pei fever (Loeven, 1994). Although reported rarely in humans, no published reports of peripheral neuropathy in veterinary patients were found.

A more common yet fortunately still rare cause of neurotoxicosis in veterinary medicine is metronidazole. A nitroimidazole antibacterial and antiprotozoal agent used to treat *Giardia* and anaerobic intestinal bacterial overgrowth in small animals (Plumb, 2005), metronidazole can result in a sensory peripheral neuropathy manifesting as proprioceptive deficits (Gupta *et al.*, 2000) as well as CNS effects including ataxia, nystagmus, head tilt, and seizure activity (Plumb, 2005). Myelinated fibers are most commonly affected (Anthony *et al.*, 2001). The mechanism is unknown for both peripheral and central effects.

A final example of a class of agents that can cause axonopathy are the organophosphorus compounds (OPs), commonly used as insecticides, that can result in signs of neuropathy 7–10 days post-exposure, termed OP-induced delayed neuropathy or OPIDN. An example is the previously described TOCP contamination of Ginger Jake (Woolf, 1995). In addition to humans, hens have also been shown to be very sensitive to OPIDN (Damodaran *et al.*, 2001). The neuropathy associated with OPs is thought to involve slow axonal transport macromolecules such as actin and tubulin (Zhao *et al.*, 2005) which involves movement of the neuronal cytoskeleton at a rate of 1–4 mm/day (Anthony *et al.*, 2001).

## Myelinopathy

Myelin is produced by the oligodendrocytes of the CNS and Schwann cells of the PNS. It is composed of lipid and forms a sheath around certain axons, namely those of the cranial and spinal nerves. It functions to increase the speed of impulse conduction by creating isolated areas of heightened electrical excitability, termed nodes of Ranvier (Spencer, 2000). Myelin gives white matter its characteristic appearance. Toxicants that result in myelinopathy may affect the myelin itself or target the cells that produce myelin. The insult may result in loss of myelin (demyelination) or edema of the myelin sheath and subsequent separation of myelin lamellae. Remyelination of segmentally demyelinated areas can occur more so in the PNS than the CNS. When peripheral nerves are remyelinated, the process involves more Schwann cells compared to the initial myelination. As a result, the nodes of Ranvier in remyelinated peripheral nerves are closer (Anthony *et al.*, 2001).

Two examples of toxicants that result in intramyelinic edema and separation of the myelin lamellae are hexachlorophene and bromethalin. The former is an antibacterial agent commonly marketed decades ago as pHisoHex® and still commercially available for the treatment and prevention of *Staphylococcal* infections. The latter is a relatively new rodenticide that has been available for roughly 20 years now and is marketed under a variety of tradenames including Assault®, Sudden Death®, and Vengeance.® The mechanism by which hexachlorophene and bromethalin cause intramyelinic edema is due to the uncoupling of oxidative phosphorylation (Anthony *et al.*, 2001; Dorman, 2004). This uncoupling results in decreased $Na^+/K^+$-ATPase activity, weakened ion gradients, and retention of water in the myelin lamellae (van Lier and Cherry, 1988). The use of mannitol or diuretics early in the course of the disease may reverse mild changes, but continued swelling of the lamellae results in a dramatic increase in intracranial and cerebrospinal fluid (CSF) pressure that is typically unresponsive to therapy. Clinical signs in an acute toxic exposure include muscle tremors, hyperthermia, generalized seizures, hyperexcitability, hyperesthesia, and death within several hours of ingestion (4–18 h) for bromethalin.

Cats are more sensitive to bromethalin than dogs with a minimum lethal oral dose of 0.45 mg/kg versus 2.5 mg/kg in the dog (Dorman, 2004). At lower dosages, hind limb ataxia and paresis can develop in dogs and cats within 2–7 days of ingestion. Signs may include decreased or absent proprioception, loss of response to deep pain, upper motor neuron bladder paralysis, patellar hyperreflexia, and varying degrees of CNS depression. These sublethal effects may be spontaneously reversible with time (1–2 weeks). Histologic lesions consistent with bromethalin and hexachlorophene toxicosis include spongy degeneration (diffuse vacuolation) of the white matter of the CNS. Confirmation of a fatal bromethalin toxicosis can be accomplished by identifying the parent compound and/or its more toxic metabolite, desmethylbromethalin, in the liver. Due to their relative inability to metabolize bromethalin into desmethylbromethalin, guinea pigs are resistant to its toxic effects. Treatment of exposed susceptible species is largely aimed at initial decontamination via induction of emesis (in those species that can vomit) and administration of multiple doses of activated charcoal prior to the onset of clinical signs (Dorman, 2004).

Another toxicant which results in myelinopathy is inorganic lead. The peripheral neuropathic manifestation of lead intoxication is secondary to the segmental degeneration of myelin in distal motor fibers and is most commonly seen in veterinary medicine with chronic intoxication of horses. Sensory function is spared. Clinical signs may include dysphagia and secondary weight loss, ataxia, dysphonia, laryngeal paralysis ("roaring"), and facial nerve deficits. The CNS and other organs may be affected resulting in seizures, depression, secondary aspiration pneumonia, colic, and death (Gwaltney-Brant, 2004).

## FUNCTIONAL TOXICOSES

Most neurotoxicants exert their functional effects via the exquisitely orchestrated mechanisms involved in neurotransmission yet leave no structural footprint of their activity. This can occur at all levels within the nervous system including the CNS, PNS, and autonomic nervous system (ANS). Nervous impulses are chemically mediated across synapses by the release of neurotransmitters from the pre-synaptic terminal. These neurotransmitters then move across the synaptic cleft, bind to their post-synaptic target receptor, and effect either an excitatory or inhibitory response in the post-synaptic neuron or muscle (Anthony *et al.*, 2001). Functional neurotoxicants may exert their action by preventing synthesis, storage, release, binding, reuptake, or degradation of the neurotransmitter. Interference with axonal transmission via sodium, potassium, chloride, or calcium channels and subsequent alteration of action potentials can also result in functional toxicoses (Spencer, 2000; Hansen, 2006). The lack of structural lesions in these circumstances in no way minimizes the resulting pathology.

Examples of neurotransmitters include acetylcholine; the catecholamine neurotransmitters dopamine, norepinephrine, and epinephrine; the amino acid derivatives serotonin (5-hydroxytryptamine; 5-HT), GABA, glycine, histamine, aspartic acid, and glutamic acid; and various neuropeptides including enkephalins, substance P (a neurokinin), orexins, endorphins, vasopressin (antidiuretic hormone, ADH), and thyroid releasing hormone (TRH) (Beasley, 1999; Spencer, 2000). The complex array of neurotransmitters provides many targets for neurotoxicity, both intentional as with organophosphate insecticides and unintentional as with serotonin syndrome after an acute overdosage of a selective serotonin-reuptake inhibitor (SSRI) by the family dog. A more detailed discussion follows regarding some of the more common neurotransmitters involved in neurotoxicoses.

### Acetylcholine

Acetylcholine (ACh) is the neurotransmitter that mediates effects at the neuromuscular junction, at the pre-ganglionic neurons of both the parasympathetic and sympathetic nervous systems of the ANS, and at many of the post-ganglionic neurons of the parasympathetic nervous system. It is the target neurotransmitter of OP and carbamate insecticides, which have been marketed since the 1970s and now widely available commercially for home and agricultural use (Meerdink, 2004b). Acetylcholinesterase (AChE) is the enzyme that degrades ACh to choline and acetic acid once it is no longer needed to effect transmission. OP and carbamate insecticides bind to AChE, the former by phosphorylating the enzyme, the latter by carbamylating the enzyme, and prevent its degradative action on ACh. This results in an excess of ACh in the synaptic cleft and continued binding to the post-synaptic receptors. Examples of OPs include disulfoton, malathion, terbufos, phosmet, chlorpyrifos, tetrachlorvinphos, and parathion. Examples of carbamates include carbaryl, carbofuran, aldicarb, methomyl, and propoxur. The onset of action and severity of signs vary widely among these agents. OPs with a sulfur linkage need to be activated by p450 enzymes in the liver prior to exerting their toxic effects, a process which may take just minutes. Carbamates are active upon absorption but some of them are less likely to cross the blood–brain barrier than the more fat soluble OPs. Cholinergic effects are mediated by both muscarinic and nicotinic receptors. Classic muscarinic effects include excess *s*alivation, *l*acrimation, increased *u*rination, *d*iarrhea, *d*yspnea (due to increased bronchial secretions), and *e*mesis (abbreviated as the mnemonic SLUDDE or the alternative DUMBELS which stands for *d*iarrhea, *u*rination, *m*iosis, *b*ronchospasm,

*e*mesis, *l*acrimation, and *s*alivation). Nicotinic effects include tremors, weakness, and paralysis. CNS effects can range from coma and depression to hyperactivity and seizures (Blodgett, 2006).

The main distinction between the OPs and carbamates is the reversibility of the bond between the insecticide and AChE. OPs phosphorylate the esteratic site of the enzyme. At variable times depending on the type of OP bound, the carbon groups attached to the phosphorus are hydrolyzed and replaced by hydrogen. This process is classically referred to as "aging" and represents the point at which there is no possible functional recovery of that enzyme. The use of pralidoxime (2-PAM) as an antidote to preferentially bind the OP is useful only before the aging process has occurred. In contrast, the affinity of carbamates for AChE is much more labile and aging does not occur with carbamylation of the enzyme. Therefore, the bond between AChE and carbamates is spontaneously reversible with time ($t_{1/2} = 30$–40 min) and precludes the use of 2-PAM. Provided the receptor effects are not life threatening in the meantime, the prognosis for carbamate toxicoses is generally good. With potent carbamates such as methomyl, however, the signs may be severe enough and the duration of effect long enough for the outcome to be fatal. Management of life-threatening bradycardia and/or dyspnea due to bronchospasm and excess bronchial secretions with sufficient atropine (0.1–0.5 mg/kg intravenous (IV), intramuscular (IM), or subcutaneous (SC) to effect) to competitively inhibit the ACh at the muscarinic receptor is indicated in severe cases (Blodgett, 2006).

In addition to the chronic OPIDN (discussed under *Axonopathy*) and the acute toxicosis with SLUDDE effects, there is an intermediate syndrome that is most commonly seen with more liphophilic OPs and classically with chlorpyrifos exposure in cats. It is thought to arise from a down-regulation of muscarinic receptors with sublethal, prolonged exposures. The clinical signs as a result are predominantly nicotinic in nature because those receptors are not down-regulated. Signs which typically appear within 3–10 days of exposure include generalized weakness (including ventroflexion of the neck due to the cat's lack of a nuchal ligament), anorexia, muscle tremors, seizures, depression, and/or death (Blodgett, 2006).

Another toxicant with muscarinic effects is the mycotoxin, slaframine, produced from the fungus *Rhizoctonia leguminicola*, and primarily associated with clovers (*Trifolium* species). It is a cholinergic agonist which is responsible for the clinical picture of profuse salivation or "slobbers" in affected animals (Meerdink, 2004a). Mushrooms of the genera *Inocybe* and *Clitocybe*, among others, contain the toxic principle muscarine which, not surprisingly, results in clinical signs consistent with muscarinic stimulation (Turner and Szczawinski, 1991). Anatoxin-$a_{(s)}$, a neurotoxin produced by the cyanobacteria *Anabaena* sp., *Aphanizomenon* sp., and *Oscillatoria* sp., inhibits AChE in the PNS. Like slaframine, the toxin does not cross the blood–brain barrier (Roder, 2004c).

Several examples of neurotoxicants exist that antagonize muscarinic effects as well. The classic poisonous plant, *Atropa belladonna*, or deadly nightshade, is the source of atropine, a racemic mixture of the tropane alkaloids, D- and L-hyoscyamine (Burrows and Tyrl, 2001). Other belladonna alkaloids include scopolamine and hyoscine. *Datura* sp., commonly known as jimsonweed, thorn apple, or devil's trumpet, contains scopolamine and hyoscyamine. Excessive anticholinergic action can result in sinus tachycardia, a dry mouth manifesting as increased thirst, dilated pupils, visual disturbances, ileus, urinary retention, restlessness, muscular twitching, incoordination, delirium (as a result of crossing the blood–brain barrier and entering the CNS), respiratory paralysis, and death (Burrows and Tyrl, 2001; Pickrell *et al*., 2004).

Nicotinic cholinergic receptors are also involved with neurotoxicoses. Nature has provided several poisonous plants that stimulate these receptors including *Nicotiana* sp. (varying types of tobacco), *Conium maculatum* or poison hemlock, and *Lobelia* or Indian tobacco. The numerous alkaloids present in these plants (Table 9.1) as well as the cyanobacterial toxin, anatoxin-a, most commonly produced by *Anabaena flos-aquae* (Roder, 2004c), exert their neurotoxic effects by initially stimulating nicotinic cholinergic and neuromuscular junction receptors but ultimately resulting in a persistent neuromuscular blocking effect. Signs include muscular weakness, tremors, nausea, vomiting, ataxia, tachypnea, tachycardia, hypertension, mydriasis, staggering, seizures, respiratory failure, and death (Panter, 2004a,b,c,). Treatment is symptomatic and supportive. Prognosis is good except in cases of large overdoses.

**TABLE 9.1 Alkaloids exerting nicotinic effects (Panter, 2004a,b,c)**

| |
|---|
| *Piperidine alkaloids* |
| Anabasine (*Nicotiana glauca*) |
| Ammodendrine (*Lobelia formosus*) |
| *N*-acetyl hystrine (*Lobelia formosus*) |
| Coniine (*Conium maculatum*) |
| Conhydrine (*Conium maculatum*) |
| Pseudoconhydrine (*Conium maculatum*) |
| $\gamma$ coniceine (*Conium maculatum*) |
| *Pyridine alkaloids* |
| Nicotine (*Nicotiana species*) |
| Nornicotine (*Nicotiana species*) |
| Lobeline (*Lobelia species*) |
| Lobelanidine (*Lobelia species*) |
| Lobelanine (*Lobelia species*) |
| *Quinolizidine alkaloid* |
| Lupinine (*Lupinus species*) |
| Cytisine (*Cystisus, Thermopsis*, and *Laburnum species*) |
| Lupanine (*Lupinus species*) |
| Anagyrine (*Thermopsis* and *Lupinus species*) |
| Thermopsine (*Thermopsis species*) |

Many snake toxins are nicotinic receptor antagonists. Envenomation can result in muscular paralysis and death. They include the following $\alpha$-neurotoxins: erabutoxin (of the sea snake, *Laticauda semifasciata*), $\alpha$-cobratoxin (of the cobra, *Naja naja siamensis*), and $\alpha$-bungarootoxin (of the many-banded krait, *Bungarus multicinctus*).

Imidacloprid (Advantage®) is a chloronicotinyl nitroguanide insecticide marketed for flea control in pets and which exerts its effects via possibly both agonist and antagonist actions at the nicotinic ACh receptor. Death of the insect is effected via the same clinical signs as previously described above, yet imidacloprid has a wide margin of safety in mammals due to its poor systemic absorption when applied dermally (as labeled) and due to the higher affinity of imidacloprid for insect nicotinic receptors versus mammalian receptors. Acute oral ingestions are usually limited to nausea, salivation, and vomiting (Wismer, 2004).

Another mechanism by which ACh can be involved in toxicoses is the lack of release of the neurotransmitter from the pre-synaptic terminal as a result of the action of the botulinum toxin, produced by the anaerobic bacterium, *Clostridium botulinum*. Sources of the toxin include ingestion of food contaminated with either preformed toxin or clostridial spores, contamination of a puncture wound with spores, and ingestion of spores from the environment. Exposure can be oral, inhaled, or absorbed cutaneously through devitalized skin (Bailey, 2006). The toxin consists of a 100 kD heavy chain and a 50 kD light chain linked by disulfide and non-covalent bonds. Upon ingestion of the toxin, its heavy chain binds synaptotagmin, a pre-synaptic vesicle protein. Once the toxin is internalized within the pre-synaptic terminal, its disulfide bonds are cleaved, releasing the light chain. The free light chain is then available to cleave proteins that are responsible for the docking and release of ACh vesicles into the synapse.

Seven different botulinum toxins (A–G) have been described. Types C and D are most commonly associated with veterinary species (type C is most common in dogs); types A, B, E, and F have caused disease in humans. Although differences exist regarding which proteins are cleaved by each toxin type, the end result is the lack of ACh in the synaptic cleft of the neuromuscular junction (Roder, 2004b). The clinical result is a progressive flaccid paralysis which results in death within 3–10 days. Signs are symmetric and progress from the pelvic limbs toward the thoracic limbs. Cranial nerves may be affected, possibly resulting in megaesophagus, decreased jaw tone, facial paralysis, and/or a decreased gag reflex. Autonomic functions may be affected as well (Coleman, 1998). For obvious reasons, the disease is called limber neck in affected birds. In patients fortunate enough to be supported with artificial respiration, the duration of effect can persist for 6–8 months (Kotsonis *et al.*, 2001).

The prognosis for severely- affected animals is at best guarded but more realistically poor. Treatment can include debridement of wounds, penicillin therapy, and antitoxin (5 ml IV or IM) within the first 5 days of exposure. Administration of the antitoxin does not reverse the clinical signs attributed to neurons already affected by the toxin. Due to its equine source and antigenicity, an intradermal test dose is recommended prior to full administration IV or IM. For individuals already severely affected, assisted ventilation is indicated until the patient can breathe spontaneously, yet the duration of therapy required may not be practical in veterinary medicine. Extreme supportive care including assisted feeding, physical therapy, manual evacuation of the bladder, and intermittent manipulation of body position to prevent hypostatic congestion is critical. Some laboratories can confirm the diagnosis with analysis of serum, gut contents, and/or feed for the preformed toxin (Roder, 2004b; Bailey, 2006).

## Catecholamine neurotransmitters

The synthesis of catecholamine neurotransmitters begins with the amino acid, L-tyrosine. The enzyme tyrosine hydroxylase, present in the cytosol of neurons, converts L-tyrosine to L-dopa. The removal of a carboxyl group from L-dopa, aided by the enzyme L-dopa decarboxylase, forms dopamine. The enzyme dopamine $\beta$ hydroxylase then converts dopamine to norepinephrine which is packaged into synaptic vesicles and released from the pre-synaptic terminal via a $Ca^{2+}$-dependent mechanism (Spencer, 2000). Norepinephrine is also stored in the adrenal medulla and released into the blood with sympathetic stimulation (Capen, 2001). *N*-methylation of norepinephrine forms epinephrine, an adrenal hormone which is only nominally present in the brain. Catecholamines are inactivated by monoamine oxidase (MAO) and catechol-*O*-methyltransferase (COMT) enzymes. MAO-A deaminates norepinephrine and serotonin while MAO-B metabolizes dopamine (Beasley, 1999; Spencer, 2000).

In addition to its role at post-ganglionic sympathetic neurons, norepinephrine also mediates effects in the CNS. Adrenergic receptors include $\alpha_1$, $\alpha_2$, $\beta_1$, and $\beta_2$ receptors. Stimulation of $\alpha_1$ adrenergic receptors, present peripherally, results in vasoconstriction and mydriasis. Stimulation of $\alpha_2$ adrenergic receptors, present in the CNS, mediates inhibition of norepinephrine, resulting in effects opposite those of $\alpha_1$ stimulation, namely hypotension. Stimulation of $\beta_1$ receptors, located predominantly in the heart but also in the kidney, adipose, skeletal muscle, and eye, results most notably in an increase in the rate and force of cardiac contraction. Other effects include increased renin release, lipolysis, tremors, and increased production of aqueous humor. The $\beta_2$ adrenergic receptors present in skeletal muscles; in the smooth muscle of the bronchi,

vasculature, and uterus; and also in the liver mediate vasodilation, bronchodilation, uterine relaxation, and enhanced glycogenolysis in the liver (Landsberg and Young, 2001).

Examples of adrenergic toxicosis involving overstimulation of the $\alpha_2$ receptors include accidental ingestion of brimonidine, the active ingredient in Alphagan® eye drops used in the treatment of glaucoma; ingestion of Preventic® collars, and overdosage of the sedative/analgesic, xylazine. Exposures involving Alphagan® typically involve puncturing of the bottle with sparing amounts actually ingested. Clinical signs for all three toxicoses include profound hypotension and bradycardia, which can be specifically reversed with either of the $\alpha_2$ antagonists, yohimbine or atipamezole (Antisedan®). The half-life of the latter is longer in dogs and may require less frequent administration (Plumb, 2005).

An example of adrenergic toxicosis involving overstimulation of the $\beta_2$ adrenergic receptors involves the accidental puncture of pressurized albuterol inhalers. Albuterol is a $\beta_2$ adrenergic agonist used therapeutically to treat the bronchoconstriction associated with asthma. When excessive $\beta_2$ stimulation occurs, profound hypotension, a reflex tachycardia, and subsequent release of catecholamines can occur. Clinical signs usually include tachycardia with possible ventricular premature contractions (VPCs), tachypnea, hyper- or hypotension (depending on the timeframe relative to exposure and the predominating neurotransmitter effect), behavioral changes including restlessness, agitation, anxiety, largely due to secondary catecholamine release, weakness later in the course, and hypokalemia, sometimes severe, due to an intracellular potassium shift. Treatment is largely supportive with fluid therapy, management of severe tachycardia with a $\beta$ blocker such as propranolol, supplementation of potassium as needed, and diazepam to address behavioral changes due to secondary norepinephrine release (Rosendale, 2004).

Apomorphine, a commonly used emetic in veterinary medicine, mediates its effect by stimulating dopamine receptors in the chemoreceptor trigger zone. Particularly when administered via the IV route, vomiting is rapid and predictable. It can also be administered intramuscularly and within the conjunctival sac to effect emesis. Adverse effects associated with overdoses, in particular, include CNS depression or stimulation, cardiac and respiratory depression, and protracted vomiting. Administration into the conjunctival sac allows decontamination via saline lavage if such effects are seen. Otherwise, naloxone, an opiate antagonist, may be given to reverse the depressive effects. Protracted vomiting may be ameliorated with phenothiazine tranquilizers via their anti-dopaminergic properties (Plumb, 2005).

Sympathomimetics such as amphetamines, cocaine, pseudoephedrine, phenylpropanolamine, and ma huang mediate dopaminergic and norepinephrine-induced neurotoxic effects including hypertension, hyperexcitability, tachycardia, tremors, seizures, mydriasis, hyperesthesia, head-bobbing, piloerection, and death (APCC, 2006). Phenothiazine tranquilizers such as acepromazine and chlorpromazine are the mainstay of sympathomimetic overdose therapy due to their post-synaptic blocking effects of dopamine as well inhibition of its release and increase in its turnover in the CNS. Phenothiazines also block $\alpha$ adrenergic and cholinergic activity and are antihistaminic (Plumb, 2005).

## Serotonin

The synthetic pathway for the CNS neurotransmitter, serotonin, begins in serotonergic neurons with the amino acid, tryptophan. It is converted by tryptophan hydroxylase to 5-hydroxytryptophan (5-HTP), which is then converted to serotonin by aromatic amino acid decarboxylase. The various physiologic roles of serotonin include serving as an effector in gastrointestinal and cardiovascular smooth muscle, promoting platelet aggregation, and various roles in regulating sleep, mood, cognition, appetite, and behavior (Gwaltney-Brant *et al*., 2000; Spencer, 2000). Due to the popularity of SSRIs and over-the-counter supplements containing 5-HTP for the treatment of depression in humans, accidental exposures resulting in signs consistent with excess serotonin are fairly common in veterinary medicine. Examples of SSRIs include fluoxetine (Prozac®), fluvoxamine (Floxyfral®, Luvox®), paroxetine (Paxil®), and sertraline (Zoloft®) (PDR, 2006). The seeds of the West African legume, *Griffonia simplicifolia*, contain 6–10% free serotonin (Spencer, 2000). The collective neurologic signs of serotonin excess, termed serotonin syndrome, include tremors, seizures, depression (rarely to the point of coma), disorientation, vocalization, hyperesthesia, ataxia, and apparent blindness. Tachycardia and hypertension have also been seen (APCC, 2006). A majority of affected dogs exhibit vomiting or diarrhea. Other gastrointestinal signs such as hypersalivation, abdominal pain, flatulence, and bloat may be seen but are less common (Gwaltney-Brant *et al*., 2000). Treatment of the clinical patient is largely symptomatic and supportive. The specific serotonin antagonist, cyproheptadine, mediates the effects of serotonin syndrome. The drug can be given orally or crushed, mixed with water or saline, and administered rectally (Gwaltney-Brant *et al*., 2000; APCC, 2006).

## Glycine

Like serotonin, glycine is also an amino acid neurotransmitter. It is synthesized from serine, a reaction catalyzed by serine hydroxymethyltransferase. Glycine acts predominantly in interneurons (Renshaw cells) of the brainstem

and spinal cord as well as in spinal sensory, auditory, and visual pathways where it effects inhibitory functions. Two well-known toxicants, tetanus and strychnine, act by inhibiting glycine's inhibitory effects. Tetanospasmin, a biotoxin produced by the anerobic bacterium, *Clostridium tetani*, is responsible for the prevention of the $Ca^{2+}$-dependent release of glycine from CNS neurons in victims of tetanus (Spencer, 2000; Roder, 2004b). The lack of inhibition by glycine results in unchecked muscle contraction, largely of the powerful extensor muscles of the limbs and the masseter muscles. The stereotypical sawhorse stance and "lockjaw" result within 5–10 days of wound infection. Less severe signs include elevation of the nictitating membrane, which may be the earliest indication of toxicosis in the horse, the most sensitive species (Coleman, 1998). Contracture of facial muscles may result in abnormal wrinkles in the skin, erect ears, or an abnormal expression, sometimes referred to as a sardonic grin. Progression of the disease results in an inability to rise and possible seizures. Severe muscular contraction or seizures may be induced by external stimuli. Death is due to an inability of the muscles of respiration to relax and subsequent hypoxia (Roder, 2004b).

Diagnosis of tetanus is based on characteristic clinical signs, history of a wound in which the organism could have proliferated, and anerobic culture of an infected wound. Treatment includes penicillin, antitoxin administration, and debridement of the wound. Like botulinum toxin, the tetanus antitoxin is ineffective for toxin that is already bound and will not reverse existing clinical signs. Its administration can be repeated in 7–10 days. The dose for horses and cattle is 10,000–50,000 units SC or IM and 3000–15,000 units for sheep and swine (Roder, 2004b). Contrary to popular belief, the antitoxin may be extremely cost effective. At the time of this writing, the Colorado Serum Company (Denver, CO) sells 15,000 units for $12.95 (US) (www.colorado-serum.com). Muscle spasms and seizure activity can be managed by minimization of external stimuli as well as with tranquilizers and muscle relaxants such as acepromazine, diazepam, barbiturates, and methocarbamol (Coleman, 1998). The prognosis for severely affected individuals is poor.

Another antagonist of glycine is the neurotoxicant, strychnine. As mentioned earlier in this chapter, it is an alkaloid derived from the *Strychnos nux vomica* and *S. ignatii* trees. It binds with high affinity to the glycine receptor and blocks its effects as a result. Extreme muscle rigidity occurs as in the tetanus-intoxicated patient and can rapidly progress to intermittent or continuous seizures within 10–120 min of strychnine ingestion. Anxiety, apprehension, nervousness, and tachypnea also may be part of the clinical presentation due to this rapid progression. Death occurs due to hypoxia as a result of contracture of the diaphragm and abdominal and intercostal muscles. The oral $LD_{50}$ is 0.5–1.2 mg/kg for dogs; 0.5 mg/kg for horses and cows; 2 mg/kg for cats; and 0.5–1 mg/kg for pigs (Talcott, 2006). In the exposed but asymptomatic patient, aggressive decontamination is warranted. Treatment of the symptomatic patient is limited to symptomatic and supportive care. The agents used to treat muscle spasms, seizures, and anxiety in the tetanic patient may also be used in the strychnine-intoxicated patient. The prognosis for symptomatic patients is grave.

## GABA

GABA serves as the predominant inhibitory neurotransmitter in the brain of mammals and is synthesized from glutamic acid. Two main receptor subtypes, $GABA_A$ and $GABA_B$, exist. $GABA_A$ has atleast seven subunits ($\alpha$, $\beta$, $\gamma$, $\delta$, $\varepsilon$, $\theta$, and $\rho$) which combine with a chloride channel to form a receptor/ionophore complex. The complex contains binding sites not only for GABA but also for drugs such as the anticonvulsant benzodiazepines and barbiturates. Stimulation of these receptors results in CNS depression, somnolence, fatigue, lethargy, ataxia, and muscular incoordination. Paradoxical hyperactivity, excitement, or aggression can also occur. In overdoses, CNS and respiratory depression may be extreme leading to hypotension, hypoxia, hypothermia, and death (Rudolph *et al.*, 2001; Spencer, 2000).

Drugs of the avermectin class are commonly used in veterinary medicine for the prevention of heartworm disease (*Dirofilaria*) in dogs and cats and the treatment of endoparasitism (strongyles, ascarids, bots, threadworms, lungworms, stomach worms, and summer sores (*Habronema*, *Draschia* species) and ectoparasitism (*Sarcoptes*, *Demodex*). Examples of avermectins include ivermectin (Heartgard®), selamectin (Revolution®), milbemycin (Interceptor®), moxidectin, and abamectin. Their mechanism of action on target species was originally thought to be due to the enhanced release of GABA at pre-synaptic neurons, causing parasite paralysis and eventual death (Plumb, 2005). Mealey (2006), however, reports more recent evidence that suggests the target effects to be mediated via glutamate-gated chloride channels in the CNS. Such channels are not present in the mammalian brain, allowing for a wide margin of safety of these drugs in the host animal. P-glycoprotein, part of the blood–brain barrier, is present in the apical membrane of brain capillary epithelial cells, and serves as an efflux pump to remove certain drugs from the brain. In addition to the avermectins, other such drugs include loperamide (Imodium®), ondansetron (a $5\text{-}HT_3$ antagonist), digoxin, quinidine, tacrolimus (a macrolide immunosuppressant), cyclosporine A, doxorubicin, and the *Vinca* alkaloids (vincristine, vinblastine). In dogs with an MDR1 (multi-drug resistance) gene defect, classically the collie and other herding breeds, p-glycoprotein is not functional, allowing far greater concentrations of these drugs

to accumulate in the brain. Drugs such as cyclosporine A, ketoconazole, verapamil, and tamoxifen can inhibit p-glycoprotein action and can result in a toxicosis of substrate drugs even in an animal without a genetic p-glycoprotein defect (Mealey, 2006).

Once access to the GABAergic pathways in the brain is obtained, the clinical signs of an acute or chronic avermectin toxicosis in a p-glycoprotein deficient dog can be profound. They include ataxia, CNS depression (potentially to the point of coma), recumbency, disorientation, mydriasis/apparent blindness, muscle tremors, seizures, respiratory depression, hypothermia, bradycardia, hypoxia, and death. Signs may persist for weeks. The duration of effect is often difficult to predict based on the severity of signs. Extreme supportive care is indicated for the comatose patient. Assisted ventilation may be necessary. Repeated doses of activated charcoal can be of significant benefit due to the enterohepatic recirculation of avermectin drugs. If treated aggressively, severely affected dogs can recover without long-term sequelae (APCC, 2006; Mealey, 2006). The duration of therapy and intensity of supportive care required in such cases, however, is often a limiting factor in veterinary medicine. Confirmation of exposure can be accomplished by detecting the drug in the liver, adipose tissue, or blood. Because signs are due to concentrated amounts of the drug in the brain, serum, or plasma levels may not correlate well with clinical signs. The College of Veterinary Medicine at Washington State University can confirm the presence of the mutant MDR1 gene with polymerase chain reaction (PCR) analysis of a swab from a suspect dog's oral mucosa (Mealey, 2006). Otherwise, diagnosis is largely based on a history of exposure and consistent clinical signs.

## Glutamate/aspartate

The primary excitatory neurotransmitters of the brain are the non-essential amino acids, glutamate and aspartate. Their synthesis in the brain from glucose and other molecules is tightly regulated. The blood–brain barrier excludes excesses of these excitatory neurotransmitters except in areas of the hypothalamus (arcuate nucleus) and retina where acute cellular degeneration may occur as a result. Three main receptor subtypes exist for glutamate: AMPA (DL-α-amino-3-hydroxy-5-methyl-4-isoxazole), kainic acid (KA), and NMDA (*N*-methyl-D-aspartate) receptors. As previously mentioned (see *Neuronopathy* section), domoic acid is an example of a toxicant that stimulates kainate receptors, resulting in ASP (Jeffery *et al.*, 2004).

The potential role of glutamate and aspartate in the pathogenesis of yellowstar thistle intoxication in horses is not well defined (Spencer, 2000). The plant is known to contain both excitatory amino acids in addition to several unstable guaianolide sesquiterpene lactones which may degrade to the former to exert their neurotoxic effects (Burrows and Tyrl, 2001).

Ketamine, a dissociative anesthetic used commonly for induction of general anesthesia in veterinary medicine, blocks the open NMDA ion channel by binding to it. Seizures, due to elevation of CSF pressure, are a potential sequela of ketamine use at therapeutic doses. The anesthetic is a congener of phencyclidine (Plumb, 2005), also known as PCP or angel dust, which not only binds to NMDA receptor channels but also acts as an antagonist of nicotinic cholinergic receptors. Overdose with PCP can result in a variety of signs including delirium, aggression, coma, seizures, and death (Spencer, 2000).

## Histamine

Histamine is a physiologically active amine that is both a neurotransmitter as well as a mediator of peripheral effects relating to allergic reactions and gastric acid secretion. It is formed from the decarboxylation of L-histidine. The roles of histamine in the CNS are thought to include regulation of arousal, blood pressure, water metabolism, and nausea. Peripherally, histamine receptors are of two subtypes: $H_1$ and $H_2$. The former mediate allergic reactions in which histamine is released from the granules of mast cells, resulting in dilation and increased permeability of capillaries (wheal and flare reaction) and constriction of bronchial smooth muscle. The $H_2$ receptor stimulation increases gastric acid secretion (Beasley, 1999). Common $H_2$ blockers used in veterinary medicine to decrease gastric acidity include cimetidine, ranitidine, and famotidine (Plumb, 2005). Of the $H_2$ blockers, cimetidine is more likely to cross the blood–brain barrier. Adverse neurologic effects reported in people include confusion, slurred speech, flushing, sweating, dizziness, and delirium. Administration of these drugs may result in fever, which spontaneously resolves within 48–72 h after discontinuation. The mechanism is thought to be due to CNS receptor antagonism (Poisindex®, 2006).

Antagonists of $H_1$ receptors are commonly used to treat acute and chronic allergic reactions, to prevent mast cell degranulation during surgical excision, as well as to minimize motion sickness in veterinary and human medicine. They are divided into first- and second-generation antagonists. The first-generation antihistamines can both stimulate and depress the CNS while the second-generation antihistamines are considered non-sedating due to their exclusion from the brain at therapeutic dosages (Murphy, 2006). $H_1$ antihistamines can be further divided into piperazine, ethanolamine, ethylenediamine, propylamine, and piperidine derivatives. Examples of the piperazine derivative antihistamines include the first-generation hydroxyzine HCl (Atarax®) and meclizine HCl (Dramamine®, Bonine®) and the second-generation piperazine-derivative

antihistamines, cetirizine HCl (Zyrtec®). The first-generation ethanolamine-derivative antihistamines include diphenydramine (Benadryl®), dimenhydrinate (Dramamine®), and clemastine (Tavist®). Ethanolamine-derivative antihistamines are strictly first generation and include pyrilamine and tripelennamine (Pelamine®). The first-generation propylamine-derivative antihistamines include brompheniramine maleate (Dimetapp®) and chlorpheniramine maleate (Chlor-trimeton®). The second generation includes tripolidine HCl (Actifed®). The final class, piperidine-derivative antihistamines, are entirely second generation and include drugs such as loratadine (Claritin®), astemizole, and levocabastine HCl. Other antihistamines that do not fall neatly into the aforementioned categories include fexofenadine (second generation) and cyproheptadine (first generation). At therapeutic doses and with mild overdosages, particularly with the first-generation antihistamines, CNS depression and hypotension may be seen. Epinephrine is contraindicated for management of hypotension in these patients. Paradoxical CNS excitation including restlessness, hyperactivity, tachycardia, tremors, and seizures may also occur and the potential for it varies largely with the individual. Large overdoses can result in extreme CNS and cardiovascular (CV) stimulation to the point of seizure activity, cardiac arrhythmias, hypertension, and death. Seizures, tremors, and hyperactivity may be treated with diazepam (APCC, 2006; Murphy, 2006).

Scombrotoxism, the clinical toxicosis caused by ingestion of poorly preserved scombroid fish (tuna, mackerel, bonito, anchovies, sardines), results from bacterial production of histamine. Histidine-rich muscle is decarboxylated with the enzyme histidine decarboxylase to form histamine and imparts a peppery taste to the fish. Clinical signs include vasodilation, abdominal pain, nausea, vomiting, diarrhea, generalized erythema and urticaria, intense pruritus, tachycardia, bronchoconstriction, respiratory distress, shock, and possible death. Treatment includes antihistamines that are both $H_1$ and $H_2$ receptor antagonists (Mebs, 1995; Ludolph and Spencer, 2000).

## Mixed neurotransmitter effects

Many agents exert their effects via multiple neurotransmitters or pathways. Tricyclic antidepressants such as amitriptyline (Elavil®) and clomipramine (Clomicalm®) are structurally similar to phenothiazines and exert their effects via blocking the reuptake of norepinephrine and serotonin (Plumb, 2005). Inhibitors of MAOs (selegeline, Anipryl®), used in veterinary medicine for cognitive dysfunction, prevent the metabolism of neurotransmitters (Spencer, 2000; Plumb, 2005).

Accidental overdosage of certain human prescription drugs can result in excessive norepinephrine and serotonin effects. Venlafaxine (Effexor®), a bicyclic antidepressant, e.g. blocks the reuptake of norepinephrine and serotonin and to a lesser extent, that of dopamine. Neurologic effects from overdoses include depression, lethargy, tremors, vocalization, disorientation, mydriasis, ataxia, seizures, hyperactivity, and death. Other possible effects include tachypnea, tachycardia, hypersalivation, and retching. Treatment is symptomatic and supportive. Cyproheptadine may help alleviate signs (see *Serotonin* section; APCC, 2006).

**TABLE 9.2 Ergot alkaloids (Evans *et al.*, 2004)**

| Ergopeptine alkaloids | Ergoline alkaloids |
|---|---|
| Ergotamine | Lysergic acid |
| Ergocristine | Lysergol |
| Ergosine | Lysergic acid amide |
| Ergocrytpine | Ergonovine |
| Ergocornine | |
| Ergovaline | |

Another neurotoxicosis that involves multiple neurotransmitters is that induced by the ergot alkaloids produced by fungi of the *Claviceps* genus. The mycotoxins can be further divided into ergopeptine and ergoline alkaloids (Table 9.2). The alkaloids exert their toxic effects via effects on dopaminergic, serotonergic, and adrenergic pathways. Successful recovery is aimed at early diagnosis, when signs such as ataxia and confusion are reversible, and removal of the contaminated feed source (Evans *et al.*, 2004). The effects of the ergoline alkaloids, in particular, can also be seen with ingestion of the seeds of *Ipomoea* or morning glory and are very similar to those of the related street drug, lysergic acid diethylamide, also known as LSD (Burrows and Tyrl, 2001).

$\alpha$-latrotoxin, the toxin in the venom of the black widow spider (*Latrodectus*), has mixed neurotransmitter effects. Binding of the toxin to neurexins and $Ca^{2+}$-*i*ndependent *r*eceptor for $\alpha$-*l*atrotoxin (CIRL; latrophilin) results in the release of catecholamines and also ACh, GABA, and glutamate. The toxin mediates its catecholamine effects by forming a transmembrane pore through which $Ca^{2+}$ can influx, allowing release of pre-synaptic vesicles containing neurotransmitters. Clinical signs associated with a bite from *Latrodectus* species include abdominal pain, ataxia, muscle fasciculations, muscle rigidity, and flaccid paralysis, which can ascend to involve the respiratory muscles. Cats are more sensitive than dogs to the effects of $\alpha$-*l*atrotoxin, but all mammalian species are susceptible. Antivenin is commercially available for black widow spider bite victims. Like all products derived from equine serum, a test dose should be administered intradermally to determine if hypersensitivity to the product exists. If none exists, the contents of the antivenin vial (2.5 ml) can be diluted 4–20 $\times$ (10–50 ml) with sterile saline and administered slowly intravenously over approximately 15 min (Roder, 2004d; PDR®,

2006). A slow, IV injection of 10% calcium gluconate may be given to treat the muscle fasciculations and weakness as well. Aggressive pain management with opioids or benzodiazepines is indicated (Roder, 2004d).

## ION CHANNELS

The propagation of an impulse along an axon depends on an electrochemical gradient that is intricately regulated by various ion channels in an excitable membrane. Initiation of action potentials, release of neurotransmitters, axonal transport, and healthy muscle activity rely on control of ions such as sodium, potassium, chloride, and calcium (Spencer, 2000). Specific examples follow regarding the variety of natural and synthetic toxicants that target the ion channels of the nervous system.

### Sodium channels

The sodium channel of mammals is made up of three protein subunits, the largest of which is the transmembrane alpha ($\alpha$) subunit which contains the $Na^+$ pore and which is flanked by two smaller $\beta$ subunits. The pores are voltage-gated, allowing sodium ion entrance into the cytoplasm only when charge-dependent conformational changes occur. Nature provides us with several examples of neurotoxicants which affect the sodium channel. The TTX present in various puffer fish (*Tetraodon, Fugu, Lagocephalus, Sphaeroides, Arothon, Chelonodon, and Amblyrhynchotes* species), and in the venom of the blue-ringed octopus (*Hapalochlaena maculosa* and *H. lunulata*), among many other creatures, exhibits a profound binding affinity for the sodium channel peptide complex. Whereas the $Na^+$ ion reversibly binds to peptide complex for just nanoseconds, TTX enters, binds to the external surface of the peptide ion channel, and remains bound for tens of seconds, an eternity with respect to nerve conduction, halting the action potential. Four grades of TTX poisoning have been described in humans. The first grade, evident within minutes, involves numbness around the mouth with or without nausea. The second involves numbness of the tongue, face, and skin and early motor paralysis and incoordination manifested as slurred speech. The third grade involves more widespread paralysis resulting in dyspnea, hypotension, the inability to speak, and fixed and dilated pupils. The final grade is characterized by severe paralysis involving the respiratory muscles, hypoxia, hypotension, and cardiac arrhythmias. Consciousness may be lost followed by death due to respiratory failure as early as 17 min after ingestion but up to 24 h. Treatment is aimed at decontamination and symptomatic and supportive care (Kaku and Meier, 1995).

Saxitoxin, the causative toxin for paralytic shellfish poisoning (PSP), is produced by several dinoflagellates, most notably *Alexandrium* and *Pyrodinium* species, and causes a similar clinical picture as that of TTX toxicosis also due to its binding to the sodium ion channel (Smart, 1995).

Local anesthetics exert their pharmacologic effects by binding to the fast sodium channel complex when it is inactive, inhibiting its recovery after depolarization. Lidocaine, as an example, rapidly dissociates from the sodium channel complex and is also rapidly metabolized in the liver. Consequently, adverse events associated with therapeutic dosages are rare. In an overdose situation, however, CNS depression, ataxia, seizures, and circulatory collapse can occur (Welch, 2000; Plumb, 2005). Dibucaine HCl is 10 times more potent than lidocaine and much more likely to result in adverse effects as a result. Cats are especially susceptible to the methemoglobinemic potential of local anesthetics (Welch, 2000).

Several toxins affect the sodium ion channel by enhancing its activity, resulting in repetitive and prolonged neurotransmission. Brevetoxins, produced by the dinoflagellates *Karenia brevis* and *Gymnodinium breve*, prolong the opening of the sodium ion channels, perpetuating the propagation of the action potential, particularly in the parasympathetic and adrenergic nerves. Bossart *et al*. (1998) implicated the toxin in the death of a minimum of 149 manatees along the southwest coast of Florida in 1996. Ciguatoxins are produced by dinoflagellates and are bioaccumulated by herbivorous, and then piscivorous, fish. They mediate their toxic effect by enhancing $Na^+$ permeability through the ion channel (Spencer, 2000). The hallmark clinical sign of ciguatera fish poisoning (CFP) in people is a paresthesia (tingling sensation) in the extremities as well as a perception of heat with cold stimuli ("dry-ice phenomenon") (Kaplan, 2000). The neurologic signs are attributable to edema of the adaxonal Schwann cell cytoplasm and can persist for weeks. Gastrointestinal pain and diarrhea are also typical, particularly in the early course of the disease (Glaziou *et al*., 1995; Spencer, 2000).

Other examples of toxicants that block inactivation of sodium channels include batrachotoxin, a steroid alkaloid isolated from the skin of *Phyllobates aurotaenia*, a South American frog (Spencer, 2000); grayanotoxins, diterpene alkaloids from the plants of the *Rhododendron, Kalmia, Pieris, Leucothoe*, and *Lyonia* genera (Puschner, 2004); aconitine, present in monkshood (*Aconitum* species); and veratridine from *Veratrum album* (Spencer, 2000). The $\alpha$ scorpion toxins stabilize $Na^+$ channels in the open state and allow neuronal hyperexcitation. The $\beta$ scorpion toxins bind to a different site in the complex and allow repetitive firing. Toxins of the Sydney funnel web spider (*Atrax robustus* and *Hadronyche* species; $\delta$-atracotoxin, atraxotoxin, robustoxin) also block sodium channel inactivation (White *et al*., 1995; Spencer, 2000).

TABLE 9.3 Representative pyrethrins and pyrethroids (Volmer, 2004)

| Pyrethrins | Type 1 pyrethroids | Type II pyrethroids |
|---|---|---|
| Pyrethrin I | Allethrin | Cyfluthrin |
| Pyrethrin II | Bifenthrin | Cyhalothrin |
| Cinerin I | Permethrin | Cypermethrin |
| Cinerin II | Phenothrin | Deltamethrin |
| Jasmolin I | Resmethrin | Fenvalerate |
| Jasmolin II | Sumithrin | Flumethrin |
| | Tefluthrin | Fluvalinate |
| | Tetramethrin | Tralomethrin |

Pyrethrins, natural organic esters isolated from the flower of the *Chrysanthemum cinerariaefolium*, have natural insecticidal properties as a result of their ability to bind to $Na^+$ ion channels and slow their inactivation. The lack of environmental contamination and relative wide margin of safety of pyrethrins to mammals are advantages of these compounds. One disadvantage of the natural compounds is their relatively short duration of action, resulting in a quick knock down effect of the insect followed by recovery. Synthetic pyrethroids were developed as of 1977 to address this issue. Table 9.3 lists both natural and synthetic (pyrethroids) pyrethrins. Type I pyrethroids do not have a cyano group in the $\alpha$ position of the molecule, a characteristic of type II pyrethroids which affords the latter greater insecticidal potency. Type I compounds cause repetitive firing of the affected nerves whereas type II pyrethroids are associated with membrane depolarization. Because of their relatively rapid metabolism, pyrethrins and pyrethroids are often marketed with synergists such as MGK-264 or piperonyl butoxide to inhibit enzymes which degrade them (mixed function oxidase and esterase), thereby prolonging their insecticidal action (Volmer, 2004).

Fish and cats represent the most susceptible animals to pyrethroids. Fish typically die acutely when exposed to pyrethroids. Cats are most commonly presented to the veterinarian as a result of exposure to the highly concentrated (45–65%) spot-on permethrin products labeled for use in dogs. Within 12–18 h of application or accidental exposure, affected cats will exhibit hyperesthesia, generalized tremors, hyperthermia, seizures, and possible death. The prognosis for complete recovery, however, is excellent provided that appropriate care is administered including control of tremors with IV methocarbamol, decontamination with a warm bath using a liquid dishwashing detergent, thermoregulation, seizure management (with diazepam, barbiturates, propofol, and/or inhalant anesthetics), and supportive care. The body temperature may become critically elevated with continued tremors and seizures or subnormal following a bath. Secondary adverse effects due to hyperthermia and enhanced toxicosis of pyrethroids at lower body temperatures may complicate the clinical course and should be avoided. Other species may have dermal hypersensitivity reactions or local paresthesia effects from topical application of pyrethroids. Steady resolution with this clinical picture is expected once the dermal residue is removed with a bath and liquid dishwashing detergent (Volmer, 2004; Hansen, 2006).

## Potassium channels

Potassium channels are largely involved with repolarization of the neuron following $Na^+$-induced depolarization. The opening of these potassium channels is voltage dependent and allows $K^+$ to leave the cell, thereby restoring the resting potential of the axonal membrane. Several subtypes of potassium channels exist, the details of which exceed the scope of this text. Neurotoxicants that block $K^+$ channels include 4-aminopyridine (Avitrol®), quinidine (derived from the *Cinchona* tree; class 1A antiarrhythmic), PCP ("angel dust"), mast cell degranulating peptide in the venom of the bee, *Apis mellifera*; venom of the gaboon viper, *Bitis gabonica*; and leuirotoxin 1 from the scorpion *Leuirus quinquestriatus hebraeus*. Neurologic clinical signs of potassium channel blockage include rapid onset of seizures progressing to death as in intoxication with Avitrol® (Schell, 2004); depression, confusion, and seizures with quinidine overdose (Plumb, 2005); and pain, urticaria, and erythema as a result of an *Apis mellifera* sting. Because of the array of potassium channel functions and their various regulatory pathways (voltage, calcium, sodium, ATP, increased cell volume (Spencer, 2000)), the clinical effects of potassium channel blockade are widely varied.

## Chloride channels

As opposed to sodium and potassium channel neurotoxicants, relatively few toxicants have been identified that affect the chloride channel. Chloride ions can passively diffuse down their concentration gradient out of the neuron. Chloride ion channels regulate the entrance of chloride into the neuron and affect the membrane potential as a result. Normal resting membrane potential is $-70$ mV. Threshold potential, the potential at which an action potential is propagated, is roughly $-50$ mV. Membranes can be hyperpolarized by allowing entrance of negative chloride into the neuron and making it more difficult to reach threshold potential. Both benzodiazepines and barbiturates mediate their effects via $GABA_A$ receptors which are chloride ionophore complexes (Crystal and Schaumburg, 2000). Minute details regarding binding sites, duration, and frequency of chloride channel opening effected by barbiturates and benzodiazepines have been extensively studied (Hobbs *et al.*, 1996; Crystal and Schaumburg, 2000; Maytal and Shinnar, 2000), yet the exact mechanism remains unclear. Ultimately, the two classes of drug inhibit excitatory

neurotransmission by increasing chloride conductance into the neuron. This can occur both pre-synaptically or post-synaptically (Harrison *et al.*, 2006).

The use of potassium bromide for chronic management of idiopathic epilepsy in veterinary patients is based on the competition of the bromide ion with chloride ions for transport across cell membranes. The therapeutic action relies on hyperpolarization of the neuronal membrane and a decrease in the propagation of epileptic discharges. Bromide blood levels should be monitored routinely in treated animals and particularly in those animals exhibiting signs of bromide toxicity. Neurologic signs consistent with a bromide toxicosis include ataxia, tremors, and sedation to the point of stupor in veterinary species (Plumb, 2005). Headache, mood alterations, hallucinations, speech abnormalities, and visual disturbances have also been reported with human bromism (Spencer, 2000). Because bromide has a longer half-life than chloride, the latter is preferentially excreted by the kidney. In animals with a deficiency of dietary salt, the half-life of bromide is prolonged, enhancing the chances of neurotoxicity. Conversely, the epileptic patient with a high-dietary salt intake may have seizure activity that is poorly managed with potassium bromide treatment (Plumb, 2005).

## CONCLUSION

The health of the nervous system, and largely that of the individual, relies on the system's structural and functional integrity. From specialized nerve cells and anatomic structures to axonal transport, myelination, neurotransmitter synthesis, storage, release, binding, and degradation as well as the regulation of action potentials, maintenance of nervous system integrity is a complex task requiring significant energy expenditure. This functional complexity and structural specialization provide a plethora of targets for neurotoxicant action. The scope of this chapter precludes a detailed discussion of every known neurotoxicant. Many more mechanisms exist by which neurotoxicants exert their effects. Table 9.4 provides a more extensive, yet certainly not exhaustive, list of neurotoxicants grouped by mechanism or site of action, where known.

The extent to which a neurotoxicant exerts its effects depends on a variety of factors including species, age, genetics, underlying diseases, drug therapy, diet, stress, and concurrent toxicoses. Neurologic effects may be potentiated by a combination of two or more of these factors. Veterinary practitioners should be aware of these underlying susceptibilities and the most common mechanisms by which neurotoxicants act so that toxicoses may be avoided or appropriately diagnosed and successfully treated when they do occur.

**TABLE 9.4 Miscellaneous neurotoxicants arranged by mechanism of action (Beasley, 1999; Spencer, 2000; Burrows and Tyrl, 2001; Schulze, 2002; Plumlee, 2004)**

| |
|---|
| *Mediation by acetylcholine* |
| Amanita (muscarinic) |
| *Atropa belladonna* |
| Atropine |
| Botulism |
| Carbamates |
| *Datura* (jimsonweed) |
| Imidacloprid (nicotinic) |
| Kentucky coffee tree (*Gymnocladus dioica*) |
| Organophosphorates |
| Slaframine |
| *Producing acidosis* |
| Aspirin |
| Ethanol |
| Ethylene glycol |
| Metaldehyde |
| Methanol |
| *Stimulating cannabinoid receptors* |
| Marijuana (*Cannabis sativa*) |
| *Effect on chloride channel* |
| Avermectins |
| Picrotoxin (antagonizes GABAergic transmission) |
| Potassium bromide |
| *Mediation by GABA* |
| Avermectins (glutamate-gated ion channels in insects) |
| Baclofen (centrally acting skeletal muscle relaxant; *p*-chlorophenyl derivative of GABA) |
| Barbiturates |
| Benzodiazepines |
| Bicuculline (antagonist) |
| Fipronil (antagonist) |
| Flumazenil (antagonist) |
| *Cicuta*/water hemlock |
| Cyclodiene organochlorines (competitive inhibition) |
| *Mediation by glutamate/aspartate* |
| Domoic acid |
| +/− Yellowstar thistle (*Centaurea solstitialis*) |
| *Mediation by glycine* |
| *Calycanthus* sp. (Bubby bush) |
| Strychnine |
| Tetanus |
| Tremorgenic mycotoxins (penitrem A and roquefortine) |
| *Producing hepatic encephalopathy* |
| Acetaminophen |
| Alsike clover (horses) |
| Blue-green algae |
| Cycad palms (*Cycas, Macrozamia species*) |
| *Lantana* |
| DL-methionine |
| Non-protein nitrogen |
| NSAIDs |
| Pyrrolizidine alkaloids – *Senecio, Crotolaria, Heliotroprium* (giant hog weed), *Amsinckia* (fiddle neck, tarweed), *Symphytum* (comfrey) |
| *Mediation by histamine* |
| Brompheniramine |
| Chlorpheniramine |
| Cetirizine |

(*Continued*)

**TABLE 9.4 (Continued)**

Cimetidine
Cyproheptadine
Dimenhydrinate
Diphenhydramine
Famotidine
Fexofenadine
Hydroxyzine
Loratadine
Ranitidine
Scombroid fish poisoning

*Causing hypoglycemia*
Cocklebur (*Xanthium*)
Xylitol

*Mediation by norepinephrine*
Albuterol (β adrenergic agonist)
Atipamezole (α adrenergic blocker)
Brimonidine (α adrenergic agonist)
Doxazosin (α adrenergic blocker)
Phentolamine (α adrenergic blocker)
Phenoxybenzamine (α adrenergic blocker)
Tamsulosin (α adrenergic blocker)
Terazosin (α adrenergic blocker)
Yohimbine (α adrenergic blocker)
Atomoxetine (norepinephrine reuptake inhibitor)
Metoprolol (β adrenergic blocker)
Propranolol (β adrenergic blocker)
TCAs
MAOIs
Venlafaxine (norepinephrine/dopamine reuptake inhibitor)

*Mediation of opioid receptors*
Butorphanol
Buprenorphine
Fentanyl
Naloxone
Loperamide
Morphine
Meperidine
Heroin
Hydromorphone
Oxymorphone
Codeine
Oxycodone
Tramadol
Horse chestnut (*Aesculus hippocastanum*)

*Effect on potassium channels*
4-Aminopyridine
Organochlorines (inhibit $K^+$ efflux)

*Mediation by serotonin*
Canary grass poisoning
*Griffonia*/5-HTP supplements
*Phalaris* staggers
*Psilocybe* mushrooms
SSRIs
Tryptamine alkaloids

*Effect on sodium channels*
Ciguatoxin
Local anesthetics
Organochlorines (inhibit $Na^+$ influx)
Pyrethroids/permethrin
Saxitoxin
Sodium intoxication
Tetrodotoxin

*Sympathomimetics/catecholamines*
Amphetamines (bind to carrier)
Pseudoephedrine
Ma huang (*Ephedra*)
Phenylpropanolamine
Adderal® (amphetamine–dextroamphetamine)
Cocaine
MDMA/"Ecstasy"
Tricyclic antidepressants (inhibit transport)
Monoamine oxidase inhibitors (MAOIs)
+/− yellowstar thistle (*Centaurea solstitialis*)

*Thiamine deficiency/thiaminase excess*
*Equisetum*
*Pteridium* species (Bracken fern)
Raw fish diet
Excess dietary sulfur

*Uncoupler of oxidative phosphorylation*
Bromethalin
Trialkyltin
Hexachlorophene

*Multiple/miscellaneous MOAs*
*Astragalus/Oxytropis* (locoweed; miserotoxin – causes demyelination of the posterior spinal cord resulting in incoordination, hypermetria, ataxia, and clicking of the dewclaws – "cracker heels"; swainsonine – affected animals exhibit ataxia, emaciation, staggering, and proprioceptive deficits, belligerence or violent startling in horses)
Bovine bonkers (ammoniated feed)/4-methylimidazole
Cycad palm (*Cycas* species: β-*N*-methylamino-L-alanine; *Macrozamia* species: β-*N*-oxalylamino-L-alanine)
5-Fluorouracil/1080 (antimetabolite antineoplastic agent; forms suicide complex with thymidine synthetase)
Lead
Mercury
Methylxanthines, *Guarana* (adenosine receptor antagonists)
Sudan grass (β cyanoalanine)
Phenothiazines (anticholinergic, antihistaminic, α adrenergic blocking effects)
Yesterday, today, and tomorrow plant (*Brunfelsia*; brunfelsamidine)

*Unknown etiology/MOA*
Tick paralysis
Macadamia nuts
Diethyltoluamide (DEET)
Nitrofuran
Desert spike
Sleepy grass
White snakeroot
Essential oils
Hydrocarbons
Indoxacarb

*Metabolic*
Carbon monoxide
Cyanide
Zinc phosphide

# REFERENCES


Anthony DC, Montine TJ, Valentine WM, Graham DG (2001) Toxic responses of the nervous system. In *Casarett & Doull's Toxicology: The Basic Science of Poisons*, 6th edn, Klaassen CD (ed.). McGraw-Hill, New York, pp. 535–63.

ASPCA Animal Poison Control Center (APCC) (2006) Unpublished data, Urbana, IL.

Atchison WD (2005) Is chemical neurotransmission altered specifically during methylmercury-induced cerebellar dysfunction? *Trends Pharmacol Sci* **26**(11): 549–57.

Bailey EM (2006) Botulinum. In *Small Animal Toxicology*, 2nd edn, Peterson ME, Talcott PA (eds). Elsevier, Inc., St. Louis, pp. 603–8.

Beasley VR (1999) *Veterinary Toxicology*. International Veterinary Information Service, www.ivis.org/advances/Beasley/toc.asp.

Blodgett DJ (2006) Organophosphorus and carbamate insecticides. In *Small Animal Toxicology*, 2nd edn, Peterson ME, Talcott PA (eds). Elsevier, Inc., St. Louis, p. 941.

Bossart GD, Baden DG, Ewing RY, Roberts B, Wright SD (1998) Brevetoxicosis in manatees (*Trichechus manatus latirostris*) from the 1996 epizootic: gross, histologic, and immunohistochemical features. *Toxicol Pathol* **26**(2): 276–82.

Burrows GE, Tyrl RJ (2001) *Toxic Plants of North America*. Iowa State University Press, Ames.

Capen CC (2001) Toxic responses of the endocrine system. In *Casarett & Doull's Toxicology: The Basic Science of Poisons*, 6th edn, Klaassen CD (ed.). McGraw-Hill, New York, pp. 711–59.

Coleman ES (1998) Clostridial neurotoxins: tetanus and botulism. *Compend Contin Educ Pract Vet* **20**(10): 1089–97.

Crystal HA, Schaumburg HH (2000) Benzodiazepines. In *Experimental and Clinical Neurotoxicology*, 2nd edn, Spencer PS, Schaumburg HH (eds). Oxford University Press, New York, pp. 231–5.

Damodaran TV, Abdel-Rahman A, Abou-Donia MB (2001) Altered time course of mRNA expression of alpha tubulin in the central nervous system of hens treated with diisopropyl phosphofluoridate (DFP). *Neurochem Res* **26**(1): 43–50.

Dorman D (2004) Bromethalin. In *Veterinary Clinical Toxicology*. Plumlee KH (ed.). Mosby Inc., St. Louis, pp. 446–8.

Easton N, Marsden CA (2006) Ecstasy: Are animal data consistent between species and can they translate to humans? *J Psychopharmacol* **20**(2): 194–210.

Evans TJ, Rottinghaus GE, Casteel SW (2004) Ergot. In *Veterinary Clinical Toxicology*, Plumlee KH (ed.). Mosby Inc., St. Louis, pp. 239–43.

Francis BM (1994) *Toxic Substances in the Environment*. John Wiley & Sons, Inc., New York.

Glaziou P, Chinain M, Legrand AM (1995) Clinical toxicology of ciguatera poisoning. In *Handbook of Clinical Toxicology of Animal Venoms and Poisons*, Meier J, White J (eds). CRC Press, Inc., Boca Raton, pp. 59–74.

Gouzoulis-Mayfrank E, Daumann J (2006) Neurotoxicity of methylenedioxyamphetamines (MDMA; ecstasy) in humans: How strong is the evidence for persistent brain damage? *Addiction* **101**(3): 348–61.

Gwaltney-Brant S (2004) Lead. In *Veterinary Clinical Toxicology*, Plumlee KH (ed.). Mosby Inc., St. Louis, pp. 204–10.

Gwaltney-Brant SM, Albretsen JC, Khan SA (2000) 5-Hydroxytryptophan toxicosis in dogs: 21 cases (1989–1999). *J Am Vet Med Assoc* **216**(12): 1937–40.

Gupta BS, Baldwa S, Verma S, Gupta JB, Singhal A (2000) Metronidazole induced neuropathy. *Neurol India* **48**(2): 192–3.

Hansen SR (2006) Pyrethrins and pyrethroids. In *Small Animal Toxicology*, 2nd edn, Peterson ME, Talcott PA (eds). Elsevier, Inc., St. Louis, pp. 1002–10.

Harrison N, Mendelson WB, de Wit H (2006) *Psychopharmacology: The 4th Generation of Progress*. http://www.acnp.org/G4/GN401000173/CH169.html

Hobbs WR, Rall TW, Verdoorn TA (1996) Hypnotics and Sedatives; Ethanol. In *Goodman & Gilman's The Pharmacological Basis of Therapeutics*, 9th edn, Hardman JG, Limbird LE (eds). McGraw-Hill, New York, pp. 361–96.

Jeffery B, Barlow T, Moizer K, Paul S, Boyle C (2004) Amnesic shellfish poison. Food *Chem Toxicol* **42**(4): 545–57.

Kaku N, Meier J (1995) Clinical toxicology of fugu poisoning. In *Handbook of Clinical Toxicology of Animal Venoms and Poisons*, Meier J, White J (eds). CRC Press, Inc., Boca Raton, pp. 75–83.

Kaplan JG (2000) Ciguatoxin. In *Experimental and Clinical Neurotoxicology*, 2nd edn, Spencer PS, Schaumburg HH (eds). Oxford University Press, New York, pp. 386–90.

Kotsonis FN, Burdock GA, Flamm WG (2001) Food toxicology. In *Casarett & Doull's Toxicology: The Basic Science of Poisons*, 6th edn, Klaassen CD (ed.). McGraw-Hill, New York, pp. 1049–88.

Landsberg L, Young JB (2001) Pharmacology of the sympathoadrenal system. In *Harrison's Principles of Internal Medicine*, 15th edn, Braunwald MD (ed.). McGraw-Hill, New York, pp. 443–8.

Loeven KO (1994) Hepatic amyloidosis in two Chinese Shar Pei dogs. *J Am Vet Med Assoc* **204**(8): 1212-16.

Ludolph AC, Spencer PS (2000) Scombroid fish (histamine). In *Experimental and Clinical Neurotoxicology*, 2nd edn, Spencer PS, Schaumburg HH (eds). Oxford University Press, New York, p. 1096.

Mandella RC (2002) Applied neurotoxicology. In *Handbook of Toxicology*, 2nd edn, Derelanka MJ, Hollinger MA (eds). CRC Press, LLC, Boca Raton, pp. 371–99.

Maytal J, Shinnar S (2000) Barbiturates. In *Experimental and Clinical Neurotoxicology*, 2nd edn, Spencer PS, Schaumburg HH (eds). Oxford University Press, New York, pp. 219–25.

Mealey KL (2006) Ivermectin: macrolide antiparasitic agents. In *Small Animal Toxicology*, 2nd edn, Peterson ME, Talcott PA (eds). Elsevier, Inc., St. Louis, pp. 785–94.

Mebs D (1995) Biology and distribution of poisonous marine animals. In *Handbook of Clinical Toxicology of Animal Venoms and Poisons*. Meier J, White J (eds). CRC Press, Inc., Boca Raton, pp. 27–31.

Meerdink GL (2004a) Slaframine. In *Clinical Veterinary Toxicology*, Plumlee KH (ed.). Mosby Inc., St. Louis, pp. 262–4.

Meerdink GL (2004b) Anticholinesterase insecticides. In *Clinical Veterinary Toxicology*, Plumlee KH (ed.). Mosby Inc., St. Louis, pp. 178–80.

Murphy L (2006) Antihistamine toxicosis. *Veterinary Medicine*, **96**(10). http://www.aspca.org/site/DocServer/toxbrief_1001.pdf?docID=124&AddInterest=1101 (accessed on May 26, 2006).

Panter KE (2004a) Piperidine alkaloids. In *Clinical Veterinary Toxicology*, Plumlee KH (ed.). Mosby Inc., St. Louis, pp. 365–8.

Panter KE (2004b) Pyridine alkaloids. In *Clinical Veterinary Toxicology*, Plumlee KH (ed.). Mosby Inc., St. Louis, pp. 369–70.

Panter KE (2004c) Quinolizidine alkaloids. In *Clinical Veterinary Toxicology*. Plumlee KH (ed.). Mosby Inc., St. Louis, pp. 377–379.

PDR® (2006) *Physicians' Desk Reference*, 60th edn. Thomson PDR, Montvale, NJ.

Pickrell JA, Oehme F, Mannala SA (2004) Tropane alkaloids. In *Clinical Veterinary Toxicology*, Plumlee KH (ed.). Mosby Inc., St. Louis, pp. 381–2.

Plumb DC (2005) *Veterinary Drug Handbook*, 5th edn. PharmaVet Inc., Stockholm.

Plumlee KH (2004) *Clinical Veterinary Toxicology*. Mosby Inc., St. Louis, 2004.

Poisindex® (2006) Cimetidine and related drugs. Thomson Micromedex® Healthcare Series, Volume 128, copyright 1974.

Puschner B (2004) Grayanotoxins. In *Clinical Veterinary Toxicology*, Plumlee KH (ed.). Mosby Inc., St. Louis, pp. 412–15.

Roder J (2004a) Antineoplastics. In *Clinical Veterinary Toxicology*, Plumlee KH (ed.). Mosby Inc., St. Louis, pp. 299–302.

Roder J (2004b) Bacteria. In *Clinical Veterinary Toxicology*, Plumlee KH (ed.). Mosby Inc., St. Louis, pp. 98–9.

Roder J (2004c) Blue-green algae. In *Clinical Veterinary Toxicology*, Plumlee KH (ed.). Mosby Inc., St. Louis, pp. 100–101.

Roder J (2004d) Spiders. In *Clinical Veterinary Toxicology*, Plumlee KH (ed.). Mosby Inc., St. Louis, pp. 111–12.

Rosendale M (2004) Bronchodilators. In *Clinical Veterinary Toxicology*, Plumlee KH (ed.). Mosby Inc., St. Louis, pp. 305–7.

Rudolph U, Crestani F, Mohler H (2001) $GABA_A$ receptor subtypes: dissecting their pharmacologic functions. *Trends Pharmacol Sci* **22**(4): 188-94.

Sanders SG, Tucker RL, Bagley RS, Gavin PR (2001) Magnetic resonance imaging features of equine nigropallidal encephalomalacia. *Vet Radiol Ultrasound* **42**(4): 291–6.

Schell MM (2004) 4-Aminopyridine. In *Clinical Veterinary Toxicology*, Plumlee KH (ed.). Mosby Inc., St. Louis, pp. 443–4.

Scheumack DD, Howden ME, Spence I, Quinn RJ (1978) Maculotoxin: a neurotoxin from the venom glands of the octopus *Hapalochlaena maculosa* identified as tetrodotoxin. *Science* **199**: 188–9.

Schulze GE (2002) Fundamental neurotoxicology. In *Handbook of Toxicology*, 2nd edn, Derelanka MJ, Hollinger MA (eds). CRC Press, LLC, Boca Raton, pp. 353–70.

Shin EJ, Suh SK, Lim YK, Jhoo WK, Hjelle OP, Ottersen OP, Shin CY, Ko KH, Kim WK, Kim DS, Chun W, Ali S, Kim HC (2005) Ascorbate attenuates trimethyltin-induced oxidative burden and neuronal degeneration in the rat hippocampus by maintaining glutathione homeostasis. *Neuroscience* **133**(3): 715–27.

Smart D (1995) Clinical toxicology of shellfish poisoning. In *Handbook of Clinical Toxicology of Animal Venoms and Poisons*, Meier J, White J (eds). CRC Press, Inc., Boca Raton, pp. 33–57.

Smith WE, Smith AM (1975) Minamata. Holt, Rinehart, and Winston, New York, NY.

Spencer PS (2000) Biological principles of chemical neurotoxicity. In *Experimental and Clinical Neurotoxicology*, 2nd edn, Spencer PS, Schaumburg HH (eds). Oxford University Press, New York, pp. 3–54.

Talcott PA (2006) Strychnine. In *Small Animal Toxicology*, 2nd edn, Peterson ME, Talcott AA (eds). Elsevier, Inc., St. Louis, pp. 1076–82.

Turner NJ, Szczawinski AF (1991) *Common Poisonous Plants and Mushrooms of North America*. Timber Press, Inc., Portland.

van den Munckhof P, Gilbert F, Chamberland M, Levesque D, Drouin J (2006) Striatal neuroadaptation and rescue of locomotor deficit by L-dopa in aphakia mice, a model of Parkinson's disease. *J Neurochem* **96**(1): 160–70.

van Lier RB, Cherry LD (1988) The toxicity and mechanism of action of bromethalin: a new single-feeding rodenticide. *Fundam Appl Toxicol* **11**(4): 664–72.

Volmer PA (2004) Pyrethrins and pyrethroids. In *Clinical Veterinary Toxicology*, Plumlee KH (ed.). Mosby Inc., St. Louis, pp. 188–90.

Welch SL (2000) Local anesthetic toxicosis. *Vet. Med* **95**(9). http://www.aspca.org/site/DocServer/toxbrief_0900.pdf?docID=122 (accessed on May 26, 2006).

White J (1995) Clinical toxicology of blue ringed octopus bites. In *Handbook of Clinical Toxicology of Animal Venoms and Poisons*. Meier J, White J (eds). CRC Press, Inc., Boca Raton, pp. 171–5.

White J, Cardoso JL, Fan HW (1995) Clinical Toxicology of Spider Bites. In *Handbook of Clinical Toxicology of Animal Venoms and Poisons*, Meier J, White J (eds). CRC Press, Inc., Boca Raton, pp. 259-329.

Wismer T (2004) Novel insecticides. In *Clinical Veterinary Toxicology*, Plumlee KH (ed.). Mosby Inc., St. Louis, pp. 183–6.

Woolf AD (1995) Ginger Jake and the blues: a tragic song of poisoning. *Vet Hum Toxicol* **37**(3): 252–4.

Zhao XL, Xie KQ, Han XY, Yu LH, Zhu ZP, Zhang TL, Zhang CL (2005) Effects of methamidophos on microtubule and microfilament proteins in sciatic nerve of hens. *Chinese J Occup Hyg Dis* **23**(2): 102–4.

CHAPTER

# 10

# Liver toxicity

*Karyn Bischoff and Shashi K. Ramaiah*

## INTRODUCTION

There is continued interest concerning the higher incidence of liver toxicosis caused by prescription/injectable drugs, over-the-counter medications, or dietary supplements that are often combined with special diets and alcohol consumption, in addition to environmental chemicals/xenobiotics (Watkins and Seeff, 2006). Though alcohol consumption is not a major risk factor in most veterinary patients, other factors such as toxic pasture or house plants, cyanobacteria, pollutants, fungal toxins, and household waste may come into play. An incomplete list of hepatotoxins is found in Table 10.1. Complicating matters is the increasing population of geriatric veterinary patients, preexisting liver disease must be considered with the patient exposed to liver toxicants.

The liver is a remarkable organ that usually protects the individual against injury from foreign substances. It is very robust in its capacity to regenerate and repair the damage (Ramaiah *et al.*, 2004; Mehendale, 2005). However, if the ability to regenerate is not adequate, or if injury to the liver is very severe, liver damage may progress to liver failure and death. Chemical/drug-induced liver injury has become the most frequent cause of acute liver failure in humans in the United States and around the world, exceeding all other causes combined (Watkins and Seeff, 2006). Toxic liver disease also remains the major single reason for regulatory actions concerning drugs. Such actions may include failure of approval, withdrawal from the market, restrictions on use, and warnings to physicians (Watkins and Seeff, 2006).

Xenobiotic-induced liver injury is encountered in a variety of circumstances. Some natural toxins such as the peptides of *Amanita phalloides*, the pyrrolizidine alkaloids, the toxin of the cycad nut, and other phytotoxins are hazards posed by the environment. They may be ingested by the curious or very hungry veterinary patient. Others, such as mycotoxins, are ingested unknowingly because of feed contamination due to climatic conditions favorable to fungal growth. A striking example of this phenomenon is the recent contamination of dog food with aflatoxin resulting in dozens of canine deaths in late 2005 and early 2006. Other circumstances of exposure to hepatotoxins in the home or farm include accidental food or feed contamination with industrial chemicals or pesticides, e.g. a human outbreak of hepatic porphyria in Turkey in 1955 caused by ingestion of wheat to which the fungistatic agent, hexachlorobenzene, had been added.

Research in the last decade has focused on understanding the different mechanisms for chemical-induced liver injury. Researchers have attempted to understand the molecular basis for injury and contribution of individual cell types to ultimate hepatic pathology. Recently, techniques such as microarray-based toxicogenomics, 2D gel electrophoresis-based proteomics and metabolomics, have attempted to further design novel hypotheses to test the molecular players involved in liver damage. Idiosyncratic drug reactions remain a mystery.

The goal of this chapter is to provide a basic understanding of the liver pathophysiology and general concepts of liver injury, and to expand on the commonly occurring drug/chemical toxicoses in veterinary medicine.

## STRUCTURAL AND FUNCTIONAL ORGANIZATION OF THE LIVER

The structural and functional organization of the liver has been described by the hepatic lobule and hepatic acinus

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**TABLE 10.1 Incomplete list of hepatotoxins of veterinary interest**

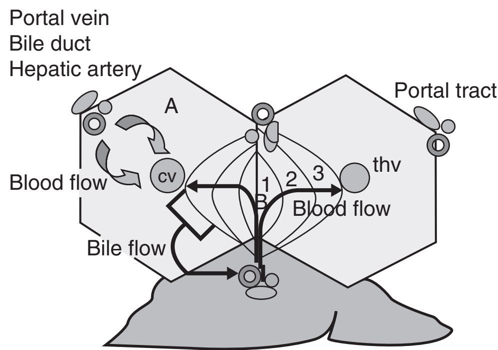
| Classification of mechanism | Toxicant | Source of toxicant | Source classification | Species affected |
|---|---|---|---|---|
| Free radical | Carbon tetrachloride | Cleaning agents | Industrial | All |
| | Yellow phosphorus | Fireworks | Industrial | All |
| | Iron | Dietary supplement | Household | All |
| | Copper | Dietary supplement | Feed, industrial | Sheep, some dog breeds |
| Electrophile | Aflatoxins | *Aspergillus* spp. | Mycotoxin | All |
| | Acetaminophen | NSAID | Household | All, cats |
| | Sesquiterpene lactones | *Helenium* spp., *Hymenoxis* spp. | Plant | Herbivores |
| Biliary obstruction | Sporodesmin | *Pithomyces chartarum* | Mycotoxin | Herbivores, ruminants |
| | | *Tribulus terrestris* | Plant | Herbivores, ruminants |
| | | *Panicum* spp. | Plant | Herbivores, ruminants |
| | | *Agave lecegilla* | Plant | Herbivores, ruminants |
| | | *Nolina texana* | Plant | Herbivores, ruminants |
| Other | Pyrrolizidine alkaloids | *Amsinkia* spp.<br>*Crotolaria* spp.<br>*Cynoglossum* spp.<br>*Echium* spp.<br>*Erechtites glomerulata*<br>*Senecio* spp. | Plant | Herbivores |
| | Glycosides of methylazocymethanol | *Cycas* spp. | Plant | Dogs |
| | Carboxyatractyloside | *Xanthium* spp. | Plant | Swine, herbivores |
| | Triterpenes | *Lantana* spp. | Plant | Herbivores |
| | Amatoxins | *Amanita* spp. | Mushroom | All |
| | Microcystins | *Microcystis* spp. | Cyanobacteria | All |
| | Nodularins | *Nodularia* spp. | Cyanobacteria | All |
| | Phenolics and coal tar derivatives | Disinfectants, clay pigeons | Household | All |
| Idiosyncratic | Sulfonamides | Antimicrobial | Drug | Dogs |
| | Carprofen | Anti-inflammatory | Drug | Dogs |
| | Diazepam | Sedative | Drug | Cats |

models. The hepatic lobule is defined histologically as a hexagonal region of parenchyma surrounding the central vein. Six portal triads, composed of branches from the portal vein and hepatic artery as well as bile ductules, border the edge of the lobule. One-cell-thick plates or cords of hepatocytes are arranged radially around the central vein and blood sinusoids form between them. The hepatic parenchyma is divided into three zones based on proximity to the central vein. The area around the central vein is termed *centrilobular*, the area closer to the portal triads is *periportal*, and the area between the centrilobular and periportal parenchyma is termed *midzonal*.

Alternately, Rappaport defined the hepatic acinus as the smallest structural and functional unit in the liver based on the microcirculation in the liver. The simple hepatic acinus is defined as a parenchymal mass organized around the portal triad (Figure 10.1). Within the acinus, blood drains from the portal triad through the sinusoids into the central hepatic vein. The cells of the acinus are arbitrarily divided into three zones: *zone 1* corresponds to the periportal zone, *zone 2* corresponds to the midzonal parenchyma, and *zone 3* corresponds to the centrilobular zone. The blood supply is mainly from the portal tract vessels: 60–80% from branches of the portal vein supplying nutrients and toxins from the gastrointestinal tract, and 20–40% from the hepatic artery supplying oxygen (Treinen-Moslen, 2001). This blood is mixed in the penetrating vessels then enters the sinusoids. Blood flows sequentially through zones 1, 2, and 3 before draining via the terminal hepatic vein (central vein) (Figure 10.1). A consequent of this structure, hepatocytes in zone 1 receive blood that is 9–13% oxygenated whereas zone 3 is relatively hypoxic, the blood is 4–5% oxygenated (Treinen-Moslen, 2001). Blood entering zone 3 is relatively nutrient depleted (Sturgill and Lambert, 1997).

Hepatocyte stem cells, termed oval cells, are located in the canals of Hering where bile canaliculi from the hepatic cords converge on bile ductules of the portal triad. New hepatocytes progress down hepatic cords to replace the aging zone 3 hepatocytes.

Sinusoids, which are located between cords of hepatocytes, are larger than capillaries and lined by specialized




Comparative terminology for hepatic functional units

| Classic lobule (A) | Acinus concept (B) |
|---|---|
| Periportal, peripheral | Zone 1, periportal |
| Mid-zonal | Zone 2 |
| Central, centrilobular | Zone 3, perivenular |
| Panlobular | Panacinar |
| Multilobular | multiacinar |

Adopted from Veterinary Laboratory Medicine, Meyer and Hervey (eds), 2nd edition, 1998, p. 159, with permission

**FIGURE 10.1** Lobular versus acinar concept in liver architecture. The hepatic lobule is defined histologically as a hexagonal region of parenchyma surrounding the central vein. The blood flows from the portal vein and hepatic artery and mixed in the penetrating vessels, and enter the sinusoids. The hepatic parenchyma is divided into centrilobular, midzonal and periportal areas based on the proximity to the central vein. The acinus represents the functional unit in the liver wherein the parenchyma is organized around the portal triad and the triads represent the corners of the acinus triangle. In this concept, blood drains from the portal triad through the sinusoids into the central hepatic vein. The cells of the acinus are divided into zone 1 which corresponds to the periportal zone; zone 2 corresponds to the midzonal parenchyma, and zone 3 corresponds to the centrilobular zone.

endothelium. The endothelial lining is discontinuous and endothelial cells have fenestrae to allow movement of fluid and molecules less than 259 kDa (Watkins, 1999; Treinen-Moslen, 2001; Plumlee, 2004). This material enters the space of Disse, located between the endothelium and the hepatocytes. Within the space of Disse, hepatocytes contact free and protein bound molecules which may be absorbed by diffusion or active transport (Watkins, 1999).

The liver is a functionally diverse organ. Blood from the gastrointestinal tract, via the portal vein, is filtered for endotoxin, ammonia, other bacterial products, and xenobiotic (Treinen-Moslen, 2001; Plumlee, 2004). The liver is directly involved in nutrient homeostasis, including glucose regulation (Treinen-Moslen, 2001; Piñeiro-Carrero and Piñeiro, 2004); cholesterol synthesis and uptake (Treinen-Moslen, 2001); synthesis of proteins such as clotting factors, albumin, and very low-density lipoprotein (Treinen-Moslen, 2001; Plumlee, 2004); storage of glycogen, lipids, minerals, and vitamins (Plumlee, 2004); and metabolism and excretion of such things as hemoglobin breakdown products (Plumlee, 2004), steroid hormones (Brown, 2001), and drug metabolites.

There is remarkable metabolic diversity of the hepatic zones in order to accommodate its numerous functions. The liver is a predominant organ for the metabolism of xenobiotics, compounds that are foreign to the body. Hepatocytes of zone 3 in particular are rich in enzymes. These enzymes are divided into two main categories although phase III transporters are recently implicated in drug metabolism and transport (Xu *et al.*, 2005). Phase I enzymes are involved in oxidation, reduction, and hydrolysis reactions, and phase II enzymes are involved in conjugation reactions. These enzymes will be described in more detail later. Recently it has been shown that pretreatment with several inducers alter the expression of phase III transporters, alters the excretion of xenobiotics, and provides an important means to protect the body from xenobiotic insults. Zone 3 hepatocytes are involved in glycolysis and lipogenesis (Plumlee, 2004), and zone 1 hepatocytes are mitochondria rich (Treinen-Moslen, 2001; Plumlee, 2004). Functions of zone 1 hepatocytes include bile salt extraction, fatty acid oxidation, gluconeogenesis, and protein synthesis (Treinen-Moslen, 2001; Piñeiro-Carrero and Piñeiro, 2004; Plumlee, 2004). Zone 1 hepatocytes have the highest levels of glutathione (GSH) (Treinen-Moslen, 2001).

Bile secretion is a major function of the liver. Bile is composed of bile salts, bilirubin, GSH, phospholipids, cholesterol, proteins, organic anions, metals, and conjugated xenobiotics (Treinen-Moslen, 2001; Piñeiro-Carrero and Piñeiro, 2004). Bile salts and bilirubin enter bile canaliculi via active transport through hepatocyte membranes (Treinen-Moslen, 2001). Canaliculi are dynamic structures located between hepatocytes and formed by hepatocyte membranes (Treinen-Moslen, 2001; Plumlee, 2004). Energy-dependent transport exists for certain hormones, drugs, and other xenobiotics (Treinen-Moslen, 2001). These include a group of multiple drug resistance *p*-glycoproteins that transport lipophilic cationic drugs, estrogens, and phospholipids, and canalicular multiple organic anion transporters involved in movement of molecules conjugated to GSH, glucuronide, and sulfate (Treinen-Moslen, 2001). Metal and mineral transport is important for mineral homeostasis and occurs through facilitated diffusion and receptor-mediated endocytosis across the sinusoidal membrane. Lysosomes are involved in storage and export of metals and minerals into canaliculi (Treinen-Moslen, 2001).

Canaliculi enter canals of Hering in the portal triad and lead to intrahepatic bile ducts which coalesce to form the hepatic bile duct. Most species have a gall bladder for bile storage, but many that eat frequently lack a gall bladder, including pigeons, rats, deer. Ratites lack a gall bladder, as do many parrots. Horses and related species, the rhinoceroses and tapirs, lack a gall bladder, as do elephants, camels (New and Old World), and porcupines. Most carnivores have a gall bladder, the exception being dolphins and other cetaceans. Gall bladders may or may not be present in some species such as the giraffe and hippopotamus

(Oldham-Ott and Gilloteaux, 1997). The gall bladder stores and concentrates bile and then empties into the duodenum. Bile that is excreted into the small intestine enhances nutrient uptake, protects enterocytes from oxidation, and facilitates excretion of xenobiotics and endogenous waste in the feces (Treinen-Moslen, 2001).

Seven intrinsic cell types have been identified in the liver: the parenchymal cells or hepatocytes, and the oval cell or hepatocytic stem cells, both described above, and four types of non-parenchymal cells. As well as the endothelial cells lining sinusoids and bile duct epithelium, mentioned above, the non-parenchymal cells include the Kupffer cells, which are resident macrophages, the stellate cells, also called ito cells or fat storing cells, and the pit cells or large granular lymphocytes. In the rat, hepatocytes represent for about 60% of the total cell number and 80% of hepatic tissue volume. Non-parenchymal cells in the rat are estimated to constitute about 30% of total cellular population, but comprise only 6–7% of tissue volume due to their small size relative to hepatocytes (Jones, 1996).

Kupffer cells represent 80% of the fixed macrophages in the body (Treinen-Moslen, 2001). These cells are mostly located within the sinusoidal lumina in close association with endothelial cells. Kupffer cells function as phagocytes, ingesting foreign material which may arrive through the portal circulation (Treinen-Moslen, 2001; Plumlee, 2004) as well as apoptotic or necrotic hepatocytes. Kupffer cells have other immune functions in that they act as antigen presenting cells and secrete various cytokines (Treinen-Moslen, 2001; Plumlee, 2004). They are involved in destruction of metastatic neoplastic cells (Plumlee, 2004). Kupffer cells may store minerals and are also involved in the pathogenesis of a variety of liver diseases induced by toxins such as ethanol (Laskin, 1990; Thurman *et al*., 1998).

Stellate cells are located within the sinusoids and store fat and vitamin A (Treinen-Moslen, 2001; Piñeiro-Carrero and Piñeiro, 2004; Plumlee, 2004). When there is liver injury, stellate cells may become activated to myofibroblast-like cells (Plumlee, 2004; Maddrey, 2005). Activated stellate cells produce collagen and play a role in hepatic fibrosis (Treinen-Moslen, 2001; Plumlee, 2004).

Pit cells are natural killer cells which have antineoplastic actions (Treinen-Moslen, 2001; Plumlee, 2004). They are also involved in granuloma formation (Plumlee, 2004).

# FACTORS INFLUENCING TOXIC LIVER INJURY

## Uptake and concentration

The liver has a dual blood supply as noted above. The hepatic artery brings material from the systemic circulation and the portal blood flow brings material directly from the gastrointestinal system. The portal system is involved in what is described as the "first pass effect", meaning that nutrients and xenobiotics that are absorbed from the stomach and intestines are filtered through the liver before entering the systemic circulation (Treinen-Moslen, 2001).

The space of Disse allows close contact between circulating plasma, plasma proteins, and hepatocytes, allowing for rapid diffusion of lipophilic compounds across the hepatocyte membrane (Treinen-Moslen, 2001). Some compounds are specifically taken up by sinusoidal transporters, including phalloidin from several species of mushrooms in the genus *Amanita*, microcystin produced by the cyanobacteria *Microcystis aeruginosa*, and bile acids (Treinen-Moslen, 2001).

Liver cells may accumulate high levels of metals and vitamins, which can lead to toxic injury. Excessive storage of vitamin A in stellate cells acutely leads to engorgement, activation, and proliferation of these cells (Treinen-Moslen, 2001). Chronic high levels lead to hepatic fibrosis and portal hypertension, precipitating increased fibrosis (Zimmerman, 1999; Piñeiro-Carrero and Piñeiro, 2004; Maddrey, 2005). Cadmium is sequestered by hepatic metallothioneins but may produce pathology when storage capacity is exceeded (Treinen-Moslen, 2001). The liver is also responsible for iron homeostasis. There is receptor-mediated uptake of iron from the sinusoids and sequestration in storage proteins such as ferritin. High levels of iron cause lipid peroxidation of zone 1 hepatocytes (Treinen-Moslen, 2001). Of veterinary relevance is the copper-mediated liver damage noted in certain breeds of dogs (Bedlington terriers, Dobermans, and Dalmatinans) where copper is stored within the lysosomes of hepatocytes resulting in progressive accumulation of copper resulting in liver necrosis (Rolfe and Twedt, 1995). Sheep are also particularly susceptible to elevated hepatic copper levels.

## Bioactivation and metabolism

Most xenobiotic agents absorbed by the small intestine are highly lipophilic. Renal excretion is the primary mechanism of removal for xenobiotics, but renal excretion of lipophilic compounds, which are frequently protein bound in the circulation, is poor (Dahm and Jones, 1996; Sturgill and Lambert, 1997; Watkins, 1999). Such lipophilic compounds must be metabolized to increase their water solubility for excretion (Dahm and Jones, 1996; Sturgill and Lambert, 1997; Zimmerman, 1999). Enzymes within the liver add functional groups or conjugate xenobiotics to water-soluble molecules to promote excretion. While these reactions often function in the detoxification of compounds, there is significant potential for toxification (Zimmerman, 1999). Phase I reactions involve oxidation, reduction, and hydrolysis, and are ubiquitous in mammals (Brown, 2001). Phase I enzymes, which are predominantly located in zone 3, may produce active metabolites.

Many hepatic enzymes are present in the smooth endoplasmic reticulum of the hepatocyte. When liver tissue is homogenized, the endoplasmic reticulum breaks down into small vesicles known as microsomes, thus these enzymes are termed microsomal enzymes. Many of the phase I enzymes are microsomal enzymes. As a rule, microsomal enzymes require oxygen and NADPH to function (Dahm and Jones, 1996; Brown, 2001).

Most phase I enzymes contain heme, giving them a red coloration, and they absorb light at a wavelength of 450 nm. These enzymes are known as cytochrome P450s, the P for pigment and 450 for the wavelength of absorbed light. Most cytochrome P450s act as mixed function oxidases (MFOs). Genes for cytochrome P450s are highly conserved in mammals. There are 3 gene families, CYP1, CYP2, and CYP3, and more than 36 cytochrome P450 isoenzymes have been identified in animals (Dahm and Jones, 1996; Watkins, 1999).

Oxidation is the major phase I reaction produced by the group of cytochrome P450s known as MFOs. Important substrates include steroid hormones and lipid soluble drugs (Brown, 2001). Oxidative reactions frequently lead to the formation of highly reactive epoxides (Watkins, 1999). These toxic metabolites are very transient and usually detoxified rapidly by phase II conjugation or other mechanisms, such as microsomal epoxide hydrolases (Watkins, 1999; Piñeiro-Carrero and Piñeiro, 2004). If an individual is exposed to the xenobiotic in doses too high for these mechanisms to compensate reactive phase I metabolites are not removed quickly enough to prevent injury (Watkins, 1999).

Non-cytochrome P450 enzymes may be involved in oxidative reactions. One such enzyme is alcohol dehydrogenase whose substrates include vitamin A, ethanol, and ethylene glycol. Aldehyde dehydrogenase is another.

Most reduction reactions also involve microsomal enzymes, with the exception of ketone reduction. Nitro compounds are reduced to amines and volatile anesthetics undergo dehalogenation by microsomal enzymes. Hydrolysis reactions are involved in metabolism of compounds with amide bonds or ester linkages, as in the conversion of aspirin to salicylate (Brown, 2001).

Phase II enzymes may be cytosolic or microsomal (Dahm and Jones, 1996; Brown, 2001). Phase II enzymes are predominantly involved in conjugating phase I metabolites or xenobiotics with functional groups. Phase II metabolites are rarely reactive, but there are exceptions, such as the glucuronide of the non-steroidal anti-inflammatory drug (NSAID) diclofenac and the GSH conjugate of $\alpha$-napthothiourea (ANIT). Phase II enzymes conjugate a polar group to the substrate at a hydroxyl group, carboxyl group, amino group, or sulfhydryl group that may have been produced through the actions of phase I enzymes. Polar molecules that are added to the substrate include glucuronic acid, sulfate derived from sulfuric acid ester, acetate, GSH, methyl groups derived from methionine, and amino acids such as glycine and cysteine. These polar groups enhance water solubility of the substrate and allow renal or biliary excretion. Disruption of phase II reactions may lead to accumulation of reactive phase I metabolites (Sturgill and Lambert, 1997).

Glucuronidation is the most common phase II reaction in humans, though it is deficient in the neonate and, of significant veterinary importance, in cats (Sturgill and Lambert, 1997; Brown, 2001; Piñeiro-Carrero and Piñeiro, 2004). Substrates with an atomic mass greater than 500 amu frequently undergo glucuronidation. Such substrates include steroid hormones, thyroxin, and bilirubin as well as many drugs, including salicylates and acetaminophen (Brown, 2001). Glucuronyl transferases are microsomal enzyme that catalyzes the transfer of glucuronide from uridine 5′-diphosphate (UDP) (Watkins, 1999). UDP may be depleted in patients overdosed with acetaminophen or other drugs that undergo this detoxification pathway. Products of glucuronidation may be excreted in the bile or urine. Those excreted in the bile may undergo hydrolysis in the intestine, which leads to reabsorption of the parent compound in a phenomenon called enterohepatic cycling (Brown, 2001). Some agents induce glucuronyl transferases, such as phenobarbital (Sturgill and Lambert, 1997).

Sulfation is the primary conjugation reaction for substrates with phenol groups or aliphatic alcohols (Sturgill and Lambert, 1997; Brown, 2001). These reactions are catalyzed by sulfotransferases in the cytoplasm. Agents that undergo sulfation include acetaminophen, morphine, ascorbic acid, and endogenous compounds like chondriotin, heparin, and some steroids. The pool of available sulfates may become saturated in drug overdoses (Brown, 2001).

Drugs with amine and hydrazine groups may be conjugated to acetate (Sturgill and Lambert, 1997). Sulfonamides often undergo acetylation (Brown, 2001). *N*-acetyltransferase is an enzyme in the cytoplasm involved in acetylation reactions.

GSH and cysteine both have sulfhydryl groups which readily bind many phase I metabolites (Brown, 2001). GSH is a free radical scavenger that prevents membrane damage from reactive metabolites. These reactions may be spontaneous or catalyzed by GSH peroxidases and selenium-dependent enzymes. Because these enzymes are cytosolic, damaged membrane phospholipids must be released by phospholipase A2 for detoxification. GSH is also involved in reduction and recycling of other antioxidants such as vitamins E and C (Dahm and Jones, 1996). When oxidized, GSH forms a dimer which must be reduced by GSH reductases, NADPH-dependent enzymes (Dahm and Jones, 1996). GSH may be depleted in the overdosed patient or due to fasting (Dahm and Jones, 1996; Sturgill and Lambert, 1997; Piñeiro-Carrero and Piñeiro, 2004). *N*-acetylcysteine is frequently used to replenish GSH.

Significant species differences exist in phase II enzymes. Glucuronyl transferase is deficient in cats, which significantly affects their ability to detoxify certain compounds including acetaminophen (Brown, 2001). Swine have reduced sulfate conjugation abilities (Brown, 2001). Dogs do not have the ability to acetylate aromatic groups (Brown, 2001; Trepanier, 2004). This may interfere with their ability to metabolize sulfonamide antimicrobials (Trepanier, 2004).

In addition to phase I and II biotransformation enzymes, studies suggests the involvement of hepatic transporter systems involved in drug efflux from hepatocytes as a means for the liver to rid itself of foreign chemicals. This is termed phase III transporter systems. Several transporter families which mediate uptake of chemicals into liver and excretion of chemicals from liver into blood and/or bile have been cloned and identified. In general, the Organic anion transporting polypeptide family (Oatps) along with Organic cation transporter 1 (Oct1) and organic anion transporter 2 mediate uptake of a large number of xenobiotics from blood into liver. Conversely, Multidrug resistance proteins (Mdrs), Multidrug resistance associated proteins (Mrps), and Breast cancer resistance protein (Bcrp) mediate efflux of xenobiotics from liver into bile or blood (Klaassen and Slitt, 2005).

Reactive metabolites of phase I or occasionally phase II processes might act as free radicals, substances with unpaired electrons. Free radicals act by the generation of reactive oxygen species such as superoxide ($O_2^-$), hydroxyl radicals (OH) and hydrogen peroxide ($H_2O_2$) through interactions with other molecules. Free radicals reacting with phospholipids of the plasma membrane, endoplasmic reticulum membrane, or mitochondrial membranes cause a self-propagating chain reaction that may lead to cell death (Dahm and Jones, 1996; Watkins, 1999).

Electrophiles are molecules with electron seeking properties, often epoxides. They form covalent bonds with nucleophilic molecules, often binding to thiol groups on proteins (Dahm and Jones, 1996; Watkins, 1999; Zimmerman, 1999). Electrophiles produced by phase I reactions may cause cell death by damage to critical proteins, such as calcium transport proteins in membranes (Dahm and Jones, 1996). Aflatoxin $B_1$ binds to guanine residues in the DNA, which may lead to defects in protein transcription and cell death, or to carcinogenesis (Dahm and Jones, 1996).

# TYPE OF CHEMICALLY MEDIATED LIVER INJURY

## Hepatic steatosis/fatty liver

Hepatic steatosis is simply the accumulation of fat vacuoles within the hepatocytes. Steatosis is a common response noted with a variety of liver toxicants and represents a potentially reversible injury to hepatocytes (Treinen-Moslen, 2001). Grossly, the affected liver will be swollen with rounded edges, friable, and light brown to yellow in color. Due to the fat accumulation, sections of the affected liver will float in formalin (Plumlee, 2004). Compounds that produce prominent steatosis associated with mortality include the antiepileptic drug valproic acid and the antiviral agent fialuridine. Other toxins that may cause hepatic steatosis include aflatoxin and white or yellow phosphorus. Although steatosis has been considered a benign and reversible situation, there are recent reports that suggest that several biochemical alterations occur during the steatosis stage resulting in steatohepatitis, fibrosis and cirrhosis (Apte *et al.*, 2004; Ramaiah *et al.*, 2004). Recently, there is a syndrome noted in obese individuals, who are often type II diabetics, called non-alcoholic fatty liver disease (NAFLD) where hepatocytes are markedly steatotic and there is marked inflammatory component (Diehl 2002). Other disorders that result in fatty livers include ethanol and carbon tetrachloride. It should be noted however that several endocrine abnormalities result in steatosis, thus assigning the cause to a specific etiology should be done with caution. Serum transaminase levels during steatosis are usually variable and a mild to moderate increase in serum transaminase is not unexpected. Hepatic steatosis can cause cholestasis if lipid accumulation is severe.

Steatosis is termed microvesicular if the fat droplets are small and do not displace the nucleus. Microvesicular steatosis often indicates a slow lipid accumulation (Bastianello *et al.*, 1987; Plumlee, 2004). This form of steatosis may indicate a deficiency in mitochondrial $\beta$-oxidation of fatty acids. It is a relatively severe form of steatosis and has been associated with some toxins including aflatoxin in primates and dogs (Bastianello *et al.*, 1987; Zimmerman, 1999) and valproic acid in humans (Sturgill and Lambert, 1997; Zimmerman, 1999).

Macrovesicular steatosis describes hepatocytes containing large, usually single fat droplets that displace the hepatocyte nucleus to the periphery of the cytoplasm. This is the more common form of steatosis in domestic animals (Plumlee, 2004). Macrovesicular steatosis indicates an imbalance between fatty acid uptake and the secretion of very low-density lipoproteins. This may be due to increased triglyceride mobilization, decreased fatty acid oxidation, decreased synthesis of very low-density lipoproteins, or other metabolic anomalies (Sturgill and Lambert, 1997; Zimmerman, 1999; Treinen-Moslen, 2001; Plumlee, 2004). Macrovesicular steatosis has been reported in dogs with subacute aflatoxicosis (Bastianello *et al.*, 1987). Zone 1 macrovesicular steatosis is reported with yellow phosphorus toxicosis (Zimmerman, 1999). Macrovesicular steatosis is relatively common in veterinary medicine and occurs with a variety of metabolic diseases. Steatosis is common in obese cats with anorexia and preparturient cattle due to mobilization of lipids.

## Steatohepatitis

Steatohepatitis is the accumulation of lipid and the presence of inflammatory cells within hepatic parenchyma. Steatohepatitis is usually the next stage of steatosis if untreated (Lieber, 1994; Bautista, 2002; French, 2003). The inflammatory cells are usually neutrophils and mononuclear leukocytes. There is no specificity noted with this pathologic stage and the distribution of lipidosis and inflammation is random and multifocal. Conditions usually associated with steatohepatitis are alcoholic liver disease, NAFLD and endotoxemia secondary to intestinal disease. Any toxic compounds that cause steatosis can also result in steatohepatitis if condition is left untreated. It should be noted that steatohepatitis may progress to fibrosis/cirrhosis and hepatocellular carcinoma if the inciting cause is not removed or treated (Diehl, 2002). The clinical biochemistry alterations of the liver usually include elevated serum transaminases.

## Cell death

Two forms of cell death are described, apoptosis and necrosis. Apoptosis is often called "programmed cell death" and is a normal physiologic process. Individual cells are affected (Dahm and Jones, 1996), cell death is not associated with inflammation, and normal architecture of the hepatic parenchyma is maintained allowing regeneration (Treinen-Moslen, 2001; Piñeiro-Carrero and Piñeiro, 2004). Apoptotic cells undergo cell shrinkage, nuclear condensation, and pyknosis (Dahm and Jones, 1996; Treinen-Moslen, 2001), but mitochondrial function is maintained (Piñeiro-Carrero and Piñeiro, 2004) and the cell membrane remains intact (Zimmerman, 1999). Apoptotic cells are occasionally seen in the centrilobular area but are rapidly phagocytized by macrophages and other hepatocytes (Plumlee, 2004). Apoptosis may be induced by xenobiotics due to oxidative stress (Piñeiro-Carrero and Piñeiro, 2004), decrease in apoptotic suppressors, or enhanced expression of apoptosis genes (Dahm and Jones, 1996).

Necrosis is the predominant form of cell death in most toxic insults. The term "necrotic" is used to describe "dead and dying" cells which are often identified by homogenous eosinophilic cytoplasm on hematoxylin and eosin (H&E) stained liver sections with variable loss of nuclear and cellular detail. Degenerative changes to the hepatocyte may precede necrosis. Microscopically evident changes may include ballooning degeneration, hyaline degeneration, and the presence of Mallory bodies (Zimmerman, 1999). Cells lose osmotic homeostasis and there is swelling of hepatocytes on a microscopic basis and organelles on an ultrastructural basis (Dahm and Jones, 1996; Treinen-Moslen, 2001). Energy production fails due to loss of calcium homeostasis (Dahm and Jones, 1996; Zimmerman, 1999). Eventually there is rupture of the cell membrane and leakage of cell contents (Dahm and Jones, 1996; Zimmerman, 1999; Treinen-Moslen, 2001). Typically, necrosis results in elevation of serum transaminases such as alanine transaminase (ALT), aspartate transaminase (AST) and sorbitol dehydrogenase (SDH). Depending on the extent of liver necrosis, the liver function may or may not be affected.

Necrosis is often initiated by damage to membranes, either the plasma membrane of the cell or the membranes of organelles, particularly the mitochondria (Zimmerman, 1999). Cell membrane damage is often caused by peroxidation of membrane phospholipids (Zimmerman, 1999). Damage to the plasma membrane interferes with ion regulation, damage to the membranes of the mitochondria interfere with calcium homeostasis and energy production, and damage to the smooth endoplasmic reticulum membrane diminishes the ability of that organelle to sequester calcium (Zimmerman, 1999). Inhibition of protein synthesis is an alternate mechanism that may cause cell necrosis. Toxins that act in this way include phalloidin and related mushroom toxins, which inhibit the action of RNA polymerase and therefore mRNA synthesis (Piñeiro-Carrero and Piñeiro, 2004).

Necrotic liver injury can be focal, zonal, bridging, or massive and panlobular. Focal necrosis is randomly distributed and involves hepatocytes either individually or in small clusters (Treinen-Moslen, 2001). Zonal necrosis is common and usually occurs in zone 3, the centrilobular zone (Zimmerman, 1999; Treinen-Moslen, 2001; Plumlee, 2004). Grossly, the liver will have a reticulated pattern with dark red central areas separated by brown to yellow areas (Plumlee, 2004). Bridging necrosis describes confluent zones of necrosis that extend between zones of the lobule and between lobules (Treinen-Moslen, 2001). Panlobular or massive necrosis denotes hepatocyte loss throughout the lobule with loss of lobular architecture (Treinen-Moslen, 2001; Plumlee, 2004). The liver is grossly swollen and friable with panlobular injury (Plumlee, 2004).

Although necrosis and apoptosis are considered separate entities, an alternate view is emerging that apoptosis and necrosis are frequently the consequence of the same initiating factors and signaling pathways. Rather than being separate entities, apoptosis and necrosis in their pure form may represent extremes on a continuum of cell death. Thus necrosis resulting from oncotic event or oncosis is termed "oncotic necrosis" and that originating from apoptosis is termed "apoptotic necrosis" (Levin *et al.*, 1999; Jaeschke *et al.*, 2004; Malhi *et al.*, 2006).

The nature of the lesions can tell us something about the mechanism of injury. Several drugs/chemicals such as carbon tetrachloride ($CCl_4$), acetaminophen, thioacetamide, allyl alcohol and ethanol result in hepatocyte oncotic necrosis. Carbon tetrachloride, acetaminophen, and thioacetamide cause centrilobular necrosis, a commonly seen

pattern while the necrosis caused by allyl alcohol may fit the more rarely seen midzonal pattern. Recently, we have identified midzonal necrosis in a rat model of endotoxin-mediated hepatitis. Ethanol ingestion results in multifocal, random hepatic necrosis. Necrosis may be a primary event such as that caused by reactive intermediates (e.g. acetaminophen, thioacetamide and carbon tetrachloride), or may be a secondary event following infiltration of inflammatory cells as seen with ethanol.

## Pigment accumulation

Various substances may accumulate within hepatocytes or Kupffer cells. These substances may be visible by microscopy as pigment. Occasionally, these pigments lend a grossly visible tint to the liver. Bile pigment may accumulate in canaliculi and bile ducts, particularly in zone 3, leading to a yellow to green color (Zimmerman, 1999; Plumlee, 2004). Iron in the form of hemosiderin is stored in the liver as a yellow-brown pigment which may be visualized using Pearl's Prussian Blue (Plumlee, 2004). Copper may be yellow-brown and is visualized using Rhodenase (Plumlee, 2004). Lipofuscin may be present within hepatocytes as a senile change. This yellow-brown pigment represents lipid accumulation within lysosomes (Plumlee, 2004).

## Cholestatic liver injury

Cholestasis may be transient or chronic (Treinen-Moslen, 2001). If severe, bile pigments give the liver a grossly yellow to yellow-green tint (Plumlee, 2004). Cholestasis may be subdivided into canalicular cholestasis and cholangiodestructive cholestasis.

Canalicular cholestasis can be produced by drugs/chemicals that damage the bile canalicular structures and function. Cholestasis may be primary or secondary. A key component of bile secretion involves several ATP-dependent export pumps such as the canalicular bile salt transporter that moves bile salts and other transporters that export bile constituents from the hepatocyte cytoplasm to the lumen of the canaliculus. Some of the drugs bind these transporter molecules resulting in the arrest of bile formation or movement within the lumen of the canalicular system (Klaassen and Slitt, 2005). Secondary bile injury can result if there is cholestasis due to the detergent action of bile salts on the biliary epithelium or hepatocytes in areas of cholestasis. Cholestasis can occur simply as a result of physical obstruction of canaliculi within the liver parenchyma (intrahepatic) or outside the liver (extrahepatic). Causes of cholestasis may include hepatobiliary tumors, endotoxemia, hepatocyte swelling, and intraductal crystals such as calcium salts of plant saponins, e.g. those found in *Tribulus* spp. Disruption of actin filaments within the hepatocyte may cause cholestasis by preventing the normal pulsatile contractions that move bile through the canalicular system to the bile ducts. Drugs that bind to actin filaments such as phalloidin, those that affect cytoskeletal assembly such as microcystin, and those that affect calcium homeostasis and cellular energy production can generate this type of injury. Cholestatic disorders typically result in elevation of serum alkaline phosphatase, $\gamma$-glutamyl transferase and elevation in serum bilirubin. Toxic hepatopathies may be primarily cholestatic, necrotic, or mixed.

Cholangiodestructive cholestasis is caused by bile duct obstruction which may be intrahepatic or extrahepatic. Bile duct injury may lead to sloughing of epithelial cells into the lumen, cell edema, and inflammation, which may contribute to obstruction (Treinen-Moslen, 2001; Plumlee, 2004). Chronic lesions associated with cholangiodestructive cholestasis typically include bile duct proliferation and periductular fibrosis (Treinen-Moslen, 2001; Plumlee, 2004). Vanishing bile duct syndrome, characterized by a loss of bile ducts, has been seen in chronic cholestatic disease in humans (Zimmerman, 1999; Treinen-Moslen, 2001) and has been produced experimentally in dogs (Uchida *et al.*, 1989).

## Hepatic fibrosis/cirrhosis

Fibrosis is a non-specific lesion which usually results from chronic inflammation. Chronic inflammation can be the result of continuous exposure to a variety of hepatotoxic chemicals such as organic arsenicals, vinyl chloride, or high doses of vitamin A (Zimmerman, 1999), chronic ethanol ingestion and NAFLD. Fibrosis usually occurs around the portal area, in the space of Disse, and around the central veins. This results in loss of liver architecture and function. The hepatocytes are replaced with fibrous material and thus there is hepatocyte loss. Periportal fibrosis may lead to portal hypertension (Zimmerman, 1999).

## Cirrhosis

Hepatic cirrhosis is typically the end stage liver disease. Cirrhosis describes irreversible changes characterized by accumulation of excessive collagen deposition in the form of bridging fibrosis which disrupts the hepatic architecture (Treinen-Moslen, 2001). Hepatocytes entrapped by fibrosis undergo random mitosis and growth, termed nodular regeneration. Cirrhosis may be micronodular or macronodular depending on the amount of fibrosis and tissue regeneration. The surface of a liver with micronodular fibrosis will have a cobblestone appearance. The liver will be firm and difficult to cut with a knife.

When the liver reaches this stage, the serum transaminase enzymes are usually low due to the lack of functional

hepatocytes and the liver function parameters such as bile acids and ammonia are highly elevated. Prognosis for recovery is poor. Liver transplantation is the only solution to restore adequate liver function in human medicine.

## Hepatic neoplasia

Chemically induced tumors can originate from hepatocytes, biliary epithelium and very rarely from endothelium of hepatic sinusoids. Neoplasias occur months to years after toxin exposure (Plumlee, 2004). If a toxin causes direct damage to DNA, a single exposure may lead to neoplastic changes (Zimmerman, 1999; Piñeiro-Carrero and Piñeiro, 2004). Aflatoxin $B_1$ is an agent that acts by alkylating DNA and is associated with hepatocellular carcinoma in humans infected with hepatitis B virus. Aflatoxin B1 has also been implicated in liver cancers in trout and laboratory animals (Newberne and Butler, 1969). Non-genotoxic agents must be given at high doses for long periods of time to induce cancer (Piñeiro-Carrero and Piñeiro, 2004). Examples of non-genotoxic agents include phthalate esters in plasticizers, phenoxy acid herbicides, and hypolipidimic drugs (Piñeiro-Carrero and Piñeiro, 2004).

Hepatic adenomata are benign neoplasms of the hepatocytes that have been associated with contraceptive steroid use in humans (Zimmerman, 1999). Hepatocellular carcinomata are of more concern. These malignant neoplasms of the hepatic parenchyma may be induced by various chemical and botanical toxins. They have been found in humans affected with hepatitis B virus and exposed to aflatoxin $B_1$, as noted above, and associated with anabolic steroid abuse (Zimmerman, 1999). Other risk factors for hepatocellular carcinomata include viral hepatitis C and D, ethanol abuse, and microcystin exposure.

Biliary carcinomata or cholangiocarcinomata are not commonly associated with exposure to drugs/chemicals. Biochemical indicators noted include normal, elevated or low levels of serum transaminases and loss of liver function resulting in increased bile acids and ammonia levels.

Angiosarcomata or hemangiosarcomata derives from sinusoidal epithelium (Zimmerman, 1999; Treinen-Moslen, 2001; Plumlee, 2004). This neoplasm is rare but rapidly lethal and has been associated with exposure to vinyl chloride, inorganic arsenics (Zimmerman, 1999), and Thorotrast, a form of radioactive thorium dioxide once used in radiographic contrast studies.

# MECHANISMS OF LIVER DAMAGE

Mechanisms of liver injury have been divided into two classifications, intrinsic and idiosyncratic. Intrinsic injury may lead to steatosis, necrosis, cholestasis, or a mixed form of damage, often with minimal inflammation (Cullin and Ruenber 1991; Sturgill and Lambert, 1997). Intrinsic liver injury is a predictable, reproducible, dose-dependent reaction to a toxicant (Dahm and Jones, 1996; Sturgill and Lambert, 1997; Zimmerman, 1999; Piñeiro-Carrero and Piñeiro, 2004). A threshold dose exists for xenobiotics causing intrinsic liver injury (Sturgill and Lambert, 1997). There is commonly a predictable latent period between the time of exposure and clinical evidence of liver injury (Dahm and Jones, 1996). This type of liver injury accounts for the vast majority toxic liver injury and is often caused by reactive products of xenobiotic metabolism, most commonly electrophiles and free radicals. A few drugs cause intrinsic liver injury without bioactivation (Zimmerman, 1999). An abbreviated summary of mechanisms of intrinsic liver injury is illustrated in Figure 10.2.

Idiosyncratic responses are, by contrast, unpredictable responses to a drug or other toxicant. They are rare, non-dose dependent, and often associated with extrahepatic changes (Sturgill and Lambert, 1997; Zimmerman, 1999; Piñeiro-Carrero and Piñeiro, 2004; Shenton *et al.*, 2004).

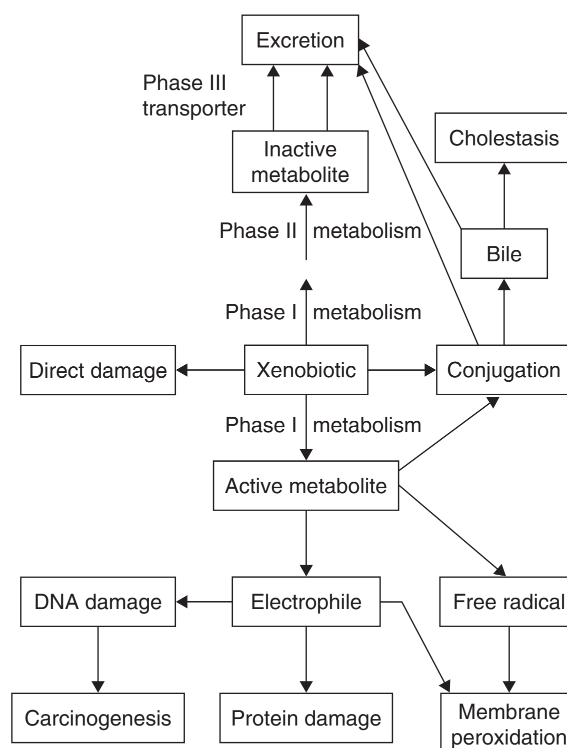



**FIGURE 10.2** Basic mechanisms of hepatic injury showing the relationship between multiple pathways for metabolism and toxicity for any compound. The liver metabolizes xenobiotics (and some endogenous compounds) to form water-soluble products appropriate for urinary or biliary excretion. Some compounds are activated through these metabolic processes to free radicals, electrophiles, or other toxic products that may induce hepatic injury.

Idiosyncratic drug reactions often occur after sensitization followed by reexposure to a drug (Sturgill and Lambert, 1997; Dahm and Jones, 1996; Zimmerman, 1999; Treinen-Moslen, 2001). There is usually a delay of 1–5 weeks (Zimmerman, 1999), and occasionally several months, between the time of the first dosing and the time clinical signs become evident, but onset is expedited with rechallenge (Dahm and Jones, 1996; Sturgill and Lambert, 1997; Watkins, 1999). Hepatic changes associated with idiosyncratic drug reactions include necrosis, cholestasis, or both, and there is often an inflammatory response involving macrophages and eosinophils (Sturgill and Lambert, 1997). Extrahepatic clinical signs may include pyrexia, rash, and peripheral eosinophilia (Sturgill and Lambert, 1997; Watkins, 1999; Zimmerman, 1999; Piñeiro-Carrero and Piñeiro, 2004). Some idiosyncratic drug reactions resemble serum sickness (Zimmerman, 1999).

Liver injury can also be classified as acute or chronic. Acute responses to liver toxins may be cytotoxic, cholestatic, or both. These classifications are also present in chronic hepatotoxin responses, with the addition of veno-occlusive disease, often associated with thrombosis of the hepatic vein (Piñeiro-Carrero and Piñeiro, 2004).

Mechanisms of liver injury are described in more detail below.

## Oxidative stress mediated by free radicals

Free radicals are generated from within hepatocytes in several ways, such as ionizing radiation, oxidative metabolism by cytochrome P450, reduction and oxidation (redox) reaction that occur during normal metabolism, transition metals such as iron and copper, and from nitric oxide (NO) generated by variety of inflammatory cells. The reactive species generated result in lipid peroxidation of membranes, oxidative modification of proteins and lesions within DNA (Crawford, 1999).

Free radicals have unpaired electrons, making them highly reactive. They may be formed by one electron oxidation or reduction reactions, leading to cationic or anionic radicals, respectively (Dahm and Jones, 1996). Alternately, hemolytic bond scission leads to neutral radical formation (Dahm and Jones, 1996). Oxygen free radicals result from metabolic processes, leukocytic respiratory burst, or to the effects of ionizing radiation. Hydroxyl radicals, superoxide-radicals, and hydrogen peroxide are major reactive oxygen species (Dahm and Jones, 1996). The free radical NO, an important cell-signaling agent released by leukocytes, may react with superoxide to form peroxinitrite.

Free radicals cause peroxidation of phospholipids within the plasma membrane of the cell as well as the membranes of the mitochondria and endoplasmic reticulum. The radicals act by removing a proton ($H^+$) from a methylene carbon within a polyunsaturated fatty acid, forming a lipid free radical. This lipid free radical may then abstract a proton from a neighboring polyunsaturated fatty acid, generating more lipid free radicals. It is estimated that this can occur 4–10 times per initiation (Dahm and Jones, 1996). Effects of lipid peroxy radicals on the cell membrane include increased permeability, decreased fluidity, and inactivation of membrane proteins (Dahm and Jones, 1996). Additionally, mitochondrial membranes lose polarity (Watkins, 1999).

Lipid peroxy radicals can react with metal ions stored within the hepatocyte, generating more lipid radicals. It is estimated that propagation by this mechanism can occur in 4–10 steps per initiation (Dahm and Jones, 1996).

The most frequent mechanism of free radical production leading to hepatocellular injury involves phase I metabolism of xenobiotics and the cytochrome P450 system. Phase I metabolism may lead to bioactivation of the substrate to a high-energy reactive intermediate molecule in preparation for phase II conjugation reactions. However, in circumstances such as overdosage, phase I products may accumulate. Lesions produced by these compounds are mostly centrilobular because the cytochrome P450s responsible for metabolism is mostly situated in the centrilobular areas of the liver.

The classical examples of this process are cell death resulting from acetaminophen (Figure 10.3) and carbon tetrachloride (Figure 10.4) toxicosis. Acetaminophen is a widely used analgesic that rarely induces clinical signs at therapeutic doses, except in the particularly susceptible feline species, where acute liver failure oxidation of hemoglobin, and methemoglobinemia occur.

Acetaminophen has a hydroxyl group that can undergo immediate phase II conjugation reactions. Indeed, at therapeutic doses 90% of this substrate undergoes glucuronide or sulfate conjugation in humans (Mitchell *et al.*, 1973; Court and Greenblatt, 1997; Sturgill and Lambert, 1997). These are major metabolic pathways in most species, but glucuronyl transferase deficiency in cats in part explains the sensitivity of felines to this drug (Court and Greenblatt, 1997).

Acetaminophen itself is not considered toxic. Cellular injury is caused by the unstable metabolite, *N*-acetyl-*p*-benzoquinone imine (NAPQI). Under normal conditions in humans, 5% of a dose of acetaminophen is oxidized to NAPQI, which is rapidly neutralized by conjugation with GSH (Sturgill and Lambert, 1997; Maddrey, 2005). Toxic levels of NAPQI may accumulate under certain conditions, as when large amounts of substrate are available for metabolism due to either large ingestions or inadequate glucuronidation. Metabolism of acetaminophen to NAPQI is increased in individuals who regularly consume alcohol or take medications that induce microsomal enzymes, e.g. antiepileptic therapy. The hepatic pool of GSH becomes overwhelmed and depleted permitting the accumulation of NAPQI. Possible additional risk factors that lower the

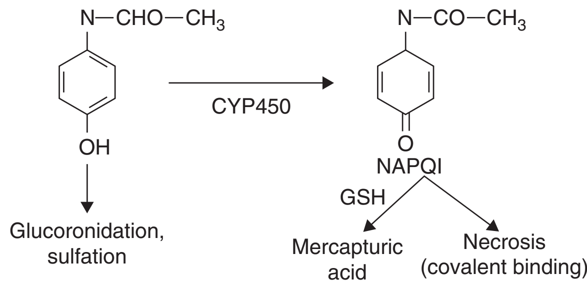



**FIGURE 10.3** Metabolism and mechanism of acetaminophen toxicity. Bioactivation of acetaminophen by P450 enzymes results in the formation of the reactive intermediate (NAPQI) which form covalent protein adducts with GSH which is then converted to mercapturic acid. When the amount of the reactive metabolite formed exceeds the GSH available for binding, the excess metabolite binds to tissue molecules resulting in centrilobular hepatic necrosis.

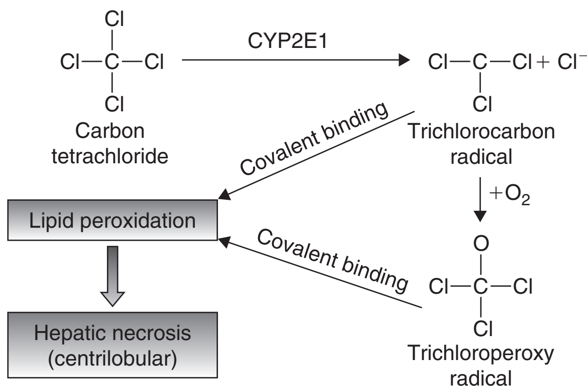



**FIGURE 10.4** Metabolism and mechanism of carbon tetracholoride toxicity. Carbon tetrachloride metabolism by CYP450 leads to free radicals such as trichlorocarbon and trichloroperoxy radical that initiates lipid peroxidation. The centrilboular location of CYP2E1 enzyme is mainly responsible for carbon tetrachloride metabolism and contributes to centrilobular necrosis similar to acetaminophen toxicity.

threshold for hepatotoxicity have been identified and include fasting and malnutrition, which deplete GSH reserves (Dahm and Jones, 1996; Sturgill and Lambert, 1996; Treinen-Moslen, 2001; Piñeiro-Carrero and Piñeiro, 2004).

The reaction that produces NAPQI generates superoxide anions as a by-product. Interactions of NAPQI with other cellular molecules also generate reactive oxygen species, leading to oxidative stress on the hepatocyte (Dahm and Jones, 1996; Zimmerman, 1999). The role of calcium and Kupffer cell activation have been implicated as contributing factors for acetaminophen-induced liver injury by producing reactive nitrogen species (Treinen-Moslen, 2001).

NAPQI also acts as an electrophile, targeting the mitochondria in particular. This reactive metabolite forms covalent adducts with cellular molecules, particularly proteins with thiol groups. Other targets besides mitochondrial proteins include plasma membrane proteins involved in calcium homeostasis and adenine nucleotides (Dahm and Jones, 1996; Sturgill and Lambert, 1997).

## Disruption of calcium homeostasis and cell membrane damage

Calcium ions ($Ca^{2+}$) are important for the mediation of hepatic injury. Cytosolic free calcium is maintained at relatively low concentrations compared to the extracellular levels. The majority of intracellular calcium is sequestered within the mitochondria and endoplasmic reticulum. Membrane associated calcium and magnesium ATPases are responsible for maintaining the calcium gradient (Farrell *et al.*, 1990). Significant and persistent increases in the intracellular calcium result from non-specific increases in permeability of the plasma membrane, mitochondrial membranes, and membranes of the smooth endoplasmic reticulum. Calcium pumps in the mitochondrial membrane require NADPH, thus depletion of available NADPH can cause calcium release from mitochondria (Cullen, 2005; Jaeschke and Bajt, 2006).

Elevated cytoplasmic calcium activates a variety of enzymes with membrane damaging effects. The major enzymes that are involved in activation by calcium include ATPases, phospholipases, proteases, and endonucleases. Thus increased calcium causes increased mitochondrial permeability and induction of apoptosis and necrosis. Calcium is required for maintenance and function of the cytoskeleton as well (Dahm and Jones, 1996; Delgado-Coello *et al.*, 2006).

Although cell injury results in increased calcium, which causes a variety of damaging effects, the cause and effect relationship of calcium in cell damage is not known. The chemicals that cause liver damage by this mechanism include quinines, peroxides, acetaminophen, iron, and cadmium.

## Mitochondrial injury

Mitochondria function in production of energy, in the form of ATP, for the cell by oxidative phosphorylation. Hepatocytes are highly metabolically active and require a continuous supply of ATP. Hepatocytes active in detoxification or replacement of damaged tissue have higher ATP requirements still (Dahm and Jones, 1996). Compounds that may disrupt mitochondrial oxidative phosphorylation include bile acids and amiodarone. Mitochondria are also critical to modulation of cell redox status, osmotic regulation, pH control, cytosolic calcium homeostasis and cell signaling. Mitochondria are important targets for virtually all types of injurious stimuli, including hypoxia and toxins. Mitochondria are targeted by oxidants, electrophiles,

lipophilic cations, and weak acids. Damage is often precipitated by increases in cytosolic calcium.

Hepatic injury is frequently accompanied by morphological change in mitochondria. Mitochondrial changes evident as structural abnormalities include greatly increased size and the development of crystalline inclusions. These changes are usually regarded as pathologic, reflecting either as a protective or degenerative response to injury. Mitochondrial damage may results in the formation of high conductance channels, the so-called mitochondrial permeability transition, in the inner mitochondrial membrane. This is an irreversible change and, because membrane potential is critical for mitochondrial oxidative phosphorylation, constitutes a deathblow to the cell.

Oxidative phosphorylation produces reactive oxygen species (Watkins, 1999). These are deactivated by antioxidants present within the mitochondrion. GSH is present within mitochondria as a scavenger for peroxides and electrophiles. Synthesis of GSH requires ATP and takes place outside of the mitochondrion. Greater than 90% depletion in GSH reserves decrease the ability of the mitochondrion to detoxify reactive oxygen species produced by oxidative phosphorylation (Watkins, 1999). GSH *S*-transferase, the enzyme required for recycling of GSH, may become overwhelmed by toxicants and reactive metabolites (Dahm and Jones, 1996).

Xenobiotics may cause cell death by their effects on mitochondrial DNA. Some antiviral dideoxynucleoside analogs can disrupt mitochondrial DNA synthesis through the inhibition of DNA polymerase gamma, leading to depletion of mitochondria and consequent hepatocyte death (Houten *et al*., 2006).

Chemicals that damage mitochondrial structure, enzymes, or DNA synthesis can disrupt $\beta$-oxidation of lipids and oxidative energy production within hepatocytes. Prolonged interruption of $\beta$-oxidation leads microvesicular steatosis which can progress to macrovesicular steatosis. This sequence of events has been noted with alcoholic and non-alcoholic steatohepatitis. The role of mitochondria has been extensively studied with NAFLD, a major issue in human medicine. Alcoholic steatosis and other forms of hepatic steatosis have been linked to impairment of ATP homeostasis and mitochondrial abnormalities have been reported in a growing body of literature.

Investigations of animals dosed with ethanol for one to several months have shown that the mechanisms of liver injury caused by ethanol ingestion are numerous and this indicates that the pathogenesis of alcoholic liver disease is complex and multifactorial (Figure 10.5) (Lieber and DeCarli, 1976). The role of CYP2E1, fatty acid metabolism, oxidative damage, endotoxin, Kupffer cell, and neutrophil infiltration has been extensively investigated in literature (DiLuzio, 1966; Bardag-Gorce *et al*., 2000; Kono *et al*., 2000; Hoek and Pastorino, 2002). Studies have shown that progression of the disease correlates well with the dose of ethanol consumed daily and the duration of alcohol consumption. Alcoholic hepatic steatosis, steatohepatitis, fibrosis, and cirrhosis represent a sequential progression in the alcoholic liver disease following ethanol ingestion. Women are more sensitive to ethanol-induced liver effects than men and experience higher incidence of liver injury (Banerjee *et al*., 2006). The histologic changes noted include fatty change, cholestasis, and inflammation. Inflammation is predominantly neutrophilic with the regions of necrosis and Mallory body formation. Although many of these changes have been observed in experimental models of alcoholic hepatitis, progression to cirrhosis has been seen only rarely.

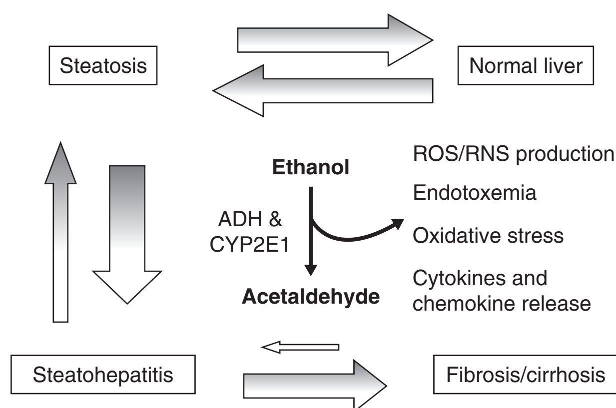



**FIGURE 10.5** Progression of alcoholic liver disease. Consumption of ethanol produces hepatic pathology in a sequence ranging from steatosis (fatty liver) on one extreme to fibrosis/cirrhosis on the opposite end of the spectrum. Steatosis and steatohepatitis represent acute stages of alcoholic liver disease. ROS = reactive oxygen specie; RON = reactive nitrogen specie

There are several drugs that inhibit $\beta$-oxidation of fatty acids in mitochondria leading to lipid accumulation, such as aspirin, valproic acid, tetracyclines.

## Disruption of cytoskeleton

Changes in intracellular calcium homeostasis produced by active metabolites of xenobiotics may cause disruption of the dynamic cytoskeleton. There are a few toxins that cause disruption of the cytoskeleton through mechanisms independent of biotransformation. Microcystin is one of these toxins. Microcystin is produced by the cyanobacterium *Microcystis aeruginosa*. Similar toxins are produced by other species of cyanobacteria. The hepatocyte is the specific target of microcystin, which enters the cell through a bile acid transporter. Microcystin covalently binds to serine/threonine protein phosphatase, leading to the hyperphosphorylation of cytoskeletal proteins and deformation of the cytoskeleton (Treinen-Moslen, 2001).

Phalloidin and related toxins found in some mushrooms, including *Amanita phalloides*, act by binding tightly to actin filaments and preventing cytoskeletal disassembly (Treinen-Moslen, 2001).

## Cholestasis

Sinusoidal transporters and canalicular transporters are involved in the movement of bile salts from the sinusoids into the canaliculi. Within the hepatocyte, transcytosis is mediated by cytoskeletal transport mechanisms. Bile is moved within the canaliculi through actions of the hepatocyte cytoskeleton causing contraction of the canalicular lumina (Treinen-Moslen, 2001). Xenobiotics acting on any of the above systems may influence bile transport and secretion.

Most chemicals that cause cholestasis are excreted in the bile, including the mycotoxin sporodesmin which concentrates 100-fold in the bile (Treinen-Moslen, 2001).

ANIT is a hepatotoxicant that damages bile duct epithelium and hepatocytes. The drug is used experimentally in rodents as a model of intrahepatic cholestasis. A single dose of ANIT induces acute cholangitis; prolonged exposure causes bile duct hyperplasia and biliary fibrosis. Although the biochemical and histological features of ANIT toxicity are well documented, the mechanism by which ANIT causes liver injury remains uncertain. *In vivo*, ANIT does not cause liver damage until it appears in bile (Jean and Roth, 1995).

This drug is initially detoxified in hepatocytes by conjugation with GSH. ANIT–GSH complexes are secreted into bile, but they are unstable and rapidly dissociate. This exposes biliary cells to high concentrations of the parent compound, which presumably causes direct cytotoxicity. The reappearance of ANIT in bile also leads to enterohepatic cycling, reuptake of the drugs in the intestine and repetitive rounds of GSH conjugation and secretion. This not only delays elimination of the drug but depletes GSH progressively from hepatocytes and leads to hepatocellular damage. In addition ANIT is also known to cause hepatotoxicity by neutrophil- and platelet-dependent mechanisms (Jean and Roth, 1995).

## Hepatogenous photosensitization

Cholestatic diseases in herbivores, and ruminants in particular, may lead to dermal photosensitization. The presentation of photosensitization is similar in appearance to that of sunburn, but it is more rapid onset and associated with different wavelengths of light (Rowe, 1989). Photosensitization is caused by circulation of photoactive compounds. There are three classifications of photosensitization. Primary photosensitization involves ingestion or dermal absorption of a photodynamic compound which enters the circulation. An example is hypericin from *Hypericum perforatum*, or St. John's wort. Aberrant pigment synthesis occurs when the photoactive compound is produced by the body, as is the case with inherited porphyria. The third type of photosensitization is hepatogenous photosensitization.

Hepatogenous photosensitization occurs secondary to bile stasis. Herbivores ingest large quantities of chlorophyll. Metabolism of chlorophyll by bacteria in the gastrointestinal tract produces the photoactive compound phylloerythrin. Absorbed phylloerythrin is readily excreted in the bile with minor urinary excretion (Rowe, 1989; Burrows and Tyrl, 2001). Upon exposure to light of wavelengths 320–400 nm, singlet oxygen is generated causing lipid peroxidation in areas of skin unprotected by hair or melanin (Burrows and Tyrl, 2001). Not all causes of cholestasis produce photosensitization, thus icterus may occur in the absence of photosensitization (Rowe, 1989).

Hepatocyte swelling may lead to cholestasis. Agents that may cause photosensitization through hepatocyte damage include the toxic plant *Lantana camera* which causes steatosis, and subacute poisoning by microcystin. Because significant hepatocyte damage must occur before the individual presents with photosensitization, damage of this type usually carries a poor prognosis (Rowe, 1989).

Agents that act by damage to the bile duct include the mycotoxin sporodesmin and sapongenins from the plant *Tribulus terrestris*. Sporodesmin, produced by *Pithomyces chartarum* and associated with ryegrass pastures in Australia and New Zealand, acts by direct damage to the biliary epithelium leading to cell necrosis and degeneration. Bile ducts become occluded with cell debris and inspissated bile. Periductular fibrosis also occurs and may further occlude the bile ducts (Rowe, 1989).

Several plant species cause bile stasis through precipitation of calcium salts of sapogenic glucuronide within bile ducts. The major toxic sapongenins from *T terrestris* are diosgenin and yamogenin. These compounds are hydrolyzed in the gastrointestinal tract to sapongenins which are further metabolized before glucuronidation in the liver (Burrows and Tyrl, 2001). The sapogenins cause cholestasis through direct occlusion of the bile duct and damage to the canalicular membrane. It should be noted that while *T terrestris* poisoning is reported with some frequency in South Africa and Australia, it is believed that the toxic sapogenins are absent or present in much lower levels in *T terrestris* present in the United States (Burrows and Tyrl, 2001).

## Inhibition of tissue repair response

Tissue repair is a dynamic response involving compensatory cell proliferation and tissue regeneration which is stimulated in order to overcome acute toxicity and recover hepatic structure and function. Extensive evidence in rodent models using structurally and mechanistically diverse hepatotoxicants such as acetaminophen, carbon tetrachloride, chloroform, thioacetamide, trichloroethylene, and allyl alcohol have demonstrated that tissue repair plays a critical role in determining the final outcome of toxic insult, i.e. recovery from injury and survival or

progression of injury leading to liver failure and death (Mehendale, 2005).

Thioacetamide, originally used as a fungicide, is a potent hepatotoxicant. Studies have shown that this compound is bioactivated by cytochrome P450 and/or flavin-containing monooxygenase (FMO) systems to sulfine (sulfoxide) and sulfene (sulfone) metabolites, which cause centrilobular necrosis. Studies suggest that thioacetamide sulfoxide, a relatively stable intermediate of thioacetamide metabolism, is obligatory for the hepatotoxic effects of this compound, indicating that it is the penultimate reactive metabolite. Accordingly, it has been reported that the hepatotoxic effects of thioacetamide are only expressed after metabolic conversion to thioacetamide *S*-oxide, which undergoes further metabolic conversion to an as yet unidentified metabolite, probably the reactive unstable thioacetamide sulfone. Recently, it has been reported that thioacetamide bioactivation is primarily mediated by the cytochrome P450 enzyme CYP2E1. The reactive intermediate of thioacetamide binds to liver proteins with the formation of acetylimidolysine derivatives that are responsible for thioacetamide-induced hepatotoxic effects. The progression of thioacetamide-induced liver injury has also been attributed to inhibited tissue repair response. Studies have shown that diet restriction increases the liver injury of thioacetamide by diminishing tissue repair response (Ramaiah and Mehendale, 2000).

Tissue repair is a complex process governed by intricate cellular signaling involving a number of chemokines, cytokines, growth factors, and nuclear receptors leading to promitogenic gene expression and cell division. Tissue repair also encompasses regeneration of hepatic extracellular matrix and angiogenesis, the processes necessary to completely restore the structure and function of the liver tissue lost to toxicant-induced initiation, progression of injury. New insights have emerged over the last quarter century indicating that tissue repair follows a dose response. Tissue repair increases with xenobiotic dose until a threshold dose is reached beyond which repair is delayed and impaired due to inhibition of cellular signaling. This may result in runaway secondary events causing tissue destruction, organ failure, and death. Prompt and adequate tissue repair response to toxic injury is critical for recovery from toxic injury. Tissue repair is modulated by a variety of factors including species, strain, age, nutrition, and disease condition causing marked changes in susceptibility and toxic outcome (Ramaiah *et al.*, 1998; Mehendale, 2005).

## Idiosyncratic reactions

Many idiosyncratic drug reactions are believed to be immune mediated. Neoantigens may result from adducts formed from the interaction of reactive drug metabolites with cellular proteins. These neoantigens may be processed by Kupffer cells or other antigen presenting cells, transported to the cell surface, and presented as antigens (Reynold and Moslen 1980). Cell and antibody-mediated immune response may cause severe liver damage. Various drugs are believed to cause immune-mediated idiosyncratic reactions in humans, including halothane, diclofenac, phenytoin, and sulfonamides (Sturgill and Lambert, 1997; Watkins, 1999; Zimmerman, 1999; Treinen-Moslen, 2001). The idiosyncratic reaction to halothane has been well studied (Figure 10.6). A few compounds have been implicated in idiosyncratic reactions in domestic animals, as will be described below.

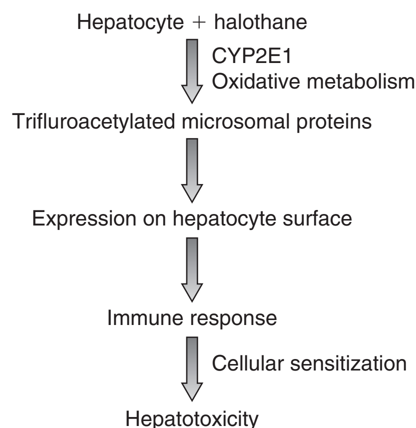



**FIGURE 10.6** Postulated mechanism of immune-mediated halothane hepatotoxicity. Halothane-mediated hepatitis is the best example for immune-mediated liver damage. Oxidative pathway yields trifluoroacetylchloride, which can react with microsomal proteins to form a neoantigen which then can generate immune response leading to hepatic injury.

Liver injury can be a result of both direct cytotoxicity and antibody-dependent cellular toxicity. Alcoholic liver disease is another example of possible immune-mediated damage. Acetaldehyde, produced by metabolism of ethanol, forms adducts with hepatic proteins similar to halothane, resulting in higher antibody titers to which some of the liver damage following ethanol ingestion may be attributed (Ramaiah *et al.*, 2004). However, the role of immune-mediated liver damage following ethanol ingestion is minimal compared to other known mechanisms of alcohol liver damage.

A few idiosyncratic drug reactions have been reported in veterinary medicine. The most well known is probably carprofen in dogs, as characterized by MacPhail *et al.* (1998). Carprofen is a non-steroidal anti-inflammatory agent. Clinical signs of idiosyncratic reaction to carprofen occur 20 days on average following the first exposure to the drug and include anorexia, vomiting, and icterus. Signs do not correlate with drug dose. Affected dogs have elevated ALT, AST, ALP, and serum total bilirubin. There is mild to severe bridging degeneration and necrosis with evidence of apoptosis. Mild to moderate periportal

inflammation is reported. Spayed female dogs are over-represented amongst those affected (MacPhail *et al*., 1998).

Other idiosyncratic reactions of veterinary importance include diazepam in cats (Center *et al*., 1996) and sulfonamides in dogs (Trepanier *et al*., 2003; Shenton *et al*., 2004; Trepanier, 2004). The clinical presentation associated with diazepam in cats is similar to that described with carprofen in dogs. Idiosyncratic reactions to sulfonamides in dogs may occur in 0.25% of dogs and a variety of organ systems may be affected. Hepatopathy is the third most reported reaction to sulfonamides and other effects may include polyarthropathy, which may predominate in Doberman pinchers, thrombocytopenia, pyrexia, and dermal drug eruptions (Trepanier, 2004).

## ON THE HORIZON

With the advent of "omics" approaches such as genomics, proteomics and metabolomics, there is increasing interest in the detection of novel genes, proteins, and metabolites associated with hepatotoxic chemicals. This technology has recently resulted in the generation of novel set of hypotheses for mechanism of action of hepatotoxic agents. Furthermore similar approaches have also led to identify signature biomarkers to diagnose hepatotoxicosis. For example serum proteomic analysis data recently revealed increased levels of vitamin D-binding protein, malate dehydrogenase and purine nucleoside phosphorylase in several models of chemically induced hepatotoxicants. These enzymes were elevated in the serum before increases in conventional serum markers such as ALT, AST and histopathology (Amacher *et al*., 2005). Therefore these proteins may of potential predictive value in early diagnosis of liver disease in the future although these markers have not been validated in veterinary or human medicine. Further, research involving transgenic and knock out models are continuing to provide insight into understanding mechanistic and molecular basis of liver injury. Development of animal models to investigate idiosyncratic drug hepatotoxicity is another area of intense investigation. Clearly there is progress in the area of liver toxicology which will continue to yield new information. This is especially true with the growing trends in research collaborations between individuals from multiple disciplines relevant to drug and chemical hepatic injury.

## REFERENCES


Amacher DE, Adler R, Herath A (2005) Use of proteomic methods to identify serum markers associated with rat liver toxicity or hypertrophy. *Clin Chem* **51**: 1796–803.

Apte UM, McRee R, Ramaiah SK (2004) Hepatocyte proliferation is the possible mechanism for the transient decrease in liver injury during steatosis stage of alcoholic liver disease. *Toxicol Path* **32**: 567–76.

Banerjee A, Apte UM, Smith III R, Ramaiah SK (2006) Higher neutrophil infiltration mediated by osteopontin is the likely contributing factor for increased susceptibility of females to Alcoholic Liver Disease. *J Pathol* **208**: 473–85.

Bardag-Gorce F, Yuan QX, Li J, French BA, Fang C, Ingelman-Sundberg M, French SW (2000) The effect of ethanol-induced cytochrome p4502E1 on the inhibition of proteasome activity by alcohol. *Biochem Biophys Res Commun* **279**: 23–9.

Bastianello SS, Nesbit JW, Williams MC, *et al*. (1987) Pathological findings in a natural outbreak of aflatoxicosis in dogs. *Onderstepoort J Vet Res* **54**: 635–40.

Bautista AP (2002) Neutrophilic infiltration in alcoholic hepatitis. *Alcohol* **27**: 17–21.

Brown SA (2001) Pharmakokinetics: disposition and fate of drugs in the body. In *Veterinary Pharmacology and Therapeutics*, 8th edn, Adams R (ed.). Iowa State University Press, Ames, IA pp. 15–56.

Burrows GE, Tyrl RJ (2001). *Toxic Plants of North America*. Iowa State University Press, Ames, IA pp. 1196–200.

Center SA, Elston TH, Rowland PH, *et al*. (1996) Fulminant hepatic failure associated with oral administration of diazepam in 11 cats. *J Am Vet Med Assoc* **209**: 618–25.

Court MH, Greenblatt DJ (1997) Molecular basis for deficient acetaminophen glucuronidation in cats. *Biochem Pharmacol* **5**: 1041–7.

Crawford JM (1999) The liver and the biliary tract. In *Robbins: Pathologic Basis of Disease*, 6th edn, Cotran RS, Kumar V, Collins T (eds). Saunders, Philadelphia, pp. 845–901.

Cullen JM (2005) Mechanistic Classification of Liver injury. *Tox Pathol* **33**: 6–8.

Cullen JM, Ruenber BH (1991) A histopathologic classification of chemical-induced injury of the liver. In *Hepatotoxicology*, Meeks RG, Harrison SD, Bull RJ (eds). CRC Press, Boca Raton, PA pp. 67–92.

Dahm LJ, Jones DP (1996) Mechanisms of chemically induced liver disease. In *Hepatology, A Textbook of Liver Disease*, Zakim D, Boyer TD (eds). W.B. Saunders Company, Philadelphia, pp. 875–90.

Delgado-Coello B, Trejo R, Mas-Oliva J (2006) Is there a specific tole for the plasma membrane $Ca^{2+}$-ATPase in the hepatocyte? *Mol Cell Biochem* (Epub Ahead of print).

Diehl AM (2002) Liver disease in alcohol abusers: Clinical Perspective. *Alcohol* **27**: 7–11.

DiLuzio NR (1966) A mechanism of the acute ethanol-induced fatty liver and the modification of liver injury by antioxidants. *Lab Invest* **15**: 50–63.

Farrell GC, Duddy SK, Kass GE, Llopis J, Gahm A, Orrenius S (1990) Release of calcium from the endoplasmic reticulum is not the mechanism for bile acid induced cholestasis and hepatotoxicity in the intact rat liver. *J Clin Invest* **85**: 1255–9.

French SW (2003) Alcoholic liver disease. In *Hepatology, A text book of liver disease*, Zakim D, Moyer TD (eds). W.B. Saunders, Philadelphia, pp. 839–922.

Hoek JB, Pastorino JG (2002) Ethanol, oxidative stress, and cytokine-induced liver cell injury. *Alcohol* **27**: 63–8.

Houten BV, Woshner V, Santos JH (2006) Role of mitochondrial DNA in toxic responses to oxidative stress. *DNA Repair* **5**: 145–52.

Jaeschke H, Bajt ML (2006) Intracellular signaling mechanisms of acetaminophen-induced liver cell death. *Tox Sci* **89**: 31–41.

Jaeschke H, Gujral JS, Bajt ML (2004) Apoptosis and necrosis in liver disease. *Liver Int* **24**: 85–9.

Jean PA, Roth RA (1995) Napthylisothiocyanate disposition in bile and its relationship to liver glutathione and toxicity. *Biochem Pharmacol* **50**: 1469–74.

Klaassen CD, Slitt AL (2005) Regulation of hepatic transporters by xenobiotic receptors. *Curr Drug Metab* **6**: 309–28.

Kono H, Rusyn I, Connor H, Mason RP, Thurman RG (2000) Allopurinol prevents early alcohol-induced liver injury in rats. *J Pharmacol Exp Therapeut* **293**: 296–303.

Laskin DL (1990) Nonparenchymal cells and hepatotoxicity. *Semin Liver Dis* **10**: 293–304.

Levin S, Bucci TJ, Cohen SM, Fix AS, Hardisty JF, LeGrand EK, Maronpot RR, Trump BF (1999) The nomenclature of cell death: recommendations of an *ad hoc* Committee of the Society of Toxicologic Pathologists. *Toxicol Pathol* **27**: 484–90.

Lieber CS (1994) Alcohol and the liver: 1994 update. *Gastroenterology* **106**: 1085–105.

Lieber CS, Decarli LM (1976) Animal models of ethanol dependence and liver injury in rats and baboons. *Fed Proc* **35**: 1232–6.

MacPhail CM, Lappin MR, Meyer DJ, *et al*. (1998) Hepatocellular toxicosis associated with administration of carprofen in 21 dogs. *J Am Vet Med Assoc* **12**: 895–1901.

Maddrey WC (2005) Drug-induced hepatotoxicity: 2005. *J Clin Gastroenterol* **39**: S83–9.

Malhi H, Gores GJ, Lemasters JJ (2006) Apoptosis and necrosis in the liver: A tale of two deaths? *Hepatology* **43**: S31–44.

Mehendale HM (2005) Tissue repair: an important determinant of final outcome of toxicant-induced injury. *Tox Pathol* **33**: 41–51.

Mitchell JR, Jollow DJ, Potter WZ, *et al*. (1973) Acetaminophen-induced hepatic necrosis: IV. Protective role of glutathione. *J Pharmacol Exp Ther* **187**: 211–17.

Newberne PM, Butler WH (1969) Acute and chronic effects of aflatoxin on the liver of domestic and laboratory animals: a review. *Cancer Res* **29**: 236–250.

Oldham-Ott CK, Gilloteaux J (1997) Comparative morphology of the gallbladder and biliary tract in vertebrates: variation in structure, homology in function and gallstones. *Microsc Res Tech* **38**: 571–98.

Piñeiro-Carrero VM, Piñeiro EO (2004) Liver. *Pediatrics* **113**: 2097–106.

Plumlee KH (2004) Hepatobiliary system. In *Clinical Veterinary Toxicology*, Plumlee KH (ed.). Mosby, St Louis, pp. 61–8.

Ramaiah SK, Mehendale HM (2000) Diet restriction as a protective mechanism in non cancer toxicity outcomes: a review. *Int J Toxicol* **19**: 413–24.

Ramaiah SK, Soni MG, Bucci TJ, Mehendale HM (1998) Temporal changes in tissue repair permit survival of diet restricted rats from acute lethal dose of thioacetamide. *Toxicol Sci* **45**: 233–41.

Ramaiah SK, Rivera C, Arteel G (2004) Early phase alcoholic liver disease: An update on animal models, pathology and pathogenesis. **23**: 217–31.

Reynolds ES, Moslen MT (1980) Environmental liver injury: Halogenated hydrocarbons. In *Toxic Injury of the Liver*, Farber E, Fisher MM (eds). Marcel Dekker, New York, pp. 541–96.

Rolfe DS, Twedt DC (1995) Copper associated hepatopathies in dogs. *Vet Clin North Am Small Anim Pract* **25**: 399–417.

Rowe LD (1989) Photosensitization problems in livestock. *Vet Clin North Am Food. Anim Pract* **5**: 301–23.

Shenton JM, Chen J, Uetrect JP (2004) Animal models of idiosyncratic drug reactions. *Chemico-Biol Interact* **150**: 53–70.

Sturgill MG, Lambert GH (1997) Xenobiotic-induced hepatotoxicity: mechanisms of liver injury and methods of monitoring hepatic function. *Clin Chem* **43**: 1512–26.

Thurman RG, Bradford BU, Iimuro Y, Knecht KT, Arteel GE, Yin M (1998) The role of gut-derived bacterial toxins and free radicals in alcohol-induced liver injury. *J Gastroenterol Hepatol* **13**: S39–50.

Treinen-Moslen M (2001) Toxic Responses of the Liver. In *Casarette & Doull's Toxicology The Basic Science of Poisons*, Klaassen CD (ed.). McGraw Hill, Columbus, OH pp. 471–89.

Trepanier LA (2004) Idiosyncratic toxicity associated with potentiated sulfonamides in the dog. *J Vet Pharmacol Therap* **27**: 129–38.

Trepanier LA, Danhof R, Toll J, *et al*. (2003) Clinical findings in 40 dogs with hypersensitivity associated with administration of potentiated sulfonamides. *J Vet Intern Med* **17**: 647–52.

Uchida H, Tomikawa S, Nishimura Y, *et al*. (1989) Vanishing bile duct syndrome in canine liver allotransplants. *Transplant Proc* **21**: 404–6.

Wallace KB, Eells JT, Madeira VM, (1997) Mitochondria-mediated cell injury. Symposium Overview. *Fundam Appl Toxicol* **38**: 23–37.

Watkins PB (1999) Mechanisms of drug induced liver disease. In *Schiff's Diseases of the Liver*, Schiff ER, Sorrell MF, Maddrey WF (eds). Lippincott-Raven Publishers, Philidelphia, pp. 1065–80.

Watkins PB, Seeff LB (2006) Drug-induced liver injury: summary of a single topic clinical research conference. *Hepatology* **43**: 618–31.

Xu C, Li CY, Kong AN (2005) Induction of phase I, II, and III drug metabolism/transport by xenobiotics. *Arch Pharm Res* **28**: 249–68.

Yee SB, Harkema JR, Ganey PE, *et al*. (2003) The coagulation system contributes to synergistic liver injury from exposure to monocrotaline and bacterial lipopolysaccharide. *Tox Sci* **74**: 457–69.

Zimmerman HJ (1999) Drug induced liver disease. In *Schiff's Diseases of the Liver*, Schiff ER, Sorrell MF, Maddrey WF (eds). Lippincott-Raven Publishers, Philidelphia, pp. 973–1064.

CHAPTER

# 11

# Renal toxicity

*Manu M.Sebastian, Steven I. Baskin, and Steven E. Czerwinski*

## INTRODUCTION

Kidney plays an important role in the total body homeostasis by multiple functions. The important functions include excretion of metabolic waste products and foreign chemicals, regulation of water and electrolyte balances, regulation of body fluid osmolality and electrolyte concentrations, regulation of arterial pressure, regulation of acid–base balance, secretion, metabolism and excretion of hormones and gluconeogenesis. A toxic insult to the kidney can disrupt any or all of these functions and result in acute or chronic toxic effect on body.

## ANATOMY

The kidneys in mammals are paired organs that lie ventral and adjacent to the lumbar vertebrae. Carnivores and horses have unilobar or unipyramidal kidneys. Kidneys of pigs and cattle are multilobar or multipyramidal. Bovine kidneys have external lobation. The medial side of each kidney contains an indented region called the hilum through which pass the renal artery and vein, lymphatics, nerve supply and ureter which carries the final urine from kidney to bladder. The kidney is surrounded by a tough fibrous capsule that protects its inner structures. If the kidney is bisected from top to bottom, the two major regions that can be visualized are the outer cortex and the inner region referred as the medulla. The corticomedullary ratio is usually approximately 1:2 or 1:3 in domestic animals. Normally the cortex appears dark brown and medulla has a pale gray color except in mature cats, in which the cortex is often yellow because of the large lipid content of tubular cells. Medulla is divided into multiple cone-shaped masses of tissues called renal pyramids. The base of each pyramid originates at the border between the cortex and medulla and terminates in the papilla which projects into the renal pelvis, a funnel-shaped continuation of the upper end of the ureter. The outer border of the pelvis is divided into open-ended pouches called major calyces that extend downward and divide into minor calyces, which collect urine from the tubules of each papilla. The walls of the calyces, pelvis and ureter contain contractile elements that propel the urine toward the bladder (Ellenport, 1975).

The functional unit of the kidney is the nephron and components of the nephron include Bowman's capsule, proximal tubule, Loop of Henle, distal tubule and the collecting duct. Each kidney (human) contains approximately 1 million nephrons and each of it is capable of forming urine. The nephron consists of a tuft of glomerular capillaries called the glomerulus, through which large amounts of fluid are filtered from the blood, and a long tubule in which the filtered fluid is converted into the urine. The glomerulus is made up of a network of branching and anatomizing capillaries and these capillaries have high hydrostatic pressure compared to other capillaries in the body. These capillaries are composed of endothelial cells that have attenuated and fenestrated cytoplasm which allow the passage of molecules. Those compounds up to a molecular weight of about 60,000 Da pass through the glomeruli into the tubular lumen to be excreted with urine. The glomerular capillaries are covered by epithelial cells and the total glomerulus is encased in Bowman's capsule with a space which is called the Bowmen's space. Fluid filtered from the glomerular capillaries flow into

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Bowman's space and then into the proximal tubule. The fluid collected in the proximal tubule flows into the loop of Henle, which dips into the renal medulla. Each loop is made up of an ascending and descending limb. The walls of the descending limb and the lower end of the ascending limb are very thin and are called the thin segment of the loop of Henle. After the ascending limb of the loop has returned partway back to the cortex, its walls become much thicker and is called the thick segment of the ascending limb. Approximately 25% of the filtered $Na^+$ and $K^+$ and 20% of the filtered water are reabsorbed by these segments of loop of Henle.

Macula densa which regulates the glomerular filtration rate (GFR) by signaling arteriolar constriction is composed of specialized cells located between the end of the thick ascending limb and the early distal tubule with close proximity to the afferent arteriole. The function of macula densa is to prevent massive losses of fluid/electrolytes in impaired tubular resorption. The fluids after passing this area enter distal tubule which lies in renal cortex, and move to the connecting tubule which leads to the cortical collecting tubule. The initial parts of 8–10 collecting ducts join to form a single larger collecting tubules that runs downwards into the medulla and becomes the medullary collecting duct. The collecting ducts merge to form progressively larger ducts that eventually empty into the renal pelvis through the tips of renal papillae.

Blood flow to the two kidneys is approximately 22–25% of the cardiac output. The kidney is supplied by the renal artery which enters the kidney through the hilum and then branches progressively to form the interlobar arteries, arcuate arteries, interlobular arteries (also called radial arteries) and afferent arterioles, which lead to the glomerular capillaries. The distal ends of each glomerulus coalesce to form the efferent arteriole, which leads to a secondary capillary network, the peritubular capillaries which surround the renal tubules. The cortex receives approximately 90% of the blood flow compared to the medulla or papilla and so blood-born toxic molecules reaching the kidney have more toxic effect on the cortex compared to medulla or renal papillae. The interstitial space is occupied by the fenestrated peritubular capillaries and a small number of fibroblasts-like cells. Increase in thickness of interstitial space in pathological conditions is due to edema, proliferation of fibrous tissue or infiltration of inflammatory cells (Guyton and Hall, 2006; Kriz and Bankir, 1998).

## BIOTRANSFORMATION AND EXCRETION

The xenobiotics which are absorbed through the gastrointestinal (GI) tract, lungs, skin and other special routes of administration (e.g. intravenous) eventually reach the blood. After entering the blood toxicant is distributed throughout the body. The rate of distribution to organs or tissues is depended on the blood flow and the rate of diffusion out of the primarily capillary bed into the cells of a particular organ. Hence kidney is exposed to several toxicants. Xenobiotics are often concentrated in specific sites which may be the site of their toxic action or at sites other than target organs which can be considered as a storage depot. Kidney along with liver are the two important organs in the body which concentrate toxicants. A classic example is metallothionein, a protein which binds to cadmium and zinc with high affinity in kidney and liver (Rozman and Klaassen, 2001). Kidney is a target organ for many toxicants both primary and their metabolites which are distributed through blood as it receives approximately a quarter of the cardiac output. Xenobiotics can induce their toxicity on an organ by the action of their parent compound or through the metabolite as a result of bioactivation via metabolism (biotransformation). The nephrotoxicity can be induced by the parent compound (e.g. gentamicin), the biotransformed molecules in extra renal organs like liver (e.g. hepatic biotransformation of haloalkene) and by biotransformation in kidney (e.g. chloroform). Biotransformation reactions are catalyzed by Phase I and Phase II enzymes which convert the parent compounds to more polar and hydrophilic metabolites, thereby excreted more rapidly or convert them to reactive chemical species which can induce toxicity. The important Phase I enzymes present in the kidney are cytochrome P450, prostaglandin synthase and reductases. The important Phase I enzyme cytochrome P450 is present in the kidney, but the concentration is only approximately 10% of that in liver. The enzyme is found in the highest concentration in the renal cortex (majority in the proximal tubular epithelial cells), although small amounts are found in medulla. The compounds which are biotransformed by cytochrome P450 in the kidney are acetaminophen (acute overdosage), cephaloridine, chloroform and ochratoxin (partially). Prostaglandin synthase another Phase I enzyme is found primarily in the medullary interstitial and collecting duct cells. Chronic acetaminophen nephrotoxicity is attributed to the prostaglandin H synthase biotransformation. Phase I enzymes capable of catalyzing reduction reaction (reductases) are also present in the kidney and are involved in the reduction of 5-nitro group in 5-nitrofurans to reactive chemical species leading to nephrotoxicity. The important Phase II enzymes present in the kidney are UDP-glucuronosyltransferases (UGT), sulfotransferases and glutathione-*S*-transferases. Liver possess the highest UGT concentration and kidney has much lower concentration. UGT may contribute to acute or chronic toxicity associated with clofibrate, diclofenac (a nonsteroidal antiinflammatory agent (NSAID)) and other NSAIDs. The substrates for glutathione-*S*-transferases

include haloalkenes, acrolein, etc. (Rankin and Valentovic, 2004).

Kidney is the most important organ for the excretion of xenobiotics. The different mechanisms by which the toxicants are removed by the kidney are glomerular filtration, tubular excretion by passive diffusion and active tubular secretion. The xenobiotics which enter the blood reach the kidney where they are filtered through the large pores of the glomerular capillaries (70 nm size) and reach the tubular lumen and eventually excreted through the urine. Those compounds up to a molecular weight of about 60,000 are filtered through the glomeruli into the tubular lumen to be excreted with urine. The removal of toxicants through kidney tubule is mainly depended on the water solubility of the toxicant. Those xenobiotics which are biotransformed to more water soluble products (more polar) are excreted with the urine while those toxicants with high lipid solubility (high lipid/water partition coefficient) are reabsorbed across the tubular cells into the blood stream. Toxicants can be excreted by passive diffusion through the tubules and flow of urine is important for maintenance of concentration gradient necessary for this process. Toxicants are also excreted into urine by active secretion by a transporter which is an active transport process. These transporters are localized in the proximal tubules. The important transporters are organic anion transporter (oat) which is localized at the basolateral membranes of the proximal tubular cells and organic cation transporter (oct) which are involved in uptake of organic acids and some cations, respectively, into the renal tubular cells. Multidrug resistant protein (mdr) and multiresistant drug protein (mrp) move xenobiotics from the proximal tubule cells into the lumen. The organic action transporter (octn2) and peptide transporter (PEP2) are involved in the reabsorption of chemicals from the tubular lumen into the proximal tubular cells. A classic example of the action of active transport is the difference in susceptibility of the cephaloridine in adult and newborn animals. In newborn animals the active uptake of cephlaoridine is not well developed and hence it is not absorbed and concentrated in the tubular cells to result in toxicity (Rozman and Klaassen, 2001). Thus tubules play an important role in the excretion of toxicants and a toxic insult to those cells will result in accumulation of toxicants in the body (Figure 11.1).

The proximal tubule can be divided into three discrete segments. The S1 segment (pars convoluta) is the initial portion of the proximal convoluted tubule, S2 the transition between the pars convoluta and the pars recta which consists of the end of the convoluted segment and initial portion of the straight segment, and S3 the pars recta which consists of distal portion of the proximal segment (Figure 11.1).

The toxic effect of many xenobiotic compounds in kidney is specific to the anatomical locations as proximal tubules, glomeruli, medulla/papilla or loop of Henle (Figure 11.1). The site selective injury which is observed in human when exposed to many compounds is similar to that in domestic animals. The proximal tubule is the specific target for several antibiotics, mycotoxins, some antineoplastic drugs and heavy metals. Medulla/papilla is the target site for analgesics like phenylbutazone in horses. Loop of Henle is target for fluoride ion and glomeruli for immune complexes. The cause of these site specific target injury is multifactorial and may include detoxification mechanisms, regenerative ability, difference in blood flow, transport and accumulation of the chemicals and their metabolites, etc. Proximal convoluted tubule is the most common site of toxin induced injury and there are multiple reasons for this site specific action. These reasons include cytochrome P450 and cysteine conjugate β-lyase activity localization in this area (those agents requiring bioactivation by these enzymes induce toxicity in this region, e.g. chloroform), selective accumulation of xenobiotics in this area due to a relative loose epithelium compared to distal tubules which allows compounds to get into the cells easily, increased transport of several molecules like organic anions, cations and heavy metals resulting in increased accumulation in this area and increased susceptibility of proximal convoluted tubular epithelial cells to ischemic injury. Compared to the proximal tubules, distal tubules are less affected by toxicant injury (Schnellmann, 2001). The other anatomical location which is commonly affected by toxicant injury is the renal papillae and the classic example is the renal papillary necrosis by analgesics in horses. The important factors for this site specific injury are due to high concentration of prostaglandin H synthase activity in this area (they metabolize analgesics to reactive intermediate compounds which bind to cellular macromolecule), high papillary concentration of the toxicants, inhibition of the vasodilatory prostaglandins which result in ischemia to this area (Schnellmann, 1998).

## TOXIC EFFECTS ON KIDNEY

### Acute renal failure

Toxicants produce their effect on the different components of the kidney by affecting the tubules, glomeruli, vessels and the interstitium. Toxicants are an important cause of both acute and chronic renal failure with majority of the toxicants manifesting their effect by acute renal failure. The most important manifestation of acute renal failure is the decrease in GFR which leads to an excess of urea or other nonprotein nitrogenous component in the blood (azotemia). The decrease in GFR can be due to prerenal, renal or postrenal causes. Renal toxicity generally leads to renal or sometimes postrenal azotemia. Postrenal

**TABLE 11.1 Toxicants that adversely affects renal system**

| Toxicant | Species | Site and action |
|---|---|---|
| *Metals* | | |
| Mercury | All species | Tubules, tubular degeneration and necrosis |
| Arsenic | All species | Tubules, tubular degeneration and necrosis |
| Copper | All species | Tubules, degeneration and necrosis |
| Cadmium | All species | Tubules, tubular degeneration and necrosis |
| Lead | All species | Tubules, tubular degeneration and necrosis |
| Fluorine | All species | Tubules, tubular degeneration and necrosis |
| Zinc | All species | Tubules, tubular degeneration and necrosis |
| *Mycotoxins* | | |
| Ochratoxin | All species | Tubules, tubular degeneration and necrosis |
| Citrinin | All species | Tubules, tubular degeneration and necrosis |
| Oosporein | Avian | Tubules, tubular degeneration and necrosis |
| *Plants* | | |
| Oxalate in plant | Ruminants predominantly | Tubules, tubular degeneration and necrosis by crystals |
| Halogeton | | |
| Rhubarb | | |
| Sorrel, dock (*Rumex* spp.) | | |
| Oak | Ruminants | Tubules, tubular degeneration and necrosis |
| Amaranthus | Cattle and swine | Tubules, tubular degeneration and necrosis |
| Bracken fern | Cattle | Urinary bladder, hemorrhage, neoplasms |
| Easter lily | Cat | Tubules, tubular degeneration and necrosis |
| Red maple | Equine | Tubules, tubular degeneration and necrosis |
| White snake root | Equine and ruminants | Tubules, tubular degeneration and necrosis |
| Vitamin D/analog plants | Ruminants and horses | Tubules, arteries, mineralization |
| Cestrum diurnum | | |
| *Solanum* spp. | | |
| *Trisetum* spp. | | |
| *Sorghum* spp. | Ruminants | Urinary bladder, necrosis |
| *Antifungals* | | |
| Amphotrecin B | Dogs and cats mostly | Tubules, tubular necrosis by vasoconstriction |
| *Antibacterials* | | |
| Neomycin | | |
| Gentamicin | | |
| Tetracycline | Ruminants, dogs, cats, snakes | Tubules, tubular degeneration and necrosis |
| Sulfonamide | Ruminants | Tubules, tubular degeneration and necrosis by crystals |
| Furazolidone | Goat | Tubules, tubular degeneration |
| *NSAIDs/analgesics* | | |
| Phenyl butazone | Horse | Renal papillae, necrosis by vasoconstriction |
| Flunixin meglumine | | |
| Multiple NSAIDs and analgesics | Dogs, cats and avian | Tubules and renal papillae, necrosis |
| *Antineoplastics* | | |
| Cisplastin | Dogs | Tubules, degeneration and necrosis |
| Cyclophosphamide | Dogs | Urinary bladder, necrosis |
| *Rodenticides* | | |
| Diquat/paraquat | All species | Tubules, degeneration and necrosis |
| Zinc phosphide | All species | Tubules, degeneration and necrosis |
| Cholecalciferol rodenticide | All species | Tubules, arteries, mineralization |
| *Miscellaneous* | | |
| Ethylene glycol | Dogs and cats predominantly | Tubules, tubular degeneration and necrosis by crystals |
| Pine oil | Cats | Tubules, tubular degeneration and necrosis |
| Vitamin K3 | Horses | Tubules, tubular degeneration and necrosis |
| Cyclosporine | Dogs and cats | Tubules, glomerulus |
| Blister beetle | Mostly horses, but cattle also | Urinary bladder, necrosis |
| Raisin | Dogs | Tubules, tubular degeneration and necrosis |
| Snake venom | All species | Glomerulus, mesangiolysis |

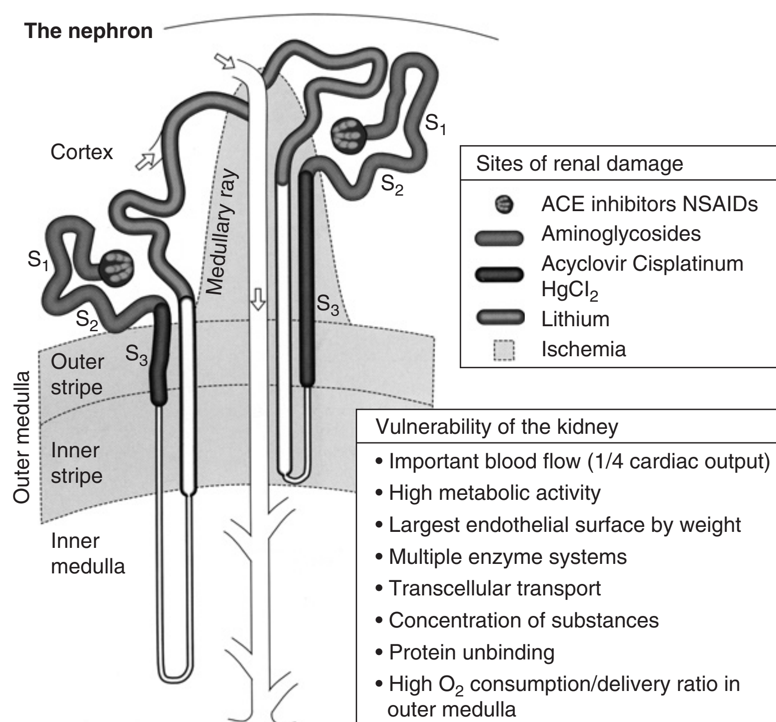


**FIGURE 11.1** Sites of renal damage, including factors that contribute to the kidney's susceptibility to damage. ACE: angiotensin converting enzyme; NSAIDs: nonsteroidal antiinflammatory drugs; $HgCl_2$: mercuric chloride (adapted from Berl and Bonaventure 1998, with permission).

azotemia is usually observed in conditions due to obstruction of renal out flow or post renal leakage. The toxic effect of chemicals acting on kidney results in reduced GFR leading to azotemia. The different mechanisms by which toxins/chemicals induce renal failure are: (1) tubular epithelial damage leading to formation of tubular casts which leads to tubular blockage, (2) tubular epithelial necrosis which leads to reabsorption of glomerular filtrate, (3) renal vasoconstriction which leads to hypoxia resulting in necrosis and (4) changes in the glomerular ultrafiltration barrier. Majority of the chemicals induce renal failure by direct action on the tubules.

The ultrastructural changes which lead to acute renal failure are due to the loss of cell-to-cell adhesion, and cell-to-matrix adhesion which eventually lead to a loss of tubular integrity.

The clinical signs associated with acute renal failure are nausea, vomiting, GI bleeding, esophagitis, gastritis and colitis which are all associated with azotemia. When azotemia is associated with a constellation of clinical signs and symptoms and biochemical abnormalities it is called uremia. There is secondary involvement of GI tract (uremic gastroenteritis), peripheral nerves (peripheral neuropathy) and sometimes heart (uremic fibrinous pericarditis).

## Chronic renal failure

Chronic renal failure is associated with long-term exposure to the toxins and is mostly related to the secondary pathological changes triggered by the initial injury. These secondary changes are compensatory mechanisms to maintain the function of the whole kidney but eventually result in reduced glomerular function leading to tubular and interstitial changes. Once the chronic changes are initiated it progresses to an end stage kidney.

Clinical signs of chronic renal failure are primarily (1) edema which is due to reduced renal perfusion leading to stimulation of renin–angiotensin system, which stimulates aldosterone secretion leading to retention of sodium and water, (2) hypocalcemia with compensatory parathyroid activity and osteodystrophy and (3) reduced red blood cell counts due to reduced synthesis of erythropoietin as a result of damage to juxtaglomerular cells.

## Pathological correlates

The kidney has extensive reserve capacity and hence renal diseases may be present with or without any clinical signs or clinicopathological abnormalities. Renal failure occurs or is manifested after substantial loss of the functional unit, the nephron. Renal failure represents clinical signs or laboratory abnormalities as a result of reduced renal function. Hence measurement of renal function will aid in diagnosing a toxic insult to the renal tubules or glomeruli. The various renal functions which can be measured to evaluate a toxic insult include urine concentration, blood urea nitrogen (BUN), urea excretion, serum creatinine, uric acid in birds, GFR, urine electrolyte clearance ratios, changes in blood levels of calcium, phosphorus,

magnesium, sodium and potassium as these elements are either due to loss or reduced excretion.

Urine analysis is one of the keys to evaluate the kidney function and to interpret the injury to the various component of the kidney. Urine analysis involves multiple physical and chemical parameters and sediment analysis. The different physical characters which are evaluated to assess the kidney function include volume, color, transparency and odor. The different chemical characters evaluated include ketones, protein, glucose, bilirubin, blood, urobilinogen, nitrate and enzymes. The different sediments which help to evaluate a toxic insult to the urinary system include epithelial cells, erythrocytes, leukocytes, casts and crystals. The important abnormal sediments which help in diagnosing an acute failure include renal epithelial cells, pigments casts (myoglobin, hemoglobin) and crystalluria (uric acid).

The other important parameters which can be measured to evaluate the function of the kidney include urine specific gravity, BUN, serum creatinine, uric acid excretion (in birds), GFR and levels of calcium and phosphorus in the serum. A reduced urine specific gravity, increased BUN (azotemia) increased serum creatinine concentration, hyperuricemia (in birds), altered GFR and altered serum calcium and phosphorus levels are all indicators of renal damage. Because of the high reserve capacity of kidney, many of these alterations are observed only when more than two third of the nephrons are damaged (Gregory, 2003).

The gross findings in renal damage are not that evident in acute damage and may include swollen cut sections, pale renal parenchyma and reddish discoloration. Mineralization and crystal deposits will be manifested by linear pale streaks in cortex and medulla with gritty consistency (mineral). Papillary necrosis is characterized by pale discoloration in the papilla and renal crest. The gross observations observed in chronic renal failure are reduced size, irregular subscapular surface, hard to section with gritty consistency. The important histopathological observations associated with renal damage are necrosis and degeneration of tubular lining epithelium mostly in the proximal convoluted renal tubular epithelium, regeneration, casts (protein, degenerated cells, globin) in the lumen, mineralization of the basement membranes of tubules, glomeruli tufts, its capsule and blood vessels, necrosis of the renal papillae, necrosis of the glomerular matrix with hemorrhage and increased cellularity of the glomerular tufts (proliferative glomerulonephritis) (Confer and Panciera, 2001).

# NEPHROTOXIC AGENTS

Specific nephrotoxic agents include mercury, cadmium, ochratoxin A, citrinin, oosporein, ethylene glycol, aminoglycosides, sulfonamides, tetracyclines, amphotericin B, cyclosporine, cisplatin, NSAIDs, Easter lily, pigweed, oak, oxalate containing plants and raisins. Other agents mentioned in this section produce toxic effects in multiple organs including kidneys.

## Mycotoxins

Ochratoxin is a mycotoxin produced by the toxic strains of *Penicillium* and *Aspergillus* spp. mostly related to improper storage conditions of grain, coffee, etc. Three different ochratoxins have been isolated and characterized (ochratoxins A, B and C). Of these ochratoxin A is the most commonly detected and most toxic among the three. The subchronic and chronic effects of this toxin are more of concern compared to the acute toxicity. Pigs are the most common species to be affected by ochratoxicosis. Clinical pathological changes include increased BUN values, reduced creatinine clearance and urine specific gravity. The gross pathological changes observed in experimental studies of pigs include enteritis, pale tan discoloration of liver, edema and hyperemia of the mesenteric and other lymph nodes. The microscopic findings are mostly confined to the kidney and GI tract which include necrosis of the renal tubular epithelium most frequently affecting the proximal convoluted tubules, dilation of the tubules, necrotizing enteritis in multiple locations, fatty degeneration of liver and focal necrosis of lymphocytes in the germinal center of the lymph nodes (Szczech *et al.*, 1973a; Zomborszky-Kovacs *et al.*, 2002). The renal pathological changes observed in experimental studies of dogs are similar to that observed in the porcine studies and included necrosis and desquamation predominantly in the proximal convoluted tubules, eosinophilic granular casts in proximal and distal convoluted tubules (Szczech *et al.*, 1973b; Szczech, 1975).

Ochratoxicosis has been reported commonly in avian species. Experimental studies in young broilers with ochratoxin A show renal edema and tubular necrosis (Wyatt *et al.*, 1975; Duff *et al.*, 1987). Chronic exposure of ochratoxin A is considered as an etiology in Balkan endemic nephropathy and urothelial tumors in human beings.

Citrinin is a benzopyran metabolite produced by the toxic strains of *Penicillium* sp. and *Aspergillus* spp. Some hepatotoxic effects have been reported for citrinin but the lethal effects are largely due to the nephrotoxic effects. The specific mechanism of action in kidney is not known. Primary effect is on the kidney and leads to acute tubular necrosis. In pigs, rats and rabbits, the proximal segments are affected. Histopathological findings in different species of animals include necrosis and desquamation of the proximal renal epithelium, basement membrane thickening, tubule dilation and proliferation of cells in interstitium (O'Brien and Dietrich, 2004). Experimental studies show that citrinin induces renal damage in turkeys and ducklings

along with hepatic degeneration and lymphoid necrosis (Mehdi *et al.*, 1984).

Oosporein which is produced primarily by *Oospora colorans* and *Caetomium trilaterale* is observed in feed stuff, cereals and peanuts. It has been reported to be toxic in poultry resulting in nephrotoxicity, visceral and articular gout. The pathological observations in oosporein toxicity include necrosis of the tubular epithelial cells in the proximal tubules with basophilic casts, hyaline casts in the distal tubules with fibrosis and interstitial pyogranulomatous inflammation, urate deposits in various tissues and proventricular enlargement with mucosal necrosis (Pegram and Wyatt, 1981; Brown *et al.*, 1987).

Fumonisin is a mycotoxin produced by the fungus *Fusarium moniliforme* primarily in corn. Brain, liver and lung are the target organs in horses and pigs. In rats and rabbits, kidney is the target organ. Histopathological changes primarily affect the renal tubular epithelium at the junction of cortex and medulla. Toxicity leads to apoptosis of epithelial cells of proximal convoluted tubules (Bucci *et al.*, 1998).

# DRUGS

## Aminoglycosides

The most important toxic effects manifested by aminoglycosides are nephrotoxicity, ototoxicity and neuromuscular blockade. Aminoglycosides accumulate in the proximal tubular epithelial cells where they are sequestered in the lysosomes and interact with ribosomes and mitochondria resulting in cellular damage by altered mitochondrial function and inhibition of protein synthesis.

Gentamicin toxicity has been reported in dogs. Grossly the kidney appears pale. Histopathological changes include tubular epithelial necrosis with regeneration and mineralization. Predominantly the proximal tubules are affected. Lesions will progress from hyaline droplet degeneration with dilated lumen to necrosis of the renal tubular lining epithelium (Sprangler *et al.*, 1980). Gentamicin toxicity has also been reported in snakes. In experimental studies conducted on gopher snakes, proximal tubules showed hydropic degeneration and this progressed to tubular necrosis 1 week after the gentamicin was discontinued. In the same experiment the snakes also developed visceral gout with urate crystals in multiple visceral organs (Montali *et al.*, 1979).

Neomycin toxicosis has been reported in calves. Experimental exposure of neomycin in calves has reported both nephrotoxicity and totality (demonstrated clinically). The clinical pathological observations included granular casts in urine, proteinuria and low specific gravity of urine, azotemia, decreased creatinine clearance, polyuria and polydipsia. The histopathological findings included renal tubular epithelial degeneration and necrosis (Crowell *et al.*, 1981).

Amphotericin B is an antifungal agent and is a drug of choice for systemic mycosis. Toxicosis of this drug can happen at recommended dosage rates. Nephrotoxicity may be related to a decrease in intrarenal blood flow associated with arteriolar vasoconstriction, a subsequent decrease in GFR and impaired tubular function. Dogs and cats are the common species affected with more susceptibility in cats. The common histopathological finding includes renal tubular necrosis, dilation of the tubules and mineralization with mild lesions in the interstitium and glomeruli (Rubin *et al.*, 1989).

## Cisplatin

Cisplatin is an antineoplastic drug which causes toxic changes in kidney, intestines and bone marrow. Cisplatin or its metabolites are eliminated through urine. Cisplatin may directly damage tubules and lead to tubular degeneration and necrosis or cause vasoconstriction leading to tubular necrosis. The exact mechanism of toxicity is not known and may involve the metabolites of cisplatin and not the platinum atom itself. The pathological findings associated with cisplatin treatment in dogs include mild renal tubular atrophy and tubular necrosis (Choie *et al.*, 1981; Forrester *et al.*, 1993).

## NSAIDs and other analgesics

NSAIDs are the most widely used analgesics in veterinary medicine, and all have some toxic potential. The common species affected are the dogs, cats and horses. NSAIDS cause toxicity of the GI tract, kidneys and liver. NSAIDs cause renal toxicity mainly by the inhibition of prostaglandin synthesis and renal blood flow resulting in renal papillary necrosis. The hepatic toxicity is mostly attributed to idiosyncratic reaction. Renal tubular or papillary necrosis is also reported in a limited number of cases in dogs (Jones *et al.*, 1996).

Horses are susceptible to phenylbutazone toxicity especially when dehydrated and the target organ is kidney. The most common gross observation in experimental studies conducted in horse dosed with phenylbutazone and deprived of water was yellow green radial streak in the renal papillae. The microscopic observations include sloughing of the renal pelvic epithelium including the terminal lining of collective ducts with coagulation necrosis of the underlying papillary interstitium, mineralization of the calyx necrotic papillary interstitium contain nuclear debris and hemorrhage and dilation of collecting ducts and cortical tubules in wedge-shaped segment of the kidney tissue above area of necrotic papillae. Other lesions observed in experimental studies of horses include ulceration and

erosions of the glandular portion of the stomach, submucosal edema of the small intestine, erosions and ulcers of the large colon, ulceration of the gastric mucosa and tongue. Pathological findings in experimental studies of horses exposed to flunixin meglumine (aminonicotinic acid) were glandular mucosal erosion and ulcer of the stomach (Gunson and Soma, 1983; MacAllister *et al.*, 1993).

Large doses of acetaminophen can cause renal toxicity along with hepatotoxicity in rats and mice. Toxicity is characterized by renal tubular necrosis in the proximal tubules (Schnellmann, 2001). Recently report of vulture mortality was reported from the Indian subcontinent related to NSAID toxicity. Diclofenac is an NSAID widely available veterinary drug in the Indian subcontinent, used in domestic livestock. Vultures were exposed to the drug when they consumed carcasses of cattle that were treated with diclofenac shortly before death. Experimental studies of this drug in vultures showed marked nephrotoxicity. The gross observations were primarily deposits of urate on the surface of internal organs related to renal failure. Histopathological finding were acute necrosis of the proximal renal tubular epithelium with minimal inflammatory response and deposits of urate crystals (Oaks *et al.*, 2004; Meteyer *et al.*, 2005). Aspirin, ibuprofen and acetaminophen are the important analgesics which are reported to cause toxicosis in veterinary medicine. Generally, toxicosis is a result of large overdoses and affects liver. Renal lesions including renal tubular necrosis and papillary necrosis have been reported in dogs.

## Sulfonamides

Renal toxicity associated with sulfonamides is rare as most of the current pharmaceutical preparations are relatively highly soluble at the pH normally occurring in the kidney. Toxicity occurs due to very high overdosage. Sulfonamides can cause both acute and chronic toxic effects. The most important organ affected in toxicity is the kidney. Animals with renal toxicity will show elevated levels of BUN and creatinine. In some animals sulfonamide crystals are observed in the renal pelvis as a gross finding and kidneys are gritty in texture when cut. Histopathological observation is primarily renal tubular epithelial degeneration and necrosis due to the direct action of the crystals and these crystals are visible with polarized light only if the kidney is processed to prevent the dissolution (Jones *et al.*, 1992).

## Tetracyclines

Tetracyclines have been reported to cause toxicity, and kidneys are the primary organs affected. Renal tubular necrosis is the common pathological finding. Animals with renal toxicity will show oliguria, marked azotemia, moderate proteinuria, tubular casts in urinary sediment with inability to concentrate urine (Lairmore *et al.*, 1984; Vaala *et al.*, 1987).

## Vitamin K3 (menadione sodium sulfate)

Toxicity is commonly reported in horses. In experimental studies the gross lesion observed was enlargement and paleness of the kidney. The microscopic lesions include diffuse or multifocal tubular necrosis and dilation with proteinaceous and cellular debris from red blood cells and neutrophils and casts, diffuse interstitial edema and lymphocytic infiltration regeneration of the tubular epithelium. In animals which survived for 3 months renal tubules showed degeneration, necrosis and dilation. In chronic renal failure following vitamin K3 administration, the kidneys were reduced in size, the capsule adherent, and pale streaks of connective tissue may be grossly apparent. Microscopic findings include connective tissue proliferation with mononuclear cell infiltration, moderate hypercellularity of glomeruli with few sclerotic glomeruli. The tubules are dilated but the lining epithelium appears normal with mineral, cellular and proteinaceous cast which may be seen in many tubules (Rebhun *et al.*, 1984).

## Cyclosporine

Cyclosporine is a macrolide antibiotic and has been used as an immunosuppressive agent. Cyclosporine can cause both renal and nonrenal toxicity. Clinically renal toxicity consists of four discrete syndromes which include acute reversible renal functional impairment, delayed renal allograft function, acute vasculopathy and chronic nephropathy with interstitial fibrosis. Proximal tubular epithelium is uniquely sensitive to the toxic effect. The toxic effect is characterized by isometric cytoplasmic vacuolations (equally sized several small vacuoles in cytoplasm), necrosis with or without subsequent mineralization, inclusion bodies (giant mitochondria) and giant lysosomes. Acute vasculopathy consists of vacuolization of the arteriolar smooth muscles and endothelial cells leading to necrosis. In some cases thrombotic microangiopathy develops characterized by thrombosis of the renal microvasculature. Long-term treatment with cyclosporine results in chronic nephropathy with interstitial fibrosis (Charney *et al.*, 2004).

## Furazolidone

Furazolidone renal toxicity has been reported in goats. The gross observations included congestion in brain, livers and kidneys. The histopathological findings included degenerative changes in the renal tubules, hepatocyte

necrosis and degeneration in the centrilobular areas (Ali *et al.*, 1984).

## METALS

### Mercury

Fungicides, preservatives and fixatives are the main sources of mercury toxicosis. Often toxicosis is related to accidental ingestion of obsolete mercury products. The lesions of mercury toxicosis are distributed in the GI tract, kidney and sometimes in the brain (alkyl organic mercury). Inorganic mercurial salts accumulate in renal cortex and are excreted primarily in urine. Inorganic mercurial salts cause direct tissue necrosis and renal tubular epithelial necrosis by two mechanisms. Mercuric ion binds covalently with sulfur and inhibits sulfhydryl containing enzymes in microsomes and mitochondria. The mercurial salts may bind to protein as mercaptides. Death occurs in acute toxicity, and oliguria and azotemia are observed in animals surviving acute exposure (Osweiler, 1996). Gross lesions include gastric ulcers, congestion of the intestinal mucosa, pale swollen kidney and pale liver. Histopathological findings in kidney are primarily renal tubular degeneration and necrosis especially in the proximal tubular epithelium. In chronic exposure glomerulonephritis is observed due to antigen–antibody complex deposition (Jones *et al.*, 1996).

Diagnosis and confirmation can be made by analytical estimation in the kidney. Differential diagnoses include toxicosis by lead, thallium, phenylarsonic feed additive and ethylene glycol toxicosis, encephalitis, polioencephalomalacia, hog cholera (pigs) and erysipelas (pigs).

### Cadmium

The other metal which is associated with rare cases of renal toxicity in domestic animals is cadmium. Cadmium exposure is by ingestion of cadmium–nickel batteries. Cadmium is concentrated in the renal tubules and to a lesser degree in hepatocytes. Cadmium induces metallothionein production and forms cadmium–metallothionein complex. This complex is filtered freely by the glomerulus and is reabsorbed by proximal tubules. Inside the tubules renal metallothionein is formed and once the renal metallothionein pool is saturated free cadmium ion initiates renal tubular injury (Osweiler, 1996; Schnellmann, 2001).

### Arsenic

Toxicosis is mostly reported in cattle and dogs by accidental exposure but all species of domestic animals can be affected. Trivalent arsenicals inhibit cellular respiration and cause capillary dilation and degeneration. Pentavalent inorganic arsenicals cause uncoupling of oxidative phosphorylation leading to cellular energy deficiency. In acute toxicosis the pathological changes are mostly limited to the GI tract. Subacute toxicity is by exposure to low dose for several days. In subacute cases kidneys are affected. The clinical signs associated with subacute toxicity are oliguria and proteinuria followed by polyuria. Watery diarrhea exhibited in acute poisoning will also be observed. Death results due to dehydration, acidosis and azotemia (Osweiler, 1996). The gross findings include pale swollen kidney and pale liver. Renal damage affects all portions of the nephron leading to severe renal tubular necrosis. Histopathological lesions consist of dilation of the vessels, submucosal congestion and edema, necrosis of the intestinal epithelium, renal tubular necrosis and fatty degeneration of the hepatocytes (Jones *et al.*, 1996). Diagnosis is by correlating the case history, pathological findings and analytical estimation of arsenic in liver, kidney and other tissues (hair). Differential diagnosis of pathological lesions includes infectious agents affecting kidney and NSAID exposure.

### Copper

Acute copper toxicosis results from accidental ingestion or administration of copper containing formulations like anthelmintics, feed additives, foot baths used for livestock, pesticides including fungicides and algaecides at a toxic dose. Copper toxicosis is a problem observed frequently in the sheep as they are sensitive to excessive accumulation of copper. Sheep accumulate copper in the liver in proportion to intake, where there is slow build up of the copper and then there is sudden release which results in excess copper in the circulation. This excess copper in the circulation oxidize the erythrocyte membrane resulting in intravascular hemolysis. Clinical signs in sheep are due to acute anemia and include weakness, anorexia, fever, pale mucous membranes and dyspnea. Grossly the kidney has a dark red or bluish black color (gun metal kidneys) and the liver will be pale yellow and friable. Microscopic findings include necrosis of renal tubular epithelium and hemoglobin casts (Osweiler, 1996; Maiorka *et al.*, 1998). Diagnosis is by correlating liver copper values with pathological findings and case history.

### Fluoride

Fluoride toxicosis can be acute or chronic and acute toxicosis is by exposure to insecticides/rodenticides and exposure to volcanic dust exposure. Fluoride is metabolized via renal excretion and is preferentially deposited in bones

and teeth. Acute fluoride intoxication produces clinical signs and lesions of gastroenteritis and renal tubular necrosis. In a study conducted in sheep exposed to fluoroacetate, the microscopic findings in acute toxicosis included degeneration and necrosis of myocardial fibers. In a study conducted in sheep poisoned by sodium fluoride the pathological findings included necrosis of the proximal tubular epithelial cell, necrotizing rumenitis, reticulitis and abomasitis (Shupe, 1980; O'Hara *et al*., 1982; Maylin *et al*., 1987).

## Lead

Lead toxicosis is caused by exposure to lead paint, lead containing objects like toys foils, etc., plumbing material, tiles and linoleum. Lead toxicosis affects hemoglobin synthesis by inhibiting the enzymes delta-aminolevulinic acid synthetase and ferrochelatase resulting in anemia. Clinical signs include signs of central nervous system involvement, anorexia, anemia and proteinuria. Clinical pathological findings suggestive of lead toxicosis are basophilic stippling of erythrocytes and metarubricytosis with minimal polychromasia. Anemia may be microcytic hypochromic to normocytic normochromic (Prescott, 1983). Animals dying of acute lead poisoning have very few microscopic lesions other than mild degenerative neuronal necrosis in the cerebral cortex and mild renal epithelial degeneration.

In chronic cases the kidney histopathological changes are characterized by degeneration and necrosis of the renal tubular epithelium which may show regeneration. Degenerative changes of the renal epithelial cells and hepatocytes have been reported in chronic lead poisoning in dogs. Intranuclear inclusions may be visible in the renal tubular epithelial cells, but their absence do not rule out lead toxicosis (Jones *et al*., 1996; Osweiler, 1996).

In birds especially water fowls, lead toxicosis has been reported and the lead shots may be found in the gizzard. The birds are generally emaciated with extensive muscle wasting with atrophy of the breast muscles. Changes in the kidney are primarily renal tubular degeneration or necrosis (with or without intranuclear inclusions) (Locke and Thomas, 1996). Diagnosis is by analytical estimation and history of exposure. Liver and kidney are the preferred samples for analysis of lead.

## Zinc

Zinc toxicosis has been reported in dogs as a result of ingestion of zinc containing hardwares, pennies which contain zinc, drinking water, acidic food material from galvanized tanks and containers. Severe intravascular hemolysis is the most prominent clinical pathological finding associated with zinc toxicosis in dogs. The gross observation in dogs and cats exposed to zinc includes splenomegaly, icterus, enlarged diffused red kidney with hemorrhage, and histopathological observations in the kidney are renal tubular necrosis (Luttgen *et al*., 1990; Osweiler, 1996). In experimental studies conducted in sheep exposed to zinc, renal lesions observed were degeneration of the tubular lining epithelium along with pancreatic lesions (Smith and Embling, 1993).

The renal histopathological changes observed in experimental studies in veal calves include multifocal renal cortical fibrosis with necrosis in convoluted tubules and loops of Henle and intratubular mineralization (Graham *et al*., 1988). Diagnosis is by correlating case history, pathological findings and analytical estimation in liver, kidney and pancreas.

# PESTICIDES

## Paraquat and diquat

Paraquat is one of the two widely used bipyridyl broad-spectrum herbicides (the other being diquat). The most common route of intoxication is ingestion. Among domestic animals toxic exposure has been reported in cattle, sheep, horses, pigs, poultry and dogs. Paraquat primarily affects the lungs. Kidney and liver may also be affected and the histopathological change is renal tubular epithelial degeneration. Diquat toxicity results in intracerebral hemorrhage and acute renal failure (Dungworth, 1993). Diagnosis is by correlating history of exposure with pathological findings.

## Cholecalciferol/vitamin D3

Vitamin D3 toxicity in veterinary medicine is by overdosage of vitamin supplements or exposure to rodenticide. Dogs and cats are the common species affected but any species can be affected. The common clinical pathological finding is rapid increase in plasma phosphorus in acute cases and followed by an increase in plasma calcium levels. Histological findings include mineralization in multiple organs including the kidneys, lungs, myocardium, stomach and vessels. In kidneys mineralization of arteries as well as mineral deposition in the tubular basement membranes are observed. Diagnosis is by chemical evaluation of serum, plasma, bile, urine and kidney for PTH/25-hydroxycholecalceferoland correlating pathological findings (Gunther *et al*., 1988; Fooshee and Forrester, 1990; Talcott *et al*., 1991). Differential diagnosis includes ethylene glycol toxicity, hypercalcemia of malignancy, chronic renal failure and other causes of hypercalcemia.

## Zinc phosphide

Toxicosis can happen by animals feeding directly on bait or by eating tissues of zinc phosphide-poisoned animals. All domestic animals can be affected and reports are common in dogs. Zinc phosphide releases phosphine gas when in contact with acid contents of the stomach. Both phosphine and intact zinc phosphide are absorbed from the GI tract. The phosphine is believed to cause the majority of acute signs, while the intact phosphide may cause hepatic and renal damage later. Histopathological findings in kidney include renal tubular degeneration and necrosis (Casteel and Bailey, 1986). Diagnosis is by analysis of stomach contents/vomitus/liver and kidney for phosphine gas and the samples have to be submitted frozen in air-tight containers.

# PLANTS

## Easter lily

Cats are the most common species affected. The aqueous extracts of leaves and flowers are proven to be nephrotoxic and pancreatoxic. The renal tubular epithelial cells are damaged resulting in anuric renal failure. Clinical signs start with GI disturbances followed by isosthenuria, polyuria, dehydration, anuria, weakness and recumbency. The clinical pathological changes include an elevated creatinine and BUN with glucosuria, proteinuria with numerous tubular epithelial casts in urine sediment. The significant histopathological findings include acute necrosis of proximal convoluted tubules and degeneration of pancreatic acinar cells. The tubular epithelial cells show loss, degeneration and regeneration. Tubular lumen contains protein casts and cellular debris. Renal ultrastructurally changes include swollen mitochondria, megamitochondria, edema and lipidosis (Langston, 2002; Rumbeiha *et al*., 2004). Diagnosis is by correlating the clinical signs with histopathological examination and case history.

## Oxalate containing plants

### *Rheum, Halogeton, Rhubarb*

The common plants which contain toxic level of soluble oxalates are Rhubarb (*Rheum rhaponticum*), Halogeton (*Halogeton glomerulatus*) and Grease wood (*Sarcobatus vermiculatus*), beets and dock (*Rumex* spp.) and lamb quarters (*Chenopodium* spp.).

The toxic principles are oxalic acid, sodium and potassium oxalates, which complex with calcium forming calcium oxalate and crystallize in the kidney when they are excreted. Sheep, cattle and swine are the common species affected. Hypocalcemia is observed due to formation of calcium oxalate. Increased BUN values are observed in chronic cases with nephritis. The gross lesions consist of radially arranged white streaks in the kidney and hemorrhages in the GI tract. The common microscopic findings are hemorrhagic rumenitis in sheep associated with deposits of calcium oxalate in the walls of blood vessels, oxalate nephrosis characterized by irregular rhomboid crystals which are visible in sharply reduced or polarized light, protein casts in tubular lumen with varying degrees of renal tubular epithelial degeneration and necrosis (Dickie *et al*., 1978; Panciera *et al*., 1990).

## *Amaranthus* spp.

The toxic principles include an unknown agent which causes perirenal edema and nephrosis. The most common species affected are cattle and swine. The significant clinical pathological findings include increased BUN, proteinuria, CK and creatinine.

The most striking gross lesion is retroperitoneal edema in the perirenal connective tissue. Edema is often present in the ventral abdominal wall and per rectal area. Kidneys are normal or small in size and pale with subcapsular petechiae. In calves free straw colored fluid is observed in thoracic and peritoneal cavities. Histopathological findings include degeneration and necrosis of the tubular epithelium of the proximal tubules with interstitial edema, dilation of the renal tubules with protein casts and some may have oxalate crystals (Stuart *et al*., 1975; Kerr and Kelch, 1998). Diagnosis is by evidence of consumption of the plant material with appropriate clinical signs and pathological findings.

## Oak (*Quercus* spp.)

Oak (*Quercus* spp.) poisoning has been reported from many regions of the world, and more than 60 species of oak have been identified in North America, Tannins and their metabolites (digallic acid, gallic acid and pyrogallol) are responsible for the toxicity. High levels of toxic component are in young leaves and the shells of green acorns. Cattle and horses are the common species affected with reports more common in cattle.

Kidney is the target organ with pathological findings also in GI tract. Oak causes GI irritation which manifests in the early course of toxicosis, characterized by anorexia, constipation and colic. After a few days, constipation is followed by diarrhea, and fragments of acorns may be present within stools.

The gross findings include swollen pale kidney with petechial hemorrhage, perirenal edema, hemorrhage and

ulcers in the GI tract (can extend from pharynx to the lower intestinal tract). The histopathological findings include degeneration and necrosis of tubular epithelium, regeneration of tubular epithelium, dilated tubules with erythrocytes, homogenous and granular casts and sloughed necrotic tubular epithelial cells and neutrophil infiltration. The renal interstitium has multifocal areas of hemorrhage and edema. Secondary lesions related to uremia and renal failure may also be observed in stomach and tongue. Oak toxicosis has high mortality due to renal failure (Anderson *et al*., 1983; Plumlee *et al*., 1998). Diagnosis is by evidence of consumption of the plant material with appropriate clinical signs and pathological findings.

## Red maple (*Acer rubrum*)

The most common species affected is equine. An unidentified toxin creates an acute hemolytic anemia associated with methemoglobinemia and/or Heinz body formation. Clinical pathological findings are hemolytic anemia and blood smears may show eccentrocytes, spherocytes, anisocytes and Heinz bodies. It produces generalized icterus, spleenomegaly and enlarged liver with pale centrilobular areas, dark brown black discolored kidney, increased pleural and pericardial fluid, petechiae and ecchymosis in the serosal surfaces. The histopathological findings in kidney include renal tubular epithelial degeneration with hemoglobin casts (Figure 11.2) (George *et al*., 1982; Stair *et al*., 1993). Diagnosis is by evidence of consumption of the plant material with appropriate clinical signs and pathological findings. Differential diagnosis includes nitrate toxicosis, equine infectious anemia and Babesiosis.

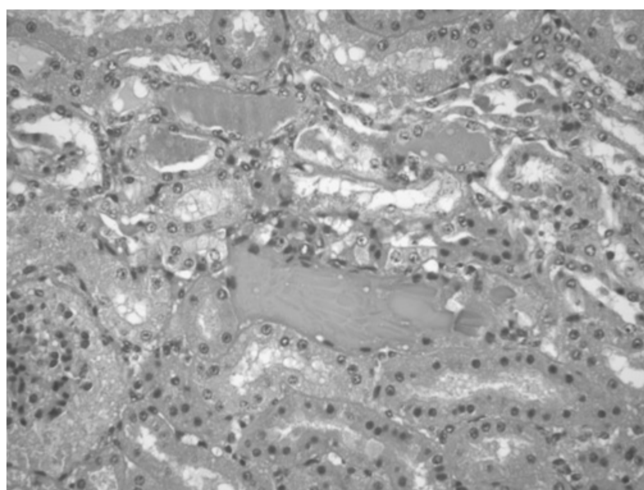

**FIGURE 11.2** Red maple poisoning, equine kidney, tubules are dilated and lined by degenerate epithelium. The tubular lumen is filled with casts. H&E stain 40×. This figure is reproduced in color in the color plate section.

## Vitamin D containing plants

The most common plants which contain calcitriol that accounts for pathogenesis are *Cestrum diurnum*, *Trisetum flavescens* and *Solanum malacoxylon*. The toxicityin Cestrum is attributed to 1,25-dihydroxy-vitamin D-glycoside and that in *Solanum malacoxylon* to a molecule similar to or identical to 1,25-dihydroxy vitamin D. The plant (*Cestrum diurnum*) is seen in warmer parts of the USA like Florida and is grown as an ornamental plant. *Solanum* spp. is seen in Brazil and Argentina, and *Trisetum* spp. is seen in Germany and Austria. The common species affected are cattle and horses. *Solanum malacoxylon* toxicity is commonly reported in sheep and cattle. Other animals are susceptible experimentally. Adult cows are more susceptible than calves. The clinical pathological observation in the early stage is hypercalcemia and hyperphosphatemia and later the levels decrease resulting in normal calcium and phosphorus levels. The gross findings include white to tan gritty foci in multiple organs including kidney. In cattle the microscopic findings include cardiac mineralization commonly in the endocardium of left atrium, mineralization of the aorta primarily involving tunica intima and media. Mineralization is observed in several major and minor blood vessels. Other locations in which microscopic evidence of mineralization are observed include kidneys, alveolar septa of lung, bronchiolar epithelium, muscles of the intestine and stomach. Diagnosis is by correlating history of exposure, pathological findings and estimation of 1,25-dihydroxy cholecalciferol levels in serum (Jones *et al*., 1996).

## White snake root (*Eupatorium rugosum*)

The common species affected by white snake root (*Eupatorium rugosum*) are cattle, horses, sheep and goats. The toxic component is tremetol and has been described as a fat soluble, high molecular weight alcohol. The toxic principle is a cumulative poison and repeated exposure to small amounts results in intoxication. Clinical pathological changes associated with liver and muscle damage is observed. Myoglobinuria or hemoglobinuria is also observed. The gross findings are observed primarily in heart and skeletal muscles with congestion in kidneys. Histopathological changes in the kidney consist of degeneration and regeneration of the tubular epithelium with hemoglobin or myoglobin casts and congestion (Olson *et al*., 1984; Jones *et al*., 1996). Diagnosis is made by correlating clinical signs, history of exposure, pathological findings and chemical analysis of the toxin in liver and kidney. The differential diagnosis includes ionophore toxicity, selenium/vitamin E deficiency and oleander toxicity.

## MISCELLANEOUS AGENTS

### Ethylene glycol

Intoxication with ethylene glycol is caused by consumption of antifreeze solution which contains up to 95% ethylene glycol. Mostly cats and dogs are the affected species but there are rare reports of intoxication in other species. The common clinical pathological findings in cats and dogs include neutrophilia, lymphopenia, azotemia, hyperphosphatemia, hypocalcemia, hyperglycemia and decreased whole blood bicarbonate. The common findings in urine analysis include proteinuria, glucosuria, hematuria, calcium oxalate and hippurate crystalluria, and the presence of renal epithelial cells, white blood cells and granular and cellular casts in the urine sediment.

Grossly the kidneys are firm with pale streaks at the corticomedullary junction. In some cases pulmonary edema and hyperemia of the gastric and intestinal mucosa are observed. The microscopic findings include marked renal interstitial fibrosis (in cases which survive for long), mild lymphocytic infiltration in the interstitium, atrophy, degeneration and necrosis and of the renal tubular epithelium with birefringent crystals (Figure 11.3), mineralization and glomerular atrophy in some cases. Microscopic demonstration of birefringent crystals in renal tubules using polarized light (Figure 11.4) is pathognomonic for ethylene glycol toxicity in dogs and cats (Jones *et al*., 1996). The antemortem diagnosis is by clinical pathological evaluation (increased anion and osmolal gaps, suggestive), colorimetric test kit, glycolic acid assay in serum and postmortem diagnosis is by pathological evaluation or chemical analysis of kidneys.

### Snake bite

Most of the poisonous snakes in USA are of Crotalidae family which includes the rattle snakes, water moccasins and copper heads. The Crotalidae family produces venoms which are mostly necrolytic and hemolyzing. Hemorrhagins in crotalid venom are toxic to the vessels and hence cause hemorrhage and edema at the wound site, in addition to systemic hemorrhage and shock. Marked anemia is observed due to hemolysis and extravasations. Disseminated intravascular coagulation is observed in some cases. The common initial clinical pathological changes include echinocytosis, thrombocytopenia, leukocytosis and prolonged activated clotting time. Generally edema and erythema along with fang marks (Crotalidae family) may be observed at the site of bite although it is tough to identify due to thick hair coat in animals. Major route of venom excretion is through the kidneys and hence kidney failure is observed in many cases of crotalid snake bites characterized by renal tubular necrosis with protein casts, hemorrhage and necrosis in the glomeruli (mesangiolysis). Mesangial proliferative glomerulonephritis is observed in patients who survive (Dickinson *et al*., 1996; Sitprija and Chaiyabutr, 1999; Hackett *et al*., 2002).

### Raisins

Grape and raisin ingestion has been reported to cause renal toxicity in dogs. The specific mechanism is not known. Clinical pathological findings include hypercalcemia, hyperphosphatemia, increased Ca $\times$ $PO_4$ product elevated BUN and serum creatinine concentrations, all related to renal damage.

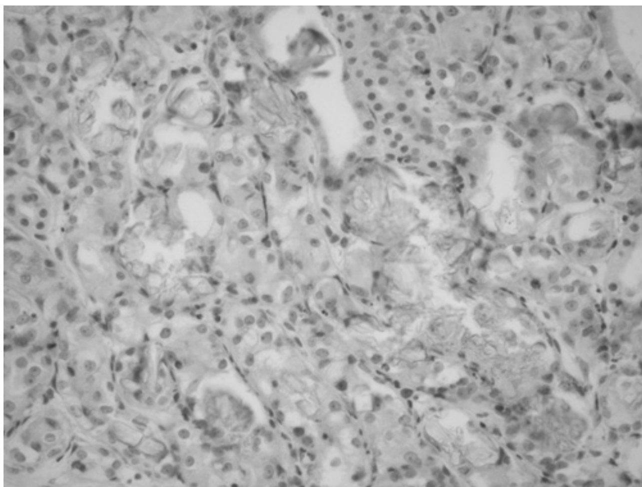

**FIGURE 11.3** Ethylene glycol poisoning, canine kidney, tubules are lined by degenerate epithelium and multiple tubules contain crystalline material. H&E stain 40×. This figure is reproduced in color in the color plate section.

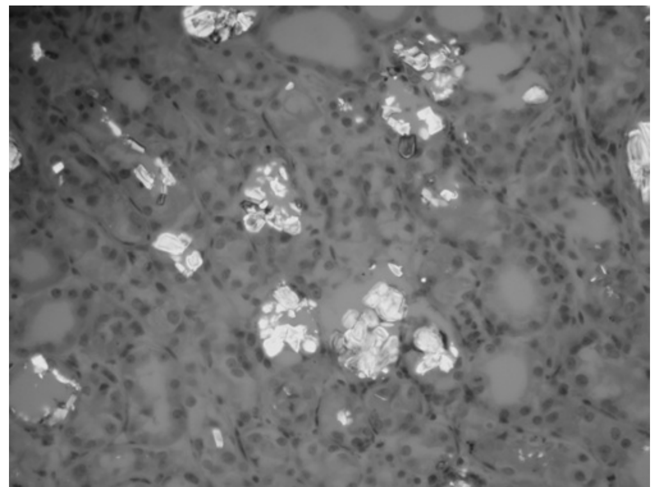

**FIGURE 11.4** Ethylene glycol poisoning, canine kidney, multiple tubules contain birefringent crystalline material. H&E stain 40× under polarized light. This figure is reproduced in color in the color plate section.

Gross lesions are absent and microscopic findings include degeneration and necrosis of proximal renal tubular epithelium with intact basement membrane, regeneration of the tubular epithelium and mineralization. The tubules contain granular and protein casts. In few animals fibrinous arteritis of the large colon were also observed (Eubig *et al.*, 2005; Morrow *et al.*, 2005).

## Pine oil

It is a component of many house hold cleaners and disinfectants. Renal toxicity has been reported in cats exposed to pine oil. The histopathological findings include renal cortical necrosis and centrilobular necrosis (Rousseaux *et al.*, 1986).

## Lower urinary tract

The anatomical components of this area include the ureters, urinary bladder and urethra. Only very few agents are reported to induce damage to these areas. Transitional epithelium lines these areas and the reduced toxic insult to this area may be due to reduced metabolic activity of transitional epithelium and reduced time of exposure to these cells as urine is excreted rapidly except for the time retained in the urinary bladder. The important agents which induce changes in the lower urinary tract are blister beetle, bracken fern, *Sorghum* spp. and cyclophosphamide.

## Black blister beetle (*Epicauta* spp.)

Toxicosis is due to ingestion of blister beetle itself. Toxicity occurs primarily when beetles are present in alfalfa hay mostly in southwestern US and occasionally in Eastern and Midwestern states of United States. The toxic principle is cantharidin, bicyclic terpenoid, which is contained in the hemolymph, genitalia and other tissues of beetle. This is a very stable compound and remains toxic even in dead beetle. Ingested cantharidin causes irritation of oral and GI mucosa leading to colic. Horses are the most common species affected.

Clinical pathological findings include hemoconcentration, neutrophilic leukocytosis and hypocalcemia. Lesions are confined to GI tract, urinary tract and heart. Gross lesions are rare and may include ulceration and hyperemia in the entire GI tract and urinary bladder. Histopathological findings include ulceration and necrosis of the mucosal epithelium in the esophagus, stomach, urinary bladder and myocardial necrosis (Schoeb and Panciera, 1979; Schmitz, 1989; Helman and Edwards, 1997). Diagnosis is by clinical signs, pathological findings confirmed by analysis of urine and gastric contents for cantharidin.

## Bracken fern (*Pteridium aquilinum*)

Most common species affected are horses and cattle. Several toxic principles are recognized and poisoning in nonruminants is due to presence of thiaminase that leads to thiamine deficiency. In ruminants the toxins include a bone marrow toxin and the suspected carcinogen ptaquiloside which induce the bladder tumors in cattle.

In cattle different clinical manifestations of the poisoning by bracken fern are reported. When large amount of plants are consumed in a short period of time aplasia of the bone marrow develops and results in mortality. Thrombocytopenia, neutropenia and anemia develop and early myeloid cells are destroyed. Those animals consuming less quantity for longer period develop hematuria and are called enzootic hematuria. They also develop urinary bladder tumors and squamous cell carcinoma in the GI tract. All these lead to death due to anemia and chronic wasting. The pathological findings include widespread petechial and ecchymotic hemorrhage in intestines, serosal surfaces of several internal organs including urinary bladder, gall bladder, heart, subcutaneous tissue and muscles. Some animals develop abomasal ulcers. Hyperplasia and hemorrhage are observed in the urinary bladder and also ureter or renal pelvis. Chronic cystitis with hemangiomas develops which bleed resulting in hematuria. Adenocarcinomas, transitional cell carcinomas, squamous cell carcinomas, papillomas, fibromas and adenomas are reported in the urinary bladder, renal pelvis and ureters, related to exposure of bracken fern. Tumors in the GI tract are mostly squamous cell carcinomas located in pharynx, base of tongue, esophagus and rumen (Pamukcu *et al.*, 1976; Jones *et al.*, 1996; Gava *et al.*, 2002; Carvalho *et al.*, 2006). Diagnosis is by evidence of consumption of the plant material with appropriate clinical signs and pathological findings.

## *Sorghum* spp. (Equine Cystitis Ataxia syndrome)

In horses, especially females, a syndrome of cystitis and ataxia has been associated with the consumption of hybrid strain of *Sorghum* spp. Gross observations include edema and hemorrhage in the bladder mucosa with occasional ulceration of the mucosa. Histological findings include focal necrosis and ulceration of the urinary bladder mucosa, degeneration of axons and demyelination with gitter cells in cervical, thoracic, lumbar and sacral spinal cord segments. Fetal malformations associated with hybrid

sudan are mentioned in the reproductive system section (Adams *et al*., 1969; Morgan *et al*., 1990).

### Cyclophosphamide

The common pathological manifestation of adverse reaction of cyclophosphamide therapy in dogs and cats is sterile hemorrhagic cystitis characterized by ulceration, edema and necrosis. Toxic effects of cyclophosphamide in the urinary bladder are by the action of acrolein, a metabolite of cyclophosphamide. Acrolein causes submucosal, edema, necrosis, hemorrhage and fibrosis of the urinary system epithelia (Dhaliwal and Kitchell, 1999; Charney *et al*., 2003).

## CONCLUSION

The primary functions of the kidney are get rid of the body waste materials that are either ingested or produced by metabolism and to control the volume and composition of the body fluids. The toxins absorbed by the different routes are biotransformed and enter the blood which is eliminated through the urine, feces and air. Since kidney receives approximately a quarter of the cardiac output it is an important organ for the exposure of toxicants and their metabolites.

## REFERENCES


Adams LG, Dollahite JW, Romane WM, *et al.* (1969) Cystitis and ataxia associated with sorghum ingestion by horses. *J Am Vet Med Assoc* **155**(3): 518–24.

Ali BH, Hassan T, Wasfi IA, Mustafa AI (1984) Toxicity of furazolidone to Nubian goats. *Vet Hum Toxicol* **26**(3): 197–200.

Anderson GA, Mount ME, Vrins AA, *et al*. (1983) Fatal acorn poisoning in a horse: pathologic findings and diagnostic considerations. *J Am Vet Med Assoc* **182**(10): 1105–10.

Berl T, Bonaventure JV (1998) *Atlas of Diseases of Kidney*, Schrier RW (ed.). Blackwell Publishing, Philadelphia, PA.

Brown TP, Fletcher OJ, Osuna O, *et al*. (1987) Microscopic and ultrastructural renal pathology of oosporein-induced toxicosis in broiler chicks. *Avian Dis* **31**(4): 868–77.

Bucci TJ, Howard PC, Tolleson WH, Laborde JB, Hansen DK (1998) Renal effects of fumonisin mycotoxins in animals. *Toxicol Pathol* **26**(1): 160–4.

Carvalho T, Pinto C, Peleteiro MC (2006) Urinary bladder lesions in bovine enzootic haematuria. *J Comp Pathol* **134**(4): 336–46.

Casteel SW, Bailey Jr EM (1986) A review of zinc phosphide poisoning. *Vet Hum Toxicol* **28**(2): 151–4.

Charney D, Solez K, Rascusen L (2004) Nephrotoxicity of cyclosporine and the immunosuppressive and immunotherapeutic agents. In *Toxicology of Kidney*, 3rd edn, Hook JB, Tarloff JB, Lash LH (eds), CRC Press, Boca Raton, FL.

Charney SC, Bergman PJ, Hohenhaus AE, *et al*. (2003) Risk factors for sterile hemorrhagic cystitis in dogs with lymphoma receiving cyclophosphamide with or without concurrent administration of furosemide: 216 cases (1990–1996). *J Am Vet Med Assoc* **222**(10): 1388–93.

Choie DD, Longnecker DS, Del Capmo AA (1981) Acute and chronic cisplatin nephropathy in rats. *Lab Invest* **44**(5): 397–402.

Confer AW, Panciera RJ (2001) The urinary system. In *Thomson's Special Veterinary Pathology*, 3rd edn, McGavin MD, Carlton WW, Zachary JF (eds), Mosby, St. Louis, MO.

Crowell WA, Divers TJ, Byars TD, *et al*. (1981) Neomycin toxicosis in calves. *Am J Vet Res* **42**(1): 29–34.

Dhaliwal RS, Kitchell BE (1999) Cyclophosphamide. *Compend Contin Educ Vet Pract* **21**: 1059–63.

Dickie CW, Hamann MH, Carroll WD, *et al*. (1978) Oxalate (*Rumex venosus*) poisoning in cattle. *J Am Vet Med Assoc* **173**(1): 73–4.

Dickinson CE, Traub-Dargatz JL, Dargatz DA, *et al*. (1996) Rattlesnake venom poisoning in horses: 32 cases (1973–1993). *J Am Vet Med Assoc* **208**(11): 1866–71.

Duff SR, Burns RB, Dwivedi P (1987) Skeletal changes in broiler chicks and turkey poults fed diets containing ochratoxin A. *Res Vet Sci* **43**(3): 301–7.

Dungworth, DL (1993) The respiratory system. In *Pathology of Domestic Animals*, 4th edn, Jubb KVF, Kennedy PC, Palmer N (eds), Academic Press, San Diego, USA.

Ellenport (1975) Urogenital system. In *Sisson and Grossman's The Anatomy of the Domestic Animals*, 4th edn, Getty R (ed.), Saunders, Philadelphia, PA.

Eubig PA, Brady MS, Gwaltney-Brant SM, *et al*. (2005) Acute renal failure in dogs after the ingestion of grapes or raisins: a retrospective evaluation of 43 dogs (1992–2002). *J Vet Intern Med* **19**(5): 663–74.

Fooshee SK, Forrester SD (1990) Hypercalcemia secondary to cholecalciferol rodenticide toxicosis in two dogs. *J Am Vet Med Assoc* **196**(8): 1265–8.

Forrester SD, Fallin EA, Saunders GK, *et al*. (1993) Prevention of cisplatin-induced nephrotoxicosis in dogs, using hypertonic saline solution as the vehicle of administration. *Am J Vet Res* **54**(12): 2175–8.

Gava A, da Silva Neves D, Gava D, *et al*. (2002) Bracken fern (*Pteridium aquilinum*) poisoning in cattle in southern Brazil. *Vet Hum Toxicol* **44**(6): 362–5.

George LW, Divers TJ, Mahaffey EA, *et al*. (1982) Heinz body anemia and methemoglobinemia in ponies given red maple (*Acer rubrum L.*) leaves. *Vet Pathol* **19**(5): 521–33.

Graham TW, Holmberg CA, KeenCL, *et al*. (1988) A pathologic and toxicologic evaluation of veal calves fed large amounts of zinc. *Vet Pathol* **25**: 484–91.

Gregory CR (2003) Urinary system. In *Duncan and Prassse's Veterinary Laboratory Medicine: Clinical Pathology*, 4th edn, Latimer KS, Mahaffey EA, Prasse KW (eds), Iowa state University Press, Ames, IA.

Gunson DE, Soma LR (1983) Renal papillary necrosis in horses after phenylbutazone and water deprivation. *Vet Pathol* **20**(5): 603–10.

Gunther R, Felice LJ, Nelson RK, *et al*. (1988) Toxicity of a vitamin D3 rodenticide to dogs. *J Am Vet Med Assoc* **193**(2): 211–14.

Guyton AC, Hall JE (2006) *Text Book of Medical Physiology*. Elsevier, Philadephia, PA.

Hackett TB, Wingfield WE, Mazzaferro EM, *et al*. (2002) Clinical findings associated with prairie rattlesnake bites in dogs: 100 cases (1989–1998). *J Am Vet Med Assoc* **220**(11): 1675–80.

Helman RG, Edwards WC (1997) Clinical features of blister beetle poisoning in equids: 70 cases (1983–1996). *J Am Vet Med Assoc* **211**(8): 1018–21.

Jones RD, Baynes RE, Nimitz CT (1992) Nonsteroidal anti-inflammatory drug toxicosis in dogs and cats: 240 cases (1989–1990). *J Am Vet Med Assoc* **201**(3): 475–57.

Jones TC, Hunt RD, King NW (1996) *Veterinary Pathology*, 6th edn. Williams and Wilkins, Baltimore, MD.

Kerr LA, Kelch WJ (1998) Pigweed (*Amaranthus retroflexus*) toxicosis in cattle. *Vet Hum Toxicol* **40**(4): 216–18.

Kriz W, Bankir L (1988) A standard nomenclature for structures of the kidney. The Renal Commission of the International Union of Physiological Sciences (IUPS). *Kidney Intl* **33**(1): 1–7.

Lairmore MD, Alexander AF, Powers BE, *et al*. (1984) Oxytetracycline-associated nephrotoxicosis in feedlot calves. *J Am Vet Med Assoc* **185**(7): 793–5.

Langston CE (2002) Acute renal failure caused by lily ingestion in six cats. *J Am Vet Med Assoc* **220**(1): 49–52, 36.

Locke LN, Thomas NJ (1996) *Noninfectious Disease of the Wild Life*, Fairbrother A, Locke LN, Gerald GN (eds). Iowa State University Press, Ames, IA.

Luttgen PJ, Whitney MS, Wolf AM, *et al*. (1990) Heinz body hemolytic anemia associated with high plasma zinc concentration in a dog. *J Am Vet Med Assoc* **197**(10): 1347–50.

MacAllister CG, Morgan SJ, Borne AT, *et al*. (1993) Comparison of adverse effects of phenylbutazone, flunixin meglumine, and ketoprofen in horses. *J Am Vet Med Assoc* **202**(1): 71–7.

Maiorka PC, Massoco CO, de Almeida SD, *et al*. (1998) Copper toxicosis in sheep: a case report. *Vet Hum Toxicol* **40**(2): 99–100.

Maylin GA, Eckerlin RH, Krook L (1987) Fluoride intoxication in dairy calves. *Cornell Vet* **77**(1): 84–98.

Mehdi NA, Carlton WW, Boon GD, Tuite J (1984) Studies on the sequential development and pathogenesis of citrinin mycotoxicosis in turkeys and ducklings. *Vet Pathol* **21**(2): 216–23.

Meteyer CU, Rideout BA, Gilbert M, *et al*. (2005) Pathology and proposed pathophysiology of diclofenac poisoning in free-living and experimentally exposed oriental white-backed vultures (*Gyps bengalensis*). *J Wildl Dis* **41**(4): 707–16.

Montali RJ, Bush M, Smeller JM (1979) The pathology of nephrotoxicity of gentamicin in snakes. A model for reptilian gout. *Vet Pathol* **16**(1): 108–15.

Morgan SE, Johnson B, Brewer B, *et al*. (1990) Sorghum cystitis ataxia syndrome in horses. *Vet Hum Toxicol* **32**(6): 582.

Morrow CM, Valli VE, Volmer PA, *et al*. (2005) Canine renal pathology associated with grape or raisin ingestion: 10 cases. *J Vet Diagn Invest* **17**(3): 223–31.

Oaks JL, Gilbert M, Virani MZ, *et al*. (2004). Diclofenac residues as the cause of vulture population decline in Pakistan. *Nature* **427**(6975): 630–3.

O'Brien E, Dietrich DR (2004) Mycotoxins affecting the kidney. In *Toxicology of Kidney*, 3rd edn, Hook JB, Tarloff JB, Lash LH (eds), CRC Press, Boca Raton, FL.

O'Hara PJ, Fraser AJ, James MP (1982) Superphosphate poisoning of sheep: the role of fluoride. *NZ Vet J* **30**(12): 199–201.

Olson CT, Keller WC, Gerken DF, *et al*. (1984) Suspected tremetol poisoning in horses. *J Am Vet Med Assoc* **185**(9): 1001–3.

Osweiler GD (1996) *Toxicology.* Williams and Wilkins, Philadelphia, PA.

Pamukcu AM, Price JM, Bryan GT (1976) Naturally occurring and bracken fern induced bovine urinary bladder tumors. *Vet Pathol* **13**: 110–22.

Panciera RJ, Martin T, Burrows GE, *et al*. (1990) Acute oxalate poisoning attributable to ingestion of curly dock (*Rumex crispus*) in sheep. *J Am Vet Med Assoc* **196**(12): 1981–4.

Pegram RA, Wyatt RD (1981) Avian gout caused by oosporein, a mycotoxin produced by *Caetomium trilaterale*. *Poult Sci* **60**(11): 2429–40.

Plumlee KH, Johnson B, Galey FD (1998). Comparison of disease in calves dosed orally with oak or commercial tannic acid. *J Vet Diagn Invest* **10**(3): 263–7.

Prescott CW (1983) Clinical findings in dogs and cats with lead poisoning. *Aust Vet J* **60**(9): 270–1.

Rankin GO, Valentovic MA (2004) Role of Xenobiotic metabolism. In *Toxicology of Kidney*, 3rd edn, JB Hook, Tarloff JB, Lash LH (eds), CRC Press, Boca Raton, FL.

Rebhun WC, Tennant BC, Dill SG, *et al*. (1984), Vitamin K3-induced renal toxicosis in the horse. *J Am Vet Med Assoc* **184**(10): 1237–9.

Rousseaux CG, Smith RA, Nicholson S (1986) Acute Pinesol toxicity in a domestic cat. *Vet Hum Toxicol* **28**(4): 316–17.

Rozman KK, Klaassen CD (2001) Absorption, distribution and excretions of toxicants. In *Casarett and Doull's Toxicology: The Basic Science of Poisons*, 6th edn, Klaassen CD (ed.). McGraw Hill, New York, pp. 107–32.

Rubin SI, Krawiec DR, Gelberg, *et al*. (1989) Nephrotoxicity of amphotericin B in dogs: a comparison of two methods of administration. *Can J Vet Res* **53**(1): 23–8.

Rumbeiha WK, Francis JA, Fitzgerald SD, *et al*. (2004) A comprehensive study of Easter lily poisoning in cats. *J Vet Diagn Invest* **16**(6): 527–41.

Schmitz DG (1989) Cantharidin toxicosis in horses. *J Vet Intern Med* **3**(4): 208–15.

Schnellmann RG (1998) Analgesic nephropathy in rodents. *J Toxicol Environ Health B Crit Rev* **1**(1): 81–90.

Schnellmann RG (2001) Toxic responses of the kidney. In *Casarett and Doull's Toxicology: The Basic Science of Poisons*, 6th edn, Klaassen CD (ed.). McGraw Hill, New York.

Schoeb TR, Panciera RJ (1979) Pathology of blister beetle (*Epicauta*) poisoning in horses. *Vet Pathol* **16**(1): 18–31.

Shupe JL (1980) Clinicopathologic features of fluoride toxicosis in cattle. *J Anim Sci* **51**(3): 746–58.

Sitprija V, Chaiyabutr N (1999) Nephrotoxicity in snake envenomation. *J Nat Toxins* **8**(2): 271–7.

Smith BL, Embling PP (1993). Sequential changes in the development of the pancreatic lesion of zinc toxicosis in sheep. *Vet Pathol* **30**: 242–7.

Sprangler WL, Adelman RD, Conzelman GM, *et al*. (1980) Gentamicin nephrotoxicity in the dog: sequential light and electron microscopy. *Vet Pathol* **17**: 206–17.

Stair EL, Edwards WC, Burrows GE, *et al*. (1993) Suspected red maple (*Acer rubrum*) toxicosis with abortion in two Percheron mares. *Vet Hum Toxicol* **35**(3): 229–30.

Stuart BP, Nicholson SS, Smith JB (1975) Perirenal edema and toxic nephrosis in cattle, associated with ingestion of pigweed. *J Am Vet Med Assoc* **167**(10): 949–50.

Szczech GM (1975) Ochratoxicosis in Beagle dogs. *Vet Pathol* **12**(1): 66–7.

Szczech GM, Carlton WW, Tuite J, *et al*. (1973a) Ochratoxin A toxicosis in swine. *Vet Pathol* **10**(4): 347–64.

Szczech GM, Carlton WW, Tuite J (1973b) Ochratoxicosis in Beagle dogs. II. Pathology. *Vet Pathol* **10**(3): 219–31.

Talcott PA, Mather GG, Kowitz EH (1991) Accidental ingestion of a cholecalciferol-containing rodent bait in a dog. *Vet Hum Toxicol* **33**(3): 252–6.

Vaala WE, Ehnen SJ, Divers TJ (1987) Acute renal failure associated with administration of excessive amounts of tetracycline in a cow. *J Am Vet Med Assoc* **191**(12): 1601–3.

Wyatt RD, Hamilton PB, Huff WE (1975) Nephrotoxicity of dietary ochratoxin A in broiler chickens. *Appl Microbiol* **30**(1): 48–51.

Zomborszky-Kovacs M, Vetesi F, Horn P, *et al*. (2002) Effects of prolonged exposure to low-dose fumonisin B1 in pigs. *J Vet Med B Infect Dis Vet Public Health* **49**(4): 197–201.

CHAPTER

# 12

# Respiratory toxicity

*John A. Pickrell*

## INTRODUCTION

Inhalation toxicology refers to a route of exposure; toxic chemicals inhaled have a potential to produce lung disease and if the chemical is trans-located to other organs disease in those organs as well. Contrast this to pulmonary toxicity which rather than being a route is more of a target organ toxicity. The environment in which animals live is distinctly different than where humans live, with respect to quality of shelter, as well as square and volume of living areas (Pickrell, 1991). Thus, it is not surprising if both inhalation and pulmonary toxicity differ from animal to man. Air pollution is a reflection of and a description of the presence of chemicals in the air all of the time (Zemp *et al.*, 1999 cited in Witschi and Last, 2001).

To better understand environmental lung disease we need (1) more precise knowledge and an understanding about toxic doses of inhalation toxicants delivered to specific sites of the lung; and (2) the extent of chronic low-level exposures which may trigger and facilitate development of progressive lung disease. Many lung diseases have their beginnings with increases in oxidative burden or stress to the lung. There is strong evidence that oxidative burden to lung may initiate and favor development of lung diseases such as chronic bronchitis, asthma, and interstitial lung disease (Witschi and Last, 2001).

Lung toxicology requires interdisciplinary collaboration between anatomists, physiologists, immunologists, pathologists, molecular biologists, epidemiologists, clinicians and many other shared disciplines. Adaptation of pulmonary function tests to animals has led to an elucidation of mechanisms related to respiratory toxicology in both man and animals. In fact, the comparison of human and animal data especially in more than one species, provides data more relevant to mechanisms than any from individual species. Recent molecular measurements have brought home how closely tied many of these diseases are to specific protein synthesis, how complementary protein and RNA synthesis are. Such molecular analyses help to pinpoint specific molecular reactions to specific cell types. In addition, they help us describe the more relevant influence of each cell on the others in the near neighborhood. Tissue culture, organ minces and *in vivo* animal exposure studies especially with bronchoalveolar lavage washings further elucidate such interaction.

## LUNG STRUCTURE AND FUNCTION

### Nasal passageways normal function

The nasopharynx is lined by pseudo-stratified columnar ciliated epithelium with goblet cells (Dungworth, 1993). Abundant lymphoid nodules are present in the submucosa. The eustachian Tubes extend from the middle ear to the nasal cavity (Dungworth, 1993). Many animals, especially horses and small laboratory rodents, are obligate nose breathers. Other animals can breathe with either their nose or mouth. Dogs are blessed with a keen sense of smell relative to humans. Basset hounds or beagles primarily hunt by scent rather than sight.

These dogs specialize in following a smell or scent. Most of these breeds have longer, drooping ears; one theory says that this helps to collect scent from the air and keep it near the dog's face and nose. They also have large nasal cavities to better process scent. Their typically loose,

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moist lips also trap scent particles. This scent can be tracked for considerable time after it is made – sometimes even across water. They are most happy when being afforded the chance to track scents. For these reasons, they can become valuable after being trained to sniff out specific chemicals. For example, dogs are quite efficient at sniffing illegal drugs, if trained properly (Natural History, 2006).

Dogs have many more olfactory (smell) receptors than humans do – approximately 25 times more (Natural History, 2006). These receptors occur in special sniffing cells deep in a dog's snout; dogs can smell much more acutely than humans. In fact, dogs can sense odors at concentrations nearly 100 million ($10^8$) times lower than humans can. As an example, we can detect a drop of blood (0.05 ml). Dogs can detect one drop of blood in five quarts of water; $10^8$-fold lower.

As a second example, tertiary butyl mercaptan (TBM) has an odor threshold where a human can just sense its unpleasant odor at or near 0.1 parts per billion ($10^{-10}$) in air. If a dog is more sensitive by approximately a factor of $10^8$; that dogs odor threshold would be more like $10^{-18}$; a picogram of TBM (~0.01 pmol) in 800,000 l of air (800 $m^3$ of air; a cube ~ 9 m [~30 ft] on a side if well mixed) or 10 pmol (0.01 μmol) in 800,000,000 l of air (800,000 $m^3$ of air; a cube ~300 ft; 100 yd on a side). We usually don't get much information from sniffing the bare sidewalk, but the same action yields much information to a trained dog. It may be dropped food, or the scent of that poodle next door.

When a dog breathes normally, air laden with scent doesn't pass directly over the smell receptors. But when the dog takes a big sniff, air travels to the smell receptors, near the back of the dog's snout. To sniff deeply is to smell and track efficiently (Natural History, 2006). Wild dogs smell to hunt and to read smells left by other dogs. Domesticated dogs can be trained to sniff deeply and to smell certain odors (Natural History, 2006). The author has been told that dog sniffing parked cars is not legally a search, but it can be the basis for search for further physical evidence if a warrant is issued.

### *Epistaxis*

Circulatory disturbances such as epistaxis (nasal hemorrhage) or pulmonary hemorrhage can cause nosebleed and subsequent obscuring of vision. Obscured vision has the greatest consequences in horses performing in races. At necropsy, blood stained foam indicates terminal congestion, edema, and hemorrhage of the lung. Breakdown of blood vessels can have a traumatic, inflammatory, or neoplastic cause. It may be part of hemorrhagic diatheses or mycotic in origin. Infrequently it may be caused by elevated blood pressure – horses performing in races.

Although commonly caused by intense exercise, cases have been associated with envenomation by rattle snakes (Dickinson *et al.*, 1996). These horses have fever, tachycardia, cardiac arrhythmia, thrombosis, and hemorrhage, including epistaxis. Acutely bitten horses have 19% mortality. Since horses are often bitten on the nose and are obligate nose breathers, the nostril may swell. The nostril and airway must be kept patent (Dickinson *et al.*, 1996). Airborne reactive oxygen substances (ROS) can destroy the capillary barrier and reduce vasoactive nitric oxides; both actions increase the likelihood of equine exercise-induced pulmonary hemorrhage (EIPH) caused by exercise (Manohar *et al.*, 1993; Mills and Higgins, 1997).

High doses of ethylene glycol (>10 mg/kg) produced bovine epistaxis and hemoglobinuria (Crowell *et al.*, 1979). Both fungal cultures and brodifacoum can be a source of hemorrhage from the beak (Rabie *et al.*, 1975). Fungal rhinitis can cause epistaxis in cats (Whitney *et al.*, 2005).

### *Anosmia: hyposmia*

Pace and Lancet (1986) have shown that olfactory responses may be mediated by a GTP-binding protein (G protein); they have identified it in dendritic membranes (olfactory cilia preparations) of chemosensory neurons from three vertebrate species. The G protein is shown to mediate the stimulation by odorants of the highly active adenylate cyclase in these membranes. Its alpha subunit (42,000 Da) undergoes ADP-ribosylation catalyzed by cholera toxin that leads to adenylate cyclase activation. Signal amplification may be responsible for the unusual acuity of the sense of smell (Pace and Lancet, 1986).

Olfactory testing helps establish the presence or absence of such adverse influences to sense of smell. In the future, production animals may be tested to detect reasons for disability compensation and monitor function of smell (Doty, 2006). We expect attempts will be made to relate optimal production to a healthy sense of smell as a group and then individually. Numerous toxins, including environmental toxins, particularly heavy metals, influence the ability to smell. It is hoped that modern advances in technology, in conjunction with better production medicine practices, will make it possible to reliably monitor and limit occupational exposures to hazardous chemicals and their potential adverse influences on the sense of smell. Alternatively, hyposmia occurs in dogs following laryngectomy; these data suggest that both loss of airway and connections to the olfactory cortex may cause hyposmia (Netzer *et al.*, 2002; Doty, 2006).

## Conducting airways normal function

Upper (conducting) airways (bronchi, progressively smaller generations of bronchioles and terminal bronchioles) are lined with pseudo-stratified columnar epithelial cells with a few goblet cells interspersed. Their support tissue contains fibrous connective tissue and cartilaginous plates

(Dungworth, 1993). Animals performing for speed have progressively larger diameter airways to accommodate additional airflow needed.

Normal mucociliary clearance requires coordination of both the gel–sol mucus and beating of cilia to clear particles (Dungworth, 1993). Goblet mucus cells, submucous glands, and serous secretory cells coordinate to mix the mucus to lubricate exit flow. Mucus allows cilia to coordinate help clear particles. Non-ciliated (Clara) epithelial cells have P-450 monoxygenase systems that can actively metabolize foreign (xenobiotic) chemicals and active inflammatory compounds such as prostaglandins. Bronchial epithelial cells produce their intercellular adhesion molecule (ICAM 1).

Upper airways (bronchi and bronchioles) have bronchial associated lymphoid tissue (BALT); this tissue is similar to gut associated lymphoid tissue (GALT) (Dungworth, 1993). BALT is responsible for local immunity in the lung. As bronchioles become progressively smaller, resistance to airflow increases. In these small conducting airways, diseases reflecting altered airflow resistance are said to be diseases of small airways or small airway resistance. As the airways narrow, the epithelium changes from pseudostratified ciliated columnar cells with goblet cells to simple columnar epithelium, reflecting the form, function, and stresses of these smaller airways. The smallest bronchioles may collapse near the end of expiration, unless there is sufficient collateral circulation to keep them open.

### *Toxic diseases of the conducting airways*

Instillation of ragweed caused decrease in nasal cavity volume and minimal cross-sectional area without adverse systemic effects (Rudolph *et al*., 2003). Allergic rhinitis, that clinically resembles hay fever in humans, can occur in horses and cats in addition to dogs (Dungworth, 1993). Nasal granuloma is a chronic form of allergic rhinitis. Degranulation of mast cells and sensitization by albumin provide evidence that allergic stimulation is an underlying cause.

Constant exposure to dust through litter use or upper respiratory tract infections were blamed as likely causes of feline upper airway inflammation. However only certain cats become clinically ill; all cats are potentially exposed to similar conditions. These data suggest varying degrees of hypersensitivity to stimulating antigens in cat populations (Dye, 1992). The results of this study suggest that there is a different metabolic response as indicated by heart rate, blood lactate, $PaO_2$ to exercise in poor equine performers compared to good performers (Courouce-Malblanc *et al*., 2002).

Cattle, especially the channel island breeds, and occasionally sheep have a seasonal allergy that resembles this condition (Dungworth, 1993). Cattle are believed to be allergic to pollen antigens. Histologically, the epithelium is hyperplastic, eroded, and/or infiltrated with eosinophils. Seasonal eosinophilia in hamsters is believed to have a similar origin, although details are lacking.

Nasal granuloma is the chronic form of allergic rhinitis. Because this is moderately common in Jersey cattle, breed sensitivity is proposed. Less commonly, allergic rhinitis may have a fungal origin (Dungworth, 1993). Horses with chronic obstructive pulmonary disease have high transpulmonary pressures (Ammann *et al*., 1998).

A very high prevalence of inflammatory gastrointestinal tract problems in brachycephalic dogs that presented with upper respiratory problems was observed clinically, endoscopically, and histologically (Poncet *et al*., 2005). Histologic inflammatory lesions were observed that were not macroscopically visible at endoscopy. Respiratory and digestive signs correlated closely in French bulldogs, males and heavy brachycephalic dogs. These observations show a correlation between upper respiratory and gastrointestinal tract problems in brachycephalic breeds with upper respiratory disease (Poncet *et al*., 2005). Systemic inflammatory disease led to secondary pulmonary infiltration and inflammation (Bedenice *et al*., 2003). Lobeline induced highly reproducible respiratory hyperpnea without any apparent adverse effects; it may be useful in the investigation of pulmonary function in healthy horses and those with airway disease (Marlin *et al*., 2002).

Moderate smoke inhalation injury to the upper airway is usually independent of temperature damage; after as little as 1 h increases endothelin-1 in cells that line the airway increases (Cox *et al*., 2001). Such changes may contribute to the airway inflammation, mucus secretion, pulmonary hypertension, increased airway resistance, and decreased lung compliance, in our ovine model of smoke inhalation injury (Cox *et al*., 2001).

### *Therapeutic approaches to conducting airway dysfunction*

Oral pseudoephedrine or chlorpheniramine was reported to block the ragweed-induced nasal congestion. This canine model may be used to study upper airway diseases such as allergic rhinitis and to evaluate activity of nasal decongestants (Rudolph *et al*., 2003).

Steroid inhalation reduces the pressures to within the range of apparently normal horses; resistance and arterial oxygen tension became more normal (Ammann *et al*., 1998), suggesting an acute improvement of pulmonary function. Interferon alpha reduced the indication of upper airway inflammation (neutrophils and lymphocytes) in horses without affecting the proportion of lymphocytes that contain CD4-, CD5- and CD8-surface protein (Moore *et al*., 1998).

Tannin is a poly-dispersed poly-phenol from cotton bracts that can be inhaled by dogs near a cotton processor and inhibit acute inflammation's (phorbol myristic acid, PMA) activation of protein kinase C. Tannin inhibits

chloride secretion in airway epithelial cells in part by inhibiting protein kinase C (Cloutier and Guernsey, 1995).

## Gas exchange region

### *Introduction*

Pulmonary parenchyma is divided into units of structure and function called acini (Dungworth, 1993). An acinus is the gas exchange unit of the lung and of pulmonary parenchyma. The acinus includes all branches of respiratory bronchioles, alveolar ducts, and alveoli associated with ventilation; in addition, it contains the vasculature associated with perfusion. Lobules are many acini grouped together and surrounded by connective tissue; in cattle, sheep, and horses they form lobules visible at autopsy. In other species lobules are less obvious on macroscopic examination. Cells most important to the gas exchange are epithelium (type II epithelial corner cells and type I epithelial cells). Fibroblasts provide connective tissue and structural support for the alveolus; vascular endothelial cells line the pulmonary capillaries.

In birds there are many morphologic, physiologic, and mechanical differences between the bird's lung-airsac respiratory system and the mammalian bronchoalveolar lung (Brown *et al.*, 1997). The gas exchange unit is the parabronchus. Oxygen is extracted from air passing through the conducting airways to the gas exchange unit and on to the airsacs. The airsacs provide an auxiliary source of air moved back out to give the parabronchus an opportunity to extract oxygen. Because the extraction is countercurrent, air circulation is from the dorsal and ventral bronchi, the gas exchange area is about the same, and the gas exchange membrane is thinner, oxygen is extracted about twice as efficiently as in mammals. Airsacs and air membrane spaces provide a reduction in density to birds; reduced density is often helpful in staying aloft. Peak oxygen consumption measured in hovering hummingbirds is limited not by metabolic capacity, but by wing aerodynamics secondary to a low density atmosphere. The large mass-specific gas uptake by the avian respiratory system, at rest and especially during exercise, could be exploited as a very sensitive monitor of air quality. An example of this is the canary in the mine that predicts toxicity to the miners (Brown *et al.*, 1997).

### *Ventilation*

Tidal volume has a contribution from dead volume: the volume in the conducting airways that does not exchange gas (West, 2000a, b). Anatomic dead space measures the volume of the conducting airways to where oxygen becomes diluted, e.g. Fowler's method. Physiologic dead space measures the portion of the airways that do not exchange carbon dioxide ($CO_2$) (Bohr's method). The more rapid and shallow the breathing pattern, the higher the percentage of the non-contributing dead volume in each breath.

In addition the tidal volume has a contribution from the alveolar (acinar) volume (the volume in the alveolus that does exchange gas). Although we assume that all regions of the lung are ventilated equally, positional differences are seen in humans (West, 2000a, b). Such differences are minimized when humans are in the supine position. The anterior main bronchi receive more ventilation than do the rear ones in dogs.

Ozone and oxides of nitrogen and sulfur were modeled for absorption throughout the respiratory tract (Tsujino *et al.*, 2005). All three gases had higher concentration in the airways. For example, ozone was 3–12 times higher at the fifth generation bronchus. Sensitivity analysis indicated that tidal volume, respiratory rate, and surface area of the upper and lower airways significantly affected the results. Kinetics of inhaled gaseous substances vary substantially among animals and humans, and such variations are, at least partially, the result of anatomical and physiological differences in their airways.

Two anesthetized, spontaneously breathing ducks inhaled a non-toxic iron oxide aerosol with an aerodynamic mass mean diameter = $0.18\,\mu m$ at $460\,mg/m^3$ for 1.75 h; two awake, resting ducks inhaled a 10-fold less concentrated aerosol ($38\,mg/m^3$) for 6 h on two consecutive days (Stearns *et al.*, 1987). We found iron oxide particles trapped within the substance that coats the atria and infundibula; within epithelial cells of the atria and initial portions of the infundibula; and within interstitial macrophages. Only occasionally, small amounts of particles were found in the air capillaries. Both epithelial cells and interstitial macrophages phagocytized particles in avian lungs and transported them to the atria and the initial portions of the infundibula (Stearns *et al.*, 1987; Brown *et al.*, 1997).

### *Perfusion*

There are two different types of blood supply to the lung: nutrient vessels that provide nutrition for the lung and pulmonary vessels that specialize in exchanging alveolar oxygen onto the hemoglobin to be carried around and exchanged to the target organs (West, 2000a, b). Both gas exchanges operate on an oxygen gradient and occur between alveoli and capillaries. The capillaries are just large enough to admit erythrocytes and they are very short. Visualization of multiple short segments is misleading; blood really flows as a sheet, and the network of capillaries is said to resemble a miniature underground parking garage.

### *Diffusion*

Diffusion is driven by the properties of the blood gas barrier, not by the amount of blood available. Thus, diffusion of certain gases (e.g. carbon monoxide (CO)) is said to be

diffusion limited. In certain other gases such as nitrous oxide which do not bind to or are taken up by the red blood cell, diffusion occurs rapidly and is said to be dependent on blood volume. Oxygen takes the middle road so that some of its diffusion is a characteristic of the blood gas barrier (diffusion limited) and some portion of its diffusion is dependent on blood volume (perfusion limited).

Chickens and rabbits die from high doses of *Crotalia* in 10–20 min; death in rabbits is delayed for 10 h. Phospholipase–crotapotin (PC) paralyzes the contractile response of isolated phrenic-hemidiaphragms of rats. A latency period 20–100 min preceded the paralysis (Breithaupt, 1976).

Thus, if the barrier becomes abnormally thick, diffusion of oxygen is said to limit gas exchange. Although this is an easily understood and therefore an attractive concept to think of a thick barrier, this is less frequent. Alternatively, a failure to match ventilation with perfusion is the cause of poor gas exchange. If the blood flow changes relative to air or air relative to blood flow a mismatch of ventilation (availability air and oxygen) with perfusion (availability of blood) is said to have occurred (West *et al.*, 2000).

If, for example, the lung is heavily perfused with minimal ventilation because of a blocked airway, the amount of oxygen that can be exchanged is limited by the mismatch of poor ventilation with good perfusion. Alternatively, if the lung is efficiently ventilated portion with little or no perfusion, gas exchange is limited, because of the lack of blood and there is said to be a mismatch of good ventilation with poor perfusion (West *et al.*, 2000).

#### *Mismatch of ventilation with perfusion*

Beta-blocker intoxication causes dose dependent decreases of myocardial contractility in laboratory animals (Langemeijer *et al.*, 1992). Calcium probably causes direct negative inotropy of the myocardium, decreased parathormone production, and centrally mediated hypotension. In addition toxic doses of beta blockers can lead to central nervous system mediated respiratory arrest and death. To prevent death, early initiation of ventilation, as well as administration of beta-antagonist can be essential to correct the drop in serum calcium concentration. Phosphodiesterase inhibitors and glucagon are given to improve myocardial contractility (Langemeijer *et al.*, 1992).

Dogs inhaling smoke from a burning charcoal tip, where tobacco does not burn, developed the same coronary artery narrowing as if they had inhaled smoke from tobacco (Gering and Folts, 1990).

Dogs that inhaled 1% nitric acid on alternate days for 4 weeks had increased pulmonary resistance and decreased dynamic compliance. Anatomically, there was bronchial obstruction, diffuse chronic airway inflammation, slight epithelial changes, slight peribronchiolar fibrosis, and an increase in smooth muscle (Peters and Hyatt, 1986).

#### *Allergic lung disease in swine and poultry confinement operations*

Raising pigs and poultry indoors in large confinement facilities is increasingly common. High endotoxin, ammonia ($NH_3$), dust and endotoxin levels caused both the human caretakers and animals in those buildings to develop bronchitis and asthma exacerbation (Von Essen and Donham, 1999; Jones *et al.*, 1984).

## GENERAL PRINCIPLES IN THE PATHOGENESIS OF LUNG DAMAGE CAUSED BY CHEMICALS

### Oxidant burden

Oxidant burden in the lung is frequently associated with airborne pro-oxidants such as nitrogen dioxide, sulfur dioxide, oxidants such as ozone, free radicals, tobacco smoke, or an overzealous defense by phagocytic cells (Pickrell *et al.*, 1987; Pickrell and Mageed, 1995; Witschi and Last, 2001). Free radical damage in lung is provided by varied sources of evidence. Exposure to oxidants can lead to changes in lung structure and biochemistry (Pickrell *et al.*, 1987; Witschi and Last, 2001). Pivotal roles have been established for superoxide, pro-oxidant peroxy nitrites, and hydroxyl radicals. Responses depend on the oxidant burden in combination with the glutathione or biological antioxidant concentration. For example, in the presence of significant oxidant burden but high glutathione lung defensive metabolic enzymes are activated. At intermediate glutathione levels, inflammation is activated using nuclear factor kappa beta. At lower levels of glutathione, mitochondrial enzymes are activated. The relation of programmed cell death (apoptosis) to mitochondrial enzyme activation is being investigated (Nel *et al.*, 2006). Among animal cells, macrophages, monocytes, and neutrophils efficiently generate free radicals and oxidants; this is believed to relate to phagocytosis. With sufficient dose and depletion of glutathione, all lung toxins have an inflammatory disease component.

### Toxic inhalant gases and dose

#### *Chlorine*

Chlorine and chlorinated chemical toxicities in humans are thought to originate from manufacture of pulp, paper, plastics, and chlorinated chemicals (Witschi and Last, 2001). Animals exposed to pulp, paper, and plastics manufacture are generally considered to be small in number. Somewhat more frequently, animals are exposed to chlorine by chemical spills.

Chlorine gas is irritating to the upper airways and can cause hemoptysis, dyspnea, tracheobronchitis, or even bronchopneumonia in animals inhaling sufficient concentrations as a result of accidents and spills (Witschi and Last, 2001). Chlorine is a respiratory toxin. Symptoms from sensory irritation include bronchospasm, cellular changes to bronchioles and alveoli, to development of pulmonary disease. Full recovery from such injuries is likely; permanent loss of function is possible in cases of exposure to very high levels of chlorine (Winder, 2001). Chlorine escaping from rail car accidents leads to upper airway irritation (LeVecchio *et al.*, 2005). Complaints included shortness of breath, eye irritation, nasal complaints, cough, and skin complaints in animals and their caretakers near the chemical spill. Peak symptoms were reached in 30–90 min. Patients only infrequently required more than monitoring and rudimentary support measures (LeVecchio *et al.*, 2005). Humans exposed to similar atmospheres had a limitation of forced expiratory volume to forced vital capacity that was less than that found in normal subjects (Mehta *et al.*, 2005). This limitation is frequently reversible and less than that of seasonal allergic rhinitis (Shusterman *et al.*, 2004).

#### *Ammonia*

$NH_3$ occurs frequently in swine confinement facilities to both animals and caretakers (Carson, 2004). At greater than 100 ppm, $NH_3$ irritates eyes and respiratory membranes. $NH_3$ affects the course and intensity of microbial or parasitic infections in animals in these facilities. The degree to which it reduces growth rate in addition to infectious or parasitic disease varies from minimal to modest reductions. In poultry, $NH_3$ predisposes chickens to respiratory infections. Eye irritation causes them to close their eyes, become listless, and grow more slowly. Levels greater than 60 ppm $NH_3$ cause kerato-conjunctivitis in broilers; reduced bacterial clearance, and enhanced sensitivities to bacterial infections are shown in turkeys (Carson, 2004). High levels of $NH_3$ found in poultry houses have about the same level of toxicity to birds as they would in other animals (Brown *et al.*, 1997).

#### *Anhydrous ammonia*

Anhydrous $NH_3$ is injected from pressurized tanks into the ground as a fertilizer nitrogen source. It can be released in sufficient quantity to be lethal to animals and man if pressurized tanks used to apply fertilizer to farms are breached or large containers are spilled in transit (Carson, 2004). $NH_3$ goes to the first moisture it encounters to form a vapor cloud which can either remain for several hours or disperse efficiently, depending on atmospheric parameters such as wind velocity or humidity. In animals and man, the eyes and upper respiratory tract are prime targets. If $NH_3$ is higher than 5000 ppm, it can cause a fatal apnea, or laryngeal edema. Survivors may be blinded by corneal lesions and sloughed epithelium. Removal of animals is important if the cloud has not dispersed. Supportive therapy may be curative in moderate exposures (Carson, 2004). Loss of olfactory ability was associated with exposure to anhydrous $NH_3$, a history of wheezing and asthma, and of flu-like illness after farm work in humans (Snyder *et al.*, 2003); the extent of such illness in animals has yet to be explored.

#### *Carbon dioxide*

$CO_2$ is well tolerated at 50,000 ppm, stimulating the rate and depth of respiration (Carson, 2004). It is being explored as a novel human stressor (Kaye *et al.*, 2004). Because it is heavier than air it collects in the lower portion of animal facilities and may be an asphyxiant at 400,000 ppm (Carson, 2004). It is used as an euthanasia agent in some laboratory animal species.

#### *Carbon monoxide*

CO is a product of incomplete combustion of hydrocarbon fuels. It has background levels of about 0.02 ppm in rural areas, 13 ppm in urban areas, and 40 ppm in areas of high urban traffic. Improperly vented heaters, space heaters, or improperly adjusted heaters are frequent sources of CO. Fires especially those burning more coolly are also a good source.

CO binds about 250 times as tenaciously to hemoglobin as oxygen, forming carboxyhemoglobin (COHb). Oxygen is displaced from CO, limiting ability to take up oxygen and give off $CO_2$, the main function of lungs. Because of their high respiratory exchange efficiency, birds are unusually sensitive to CO (Brown *et al.*, 1997). Often they have smaller sizes and more active metabolism, requiring more oxygen. CO binds hemoglobin more tenaciously than oxygen, displacing it from hemoglobin and limiting hemoglobin's ability to carry oxygen.

CO forms COHb in blood; measurement of COHb is diagnostic of intoxication (Carson, 2004). In humans, $<3\%$ COHb is considered normal; 6–8% causes drifting of attention; 10–20% headaches, 20–30% dizziness; 30–60% tachypnea, tachycardia, and confusion; and 60% fatality. Birds respond more acutely than mammals because of their more efficient gas exchange. For this reason, canaries have been used as sentinels for miners (Brown *et al.*, 1997; Carson, 2004). Treatment requires a minimum fresh circulating air; 100% oxygen may be lifesaving. Prognosis depends on the amount of COHb and the hypoxic brain damage. Monitor cardiac and pulmonary function for at least 2 weeks and in some cases 6 weeks. Attention to outside ventilation connections can often be lifesaving. Aging *"white"* (unventilated) space heaters are often an unnoticed source of problems (Carson, 2004).

### *Methane*

Methane ($CH_4$) becomes asphyxiant at >85%; it is an explosion hazard at 10–15%. It is substantially lighter than air and will flow above water in a swamp source (Carson, 2004).

### *Hydrogen sulfide*

Hydrogen sulfide ($H_2S$) is heavier than air and insoluble in the water of manure pits (Carson, 2004). Thus it will exist as bubbles in swine manure pits waiting to gas animals or humans on agitation. Since $H_2S$ is insoluble in water, it may expose the deepest recesses of the lung; at 50–150 ppm $H_2S$ causes pulmonary edema (Carson, 2004). *In vitro*, $H_2S$ induces apoptosis of aorta smooth muscle cells, which is potentiated by the endogenous $H_2S$ level. Externally regulated mitogen associated protein kinase (ERK-MAPK) mediating $H_2S$-induced apoptosis of smooth muscle cells by activating caspase-3. These findings may help reveal novel mechanisms for many diseases linked to $H_2S$-related abnormal cellular proliferation and apoptosis (Yang *et al.*, 2004). $H_2S$ is less toxic to birds than to other animals; 2000–3000 ppm will change respiratory rate and depth, while 4000 ppm will kill them. The mechanism for this decreased sensitivity is not defined, but may relate to the greater efficiency of parabronchi at transferring oxygen (Brown *et al.*, 1997).

$H_2S$ ability to paralyze the respiratory tract is its greatest danger to animals and man (Carson, 2004). Above 500–2000 ppm, animals are said to take the second, but not the third, breath. Above 500 ppm, it begins to cause permanent neurologic change. Data from rats trained to run a reversed contingency maze suggested that $H_2S$ may impair learning by increasing the animals' susceptibility to interference from irrelevant stimuli (Partlo *et al.*, 2001).

$H_2S$ is readily detectable as a rotten egg smell. Humans and presumably animals can detect $H_2S$ at 0.025 ppm (Carson, 2004). Above 200 ppm $H_2S$ paralyzes the olfactory apparatus so it may not be detectable by smell. Higher concentrations seem to paralyze more rapidly. Thus, animals or man may have only a very brief instant to smell $H_2S$ at high concentrations; it is dangerous to discount the smell because it is so brief. People or animals not breathing can be dragged outside. Breathing is not re-established spontaneously, but if some artificial respiration is given, they may spontaneously recover (Carson, 2004).

### *Nitrogen dioxide and ozone*

Nitrogen dioxide ($NO_2$) is considered with ozone ($O_3$), because $NO_2$ is a pro-oxidant and $O_3$ an oxidant. In agriculture, $NO_2$ can come from relatively airtight ensilage; $NO_2$ is usually found at the top of silos (Carson, 2004). Indoor air $NO_2$ and $O_3$ can come from second hand cigarette smoke. Nitrogen dioxide exposure from newly opened silage bags may modestly affect hungry cattle if exposure levels are unusually high (i.e. in large confined animal feeding operations (CAFO) dairies), but usually they are more likely to affect caretakers in upright silos. Nitrogen dioxide can pass through the upper airway and permanently damage pulmonary parenchyma where residence times are longer. At ambient $NO_2$ (2–3 ppm) there is little damage or clinical signs. At higher levels, e.g. 20 ppm, $NO_2$ animals have coughing, some fluid in the lungs, death of type I epithelial cells significantly enlarged air spaces and an increased collagen production but no morphologic evidence of fibrosis (Gregory *et al.*, 1983; Mauderly *et al.*, 1987; Pickrell *et al.*, 1987a; Carson, 2004). Animals that die at varying times after exposure have evidence of pulmonary edema and emphysema (Carson, 2004).

Birds are unusually sensitive to $NO_2$ and $O_3$ depending on exposure level. Caged pet birds may be sensitive to second hand cigarette smoke, especially in a kitchen. Newly hatched chickens die after 5 days exposure to 1–4 ppm $O_3$; exposure to 0.3–0.7 ppm $O_3$ causes pulmonary hemorrhage in these chickens (Brown *et al.*, 1997). Pulmonary hemorrhage in mammals is typical of acute high-level oxidant exposure of a somewhat more intense nature than pulmonary edema (Pickrell *et al.*, 1987a).

## Particle size and clearance

### *Particle size and deposition mechanisms*

Inhaled aerosol particles are frequently of multiple sizes (polydispersed) (Witschi and Last, 2001). The distribution is described by geometric mean and the scatter by geometric standard deviation. Although this sounds complicated, it is even more complicated than one might imagine, because geometric mean and geometric standard deviation can be for particle number (count median), for geometric mean for mass (mass median), geometric mean for volume (volume median), and lately, for surface area (geometric surface area median). An example of this is shown for aluminum nanoparticles (NP) where you can read number (count), surface area, and volume medians from a single chart (Figure 12.1).

In this figure, number (count) median would be approximately 150–200 nm, surface area median would be approximately 700–800 nm, and volume median would be approximately 10 μm (10,000 nm) in diameter. These numbers represent varying degrees of agglomeration of the aluminum NP; NP have large surface areas relative to mass and aggregate readily (Powers *et al.*, 2006).

Particles of different aerodynamic sizes deposit in different anatomic areas of the mammalian avian respiratory tracts. In mammalian lungs, particles 5–30 μm travel mostly by momentum (inertia); they do not turn corners well and they deposit by inertial impaction in the nasopharyngeal region (Witschi and Last, 2001). Inertial impaction in bird

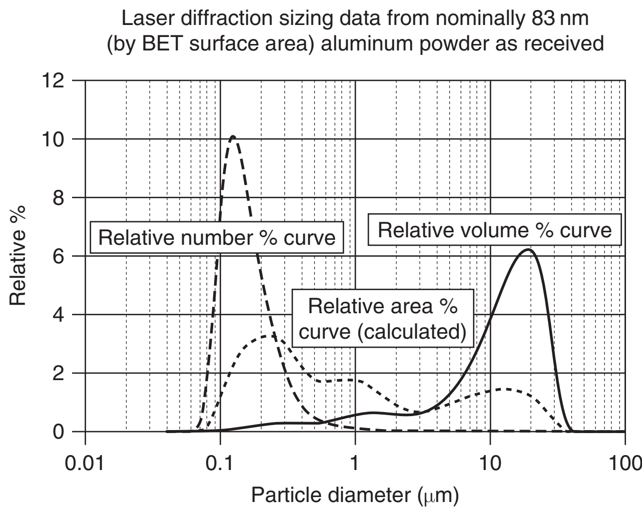


**FIGURE 12.1** Laser diffraction size data for "nanoscale" aluminum powder used for *in vitro* toxicity experiments. Note the apparent difference in size when depicted as a number distribution versus an area or volume distribution. This occurs because volume scales as the cube of the particle diameter and calculated area scales as the square. Each curve, if presented alone, would give an incomplete picture of the particle size distribution/state of agglomeration of the sample. The three curves will overlay only for an ideal spherical, monodisperse, unagglomerated system. By comparing changes in particle size distribution to that of an "ideal" dispersion, a qualitative assessment of the degree of agglomeration can be made (Reproduced with permission of Powers *et al*., 2006).

respiratory tracts is influenced by flow rate and Reynolds numbers (*Re*) (trachea 100 cm/sec at $Re = 700$; primary bronchi 130 cm/sec and $Re = 600$; narrowed primary bronchus 200 cm/sec and $Re = 550$). The expected sites of inertial impaction in birds are similar to those in mammals (Brown *et al*., 1997).

Slightly smaller particles (1–5 μm in diameter) deposit in the conducting airways – the trachea, bronchi, and bronchioles deposit by sedimentation. In the human lung, sedimentation is an important mechanism for deposition at sizes greater than 0.2 μm in diameter (Witschi and Last, 2001). In birds, deposition probability is a product of (residence time) × (aerodynamic diameter)$^2$. In parabronchi where flows are as low as 3 cm/s ($Re = 2$) and residence times may be up to 1 min for complete change of airsac volume at rest, deposition probabilities can be quite high (Brown *et al*., 1997). Diffusion, like sedimentation, is most important in the parabronchi and airsacs where residence time is long.

Particles <1 μm in aerodynamic diameter have a good probability to deposition by Brownian diffusion (Witschi and Last, 2001). The probability of diffusion increases with decreasing particle size, so that those particles whose aerodynamic diameter is less than 0.5 μm is largely by diffusion. Breathing pattern can be important. During exercise impaction of larger particles that acquire higher velocity, momentum and inertia at levels higher up the respiratory tract may occur. Alternatively, breath holding causes more deposition by gravitational sedimentation and diffusion. Brownian diffusion in birds is a function of (residence time ($t$)/spherical volume with the same diffusion displacement as the particle ($D_{te}$)). Diffusion, like sedimentation, is most important in the parabronchi and airsacs where residence time is long (Brown *et al*., 1997). The influence of greater numbers and volumes of ultra-fine particles has not as yet been defined, but it is expected that deposition in bird parabronchi may occur.

### *Particle clearance*

Particle clearance in mammals is important to the defense of the lung (Witschi and Last, 2001). Rapid clearance lessens exposure time and extent of injury. Clearance of intact particles depends heavily on pulmonary alveolar macrophages and the mucociliary escalator – mucus and movement by the cilia of epithelial cells of the upper airway. Particles can leave the lung and enter the vascular system, causing a risk to organs downstream from the lung (Nemmar *et al*., 2001; Kendall *et al*., 2002; Borm *et al*., 2006). Alternatively particles soluble in aqueous media can dissolved, especially small (fine and ultra-fine) particles. Ultra-fine (nano) particles with much of their mass in contact with the surface are especially subject to such dissolution. Factors affecting this solubility are quite complex; they include but are not limited to composition of the aqueous fluid, particle size, chemical particle composition, effect of curvature, steepness of concentration gradient, effect of agglomeration aspect ration (Nemmar *et al*., 2001; Kendall *et al*., 2002; Borm *et al*., 2006).

Bicarbonate in lung simulant fluid can influence solubility in particles moderately soluble in aqueous media. Solubility and chemical reactivity are thought to be a function of environment; they should be measured in an environment as near to that of the biological environment as possible. This can sometimes be accomplished with *in vitro* conditions closely simulating biological solutions (Powers *et al*., 2006). Solubility is thought to be a function of chemical activity, specific surface area, radius and curvature, agglomeration, and specific chemicals that are adsorbed to the nano-sized particles (NSP) (Borm *et al*., 2006). It is important to consider not just solubility in water, but also in fluids that bear significant resemblance to that of the epithelial lining fluid (ELF). ELF is the fluid that lines the epithelial cells in the respiratory tract. A minimal volume is predicted –40 to 100 ml for mature humans. pH varies from 6.9 at the end of inspiration to 7.5 at the end of expiration. Bicarbonate is a major buffer; the variation is caused by removing $CO_2$ from expiration (Langmuir, 1965). Bicarbonate concentration in lung simulant fluid increases the solubility of magnesium oxide (MgO) (Pickrell *et al*.,

2006). Stoichiometry of the likely chemical species suggests a conversion of MgO to magnesium hydroxide ($Mg(OH)_2$) in aqueous media and a subsequent conversion to the hydrated carbonate (nesquehonite; Langmuir, 1965; Pickrell *et al*., 2006).

In chickens, small amount of dust was found trapped in the respiratory epithelial cell trilaminar substance; that material was assumed to be a surfactant (Brown *et al*., 1997). No particles were found in the vascular system, kidneys, and heart, suggesting that at least this size particle does not leave the vascular system. After 1 h 54% of the particles remained, while after 36 h approximately 36% remained, suggesting that appreciable clearance took place. The mechanism of the clearance is not completely clear. However, parenchymal respiratory epithelial cells could have focused particles toward the pulmonary interstitium. Particles in the mammalian lung too small to be efficiently phagocytized were also focused into the lung's interstitium. In bird lungs, there was a virtual absence of phagocytes; the absence may have caused the epithelial cells to take a larger role. Following experimental introduction of Sephadex or Freund's adjuvant, phagocytes with physiological features similar to macrophages can enter the bird's pulmonary spaces. We do not as yet quite know what to make of the fact that they can survive in bird lung parenchymal spaces, but are not naturally found there. This raises the question, do macrophages appear in bird conducting airways and parenchyma only when needed, or do other cells perform this function?

Birds living in dusty environments – kiwis near the desert with abundant sand and poly-dispersed loose dust or birds living near or flying over volcanic ash – show significant pathology and signs after only short exposures. CAFO with poultry raised in high population densities often show reduced production and pathology at necropsy (Brown *et al*., 1997). It is not clear whether a lack of phagocytes or a delay in their introduction best correlates to heightened sensitivity of birds to small dust after relatively short exposures.

## ACUTE RESPONSES OF THE LUNG TO INJURY

### Airway reactivity

Mammalian airway reactivity is bounded by the large conducting airways surrounded by bronchial smooth musculature (BSM; Witschi and Last, 2001). BSM tone is balanced by the autonomic nervous system. Reflex contraction occurs after stimulation by irritants (air pollutants and second hand cigarette smoke – the major indoor air pollutant); bronchoconstriction can be provoked by cholinergic drugs. This stimulation is used in diagnosis as a basis to assess the "*twitchiness*" of BSM – at how low a level the bronchi can be induced to constrict. These agents bind to receptors and trigger increased intracellular cyclic monophosphates – adenosine and guanosine, respectively (cAMP and cGMP). Histamine, prostaglandins, nitric oxide, and leukotrienes also influence bronchoconstriction (Witschi and Last, 2001).

### Pulmonary edema

Pulmonary edema is exudative; it signals acute lung injury (Witschi and Last, 2001). Edema has leakage or interruption as well as thickening of the alveolar capillary membrane. Edema fluid creates a thicker diffusion barrier or causes a mismatch of ventilation to perfusion. The mismatch limits the rate of oxygen exchange from the air of the alveoli to the blood of the alveolar capillaries, even if the alveolar capillary unit is normal in all other ways.

Pulmonary edema is a less common complication in lower airway disease in the horse (Wilkins, 2003). Even something as innocent as hyperhydration prior to overexertion in horses in a field trial can cause arterial hypoxemia during the intense exercise phase was some degree of pulmonary edema, from the extravasation of the administered fluid. Hyperhydration prior to exercise may be detrimental to respiratory function (Sosa *et al*., 2002).

Fatal *Taxus* (Japanese yew) poisoning in horses led to pulmonary congestion, hemorrhage, and edema (Cope *et al*., 2004). Very high levels of fumonisin (>100 ppm in diet) caused fatal pulmonary edema in pigs 1–4 h after exposure (Marasas, 2001). A human took 3 times the dose of monensin (a sodium–potassium ionophore) that was fatal to cattle (Caldeira *et al*., 2001). Calves given *Nerium oleander* (Oleander) clippings developed tachycardia, pulmonary edema, and died suddenly (Galey *et al*., 1996). Dogs and cats with non-cardiogenic pulmonary edema resulting from airway obstruction were the most abnormal on radiograph; others with cranial trauma, seizures, or electric shock also had fatal pulmonary edema (Drobatz *et al*., 1995).

Even brief edema may compromise the alveolar capillary structure, damaging or denuding epithelium or endothelium or both (Witschi and Last, 2001). Frequently, very intense pulmonary edema will include some evidence of small pinpoint pulmonary hemorrhage. As an example, small Teflon particulates from frying pans overheated in the last 1–5 min often cause caged companion birds in the same room to die with pulmonary edema and hemorrhage (Brown *et al*., 1997). Laboratory rodents receiving very high doses of paraquat may die with pulmonary edema and hemorrhage.

Some toxic agents, such as alloxan which denude the alveolar capillary unit, reduce the likelihood of recovery (Witschi and Last, 2001). Such denuding may lead to significant collagen deposition (Pickrell and Villegas (1994), *Research Communication*). If the changes are intense and

architecture is severely compromised, less frequently pulmonary fibrosis may result. From some insults (histamine) an uneventful recovery may result. Several other insults (paraquat) cause sufficient injury to lead to healing by secondary intent (fibrosis) which depending on dose and intensity either fails to interrupt pulmonary architecture and is beneficial because it heals with only modest increases in collagen and little if any change in pulmonary function. Alternatively, with more severe injuries and edema, pulmonary architecture is interrupted, anatomic structure changed, increased total lung collagen causing more extensive and irreversible pulmonary fibrosis developed with detrimental consequences.

We determined the extent of pulmonary edema by relating lung wet weight to body weight (Witschi and Last, 2001). Since pulmonary edema may lead to weight loss in experimental animals, this ratio may slightly overestimate the extent of the edema, but is considered to be a useful indicator. Alternatively, lungs, lobes, or slices can be weighed before and after drying. The accuracy of the second procedure is limited by the uniformity of the edema, but again, this may be a useful indicator. Both of these methods have been compared to morphometric estimates under certain conditions.

Acute respiratory distress, particularly acute pulmonary edema, is treated with diuretics such as furosemide, intranasal oxygen, bronchodilators, corticosteroids, and alleviation of the underlying cause (Foreman, 1999). It is especially important to address the underlying cause in many of these conditions. Furosemide was formerly used in North America as a race-day preventative for EIPH. The difficulty with using furosemide as a preventive was that recent data have shown that furosemide may also be a performance-enhancing agent in addition to its therapeutic action.

## Mechanisms of respiratory tract injury

### *Nasal and upper airway reactivity*

Upper airway irritant gases such as formaldehyde will react differently with nasal epithelium in rats (Witschi and Last, 2001). Rats are much more heavily dependent on scent than are humans; they have more than 10-fold greater cross-sectional areas ($mm^2$) in their nasal epithelium than do humans. Thus, rats can attract much greater masses of formaldehyde in the water of their linings. Scent hounds have roughly the same area and capability, but exercise it intermittently, reducing the amount of water their linings can attract (Natural History, 2006). When this enhanced sensitivity is exercised, it warns and allows strategic shielding action such as tucking the nose under an armpit. Alternatively, identifying an odor allows considerable desensitization to it; the effect of the desensitization on strategic shielding action is continuing to be investigated.

Upper airway irritant gases and particulates, especially ultra-fine particulates with greatly increased surface area, stimulate nasal (trigeminal) nerve endings (Witschi and Last, 2001). These also elicit strategic avoidance reactions. The relation to the nasal cross-sectional area supplied by trigeminal nerve endings has not been defined. Phosphine ($PH_3$) is liberated after ingesting zinc phosphide rodenticide; $PH_3$ irritates nasal and upper airway nerve endings resulting in a different sort of strategic avoidance reaction in dogs, cats, or pigs consuming it. Breath holding and subsequent paroxysmal breathing are unusual enough strategies that they are of value in diagnosing exposure to zinc phosphide (Knight, 2006). Other agents, HCl, $NH_3$, $NO_2$, and phosgene ($COCl_2$), appear to bombard the epithelial barrier in lung for several hours and initiate leakage from the serum. Toxicity to either type I epithelial cells or endothelial cells or both is thought to play a role.

Highly reactive molecules cause their reactions in a different manner (Witschi and Last, 2001). It is unlikely that ozone can penetrate fluid layers to cause direct toxicity; it is more likely that reactive molecules initiate a cascade of ROS such as aldehydes and hydroperoxides. ROS are thought to be formed by ozonolysis of substances in the lung's ELF (e.g. lipids, fatty acids, and other chemical substrates). In addition, ROS have been implicated in oxygen, paraquat, bleomycin, and asbestos toxicities.

Metabolism of xenobiotics (substances foreign to the body) mostly reduces their toxicity; alternatively, in some instances they become more toxic, more carcinogenic, or are transformed into substances that can avoid further metabolism and cause harm to the respiratory tract (Witschi and Last, 2001). The lung contains most of the detoxifying P450 isoenzymes often at lower concentrations than in the rest of the body. These P450 isoenzymes are highly inducible. Activation of these enzymes is an initial defensive reaction which may heal the lung in the presence of relatively high amounts of antioxidants (Nel *et al.*, 2006). Although these isoenzymes are clearly activated after exposure, it is not clear whether the response is sufficiently linear to use them as a biomarker of the exposure. Cytochrome P450 2B1, the isoenzyme that metabolizes phenobarbital, is not inducible in lung. Glutathione-*S*-transferase and glutathione peroxidase are present in lung. In fact, the lung has been found to contain several forms of glutathione-*S*-transferase.

## Mediators of lung toxicity

Studies measuring releases of cytokines (tumor necrosis factor alpha (TNF$\alpha$ ), transforming growth factor B (TGF-B), and interleukin 1B (IL-1B)) in lavage fluid were used to guide the design of the culture whole lung tissue and of

specific lung cells (lung epithelial cells and fibroblasts) (Witschi and Last, 2001). These experiments were designed to elucidate the release of specific molecules signaling toxicant-induced lung damage. For example TNF$\alpha$ and IL-1B signaled acute lung injury. TGF-B and the cytokines signaling acute lung injury were both part of the pathogenesis of pulmonary fibrosis. IL 1, IL 2, IL 5, and IL 8 are thought to be essential components of epithelial lung cell injury. Finally, IL 4, IL 5, and IL 13 are thought to be associated with allergenic responses. Cytokines were thought to be so potent that they must be compartmentalized to control healthy lung responses.

### Cell proliferation

Toxicants can cause either reversible or irreversible changes in lung (Witschi and Last, 2001). Toxic changes that cause major alterations in lung architecture usually cause irreversible lung injury. Severe tissue injury is followed by either tissue loss (emphysema) or healing by secondary intent (fibrosis). We do not know how the lung repairs or attempts to repair itself in a post exposure period when animals inhale filtered air.

When type I lung epithelial cells are injured and die following exposure to an oxidant gas, or some toxicant that causes a high oxidant load, the type II lung epithelial cells associated with them proliferate, begin to flatten and stretch to cover the void left by the type I lung epithelial cell death. Clara cells proliferate following type II proliferation to fill the epithelial void left by type I cell death (Witschi and Last, 2001). Migration of blood monocytes across the alveolar capillary barrier may trigger responses from other cells in the parenchymal lung unit, e.g. interstitial fibroblasts, myofibroblasts, and endothelial cells. The lung may heal with a relatively normal appearance, a higher total lung collagen, and minimal if any alteration of pulmonary function (Witschi and Last, 2001).

## CHRONIC RESPONSES OF THE LUNG TO INJURY

### Fibrosis

Healing by secondary intent, often called pulmonary fibrosis, may be either reversible or irreversible depending on the degree of injury that is required to heal the lung. When lung injury is too high, intense, or persists too long to heal spontaneously e.g. with high levels of paraquat, fibroblasts will attempt to fill the role of lung epithelial cells. Specifically they will normally proliferate to fill the void caused by type I epithelial cell death. Fibroblast proliferation would be expected to impair gas exchange – ventilation and perfusion – making the lung less functional than would be expected by epithelial proliferation. In addition, there would be expected to be greater alteration of pulmonary architecture and increased numbers of interstitial fibroblasts. Increased fibroblasts making normal amounts of collagen would be expected to collectively make more collagen. If minimal to moderate alterations in pulmonary architecture are present the lung will spontaneously resolve (Pickrell *et al.*, 1983). If greater alterations have been made to pulmonary architecture in response to high levels of alveolar damage, the interstitial fibrosis may become irreversible and form dense fibrous scars. The lung becomes stiffer and its movement is restricted. While it is possible to produce fibrosis experimentally in laboratory rodents and dogs, there are few examples of models in nature that have been studied (Pickrell, 1991; Pickrell *et al.*, 1983; Witschi and Last, 2001).

There are at least 19 genetically distinct collagen types in animal and man. Collagen types I and III are major interstitial collagens in the lung. Type III collagen is more compliant than type I; thus, an increase in type I relative to type III which has been shown in experimental models of fibrosis caused by paraquat and asbestos would make a stiffer lung even more stiff than if only an increased amount of collagen was present and the ratios of type I to type III collagen were unchanged (Pickrell, 1991; Witschi and Last, 2001).

Fibrosis is associated with compensatory emphysema. This association illustrated that injury was often local and heterogeneous. If doses were sufficiently low, the lung healed normally. In an adjacent area where injury was somewhat higher fibroblast proliferation and the excess production of collagen resulted in fibrosis. In a still higher level of injury, even fibroblasts could not effectively fill the void and healing by secondary intent failed, cells died and lung tissue was lost, and emphysema was formed. Loss of an inability to produce the collagen needed to heal by secondary intent. We do not fully understand the extent to which this varied response is related to variations in local dose, the reserve of biological antioxidants, or the balance of proteinase–antiproteinase (Pickrell *et al.*, 1983; Pickrell *et al.*, 1987a, b; Pickrell, 1991).

### Emphysema

Emphysema has been defined as enlarged air space with tissue destruction (Witschi and Last, 2001). Loss of gas exchange membrane, specifically alveolar capillary membrane, causes a reduction in gas exchange area; as the alveoli collapse, air spaces become distended, irregular, and the possibility of trapping air increases. This makes it difficult to get enough air (sometimes called air hunger) and even more difficult to expel the air once the air is in the distended alveoli. In horses with emphysema from hay contamination or in humans with developing emphysema,

the patients become emaciated, have increasingly forced expirations and develop heave lines, signifying the difficult expirations (Lowell, 1990). *Nerium oleander* produced widespread congestion and hemorrhage as well as pulmonary emphysema. By the time death occurred at 3–14 days, liver and kidney pathology was most important (Ada *et al.*, 2001).

Some humans have alpha 1 antitrypsin deficiencies that shift the balance of proteinolysis to antiproteinase toward lung tissue digestion, loss of membranes (tissue damage) with distension (enlargement) of air spaces. We know of no natural model of alpha 1 antiproteinase deficiency. Mice with deficiencies in processing (modifying) collagen or elastin develop emphysema (O'Byrne and Postman cited in Witschi and Last, 2001). These data suggest that elastin synthesis may be important to the development of emphysema and that the elastase–antiproteinase model alone cannot explain the development of emphysema (Pickrell *et al.*, 1987a; Witschi and Last, 2001).

In experimental elastase-induced emphysema, instillation of fibroblast growth factor 2 (or injection by microsphere) restored vascularity to lung and caused it to attain normal physiological function (Morino *et al.*, 2005).

## Asthma

In asthma, small conducting airways can be made to constrict at lower irritant concentrations than in normal lungs (Witschi and Last, 2001). Bronchoconstriction reduces airway diameter with a reciprocal increase in small airway resistance. Persisting increased resistance allows our patients to speak to us with symptoms of persistent cough, wheeze, and progressive dyspnea. Irritant gases with moderate solubility also provoke these symptoms.

Guinea pigs are believed to be a natural animal model of human bronchial asthma. Stated alternatively, guinea pigs are perhaps the most sensitive of the species to upper airway irritation and to subsequent development of bronchial asthma. In addition, horses, dogs, and cats develop symptoms like those of asthma (Padrid, 2000; Davis and Rush, 2002; McCue *et al.*, 2003; Pirie *et al.*, 2003; Reinero *et al.*, 2006). Diagnostic approaches are being standardized (Padrid, 2000). Inhaled endotoxin and organic dust particulates were synergistically related to heaves in horses (Pirie *et al.*, 2003).

Potentially detrimental effects of airborne endotoxins on the welfare and exercise performance of stabled horses can be reduced by maintaining horses in "low dust" stables or at pasture, since these environments had significantly lower airborne dust and endotoxin (McGorum *et al.*, 1998). Specific allergen immunotherapy is a potential cure for allergy. No studies have evaluated its efficacy in feline allergic asthma (Reinero *et al.*, 2006). Reinero demonstrated that Rush Immunotherapy (RIT) blunted eosinophilic airway inflammation in experimental feline asthma. British anti-lewisite (BAL) cytokine profiles favoring a Th2 response developed after giving Bermuda grass antigen. RIT shifted the response to increased IFN-g and IL-10 thereafter. The mechanism of RIT may involve changes in allergen-specific immunoglobulins, induction of hyporesponsive lymphocytes, or alteration of cytokine profiles.

Bird fancier's disease is a hypersensitivity pneumonitis in the caretakers of companion birds; care must be taken to differentiate this from aspergillus exposure or from exposure to a humidifier containing mold in its water (Zacharisen and Schoenwetter, 2005). Animal poultry farmers had significantly higher incidences of chronic bronchitis (Radon *et al.*, 2002). The major risk factor for respiratory symptoms was shown to be the adequacy of ventilation of the animal houses; intervention studies are testing the effectiveness of improved ventilation on caretaker respiratory health. Airborne endotoxin can cause flu-like symptoms in birds; this condition must be differentiated from infectious avian influenza, a potential pandemic (Joseph and Subbarao, 2005).

## Lung cancer

Lung cancer was an extremely rare disease in humans at the beginning of the 20th century. In laboratory animals, spontaneously occurring malignant pulmonary tumors are rare, except for very old animals. If lung tumors do develop in laboratory rodents, they are generally peripheral adenomas instead of the more central tumors seen in humans. Adenomas usually originate from type II pulmonary epithelial cells or Clara cells. These adenomas rarely have sufficient time to develop into carcinomas. Occasionally a different kind of injury causes bronchogenic adenocarcinoma that is an unusual lung tumor in cats; it has metastasized to the digits and to the abdominal wall. Average survival time for tumors metastasizing to the digits is only ~5 weeks (van der Linde-Sippman and van den Ingh, 2000; Petterino *et al.*, 2005).

Certain mouse strains (strain A and Swiss-Webster mice) with mutations in the K-ras genes similar to human carcinomas develop adenomas that increase in incidence after the inhalation of some inhalation toxicants or carcinogens. These mice are being studied to see how close they model the pathogenesis of human lung carcinogenesis (Witschi and Last, 2001).

Rat lungs occasionally contain lesions of epithelial cells surrounding material identified as keratin (Witschi and Last, 2001). The cells may compress lung parenchyma and occasionally invade it. These lesions are found more frequently in long term tests of animals exposed to substances not considered to be carcinogens such as carbon black, titanium dioxide, and certain man-made fibers. Controversy exists as to whether these are cysts filled with keratin or can be classified as tumors.

In humans, it has been estimated that 80–90% of all lung tumors are caused by inhaling cigarette smoke (Witschi and Last, 2001). Dogs are companion animals that live with their owners over long periods of time. The most frequent tumors in dogs are mammary tumors which may metastasize to the lung. Spontaneous lung tumors in companion animals such as dogs are less frequent. Although infrequent, some dogs developed either nasal or lung tumors, after being exposed to owner's second hand cigarette smoke (Reif *et al.*, 1992). In both cases, the tumors were carcinomas – epithelial in origin and malignant. Dogs with long noses (dolocephalic breeds) tended to deposit the tumor causing (carcinogenic) particles in the nasal cavity and develop nasal carcinomas. Alternatively dogs with shorter noses (brachycephalic breeds) tended to allow the cigarette smoke particles to deposit in the upper airways and develop bronchogenic carcinomas.

# AGENTS KNOWN TO PRODUCE LUNG INJURY IN ANIMALS

## Airborne agents that produce lung injury in animals

### *Urea*

When a flock of goats consumed 4.2% urea, 18 of 54 goats showed acute respiratory embarrassment and sounds suggestive of pulmonary edema from $NH_3$ toxicosis (Ortolani *et al.*, 2000). Ten goats died within 1 h; while five with convulsions recovered when given vinegar and saline, diuretics, and atropine. Three goats with mild signs recovered spontaneously within 1 h without treatment. Generalized congestion, intense pulmonary edema, and slight tubular nephrosis were found in three surviving goats at necropsy. No more cases occurred when the diet was removed. Approximately 200 chickens inadvertently given zinc phosphide were found dead with no clinical signs after the flooring of a slat-and-litter house was breached (Tiwary *et al.*, 2005). Gross necropsy revealed intense congestion of the viscera; histopathologic examination revealed severe pulmonary edema and congestion of the chickens' lungs, hearts, livers, and kidneys.

### *Smoke*

Cardiac dysfunction after the combined burn and smoke injury in sheep had an initial depression was mostly related to hypovolemia from burns; this was improved by a large amount of fluid resuscitation. The later myocardial contractile dysfunction seemed to be correlated with smoke inhalation injury (Soejima *et al.*, 2001). In normal (control) chickens, the adjacent parabronchi were separated by a minimal septal space (Weidner and Kinnison, 2002). Comparatively, in "volume-loaded" birds, the inter-parabronchial septal spaces were measurably thickened and engorged as a result of hydrostatic pulmonary edema, similar to the effect of hydrostatic pulmonary edema in mammals. Under similar conditions, airsacs thickened; microvilli had increased density (Weidner, 2000).

Environmental tobacco smoke (ETS) from the burning of tobacco indoors can cause a health hazard for companion animals; because its aerosol can contain more than 4000 toxicants into the airway (Sullivan *et al.*, 2006). Some of them are carcinogens; the EPA has classified ETS a group A carcinogen, meaning that there is sufficient evidence to indicate that it will produce cancer in humans.

Inhaled cigarette smoke often did not cause significant increases in numbers of malignant respiratory tumors in rats, mice, hamsters, dogs, or non-human primates even when experimentally exposed for long periods of time to very high concentrations of mainstream cigarette smoke. The results are different from the epidemiological evidence in human smokers. It is difficult to reconcile this major difference between observational studies in humans and controlled laboratory studies.

Dogs living for protracted periods of time in a home with a smoker tended to get more lung cancer than those in homes of non-smokers (odds ratio: 1.6, 95% confidence interval: 0.7–3.7). No relation to increasing the number of packs of cigarettes smoked per day or the proportion of time the dog spent within the home was found. However, dog breeds with short and medium length noses had an increased tendency to get lung cancer (odds ratio: 2.4, 95% confidence interval: 0.7–7.8). Dogs living for protracted periods of time in the homes of owners that smoke have a greater chance of getting lung cancer (Reif *et al.*, 1992).

### *Naphthalene*

Naphthalene, a miscellaneous indoor toxicant, occurs in tars, petroleum, tanning preparations, and mothballs (Witschi and Last, 2001; Oehme and Kore, 2006). It is also released in cigarette smoke and from termites parasitizing houses. The lowest canine lethal dose is 400 mg/kg, but cats are more sensitive. Mothballs weigh near 2.7 or 4 g. Exposed animals have naphthalene scented breath, methemoglobinemia, Heinz body anemia, red blood cell lysis, hepatic injury, and possible nephrosis from red blood cell debris. Dogs and cats can be treated with emesis and if needed charcoal. Dogs can be given 4 mg and cats one dose of 1.5 mg of 1% methylene blue. Intravenous bicarbonate containing saline will help reduce hemoglobin or possibly cell debris precipitation in kidney tubules. Other causes of hemolysis in small animals include zinc (from pennies), onion and copper poisoning, and rattlesnake bites. Prognosis is good if no liver or kidney damage can be detected (Oehme and Kore, 2006; Talcott, 2006).

### *Overheated frying pans*

Exposure of caged birds in the room with overheated cookware has been shown to result in a high percentage of deaths (Brown *et al.*, 1997). Most of these birds have pulmonary edema and hemorrhages. Birds in adjacent rooms, even at the same level of mass exposure have only minimal pulmonary reactions. The mechanism of these reactions is not known at this time. Best guesses suggest that the overheating may imbue unique surface characteristics to the teflon or polytetrafluoroethylene aerosols. Alternatively, aerosols in the same room are less than 25 nm in diameter, where those in adjacent rooms are nearly 100 nm in diameter. Thus, birds in the same room would be exposed to >10-fold more surface areas per unit mass than those in adjacent rooms. Additionally, concentrations would be expected to reduce with diffusion to adjacent rooms. Finally, surface characteristics of the teflon particles would change.

### *Tryptophan–3 methyl indole: perilla mint*

Tryptophan toxicity is associated with cattle consuming tryptophan in lush green forages *per os* (Pickrell and Oehme, 2004). The rumen converts tryptophan to 3 MI which is metabolically activated by cytochrome P450 to a reactive compound that is concentrated in the lung and is pneumotoxic. Perilla ketone and 4-ipomeanol from Perilla frutescens damage endothelial cells and type I pulmonary epithelium (Nicholson, 2004; Pickrell and Oehme, 2004).

At high doses, 3 MI leads to pulmonary edema, emphysema, and death within 6–24 h after the onset of clinical signs (Pickrell and Oehme, 2004). Several pounds of green forage containing 4-ipomeanol may be fatal to a cow within 1–2 days (Nicholson, 2004). At lower exposure levels, animals may have respiratory signs (depression, respiratory grunt, wheeze, and froth flowing out of the nostrils), but will recover over 24–72 h. These animals will recover in 24–72 h which have minimal to no physiological impairment. However, proliferation of type II alveolar cells may persist with no demonstrable physiological consequences, if they die of other causes. If exercised, cattle with intermediate signs can be pushed into the acute syndrome; they will worsen rapidly, develop significant lung edema, and die (Kerr LE cited in Pickrell and Oehme, 2004).

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## REFERENCES

Ada SE, Al-Yahya MA, Al-Farhan AH (2001) Acute toxicity of various oral doses of dried *Nerium oleander* leaves in sheep. *Am J Chin Med* **29**(3–4): 525–32.

Ammann VJ, Vrins AA, Lavoie JP (1998) Effects of inhaled beclomethasone dipropionate on respiratory function in horses with chronic obstructive pulmonary disease (COPD). *Equine Vet J* **30**(2): 152–7.

Bedenice D, Heuwieser W, Brawer R, Solano M, Rand W, Paradis MR (2003) Clinical and prognostic significance of radiographic pattern, distribution, and severity of thoracic radiographic changes in neonatal foals. *J Vet Intern Med* **17**(6): 876–86.

Borm P, Klaessig FC, Landry TD, Moudgil BM, Pauluhn J, Thomas K, Trottier R, Wood, S (2006) Research strategies for safety evaluation of nanomaterials. Part VI. Characterization of nanoscale particles for toxicological evaluation. *Toxicol Sci* **90**: 23–32.

Breithaupt H (1976). Neurotoxic and myotoxic effects of crotalus phospholipase A and its complex with crotapotin *Naunyn Schmiedebergs Arch Pharmacol* **292**(3): 271–8.

Brown RE, Brain JD, Wang N (1997) The avian respiratory system: a unique model for studies of respiratory toxicosis and for monitoring air quality. *Environ Health Perspect* **105**(2): 188–200.

Caldeira C, Neves WS, Cury PM, Serrano P, Baptista MA, Burdmann EA (2001) Rhabdomyolysis, acute renal failure, and death after monensin ingestion. *Am J Kidney Dis* **38**(5): 1108–12.

Carson TL (2004) Gases. In *Clinical Veterinary Toxicology*, Plumlee KH (ed.). Mosby, St Louis, MO, pp. 155–61.

Cloutier MM, Guernsey L (1995) Tannin inhibition of protein kinase C in airway epithelium. *Lung* **173**(5): 307–19.

Cope RB, Camp C, Lohr CV (2004) Fatal yew (Taxus sp) poisoning in Willamette Valley, Oregon, horses. *Vet Hum Toxicol* **46**(5): 279–81.

Cox RA, Soejima K, Burke AS, Traber LD, Herndon DN, Schmalstieg FC, Traber DL, Hawkins HK (2001) Enhanced pulmonary expression of endothelin-1 in an ovine model of smoke inhalation injury. *J Burn Care Rehabil* **22**(6): 375–83.

Courouce-Malblanc A, Pronost S, Fortier G, Corde R, Rossignol F (2002) Physiological measurements and upper and lower respiratory tract evaluation in French Standardbred Trotters during a standardised exercise test on the treadmill. *Equine Vet J Suppl* **34**: 402–7.

Crowell WA, Whitlock RH, Stout RC, Tyler DE (1979) Ethylene glycol toxicosis in cattle. *Cornell Vet* **69**(3): 272–9.

Davis E, Rush BR (2002) Equine recurrent airway obstruction: pathogenesis, diagnosis, and patient management. *Vet Clin North Am Equine Pract* **18**(3): 453–67.

Dickinson CE, Traub-Dargatz JL, Dargatz DA, Bennett DG, Knight AP (1996) Rattlesnake venom poisoning in horses: 32 cases (1973–1993). *J Am Vet Med Assoc* **208**(11): 1866–71.

Doty RL (2006) Olfactory dysfunction and its measurement in the clinic and workplace. *Int Arch Occup Environ Health* **79**(4): 268–82.

Drobatz KJ, Saunders HM, Pugh CR, Hendricks JC (1995) Noncardiogenic pulmonary edema in dogs and cats: 26 cases (1987–1993). *J Am Vet Med Assoc* **206**(11): 1732–6.

Dungworth D (1993) The respiratory system. In *Pathology of Domestic Animals*, 4th edn, Jubb KVF, Kennedy PC, Palmer N (eds). Academic Press, New York, pp. 539–699.

Dye JA (1992) Feline bronchopulmonary disease. *Vet Clin North Am Small Anim Pract* **22**(5):187–201.

Galey FD, Holstege DM, Plumlee KH, Tor E, Johnson B, Anderson ML, Blanchard PC, Brown F (1996) Diagnosis of oleander poisoning in livestock. *J Vet Diagn Invest* **8**(3): 358–64.

Gering SA, Folts JD (1990) Exacerbation of acute platelet thrombus formation in stenosed dog coronary arteries with smoke from a non-tobacco-burning cigarette. *J Lab Clin Med* **116**(5): 728–36.

Gregory RE, Pickrell JA, Hahn FF, Hobbs CH (1983) Pulmonary effects of intermittent subacute exposure to low-level nitrogen dioxide. *J Toxicol Environ Health* **11**(3): 405–14.

Foreman JH (1999) Equine respiratory pharmacology. *Vet Clin North Am Equine Pract* **15**(3): 665–86, ix–x.

Jones W, Morring K, Olenchock SA, Williams T, Hickey J (1984) Environmental study of poultry confinement buildings. *Am Ind Hyg Assoc J* **45**(11): 760–66.

Joseph T, Subbarao K (2005) Human infections with avian influenza viruses. *Md Med* **6**(1): 30–2.

Kaye J, Buchanan F, Kendrick A, Johnson P, Lowry C, Bailey J, Nutt D, Lightman S (2004) Acute carbon dioxide exposure in healthy adults: evaluation of a novel means of investigating the stress response. *J Neuroendocrinol* **16**(3): 256–64.

Kendall M, Tetley TD, Wigzell E, Hutton B, Nieuwenhuijsen M, Luckham P (2002) Lung lining liquid modifies $PM_{2.5}$ in favor of particle aggregation: a protective mechanism. *Am J Physiol Lung Cell Mol Physiol* **282**: L109–14.

Knight M (2006) Zinc Phosphide. In *Small Animal Toxicology*, Peterson ME, Talcott PA (eds). Elsevier-Saunders, St. Louis, MO, pp. 1103–16.

Langemeijer JJ, de Wildt DJ, de Groot G, Sangster B (1992) Intoxication with beta-sympathicolytics. *Neth J Med* **40**(5–6): 308–15.

Langmuir D (1965) Stability of carbonates in the system MgO-$CO_2$-$H_2O$. *J Geol* **73**: 730–54.

LeVecchio F, Blackwell S, Stevens D (2005) Outcomes of chlorine exposure: a 5-year poison center experience in 598 patients. *Eur J Emerg Med* **2**(3): 109–10.

Lowell FC (1990) Observations on heaves. An asthma-like syndrome in the horse. 1964. *Allergy Proc* **11**(3): 149–50; Discussion 147–8.

Manohar M, Hutchens E, Coney E (1993) Pulmonary haemodynamics in the exercising horse and their relationship to exercise-induced pulmonary haemorrhage. *Br Vet J* **149**(5): 419–28.

Marasas WF (2001) Discovery and occurrence of the fumonisins: a historical perspective. *Environ Health Perspect* **109**(Suppl. 2): 239–43.

Marlin DJ, Roberts CA, Schroter RC, Lekeux P (2000) Respiratory responses of mature horses to intravenous lobeline bolus. *Equine Vet J 2000* **32**(3): 200–7.

Mauderly JL, Bice DE, Carpenter RL, Gillett NA, Henderson RF, Pickrell JA, Wolff RK (1987) Effects of inhaled nitrogen dioxide and diesel exhaust on developing lung. *Res Rep Health Eff Inst* **8**: 3–37.

McCue ME, Davis EG, Rush BR, Cox JH, Wilkerson MJ (2003) Dexamethasone for treatment of multisystemic eosinophilic epitheliotropic disease in a horse. *J Am Vet Med Assoc* **223**(9): 1281, 1320–3.

McGorum BC, Ellison J, Cullen RT (1998) Total and respirable airborne dust endotoxin concentrations in three equine management systems. *Equine Vet J* **30**(5): 430–4.

Mehta AJ, Henneberger PK, Toren K, Olin AC (2005) Airflow limitation and changes in pulmonary function among bleachery workers. *Eur Respir J* **26**(1): 133–9.

Mills PC, Higgins AJ (1997) Oxidant injury, nitric oxide and pulmonary vascular function: implications for the exercising horse. *Vet J* **153**(2): 125–48.

Moore BR, Krakowka S, Cummins JM, Robertson JT (1998) Changes in airway inflammatory cell populations in standardbred racehorses after interferon-alpha administration. *Vet Immunol Immunopathol* **49**(4): 347–58.

Morino S, Nakamura T, Toba T, Takahashi M, Kushibiki T, Tabata Y, Shimizu Y (2005) Fibroblast growth factor-2 induces recovery of pulmonary blood flow in canine emphysema models. *Chest* **128**(2): 920–6.

Natural History Museum of Los Angeles County (2006) http://www.nhm.org/exhibitions/dogs/formfunction/smell.html (April 2006).

Nel A, Xia T, Madler L, Ning L (2006) Toxic potentials of materials at the nanolevel – review. *Science* **311**, 622–7.

Nemmar A, Vanbilloen H, Hoylaerts MF, Hoet PHM, Verbruggen A, Nemery B (2001) Passage of intratracheally instilled ultrafine particles from the lung into the systemic circulation in hamster. *Am J Resp Crit Care Med* **164**: 1665–8.

Netzer A, Golz A, Goldenberg D, Silberman M, Joachims HZ (2002) Hyposmia following laryngectomy: experimental model. *J Otolaryngol* **31**(1): 9–12.

Nicholson SJ (2004) Furans. In *Clinical Veterinary Toxicology*, Plumlee K (ed.). Mosby, St Louis, MO, pp. 402–3.

Oehme FW, Kore AM (2006) Miscellaneous indoor toxicants – naphthalene. In *Small Animal Toxicology*, Peterson ME, Talcott PA (eds). Saunders-Elsevier, St Louis, MO, pp. 223–43.

Ortolani EL, Mori CS, Rodrigues Filho JA (2000) Ammonia toxicity from urea in a Brazilian dairy goat flock. *Vet Hum Toxicol* **42**(2): 87–9.

Pace U, Lancet D (1986) Olfactory GTP-binding protein: signal-transducing polypeptide of vertebrate chemosensory neurons. *Proc Natl Acad Sci USA* **83**(13): 4947–51.

Padrid P (2000) Pulmonary diagnostics. *Vet Clin North Am Small Anim Pract* **30**(6): 1187–206.

Partlo LA, Sainsbury RS, Roth SH (2001) Effects of repeated hydrogen sulphide (H2S) exposure on learning and memory in the adult rat. *Neurotoxicology* **22**(2): 177–89.

Peters SG, Hyatt RE (1986) A canine model of bronchial injury induced by nitric acid. Lung mechanics and morphologic features. *Am Rev Respir Dis* **133**(6): 1049–54.

Petterino C, Guazzi P, Ferro S, Castagnaro M (2005) Bronchogenic adenocarcinoma in a cat: an unusual case of metastasis to the skin. *Vet Clin Pathol* **34**(4): 401–4.

Pickrell JA (1991) Hazards in confinement housing – gases and dusts in confined animal houses for swine, poultry, horses and humans. *Vet Hum Toxicol* **33**: 32–9.

Pickrell, JA, Mageed AA (1995) Radiation in "pulmonary fibrosis." In *Lung Biology in Health and Disease*, Phan S, Thrall R (eds), Lanfant CE (series editor). Marcel Dekker, Inc., New York, pp. 363–81.

Pickrell JA, Oehme FW (2004) Tryptophan. In *Clinical Veterinary Toxicology*, Plumlee K (ed.). Mosby, St. Louis, MO, pp. 410–11.

Pickrell JA, Villegas DK (1994) Research Communication. Comparative Toxicology Laboratories, Diagnostic Medicine/Pathobiology, Kansas State University, Manhattan, KS.

Pickrell JA, Diel JH, Slauson DO, Halliwell WH, Mauderly JL (1983) Radiation-induced pulmonary fibrosis resolves spontaneously if dense scars are not formed. *Exp Mol Pathol* **38**(1): 22–32.

Pickrell JA, Gregory RE, Cole DJ, Hahn FF, Henderson RF (1987a) Effect of acute ozone exposure on the proteinase–antiproteinase balance in the rat lung. *Exp Mol Pathol* **46**(2): 168–79.

Pickrell, JA, Hahn FF, Rebar AH, Horoda RA, Henderson RF (1987b) Changes in collagen metabolism and proteinolysis after repeated inhalation exposure to ozone. *Exp Mol Pathol* **46**(2): 159–67.

Pickrell JA, Castro SD, Gakhar G, Klabunde KJ, Hayden E, Hazarika S, Oehme FW, Erickson L (2006) Comparative solubility of nanoparticles and bulk oxides of magnesium in water and lung stimulant fluids. *Toxicol Sci* **90**: 451.

Pirie RS, Collie DD, Dixon PM, McGorum BC (2003) Inhaled endotoxin and organic dust particulates have synergistic proinflammatory effects in equine heaves (organic dust-induced asthma). *Clin Exp Allergy* **33**(5): 676–83.

Poncet CM, Dupre GP, Freiche VG, Estrada MM, Poubanne YA, Bouvy BM (2005) Prevalence of gastrointestinal tract lesions in 73 brachycephalic dogs with upper respiratory syndrome. *J Small Anim Pract* **46**(6): 273–9.

Powers KW, Brown SC, Krishna VB, Wasdo SC, Moudgil BM, Roberts SM (2006) Research strategies for safety evaluation of nanomaterials. Part VI. Characterization of nanoscale particles for toxicological evaluation. *Toxicol Sci* **90**: 296–303.

Rabie CJ, Van Rensburg SJ, Van Der Watt JJ, Lubben A (1975) Onyalai – the possible involvement of a mycotoxin produced by Phoma sorghina in the etiology. *S Afr Med J* **49**(40): 1647–50.

Radon K, Monso E, Weber C, Danuser B, Iversen M, Opravil U, Donham K, Hartung J, Pedersen S, Garz S, Blainey D, Rabe U, Nowak D (2002) Prevalence and risk factors for airway diseases in farmers – summary of results of the European Farmers' Project. *Ann Agric Environ Med* **9**(2): 207–13.

Reif JS, Dunn K, Ogilvie GK, Harris CK (1992) Passive smoking and canine lung cancer risk. *Am J Epidemiol* **135**(3): 234–9.

Reinero CR, Byerly JR, Berghaus RD, Berghaus LJ, Schelegle ES, Hyde DM, Gershwin LJ (2006) Rush immunotherapy in an experimental model of feline allergic asthma. *Vet Immunol Immunopathol* **110**(1–2): 141–53.

Rudolph K, Bice DE, Hey JA, McLeod RL (2003) A model of allergic nasal congestion in dogs sensitized to ragweed. *Am J Rhinol* **17**(4): 227–32.

Shusterman D, Balmes J, Murphy MA, Tai CF, Baraniuk J (2004) Chlorine inhalation produces nasal airflow limitation in allergic rhinitic subjects without evidence of neuropeptide release. *Neuropeptides* **38**(6): 351–8.

Snyder MC, Leopold DA, Chiu BC, Von Essen SG, Liebentritt N (2003) The relationship between agricultural environments and olfactory dysfunction. *J Agric Saf Health* **9**(3): 211–19.

Soejima K, Schmalstieg FC, Sakurai H, Traber LD, Traber DL (2001) Pathophysiological analysis of combined burn and smoke inhalation injuries in sheep. *Am J Physiol Lung Cell Mol Physiol* **280**(6): L1233–41.

Sosa Leon L, Hodgson DR, Evans DL, Ray SP, Carlson GP, Rose RJ (2002) Hyperhydration prior to moderate-intensity exercise causes arterial hypoxaemia. *Equine Vet J Suppl* **34**: 425–9.

Stearns RC, Barnas GM, Walski M, Brain JD (1987) Deposition and phagocytosis of inhaled particles in the gas exchange region of the duck, Anas platyrhynchos. *Respir Physiol* **67**(1): 23–36.

Sullivan JB, Van Ert MD, Krieger GR, Peterson ME (2006) Indoor environmental quality and health. In *Small Animal Toxicology*, Peterson ME, Talcott PA (eds). Elsevier-Saunders, St. Louis, MO, pp. 244–72.

Talcott PA (2006) Zinc. In *Small Animal Toxicology*, Peterson ME, Talcott PA (eds). Elsevier-Saunders, St. Louis, MO, pp. 1095–100.

Tiwary AK, Puschner B, Charlton BR, Filigenzi MS (2005) Diagnosis of zinc phosphide poisoning in chickens using a new analytical approach. *Avian Dis* **49**(2): 288–91.

Tsujino I, Kawakami Y, Kaneko A (2005) Comparative simulation of gas transport in airway models of rat, dog, and human. *Inhal Toxicol* **17**(9): 475–85.

van der Linde-Sipman JS, van den Ingh TS (2000). Primary and metastatic carcinomas in the digits of cats. *Vet Q* **22**(3): 141–5.

Von Essen S, Donham K (1999) Illness and injury in animal confinement workers. *Occup Med* **14**(2): 337–50.

Weidner WJ (2000) Response of air sac mesothelium to expansion of extracellular fluid volume in Gallus domesticus. *J Comp Pathol* **123**(2–3): 182–5.

Weidner WJ, Kinnison JR (2002) Effect of extracellular fluid volume expansion on the interparabronchial septum of the avian lung. *J Comp Pathol* **127**(2–3): 219–22.

West JB (2000a) *Respiratory Physiology: The Essentials*, 6th edn. Lippincott, Williams and Wilkins, Philadelphia, PA, pp. 11–61.

West JB (2000b) *Pulmonary Pathophysiology: The Essentials*, 5th edn. Lippincott, Williams and Wilkins, Philadelphia, PA, pp. 3–35.

Whitney BL, Broussard J, Stefanacci JD (2005) Four cats with fungal rhinitis. *J Feline Med Surg* **7**(1): 53–8.

Wilkins PA (2003) Lower airway diseases of the adult horse. *Vet Clin North Am Equine Pract* **19**(1): 101–21.

Winder C (2001) The toxicology of chlorine. *Environ Res* **85**(2): 105–14.

Witschi HP, Last JO (2001) Toxic responses of the respiratory system, Chapter 15. In *Cassarrett and Doull's Toxicology, The Basic Science of Poisons*, Klaassen CD (ed.). McGraw Hill, New York, pp. 515–34.

Yang G, Sun X, Wang R (2004) Hydrogen sulfide-induced apoptosis of human aorta smooth muscle cells via the activation of mitogen-activated protein kinases and caspase-3. *FASEB J* **18**(14): 1782–4.

Zacharisen M, Schoenwetter W (2005) Fatal hypersensitivity pneumonitis. *Ann Allergy Asthma Immunol* **95**(5): 484–7.

C H A P T E R

13

# Cardiovascular toxicity

*Steven I. Baskin, Steven E. Czerwinski, Jaime B. Anderson, and Manu M. Sebastian*

## INTRODUCTION

Drugs, chemicals, or plants can produce toxic effects on the whole animal as well as on the heart. Twenty chemicals and drugs were selected to show that there is no specific uniform toxicity effect across animal species. It is noted from the graphics displayed (Figures 13.1–13.3) that there does not appear to be one species that is more or less sensitive to drugs or chemicals overall. It is recognized that certain chemicals are much more or

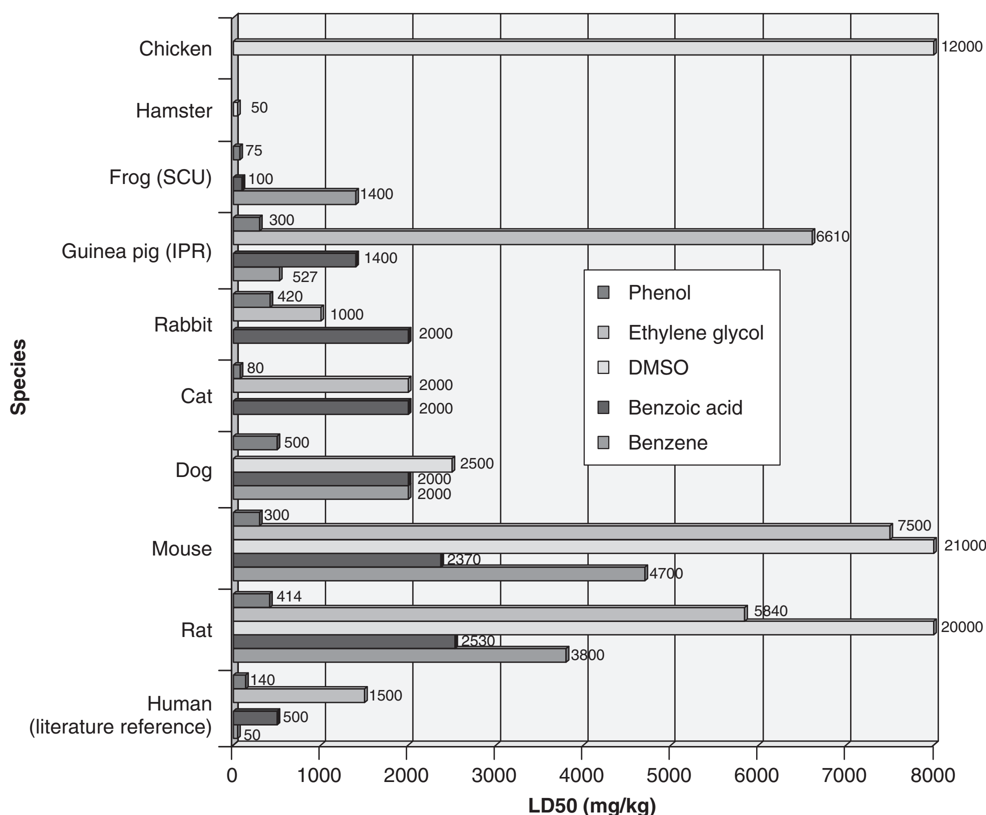


**FIGURE 13.1** Toxic effects of selected organic chemicals.

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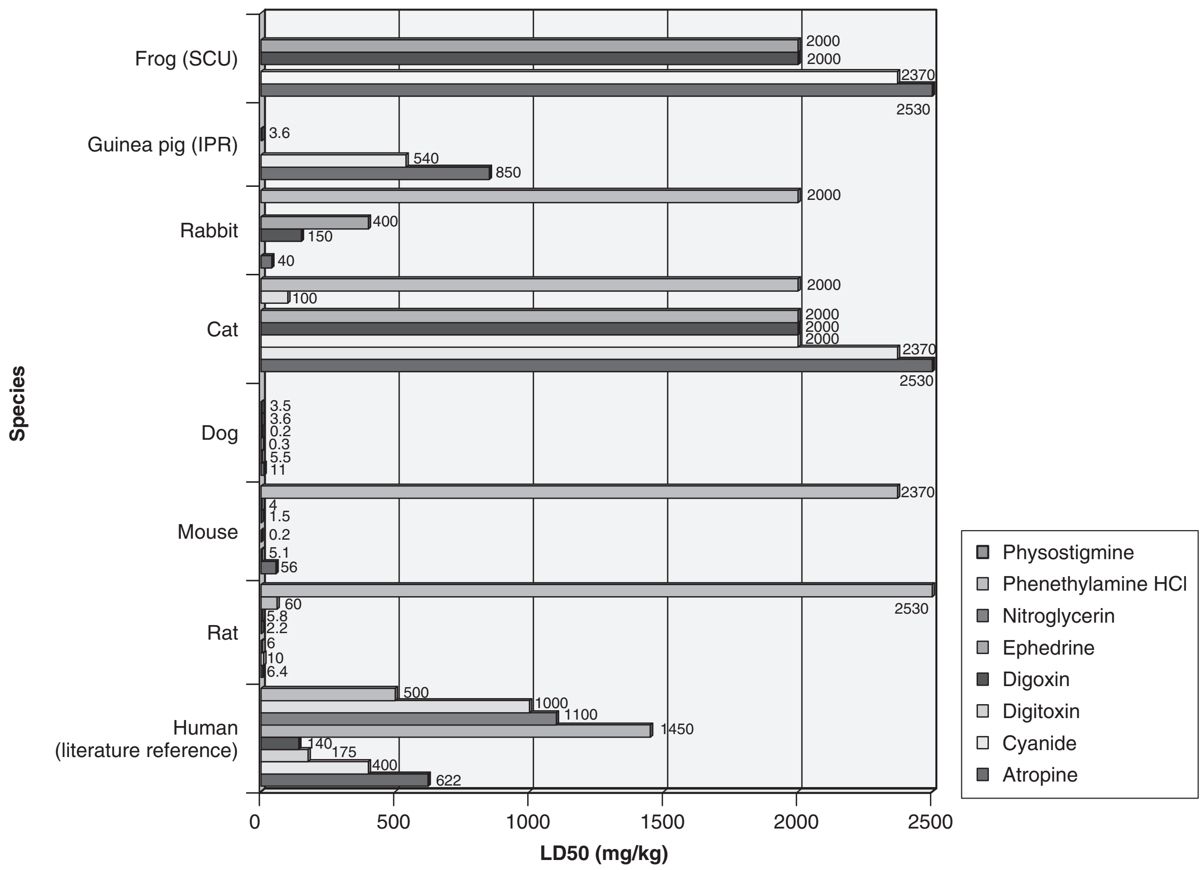


**FIGURE 13.2** Toxic effects of selected drugs.

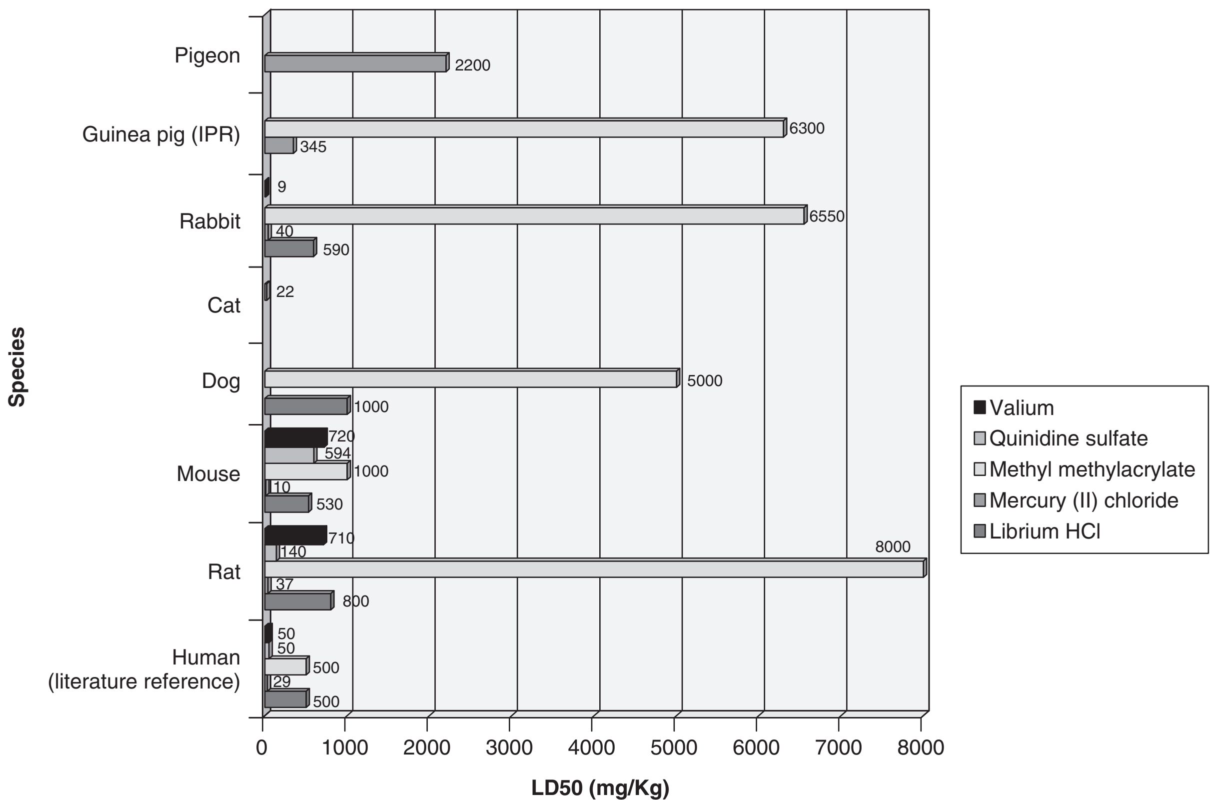


**FIGURE 13.3** Toxic effects of selected chemicals.

less toxic than others because of their inherent mechanism. Botulinum toxin, for example, is toxic because it interacts with specific protein receptors in the body. Some of the drugs that are recognized as antidotes for toxic chemicals are in and of themselves toxic. For example, *physostigmine* (Figure 13.2) (which protects against anticholinesterase agents) and *Valium*® (Figure 13.3) (both of which has anti-arrhythmic and anti-convulsant properties versus a variety of agents) also display a varied toxicity pattern across species.

## TOXIC PLANTS AFFECTING THE CARDIAC SYSTEM

Over the course of time, animals learn that certain plants should be avoided because they can induce unwanted toxic effects (Table 13.1), as in the learned experience from ingesting the bitter monarch butterfly which contains cyanogenic glycosides. Alternately, it has been proposed that certain animals will selectively graze a particular plant specifically for its medicinal effects. Many toxic plants are relatively palatable and may be the only green plants available in the pasture during certain times of the year. Toxic plants may also be hidden within hay and inadvertently fed to the livestock.

The toxicity of plants may be derived from their alkaloid compounds, proteins and amino acids (enzymes, glycoproteins), glycosides, oxalates, resins, tannins, and phenol compounds. Most plant toxins affecting the heart fall into the alkaloid and glycoside groups.

An alkaloid is a nitrogen base containing organic compound that has a pharmacologic effect in animals and humans. The alkaloid groups are now normally classified based on their common precursors if known. Historically, they have been grouped under the names of similar known compounds or by the plants or animals from which they were isolated. The toxic alkaloid compounds derived from plants usually form heterocyclic rings and generally affect the passage of ions across membranes leading to various disturbances in cardiac arrhythmia. Toxic alkaloid compounds are present in 0.0001–10% of all plants, and in general fairly evenly distributed within the plant with variable total concentrations depending on stage of growth and maturity. The alkaloid compounds present a bitter taste and so are not selectively grazed unless other more palatable forage is unavailable. Some of the alkaloids that exert an affect on the cardiovascular (CV) system are polycyclic diterpene alkaloids, steroidal alkaloids, glycoalkaloids, and alkaloid-like compounds such as tryptamine alkaloids and tropane alkaloids. Common plants containing alkaloid toxins include Larkspurs, Monkshood, Yew, Ground hemlock, and Death Camas.

A glycoside is any molecule in which a sugar group is bonded through its anomeric carbon to another group via an *o*-glycosidic bond or an *s*-glycosidic bond. Cardioglycosides exert their pharmacologic effects by inhibiting the ATPase activity of a complex of transmembrane proteins that form the sodium–potassium ATPase pump ($Na^+/K^+$-ATPase). Inhibition of the $Na^+/K^+$-ATPase in turn causes a rise not only in intracellular $Na^+$, but also in calcium, which in turn results in increased force of myocardial muscle contractions which is an indicated treatment for heart failure. However, these cardiac glycosides have steep dose–response curves; therefore minute increases in the dosage can be fatal. Table 13.1 shows a listing of some plants that contain cardiac glycosides. Cardiac glycosides produce positive ionotropy and cardiac arrhythmias by inhibition of the $Na^+/K^+$-ATPase of the heart. Table 13.1 shows milkweed, avocado, digitalis, oleander, and lily of the valley but there are many other plants containing cardiac glycosides. Cardiac glycosides from various plants show different lipophilic properties and different affinity for the $Na^+/K^+$-ATPase (Sodium Pump). There is a direct relationship across animals between inhibition of $Na^+/K^+$-ATPase and positive ionotropy (Akera *et al.*, 1973). The animals ingesting the different cardiac glycosides may present with various degrees of a rapid and weak pulse resulting from arrhythmias and ventricular fibrillations. There are differences between members of the same genus but different species could have different cardiac glycosides that have different toxic effects and time course for different animal species (i.e. Digitalis purpurea). For toxicity of digitalis, digibind is used but to act effectively against other cardiac glycosides digibind is much less effective because of its specificity.

Asclepias syriaca (courtesy of Wikipedia.com). This figure is reproduced in color in the color plate section.

**TABLE 13.1 Plants affecting the heart**

| Name | Common name | Animals typically effected | Clinical signs | MOA |
|---|---|---|---|---|
| *Asclepias* spp. | Milkweeds | Grazing animals (cattle, sheep, horse) | Trembling, staggering, pulse rapid and weak, dyspnea, salivation, bloating, mydriasis, respiratory depression, and death | Interfere with the $Na^+/K^+$-ATPase enzyme in cardiac fibers resulting in decreased intracellular $K^+$ and increased intracellular $Na^+$ causing various degrees of heart block. |
| *Persea americana* | Avocado | Grazing animals and birds | Nonspecific in pet birds, reduced activity, failure to perch, fluffed feathers, and dyspnea, death 24–48 h post exposure, | Interfere with the $Na^+/K^+$-ATPase enzyme in cardiac fibers resulting in decreased intracellular $K^+$ and increased intracellular $Na^+$ causing various degrees of heart block. |
| *Digitalis purpurea* | Foxglove | Grazing animals | Trembling, staggering, rapid weak pulse, dyspnea, salivation, bloating, mydriasis, respiratory depression, and death | Interfere with the $Na^+/K^+$-ATPase enzyme in cardiac fibers resulting in decreased intracellular $K^+$ and increased intracellular $Na^+$ causing various degrees of heart block. |
| *Convallaria majalis* | Lily of the valley | Grazing animals, pets | Vomiting, diarrhea, nausea, anorexia, abdominal cramps, delayed onset of cardiac signs for days; persistent intoxication for up to 3 weeks; paces and ventricular tachycardia may occur. Large doses cause mental confusion, weakness, depression, CV collapse, and death | Interfere with the $Na^+/K^+$-ATPase enzyme in cardiac fibers resulting in decreased intracellular $K^+$ and increased intracellular $Na^+$ causing various degrees of heart block. |
| *Apocynum* spp. | Dogbane, Indian hemp | Grazing animals | Either tachy- or bradycardia, fever, cold extremities, mydriasis, GI upset, and death | Interfere with the $Na^+/K^+$-ATPase enzyme in cardiac fibers resulting in decreased intracellular $K^+$ and increased intracellular $Na^+$ causing various degrees of heart block. |
| *Nerium* spp. | Oleander | Grazing animals | Onset 1–24 h after ingestion. Vomiting, diarrhea (diarrhea may be bloody), trembling, ataxia, weakness, cold extremities, dyspnea, paralysis, coma, and death | Interfere with the $Na^+/K^+$-ATPase enzyme in cardiac fibers resulting in decreased intracellular $K^+$ and increased intracellular $Na^+$ causing various degrees of heart block. |
| *Delphinium* spp. | Larkspurs | Grazing animals | Sudden death, especially in cattle, nervousness, staggering, falling, salivation, tremors, bloating, vomiting, abdominal pain, irregular tachycardia, respiratory paralysis, and death | Methyllycaconitine functions as a potent NM blocking agent. |
| *Aconitum* spp. | Monkshood | Grazing animals | CV disturbance including hypotension, myocardial depression, superventricular tachycardia, conduction disturbances, CNS involvement; oral irritation, nausea, dizziness, severe vomiting, diarrhea, slow/weak heartbeat, weakness, salivation, dyspnea; bloating, prostration, blenching, frothy salivation, convulsions, and death | Methyllycaconitine functions as a potent NM blocking agent. |

TABLE 13.1 (Continued)

| Name | Common name | Animals typically effected | Clinical signs | MOA |
|---|---|---|---|---|
| *Rhododendron* spp. | Azalea and rhododendron | Cattle, sheep, goats. rarely horses | Onset within 6 h of acute digestive upset, salivation, epiphora, anorexia, depression, projectile vomiting, frequent defecation; weak, ataxias, paralysis of limbs, stupor, depression of CV, CNS depression; aspiration of vomitus common in ruminants; coma, death; survival >2 days may recover | MOA similar to cardioglycoside |
| *Kalmia* spp. | Lambkill, calfkill, laurel | Grazing animals | Onset 3–14 h post ingestion, weakness, abdominal pain, salivation, epiphora, vomiting; dyspnea, bradycardia, depression, prostration; convulsions, paralysis of limbs, coma, and death within 12–14 h of onset | MOA similar to cardioglycoside |
| *Taxus* spp. | Yew | Grazing animals | Onset 1–48 h post ingestion; trembling, dyspnea, incoordination, collapse, bradycardia followed by acute cardiac failure; sub-acute meningitis, diarrhea | Inhibit conduction of depolarization through the myocardium and GI irritant |
| *Veratrum* spp. | Hellebore, Skunk cabbage | Grazing animals | Sheep: acute intoxication, onset 2–3 h post ingestion (200–400 g of stems or leaves), hypersalivation, ataxia progressing to paresis, rapid/irregular heartbeat progressing to bradycardia, dyspnea, emesis, diarrhea, diuresis, and death | Increased activity from pressor receptors inhibiting sympathetic outflow and augments vagal influence resulting in hypotension and bradycardia. Veratrum extracts are potent ruminatorics, emetics, and cause hypotension, bradycardia, increased peristalsis and hypercalcemia. Inhibition of mitosis at the neural plate development stage in embryogenesis |
| *Zygadenus* spp. | Death camas | Grazing animals | Onset 1.5–8 h post ingestion, salivation, hyperpnea, tremors, vomiting, sub-normal temperature, weak/irregular pulse, dyspnea, cyanosis, collapse, coma, and death (high mortality) | MOA similar to nonteratogenic effects of veratrum |

MOA: mode of action. *Source*: Casteel (1989).

**Asclepias** species (milkweeds) is a perennial herb 1–3-ft tall with milky sap that is classified in the subfamily Asclepiadaceae of the dogbane family Apocynaceae. There are over 140 broad and narrow leaf varieties. Most of these species occur in the United States. The plant contains cardiac glycosides that are most concentrated in the roots and shoots. Grazing animals (cattle, sheep, goats, and horse) are susceptible to intoxication. The plant is not very palatable but may be grazed during drought and retains toxicity in dried form such as in hay. Clinical signs of intoxication include trembling, staggering, rapid weak pulse, dyspnea, salivation, bloating, mydriasis, respiratory depression, and possibly death. Treatments consist of activated charcoal lavage followed by saline cathartic. Seizures are controlled symptomatically.

*Persea americana* (Courtesy of Wikipedia.com). This figure is reproduced in color in the color plate section.

***Persea americana*** (Avocado) is a tree native to Central America and Mexico and is grown commercially in the United States. It is classified in the flowering plant family, Lauraceae. The avocado toxin is lethal in pet birds (canaries, budgerigars, and cockatiels) with death occurring 24–48 h post ingestion preceded by clinical signs of reduced activity, failure to perch, fluffed feathers, and dyspnea. Nonseptic mastitis can be induced in cattle, horses, sheep, goats, and rabbits. Large doses may be lethal in goats. The toxin occurs in the fruit, bark, leaves, and seeds. However, the most likely circumstance of poisoning is the owner supplementing their pet bird's fruit and vegetable diet with avocado fruit. Cardiotoxic lesions in pet birds include hydropericardium with subcutaneous edema, and generalized congestion.

***Digitalis purpurea*** (Foxglove) is an herbaceous biennial flowering plant in the family Plantaginaceae that is originally native to most of Europe but has since become a common garden plant and now can be found throughout the United States. The leaves, flowers, and seeds of this plant are poisonous to humans and some animals and can be fatal if eaten due to the presence of the cardiac glycoside digitoxin, digoxin, and others. Dogs, cats, rabbits, and livestock are susceptible to intoxication. The plant retains toxicity in a dried form. Treatments consist of activated charcoal lavage followed by administration of a cathartic. Seizures are controlled symptomatically. Attention should be given to insure the animal maintains an adequate hydration status.

***Delphinium spp.*** (Larkspurs) is a genus of about 250 species of annual, biennial, or perennial flowering plants in the buttercup family Ranunculaceae, native throughout the Northern Hemisphere and also on the high mountains of tropical Africa. Many species are cultivated as

*Digitalis purpurea* (Courtesy of Wikipedia.com). This figure is reproduced in color in the color plate section.

*Delphinium elatum* (Courtesy of Wikipedia.com). This figure is reproduced in color in the color plate section.

garden plants. All parts of the plant contain polycyclic diterpene alkaloids such as delphinidin and methyllycaconitine which are potent neuromuscular (NM) blocking agents. Clinical signs may vary from sudden death, especially in cattle to nervousness, staggering, falling, salivation, tremors, bloating, vomiting, abdominal pain, irregular tachycardia, respiratory paralysis culminating in death. Treatment consists of administering an activated charcoal and magnesium sulfate lavage. Treat cardiac signs as a cardioglycoside intoxication. Treat NM blocking effects with physostigmine.

*Aconitum variegatum* (Courtesy of Wikipedia.com). This figure is reproduced in color in the color plate section.

**Aconitum spp.** (Monkshood) is a genus of flowering plant belonging to the buttercup family (Ranunculaceae). There are over 250 species of *Aconitum*, commonly known as aconite, monkshood, or wolfsbane. These herbaceous perennials are chiefly natives of the mountainous parts of the Northern Hemisphere. Many species of *Aconitum* are cultivated in gardens. All parts of the plant are toxic but especially the roots and seeds which contain polycyclic diterpene alkaloids such as aconitine, acinine, and others. Clinical signs consist of gastrointesinal (GI) upset, CV disturbance (to include: hypotension, myocardial depression, superventricular tachycardia, and conduction disturbances), central nervous system (CNS) involvement; oral irritation, nausea, dizziness, severe vomiting, diarrhea, slow/weak heartbeat, weakness, salivation, dyspnea; bloating, prostration, blenching, frothy salivation, convulsions, and death. Treatment is symptomatic which consist of activated charcoal lavage followed by administration of a cathartic.

***Taxus* spp.** (Yew) is a genus of yews, small coniferous trees or shrubs, the yew family Taxaceae. All species of

*Taxus baccata* (Courtesy of Wikipedia.com). This figure is reproduced in color in the color plate section.

yew contain the highly poisonous alkaloid taxine, with some variation in the exact formula of the alkaloid between the species. All parts of the tree except the arils contain the alkaloid. The arils are edible and sweet, but the seed is dangerously poisonous; unlike birds, the human stomach can break down the seed coat and release the taxine into the body. This can have fatal results if yew "berries" are eaten without removing the seeds first. Grazing animals, particularly cattle and horses, are sometimes found dead near yew trees after eating the leaves. Deer are able to break down the poisons and will eat yew foliage freely. Clinical signs occur 1–48 h post ingestion and consist of: trembling, dyspnea, incoordination, collapse, bradycardia followed by acute cardiac failure; subacute meningitis and diarrhea potentially culminating in death. Treatment is symptomatic which consist of activated charcoal lavage followed by administration of a cathartic.

*Helleborus niger* (Courtesy of Wikipedia.com). This figure is reproduced in color in the color plate section.

***Veratrum spp.*** (Hellebore, Skunk Cabbage) is a genus of 15 species of herbaceous perennial flowering plants in the family Ranunculaceae. Hellebores are widely grown in gardens for decorative purposes. Clinical signs in sheep occur 2–3 h post ingestion and consist of: hypersalivation, ataxia progressing to paresis, rapid/irregular heartbeat progressing to bradycardia, dyspnea, emesis, diarrhea, diuresis, and death. Treatment is symptomatic which consist of activated charcoal lavage followed by administration of a cathartic.

*Zgadenus fremontii* (Courtesy of Wikipedia.com). This figure is reproduced in color in the color plate section.

***Zygadenus spp.*** (Death Camas, Star Lily) is a genus of flowering plant, the family Melanthiaceae. Death Camases are perennial plants growing from a bulb or rhizome. Members of the genus are found in North America and Asia. All members of the genus are unpalatable to livestock. As the name Death Camas suggests, some are seriously toxic to both animals and humans. Clinical signs occur 1.5–8 h post ingestion and consist of: salivation, hyperpnea, tremors, vomiting, sub-normal temperature, weak/irregular pulse, dyspnea, cyanosis, collapse, coma, and death (high mortality). Treatment is symptomatic which consist of activated charcoal lavage followed by administration of a cathartic.

***Convallaria majalis*** (Lily of the Valley) is native throughout the temperate Northern Hemisphere in Asia, Europe and North America. It is the sole member of the genus

*Convallaria majalis* (Courtesy of Wikipedia.com). This figure is reproduced in color in the color plate section.

*Convallaria* in the flowering plant family Rosaceae. It is a popular garden plant. The leaves and flowers contain cardiac glycosides that have been used in medicine for centuries. All livestock, pets, and humans are susceptible. Clinical signs consist of vomiting and diarrhea, nausea, anorexia, abdominal cramps; onset of cardiac signs may be delayed. Heart condition progresses to hyperirritability with premature ventricular complexes (PVCs) and ventricular tachycardia. Large doses cause mental confusion, weakness, depression, CV collapse, end death. Treatment is symptomatic which consists of activated charcoal lavage followed by administration of a cathartic.

*Apocynum cannabinum* (Courtesy of Wikipedia.com). This figure is reproduced in color in the color plate section.

***Apocynum*** (Dogbane and Indian Hemp) is a genus in the Apocynaceae family. The genus occurs throughout most of the temperate Northern Hemisphere, except for Western Europe. It is a perennial plant 1–5-ft tall with milky sap. Cattle, sheep, and horses are susceptible. The plant retains its toxicity in dry form. Clinical signs consist of GI upset, CV disturbance including hypotension, myocardial depression, supraventricular tachycardia, conduction disturbances, CNS involvement, oral irritation, nausea, dizziness, severe vomiting, diarrhea, slow/weak heartbeat, weakness, salivation, dyspnea, bloating, prostration, blenching, frothy salivation, convulsions, and death. Treatment is symptomatic which consists of activated charcoal lavage followed by administration of a cathartic.

*Nerium oleander* (Courtesy of Wikipedia.com). This figure is reproduced in color in the color plate section.

***Nerium oleander*** (Oleander) is an evergreen shrub or small tree from 5 to 25-ft tall containing gummy sticky sap in the dogbane family Apocynaceae. It flowers in the spring or summer with white, pink, yellow, or red large clusters. All parts of the plant are toxic. Toxicity is retained in dried form. Clinical signs are seen 1–24 h after ingestion with vomiting and diarrhea (diarrhea may be bloody), trembling, ataxia, weakness, cold extremities, dyspnea, paralysis, coma, and death. Treatment is symptomatic which consists of activated charcoal lavage followed by administration of a cathartic. Attention should be given to insure the animal maintains an adequate hydration status.

***Rhododendron spp.*** (Azalea and Rhododendron) is a genus of flowering plants in the family Ericaceae. It is a large genus with over 600 species. They may be either evergreen or deciduous, and most have flower displays.

*Rhododendron ponticum* (Courtesy of Wikipedia.com). This figure is reproduced in color in the color plate section.

It includes the plants known to gardeners as azalea. Rhododendron is a very widely distributed genus, but a major concentration of diversity occurs in Asia. Several species also occur in North America and a few in Europe, and some tropical species occur as far south as Borneo and New Guinea. Many Rhododendrons have a toxin called grayanotoxin in their pollen and nectar. People have been known to become ill from eating honey made by bees feeding on rhododendron and azalea flowers. Clinical signs consist of onset within 6 h of acute digestive upset, salivation, epiphora, anorexia, depression, projectile vomiting, frequent defecation; weak, ataxias, paralysis of limbs, stupor, depression of CV, CNS depression; aspiration of vomitus common in ruminants; coma, and death; animals that survive more than 2 days may recover. Treatment is symptomatic which consists of activated charcoal lavage followed by administration of a cathartic.

*Kalmia angustifolia* (Courtesy of Wikipedia.com). This figure is reproduced in color in the color plate section.

***Kalmia spp.*** (Lambkill, Calfkill, Laurel) is a genus of about seven species of evergreen shrubs in the family Ericaceae. They are native to North America (mainly in the eastern half of the continent) and Cuba. Kalmias are popular garden shrubs, grown for their decorative flowers. The foliage is toxic if eaten, with sheep being particularly prone to poisoning, hence the name "lambkill" used for some of the species. Clinical signs occur 3–14 h post ingestion and consist of: weakness, abdominal pain, salivation, epiphora, vomiting; dyspnea, bradycardia, depression, prostration; convulsions, paralysis of limbs, coma, death within 12–14 h of onset. Treatment is symptomatic which consists of activated charcoal lavage followed by administration of a cathartic.

## PESTICIDES AND ECONOMIC POISONS AFFECTING THE CARDIAC SYSTEM

Organophosphates are used on crops and weeds all over the world. Their use provides many financial positives and negatives. Their uses allow for greater abundance of crops as well as more beautiful lawns and gardens. It is known that organophosphates can accumulate in animals and man, and can produce cardiac arrhythmias that can lead to death. The commercial organophosphates can produce atrial and/or ventricular arrhythmias which is dose dependent. The mechanism of action is the inhibition of acetylcholinesterase (AChE). The result is an increase in acetylcholine (ACh) which can depolarize the cells of the heart. It is believed one of the more sensitive areas affected is the Purkinje fibers which act to carry electric messages through the heart. ACh shows a diurnal cycle with the highest levels during 3:00 a.m. which suggests why many acute cardiac disorders seem to occur during the overnight hours. Some of the anti-arrhythmics can reduce the release of ACh which may, in part, serve as the mechanism for the anti-arrhythmic effects. Secondly, ACh does affect the passage of ions across cardiac membranes such as calcium, potassium, chloride, and sodium.

## SOLVENTS AFFECTING THE CARDIAC SYSTEM

A number of organic solvents apparently produce a negative ionotropic effect in animals. A mixture of organic solvent and a catecholamine can produce a serious arrhythmia. For example, chloroform and epinephrine or another catecholamine has been used as a model for cardiac arrhythmia. Stimulating sites such as the sinus-arterial (SA) node appear to be more sensitive than other sites. Organic solvents such as benzene (Figure 13.1) and organic chemicals such as benzoic acid, dimethyl sulfoxide (DMSO), ethylene glycol, phenol, or *o*-cresol have been shown to produce variable toxicity across different species. It is well established that the cardiac system is sensitive to organic solvents.

## DRUGS AFFECTING THE CARDIAC SYSTEM

There have been many reviews on drugs which produce cardiac toxicity (Baskin, 1991). The figures in this chapter show that there are marked differences in lethality between animals. It should be easy to guess that many different drugs have different effects including dose–response on the heart. Chemicals which are basic (Figure 13.1) can show different effects. Many chemicals exert their action through a anti-cholinergic mechanism such as atropine (Figure 13.2) and physostigmine or adrenergic mechanisms such as ephedrine or phenethylamine. Mechanical (pump) function, electrical function, endocrine function, and flow function can be changed by drugs. Certain species are sensitive or insensitive to a certain type of drug. Drugs such a cardiac glycosides, digitoxin, and digoxin often have different effects in different species. The rat heart is not very sensitive to digoxin but rat brains are sensitive to cardiac glycosides. The dog heart and brain are both sensitive to cardiac glycosides. The pigeon and frog are more sensitive to cyanide, nitroglycerin, and many other toxic gases. Historically, caged birds have been used in the mines as sentinel warning species because it is more sensitive than humans. This is just another example of species sensitivity.

## REFERENCES

### General

Akera T, Baskin SI, Tobin T, Brody TM (1973) Ouabain: temporal relationship between the ionotropic effect in the Langendorff preparation and the *in vitro* binding to and dissociation from ($Na^{+} + K^{+}$) - activated ATPase. *Naunyn Schmiedebergs Arch Pharm Exp Path* **277**: 151–62.

Baskin SI (1991) *Principles of Cardiac Toxicology*. CRC-Telford Press, FL.

Casteel SW (1989) *Toxicology Noteset*. Veterinary Medical Diagnostic Laboratory, College of Veterinary Medicine, University of Missouri, Columbia, MO.

www.wikipedia.org/wiki/GNU_Free_Documentation_License: All pictures courtesy of Wikipedia.com.

# FURTHER READING

## Atropine

Anonymous (1935) *Abdernalden's Handbuch der Biologishcen Arbietsmethoden*, vol. 4, Leipzeg, Germany, pp. 1311–89.
Diechmann WB, Witherup S, Dieker M (1952) Phenol studies XII. The percutaneous and alimentary absorption of phenol by rabbits with recommendations for the removal of phenol from the alimentary tract or skin of persons suffering exposure. *J Pharmacol Exp Ther* **105**(3): 265–72.
Gleason MN, *et al.* (1969) *Clinical Toxicology of Commercial Products – Acute Poisoning*, 3rd edn. Williams and Wilkins, Baltimore, MD, pp. 19–123.
Krause EG, Wollenberger A (1965) On the activation of phosphorylase and the glycolyserates in acutely anoxic hearts in dogs. *Biochem Z* **342**(2): 171–89.

## Benzene

Anonymous (1935) *Abdernalden's Handbuch der Biologishcen Arbietsmethoden*, vol. 4, Leipzeg, Germany, pp. 1311–89.
Gleason MN, *et al.* (1969) *Clinical Toxicology of Commercial Products – Acute Poisoning*, 3rd edn. Williams and Wilkins, Baltimore, MD, pp. 19–123.
Gusev IS (1967) Comparative assessment of the effects of small concentrations of benzene, toluene and xylene. *Gig Sanit*. **32**(2), Izdatelstvo Meditsina, Russia, pp. 3–6.
Kimura ET, Ebert DM, Dodge PW (1971) Acute toxicity and limits of solvent residue for sixteen organic solvents. *Toxicol Appl Pharmacol* **19**(4): 699–704.

## Benzoic acid

Gleason MN, *et al.* (1969) *Clinical Toxicology of Commercial Products – Acute Poisoning*, 3rd edn. Williams and Wilkins, Baltimore, MD, pp. 19–123.

## Cyanide

Diechmann WB (1969) *Toxicology of Drugs and Chemicals*. Academic Press, New York, pp. 75, 191, 475.
Ghiringhelli L (1955) Toxicity of adipic nitrile. I. Acute poisoning and mechanism of action. *Med Lav Via S Barnaba Italy* **46**(4): 221–8.
Humbert JR, Tress JH, Braico KT (1977) Fatal cyanide poisoning: accidental ingestion of amygdalin. *J Am Med Assoc* **238**(6): 482.
Marhold JV (1972) *Sbornik Vysledku Toxixologickeho Vysetrent Latek A Pripravku*. Institut Pro Vychovu Vedoucicn Pracovniku Chemickeho Prymyclu Praha, pp. 13.
Mercker H, Roser F (1957) Circulatory and metabolic reactions in specific therapy of hydrocyanic acid poisoning. *Naunyn Schmiedebergs Arch Pharmacol* **230**(2): 125–41.

## Digitoxin

Abitbol HR, Cabrera P, Camponovo I, Maldonado A, Piulats E (1966) Rescinamine and urinary excretion of catecholamines in rats. *Arch Int Pharmacodyn Ther* **160**(1): 159–60.
Gleason MN, *et al.* (1969) *Clinical Toxicology of Commercial Products – Acute Poisoning*, 3rd edn. Williams and Wilkins, Baltimore, MD, pp. 19–123.
Kimura ET, Ebert DM, Dodge PW (1971) Acute toxicity and limits of solvent residue for sixteen organic solvents. *Toxicol Appl Pharmacol* **19**(4): 699–704.
Klaassen CD (1973) Comparison of the toxicity of chemicals in newborn rats to bile duct-ligated and sham-operated rats and mice. *Toxicol Appl Pharmacol* **24**: 37–44.
MacDougal JR (1963) *A compilation of LD50 Values of New Drugs*. Department of National Health and Welfare, Food and Drug Divisions, Ottawa, Ont, p. 7.

## Digoxin

Abitbol HR, Cabrera P, Camponovo I, Maldonado A, Piulats E (1966) Rescinamine and urinary excretion of catecholamines in rats. *Arch Int Pharmacodyn Ther* **160**(1): 159–60.
Gleason MN, *et al.* (1969) *Clinical Toxicology of Commercial Products – Acute Poisoning*, 3rd edn. Williams and Wilkins, Baltimore, MD, pp. 19–123.
Klaassen CD (1973) Comparison of the toxicity of chemicals in newborn rats to bile duct-ligated and sham-operated rats and mice. *Toxicol Appl Pharmacol* **24**: 37–44.
Weiller M, Royer R, Lamarche M (1967) Comparison of digoxin toxicity in normal guinea pigs and guinea pigs treated with quinidine. *C R Seances Soc Biol Fil.* **161**(2): 427–9. *Messon et Cie Editeurs, Paris.*

## DMSO

Avery TL, Roberts D, Price RA (1973) Delayed toxicity of 4′-demethylepipodophyllotoxin 9-(4,6-O-2-thenylidene-beta-O-glucopyranoside) (NSC-122819; VM-26) in mice. *Cancer Chemother Rep* **57**(2): 165–73. US Government Printing Office, Washington, DC.
Brown VK, Robinson J, Stevenson DE (1963) A note on the toxicity and solvent properties of dimethyl sulphoxide. *J Pharm Pharmacol* **15**: 688–92.
Fox H (1960) Internal factors influencing normal and compensatory growth of the axolotl pronephros. *J Embryol Exp Morphol* **8**: 495–504.
Willson JE, Brown DE, Timmens EK (1965). A toxicologic study of dimethyl sulfoxide. *Toxicol Appl Pharmacol* **7**: 104–12.

## Ephedrine

Gleason MN, *et al.* (1969) *Clinical Toxicology of Commercial Products – Acute Poisoning*, 3rd edn. Williams and Wilkins, Baltimore, MD, pp. 19–123.
Ritter U (1927) Narcosis of experimental animals. *Naunyn Schmiedebergs Arch Pharmacol* **153**: 189.
Stecher PG, *et al.* (1960) *The Merck Index: An Encyclopedia of Chemicals and Drugs*, vol. 7. Rahway, Merck NJ, p. 403.

## Ethylene glycol

Carpenter CP, Smyth Jr HF, Pozzani UC (1949) The assay of acute vapor toxicity, and the grading and interpretation of results on 96 chemical compounds. *J Ind Hyg Toxicol Am Med Ass* **31**(6): 343–6.
Lefaux R (1968) *Practical Toxicology of Plastics*. Chemical Rubber Co., Cleveland, OH, pp. 329, 372.

Pesticide Chemicals Official Compendium (1966) Association of the American Pesticide Control Officials, Inc., Topeka, KS, pp. 502.
Union Carbide Data Sheet (1965) Industrial Medicine and Toxicology Deptartment, Union Carbide Corporation, New York.
Van Stee EW, Harris AM, Horton ML, Back KC (1975) The treatment of ethylene glycol toxicosis with pyrazole. *J Pharmacol Exp Ther* **192**(2): 251–9.

## Lithium HCl

Barnes CD, Eltherington LG (1973) *Drug Dosages in Laboratory Animals – A Handbook*. University of California Press, Berkeley, CA, pp. 71, 91.
Garattini S (1973) *The Benzodinzenines*. Raven Press, New York, p. 39.
Gleason MN, *et al*. (1969) *Clinical Toxicology of Commercial Products – Acute Poisoning*, 3rd edn. Williams and Wilkins, Baltimore, MD, pp. 19–123.
Randall LO, Schaller W, Scheckel CL, Stefko PL, Banziger RF, Pool W, Moe RA (1969) Pharmacological studies on flurazepam hydrochloride (RO 5-6901), a new psychotropic agent of the benzodiazepine class. *Arch Int Pharmacodyn Ther* **178**(1): 216–41.

## Mercury (II) chloride

Anonymous (1935). *Abdernalden's Handbuch der Biologishcen Arbietsmethoden*, vol. 4, Leipzeg, Germany, pp. 1311–89.
*Quarterly Bulletin* (1951) Association of Food and Drug Officials of the Unites States, Quart Bull **15**: 122.
Goldberg AA, Shapero M, Wilder E (1950) Antibacterial colloidal electrolytes; the potentiation of the activities of mercuric-, phenylmercuric- and silver ions by a colloidal sulphonic anion. *J Pharm Pharmacol* **2**(1): 20–6.
Troen P, Kaufman SA, Katz KH (1951) Mercuric bichloride poisoning. *N Engl J Med* **244**(13): 459–63.
Wahlberg JE (1965) Percutaneous toxicity of metal compounds. A comparative investigation in guinea pigs. *Arch Environ Health Am Med Assoc* **11**(2): 201–4.

## Methyl methylacrylate

Diechmann WB (1969) *Toxicology of Drugs and Chemicals*. Academic Press, New York, pp. 75, 191, 475.
Gleason MN, *et al*. (1969) *Clinical Toxicology of Commercial Products – Acute Poisoning*, 3rd edn. Williams and Wilkins, Baltimore, MD, pp. 19–123.
Imbriani M, Ghittori S, Pezzagno G, Huang J, Capolaligo E (1988) 1,1,1-Trichloroethane (methyl chloroform) in urine as biological index of exposure. *Am J Ind Med* **13**(2): 211–22.
Treon JF, Sigmon H (1949) The toxicity of methyl and ethyl acrylate. *J Ind Hyg Toxicol* **31**(6): 317–26.

## Nitroglycerine

Gleason MN, *et al*. (1969) *Clinical Toxicology of Commercial Products – Acute Poisoning*, 3rd edn. Williams and Wilkins, Baltimore, MD, pp. 19–123.
Holtz P (1942) Symposium on problems in the clinical pharmacology of coronary dilators. Introduction. *Naunyn Schmiedebergs Arch Pharmacol* **200**: 271.
Lorenzetti OJ, Tye A, Nelson JW (1967) Effects of glyceryl trinitrate on the aortic strip of rabbits. *J Pharmacol Exp Ther* **19**(9): 634–6.

## Phenethylamine HCl

Ferguson Jr FC, Wescoe WC (1950) The pharmacology of *N,N*-dimethyl 2-chloro-2-phenylethylamine. *J Pharmacol Exp Ther* **10**(1): 100–14.
Raaflaub J, Dubach UC (1969) Dose-dependent change in the pattern of phenacetin metabolism in man and its possible significance in analgesic nephropathy. *Klin Wochenschr* **47**(23): 1286–7.

## Phenol

Anonymous (1935). *Abdernalden's Handbuch der Biologishcen Arbietsmethoden*, vol. 4, Leipzeg, Germany, pp. 1311–89.
BIOFAX (1973) Industrial Bio-Test Laboratories, Inc., Data Sheets, Northbrook, IL, **27**, 4.
Diechmann WB, Witherup S, Dieker M (1952) Phenol studies XII. The percutaneous and alimentary absorption of phenol by rabbits with recommendations for the removal of phenol from the alimentary tract or skin of persons suffering exposure. *J Pharmacol Exp Ther* **105**(3): 265–72.
Lefaux R (1968) *Practical Toxicology of Plastics*. Chemical Rubber Co., Cleveland, OH, pp. 329, 372.

## Physostigmine

Benita S, Friedman D, Weinstock M (1986) Pharmacological evaluation of an injectable prolonged release emulsion of physostigmine in rabbits. *J Pharmacol Exp Ther* **38**(9): 653–8.
Diechmann WB (1969) *Toxicology of Drugs and Chemicals*. Academic Press, New York, pp. 75, 191, 475.
Klaassen CD (1973) Comparison of the toxicity of chemicals in newborn rats to bile duct-ligated and sham-operated rats and mice. *Toxicol Appl Pharmacol* **24**: 37–44.
Lorenzetti OJ, Tye A, Nelson JW (1967) Effects of glyceryl trinitrate on the aortic strip of rabbits. *J Pharmacol Exp Ther* **19**(9): 634–6.
Natoff IL, Reiff B (1973) Effect of oximes on the acute toxicity of anticholinesterase carbamates. *Toxicol Appl Pharmacol* **25**(4): 569–75.
Sanderson DM (1961) Treatment of poisoning by anticholinesterase insecticides in the rat. *J Pharmacol Exp Ther* **13**: 435–42.

## Quinidine sulfate

Gleason MN, *et al*. (1969) *Clinical Toxicology of Commercial Products – Acute Poisoning*, 3rd edn. Williams and Wilkins, Baltimore, MD, pp. 19–123.
Rougeot C, Jozefczak C, Mestre M, Le Fur G, Uzan A (1984) PK 10139 and quinidine: interactions with digoxin concentrations in rats and dogs. *J Pharmacol Exp Ther* **36**(5): 347–9.
Werner G (1952) Antagonism between physostigmine and a few ganglia paralyzing drugs. *Naunyn Schmiedebergs Arch Pharmacol* **216**(1–2): 175.

## Valium

Bodi T (1964) Clinical pharmacology and toxicity of a new microenema. *Curr Ther Res Clin Exp* **86**: 442–6.

Diamantis W, Kletzkin M (1966) Evaluation of muscle relaxant drugs by head-drop and by decerebrate rigidity. *Int J Neuropharmacol* **5**(4): 305–10.

Driessen JJ, van Egmond J, van der Pol F, Crul JF (1987) Effects of two benzodiazepines and a benzodiazepine antagonist on neuromuscular blockade in the anaesthetized cat. *Arch Int Pharmacodyn Ther* **286**(1): 58–70.

Gleason MN, *et al*. (1969) *Clinical Toxicology of Commercial Products – Acute Poisoning*, 3rd edn. Williams and Wilkins, Baltimore, MD, pp. 19–123.

Kimura ET, Ebert DM, Dodge PW (1971) Acute toxicity and limits of solvent residue for sixteen organic solvents. *Toxicol Appl Pharmacol* **19**(4): 699–704.

CHAPTER

# 14

# Reproductive toxicity and endocrine disruption

*Tim J. Evans*

## INTRODUCTION

Reproduction is a critical biological process in all living systems and is required for species survival. Toxicant-induced abortions, congenital defects and infertility can have devastating effects on livestock production. Wildlife species living in environments contaminated by industrial and/or agricultural chemicals have experienced impaired fertility and declining populations. There is growing concern within the scientific community and amongst government regulatory agencies about the effects of occupational and environmental exposures to reproductive toxicants on human fertility.

For the purposes of this chapter, the term "reproduction" will be used primarily in reference to vertebrate species of animals (especially mammals) and will be inclusive of "development", which is sometimes treated as a separate topic in toxicology texts. This particular book chapter will emphasize the interactions between toxicants and the male and female reproductive tracts, as well as xenobiotic-induced effects on the growth, maturation and sexual differentiation of the embryo and fetus. Since "endocrine disruption" (see definition below) is an extremely common mechanism of action for xenobiotics associated with impaired reproductive function, reproductive toxicity and endocrine disruption will be discussed together in this chapter. Efforts will be made to clarify the currently used terminology related to reproductive toxicity and endocrine disruption and to introduce the reader to normal reproductive anatomy and physiology, as well as important concepts associated with embryonic and fetal development. Endocrine disruption in wildlife species and humans and the effects of xenobiotics, including endocrine disrupting chemicals (EDCs), on reproductive function in domestic animals will be discussed along with a brief description of proposed mechanisms of action and observed effects of some selected reproductive toxicants.

Unfortunately, space constraints limit the amount of information which can be presented in this chapter, and many of the presented topics cannot be discussed at great length. There are a number of recently published textbooks and issues of journals which cover some of these subjects in greater detail and provide information which is complementary to what is presented in this chapter (Burrows and Tyrl, 2001; Plumlee, 2004; Naz, 2005; Golub, 2006a; Hood, 2006; Jobling and Tyler, 2006; Jørgensen *et al.*, 2006b; Mukerjee, 2006). The reader is directed to these publications and the other references cited in this chapter in order to gain additional insight into specific areas of reproductive function and toxicology.

It is important that the reader understand that the areas of toxicology involving reproductive toxicity and endocrine disruption, in particular, are in continual flux. New data and exceptions to "classical" mechanisms of action are being reported on a regular basis, and there continues to be ongoing debate about the various aspects of normal as well as xenobiotic-induced abnormal reproductive function. Every effort has been made to accurately represent what is currently understood about the topics of discussion in this chapter. Controversial topics or those currently still subject to debate within the scientific community have been noted wherever possible.

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# IMPORTANT DEFINITIONS AND CONCEPTS

## Reproduction

Reproduction in domestic, wild and laboratory vertebrates encompasses the wide range of physiological processes and associated behaviors and anatomical structures involved in the production of the next generation and the survival of a given species of animal (Senger, 2003). The physiological processes involved in reproduction generally include the following: (1) gametogenesis (production of sperm or ova) and the pre- and peri-pubertal changes leading up to its onset; (2) release of gametes [i.e. sperm transport and maturation, penile erection and ejaculation of sperm (mammals), copulation between a male and a female of the same species (several vertebrate classes) and ovulation of oocytes]; (3) formation of the zygote (i.e. sperm storage, capacitation and other processes leading to fertilization, or union, of a single sperm with an egg); (4) embryonic and fetal development during the incubation process in egg-bearing vertebrates or, especially in the case of mammals, during pregnancy (gestation) [i.e. activities related to the initiation and progression of zygote cleavage, blastocyst formation, separation of the germ layers, placentation (mammalian species), neurulation and organogenesis (including sexual differentiation)] and finally (5) "birth" of a single or multiple offspring (hatching in oviparous vertebrates). In the conventional sense, the reproductive process culminates with birth or parturition (mammals); however, the initiation and maintenance of milk production (lactation) for the postpartum nutrition of offspring can also be considered a critical aspect of mammalian reproduction (Evans *et al.*, 2007).

## Reproductive toxicity

For the purposes of this chapter, "reproductive toxicity" will refer to any manifestations of xenobiotic exposure reflecting adverse effects on the physiological processes and associated behaviors and/or anatomical structures involved in animal reproduction or development. This is a fairly broad definition which encompasses developmental toxicity, as well as any toxic effects of post-pubertal exposures to xenobiotics on either male or female reproduction. "Developmental toxicity" refers to any adverse effect on the developing organism associated with either pre-conception parental exposures to toxicants or post-conception xenobiotic exposures to the embryo, fetus or pre-pubertal offspring (Hodgson *et al.*, 2000; Eaton and Klaassen, 2001). Adverse effects associated with developmental toxicity of xenobiotics might not necessarily be observed until after the affected individuals have reached sexual maturity (Eaton and Klaassen, 2001).

### *Teratogenesis*

The term "teratogenesis" is derived from the Greek word for monster ("teras") and is a form of developmental toxicity (Rogers and Kavlock, 2001; Panter, 2002). "Teratogenesis" refers specifically to developmental defects induced by toxicant exposures occurring between conception and birth (Hodgson *et al.*, 2000; Eaton and Klaassen, 2001; Rogers and Kavlock, 2001). The types of abnormalities that are typically associated with teratogenesis include embryonic or fetal death; morphological, functional and/or neurobehavioral abnormalities and decreased growth rate and/or birth weight (Rogers and Kavlock, 2001; Panter, 2002).

#### *Wilson's general principles of teratology*

With respect to teratogenesis, there are six basic tenets of teratology, first defined by Wilson in 1959, which need to be kept in mind whenever gestational exposure to a teratogenic xenobiotic is suspected or when a chemical is being evaluated for its teratogenic potential. As stated by Wilson in the *Handbook of Teratology* (1977), the general principles of teratology are as follows:

1. Susceptibility to teratogenesis depends on the genotype of the conceptus and the manner in which it interacts with environmental factors.
2. Susceptibility to teratogenic agents varies with the developmental stage at the time of exposure.
3. Teratogenic agents act in specific ways (mechanisms) on developing cells and tissues to initiate abnormal embryogenesis.
4. The final manifestations of abnormal development are death, malformation, growth retardation and functional disorder.
5. The access of adverse environmental influences to developing tissues depends on the nature of the influences (agent).
6. Manifestations of deviant development increase in degree as dosage increases from no effect to the totally lethal level.

### *Mechanisms of reproductive toxicity and teratogenesis*

There are a wide range of specific mechanisms of action by which xenobiotics can adversely affect reproductive function, including embryonic and fetal development. In general, many of these mechanisms are the same as those for toxicants affecting other organ systems and essentially involve some sort of toxicant-induced cellular dysregulation and alterations in cellular maintenance which, when possible, the body attempts to repair, either successfully or unsuccessfully (Gregus and Klaassen, 2001). Oxidative damage and interference with normal enzymatic reactions are two common mechanisms by which xenobiotics

can cause the dysregulation and altered maintenance of cells within various organs and tissues.

Normal reproduction and development require, by their very nature (see review in this chapter), signaling within and between a variety of diverse organs. In sexual reproduction and mammalian pregnancy, critical communication even takes place between distinctly different organisms (i.e. male and female and mother and offspring, respectively). The dependency of reproductive function on signaling pathways inclusive of gene transcription makes this physiological process especially prone to adverse effects associated with xenobiotic-induced disruption of or interference with cell-to-cell, organ-to-organ and/or even animal-to-animal communication. Many of the mechanisms which interfere with physiological signaling activity can be classified as forms of "endocrine disruption", which will be discussed in much greater detail in this chapter.

There is a great deal of overlap between the various different mechanisms for reproductive toxicity. The level of exposure to a particular toxicant is an important determinant of what toxic effects are observed. Xenobiotics which "disrupt" endocrine pathways can do so without interactions with endogenous receptors, using mechanisms of action which can cause other forms of toxic insult at various dosages.

#### *Reproductive toxicants and teratogens*

Any xenobiotic associated with adverse effects on development or male or female reproductive function can be classified as a "reproductive toxicant". Xenobiotics capable of inducing teratogenesis are referred to as "teratogens". Although any chemicals adversely affecting animal well-being have the potential to have a negative impact on development and reproductive function, this chapter will attempt to focus on mechanisms of actions and toxicants which specifically target normal embryonic or fetal growth and maturation or have a direct effect upon the male and/or female reproductive tract.

## Hormones and hormone receptors

The term "hormone" classically refers to a substance which is secreted into the circulation by a ductless gland and which alters the function of its target cells (Hodgson *et al.*, 2000). While the traditional "endocrine" aspect of hormone action involves organ-to-organ signaling (and in the case of mammalian pregnancy animal-to-animal signaling), it is recognized that hormones can also be involved in "paracrine" (cell-to-cell) communication and signaling pathways within the same cell in which they were produced ("autocrine" function) (Evans *et al.*, 2007). In vertebrates there are a wide variety of different hormones involved in reproductive function. The major reproductive hormones are generally grouped according to their basic molecular structure and include amino acid derivatives [e.g. dopamine or prolactin inhibitory factor (PIF) and melatonin]; peptides [e.g. oxytocin, adrenocorticotropin hormone (ACTH), corticotropin releasing factor or hormone (CRF or CRH), gonadotropin releasing hormone (GnRH) and thyrotropin releasing hormone (TRH)]; proteins [e.g. activin, inhibin, insulin-like growth factors, prolactin and relaxin]; glycoproteins [e.g. follicle-stimulating hormone (FSH), luteinizing hormone (LH) and thyroid stimulating hormone (TSH or thyrotropin)]; steroids (e.g. androgens, estrogens and progestins) and eicosanoids, which include prostaglandins (Evans *et al.*, 2007).

The actions of hormones on their targets are generally mediated through receptors which initiate or inhibit some sort of signal transduction pathway or are required for hormone-induced alterations in gene expression. Hormone–receptor interactions can be modulated by a number of factors including the amount of hormone present, the affinity of the hormone for the receptor, receptor density and occupancy and interaction with other hormones, receptors and hormone–receptor complexes, as well as a variety of endogenous co-activators and inhibitors (Genuth, 2004a; Bigsby *et al.*, 2005). It should be clear by the end of this chapter that various xenobiotics are also capable, under certain exposure conditions, of modulating the interactions between endogenous hormones and their receptors.

#### *Gonadal steroid hormones and their "nuclear" receptors*

The primary gonadal steroids [i.e. androgens and estrogens (some references also include progesterone)] are also referred to as the "sex" steroids, and the imitation and/or inhibition of the actions of these hormones by xenobiotics is what was first referred to as "endocrine disruption" (Krimsky, 2000; McLachlan, 2001). The major androgens [testosterone and dihydrotestosterone (5$\alpha$-reductase conversion product of testosterone in the testes and selected non-gonadal tissues)], estrogens (estradiol and estrone) and progesterone and endogenous progestins facilitate the development and regulation of reproductive function in animal species, in large part, by interacting with (i.e. functioning as ligands for) receptors which are members of the steroid/thyroid ("nuclear") receptor superfamily, the largest family of transcription factors in eukaryotic systems (Tsai and O'Malley, 1994; Genuth, 2004a). Receptors in this superfamily are large oligomeric proteins (Genuth, 2004a), which generally consist of five domains (A/B, C, D, E and F) (Tsai and O'Malley, 1994). Although specific portions of the gonadal steroid nuclear receptor molecules can interact with a variety of co-activators as well as inhibitors, the most important domains of these receptors are generally considered to be those involved in transactivation (*N*-terminal A/B domain; also C-terminus in

estrogen receptors (ERs)); DNA-binding and hormone–receptor complex dimerization (middle portion containing two helical zinc fingers; C domain) and hormone (ligand) binding (C-terminus; E domain) (Genuth, 2004a; Bigsby *et al.*, 2005). While androgen, estrogen and progesterone receptors, which are members of the steroid/thyroid superfamily, are often thought of as being exclusively nuclear in their location, these receptors can also be located in the cytoplasm of some cells (Tsai and O'Malley, 1994; Genuth, 2004a). Cytoplasmic and nuclear gonadal steroid receptors can be bound to a variety of different heat shock proteins, which interact with the receptor's hormone-binding domain. Heat shock proteins can act as "blocking" molecules and are displaced by hormones binding to the receptors (Genuth, 2004a; Bigsby *et al.*, 2005) or as "chaperones" involved in receptor turnover and "trafficking" of these receptors between the nucleus and the cytoplasm (Pratt and Toft, 1997).

There is reportedly a single type of androgen receptor which is a member of the steroid/thyroid superfamily. In contrast, there are two types of nuclear ERs (ER$\alpha$ and ER$\beta$), which are the products of distinct genes on separate chromosomes (O'Donnell *et al.*, 2001). ER$\alpha$ and ER$\beta$ differ in their amino acid structure, tissue distribution, affinity for selective ER modulators (SERMs) and their role in female (Britt and Findlay, 2002) as well as, somewhat surprisingly, male fertility (O'Donnell *et al.*, 2001; Hess, 2003). The nuclear progesterone receptor also has two isoforms, progesterone receptor A and progesterone receptor B (PRA and PRB, respectively), which differ slightly in their amino acid sequences and their interactions with co-activators. However, unlike ER$\alpha$ and ER$\beta$, PRA and PRB are the products of a single gene (Brayman *et al.*, 2006).

#### *Genomic and non-genomic mechanisms of action of gonadal steroid hormones*

Traditionally, the receptor-mediated reproductive effects of gonadal steroids were thought to occur almost exclusively through interactions between homodimers of the hormone–nuclear receptor complexes and specific regions of DNA upstream from the basal promoter of a given gene, referred to as hormone-response elements (HREs) or, more specifically, androgen and estrogen-response elements (ARE and ERE, respectively) (Tsai and O'Malley, 1994; Genuth, 2004a). It is now understood that these "genomic" effects of gonadal steroids and their nuclear receptors, which involve alterations in gene transcription, can, in some instances, involve heterodimers of different nuclear steroid–receptor complexes, indirect binding of hormone–receptor complexes to DNA via proteins within a preformed transcriptional complexes and even ligand (hormone)-independent "activation" of nuclear gonadal steroid receptor molecules (O'Donnell *et al.*, 2001; Bigsby *et al.*, 2005; Thomas and Khan, 2005). In addition, it is also apparent that gonadal steroids can affect cellular function by non-genomic mechanisms of action involving changes in intracellular concentrations of ions, cAMP and its second messengers, and the mitogen-activated protein (MAP) kinase pathway. These non-genomic mechanisms are independent of the somewhat "time-consuming" alterations in gene expression traditionally associated with gonadal steroids and occur rapidly within seconds or minutes (O'Donnell *et al.*, 2001; Thomas and Khan, 2005). While the rapid, non-genomic effects of gonadal steroids most likely involve receptors bound to the plasma membrane, the specific identity and classification of these receptors remain unclear and might involve a number of different receptor types (Razandi *et al.*, 1999; O'Donnell *et al.*, 2001; Thomas and Khan, 2005; Warner and Gustafsson, 2006).

## Endocrine disruption

"Endocrine disruption" is a developing, multidisciplinary area of research, involving aspects of both toxicology and endocrinology (McLachlan, 2001). "Endocrine disruption" is also a potential mechanism of action for many toxicants, and this term has been defined in a variety of different ways, depending on the circumstances and the intended audience. Some of these definitions can be fairly "broad", such as the one which will be used in this chapter (see below). However, "endocrine disruption" can also be defined fairly narrowly with respect to toxicant origin (synthetic versus naturally occurring); source or site of toxicant exposure (environmental contamination versus occupational exposure); xenobiotic mechanism of action [receptor agonism and/or antagonism (see definition below) versus other mechanisms independent of direct interactions between xenobiotics and receptors]; and/or the timing of exposure (prenatal versus postnatal exposures) (Krimsky, 2000, 2001). It is critically important for one to carefully define the context in which "endocrine disruption" is being used in order to clearly and accurately discuss one's research findings or opinions with toxicologists, physiologists, wildlife biologists, medical professionals, regulatory personnel, the popular press and/or the general public (McLachlan, 2001).

Although the imitation and/or inhibition of the actions of androgens and, especially, estrogens by xenobiotics is what was first referred to as "endocrine disruption", both the multidisciplinary area of study and mechanism of action generally referred to as "endocrine disruption" have evolved over the years to encompass a wide range of specific mechanisms of action which can ultimately result in adverse effects on invertebrate and/or vertebrate animals (McLachlan, 2001). As scientists continue to investigate the effects of xenobiotics on biological systems, the paradigm of endocrine disruption will continue to "shift", and a willingness to "step out of the box" and discuss endocrine

disruption in a broader context will be necessary in order to participate in scientific discussions, to design future experiments and/or to make informed, medical or policy decisions based on "good" science (McLachlan, 2001; Guillette, 2006). For the purposes of this chapter "endocrine disruption" will refer to the effects of any synthetic or naturally occurring xenobiotic which can affect the endocrine system of exposed individuals (i.e. the balance of normal hormonal functions) and, as a result of exposure, cause physiological alterations (Keith, 1997; Hodgson *et al.*, 2000). Within the broad scope of this definition, reproduction, including prenatal and pre-pubertal development, certainly would be expected to be one of the physiological functions most profoundly affected by chemicals capable of endocrine disruption. In fact, it could be argued that the majority of reproductive toxicants interfere with endocrine function in one way or another. However, adverse effects on other, "non-reproductive" endocrine systems can also be associated with exposures to xenobiotics, and these "non-reproductive" effects need to be taken into consideration as well when describing the endocrine disruption associated with exposure to a given chemical (Guillette, 2006) (see below).

### *Mechanisms of endocrine disruption*

Endocrine disruption encompasses a wide range of mechanisms of action which can ultimately result in adverse effects on animal species. The mechanisms of action of involved in endocrine disruption can include effects which are mediated directly by interactions between the xenobiotic and an endogenous hormone receptor (i.e. the xenobiotic functions as a ligand for an endogenous receptor and a receptor–ligand complex is formed), as well as those adverse effects which alter hormonal functions without direct interactions between the toxicant and an endogenous receptor (Keith, 1997). In addition, it should be noted that some xenobiotics are capable of causing endocrine disruption by functioning as an endogenous hormone receptor ligand, as well as by mechanisms of action which are independent of the formation of a xenobiotic (ligand)–receptor complex.

#### *"Classic" receptor-mediated endocrine disruption*

"Classic" endocrine disruption can involve imitation or mimicry of the interactions between cellular receptors and endogenous hormones (i.e. receptor agonism) and/or a blockade or inhibition of the formation of receptor–hormone complexes (i.e. receptor antagonism) (McLachlan, 2001). With respect to gonadal steroids, both genomic and non-genomic physiological responses can be affected by this mimicry or blockade of endogenous hormone receptor-mediated activity (Thomas and Khan, 2005). Xenobiotics which mimic the actions of endogenous androgens or estrogens (i.e. gonadal steroid receptor agonists) are referred to, respectively, as being either "xenoandrogens" or "xenoestrogens". Conversely, reproductive toxicants which inhibit or block endogenous estrogens or androgens from interacting with their respective receptors (i.e. gonadal receptor antagonists) are generally classified as "antiandrogens" or "antiestrogens". Progestins" ("progestogens" or "progestagens" in some literature) is a generic term for endogenous or synthetic compounds which interact with progesterone receptors, and there is evidence of increasing environmental contamination with these types of EDCs.

Some xenobiotics can act as receptor agonists or antagonists, depending on the circumstances or tissues involved. "Selective ER modulators" or "SERMs" refer to a class of xenobiotics which, although originally classified as antiestrogens, can function as either ER agonists or antagonists, depending on the tissue in which estrogen-dependent responses are being discussed (Dutertre and Smith, 2000; Katzenellenbogen and Katzenellenbogen, 2000). SERMs are particularly relevant with respect to observed differences in their binding affinities to ER$\alpha$ or ER$\beta$ and their development as therapeutic agents for different types of estrogen-responsive neoplasia.

#### *Endocrine disruption independent of receptor-mediated interactions*

Endocrine disruption which is independent of interactions between xenobiotics and endogenous hormone receptors can occur in a variety of different ways. Xenobiotic exposure can result in alterations in the number of hormone receptor sites (up- or downregulation) or can cause direct or indirect hormone modifications which alter hormonal function (Keith, 1997). Xenobiotics can change the rate of synthesis or destruction of endogenous hormones and can alter how hormones are stored, how they are released into and/or transported within the circulation or even how they are eventually cleared from the body (Keith, 1997; Sikka *et al.*, 2005). Any xenobiotic which is toxic to organs or tissues producing hormones (e.g. testis and ovary) has the potential to decrease hormone synthesis and thereby indirectly cause endocrine disruption (Devine and Hoyer, 2005). It should also be noted that some of these mechanisms of endocrine disruption are not necessarily exclusive of one another. A given xenobiotic can potentially disrupt the normal balance of hormonal function by more than one mechanism which is independent of direct interactions between the toxicant and an endogenous hormone receptor.

### *"Androgenic" and "estrogenic" effects of xenobiotics*

The terms "androgenic" and "estrogenic" and their antonyms, "antiandrogenic" and "antiestrogenic" have been used in a number of different contexts. Some authors have used these terms to refer specifically to the agonistic

and antagonistic receptor interactions of xenobiotics (Hodgson *et al.*, 2000). Because the precise mechanism of endocrine disruption of a given toxicant might not always be known or might involve multiple mechanisms of action, these terms have also been used in a more general sense, especially in livestock and wildlife species, to refer to phenotypic changes which were similar to or the opposite of the effects which would be expected with exposure to endogenous androgens or estrogens (Guillette, 2006). This type of general usage can be helpful in some instances but can also be confusing, given that xenoandrogens and progestins frequently have the opposite phenotypic effects as xenoestrogens. For instance, the effects of estrogenic xenobiotics can be described as antiandrogenic or antiprogestagenic in some instances, while the effects of xenoandrogens and progestins can be referred to as being antiestrogenic in nature. Further confusion can be associated with exposures to mixtures of chemicals having different phenotypic effects, as is often the case in instances of environmental contamination, or with exposures to xenobiotics having mixed antiestrogenic and antiandrogenic effects mixed effects (i.e. methoxychlor). When the terms "androgenic", "estrogenic" or their antonyms are used within this chapter, an attempt will be made to clearly denote the intended specific or general meaning of the terms in the context in which they are used. The discretionary use of the terms "feminization" and "masculinization", as well as "defeminization" and "demasculinization", can also, in some instances, help to clarify and/or describe the phenotypic effects of a chemical suspected endocrine disruption.

### *Endocrine disrupting chemicals, endocrine disruptors and hormonally active agents*

Any reproductive toxicant capable of endocrine disruption can be considered an "EDC" or an "endocrine disruptor". Obviously, this includes a large number of xenobiotics which are used in commercially available industrial, agricultural and pharmaceutical products, as well as naturally occurring toxicants produced by plants and fungi. An effort will be made later in this chapter to discuss some of the xenobiotics most often associated with endocrine disrupting mechanisms of action.

Another term frequently used with respect to endocrine disruption, especially regarding xenobiotics which interact with endogenous hormone receptors, is "hormonally active agent" or "HAA". In most instances, "endocrine disrupting chemical", "endocrine disruptor" or "hormonally active agent" can be used interchangeably to discuss the actions of a given xenobiotic. However, whereas "endocrine disrupting chemical" and "endocrine disruptor" generally have negative connotations and imply, by virtue of the inclusion of the term "disrupt", something "dangerous" and the likelihood of adverse or toxic effects, the term "hormonally active agent" is more benign and only indicates that a given xenobiotic has the potential to affect a hormonal pathway in an animal (Krimsky, 2001). As pointed out by Krimsky (2001), a mechanism rather than a specific pathology is inferred by "hormonally active", and "hormonally active agent" is the nomenclature preferred by the National Research Council (Knobil, 1999), especially when referring to xenobiotics which interact with endogenous hormone receptors.

The circumstances and intended audience will often dictate the terms used to describe xenobiotics associated with or suspected of being having endocrine activity. "Environmental hormone" and "environmental signal" have also been used, along with "HAA", "EDC" and endocrine disruptor, to describe xenobiotics capable of interacting with endogenous hormone receptors (McLachlan, 2001; McLachlan *et al.*, 2006). However, the context in which these two terms have been routinely used generally implies environmental contaminants with documented adverse endocrine effects on animals or humans. In some instances, the term "HAA" might be more "politically correct" (Krimsky, 2001) than "EDC", "endocrine disruptor", "environmental hormone" or "environmental signal" when discussing chemicals with a suspected hormonal activity that has not been clearly associated with adverse effects on animals in a research and/or clinical setting.

### *Aryl hydrocarbon receptor-mediated endocrine disruption*

Endocrine disruption mediated by the aryl hydrocarbon receptor (AhR) is a relatively complex, species- and tissue-dependent phenomenon, involving several of the previously described mechanisms of EDC action and interactions with many important, environmentally persistent compounds. Some aspects of AhR-mediated endocrine disruption are reminiscent of the ligand-induced transcription associated with gonadal steroid receptor function. However, the unique nature of the endogenous AhR and its interactions with primarily xenobiotic agonists warrants further discussion.

#### *Aryl hydrocarbon receptor agonists*

The major agonists for the AhR protein belong to the class of environmental contaminants referred to collectively as "halogenated" or "polyhalogenated aromatic hydrocarbons" (HAHs or PAHs, respectively) and includes many highly stable and lipophilic organochlorine industrial chemicals [e.g. polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzo-difurans (PCDFs)], as well as their metabolites (Safe, 2005). In addition, other organic compounds, such as polycyclic aromatic hydrocarbons (PAHs) [e.g. 3-methylcholanthrene and benzo[a]pyrene) (BaP)] and flavones (e.g.

$\beta$-naphthoflavone), have also been shown to be AhR agonists (Parkinson, 2001).

*Mechanisms of aryl hydrocarbon receptor-mediated endocrine disruption*

Many of the mechanisms of action mediated by AhR–ligand interactions have been elucidated using 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) as a prototypical AhR agonist (Parkinson, 2001; Safe, 2005). TCDD is considered by many to be the most toxic of all of the HAHs, and it is reported to have the highest AhR-binding affinity of any of the xenobiotics in that class of chemicals (Safe, 2005). The AhR, also referred to as the "dioxin receptor", is located in the cytoplasm bound to heat shock proteins (Parkinson, 2001). Following ligand (i.e. TCDD) binding and the subsequent disassociation of the heat shock proteins, the AhR is activated by phosphorylation (Parkinson, 2001) and the activated ligand–AhR complex undergoes a rapid sequence of events involving interactions with the AhR nuclear translocator protein (Arnt) and relocation of the ligand–AhR–Arnt complex into the nucleus (Safe, 2005).

Within the nucleus, the liganded AhR/Arnt heterodimer can facilitate a variety of endocrine disrupting mechanisms. This activated heterodimer complex can interact with dioxin/xenobiotic-response elements (DREs/XREs), which function in much the same way as the previously discussed HREs, and with various co-activators to increase the expression of selected genes (Parkinson, 2001; Safe, 2005). Depending on the animal species and the tissue, multiple phase I drug-metabolizing enzymes [e.g. cytochrome P450 (CYP) enzymes (CYP1A1, CYP1A2 and CYP1B1)] and enzymes involved phase II drug-biotransformation reactions (e.g. glutathione-*S*-transferase and glucuronosyl transferase), are induced by TCDD (Safe, 2005).

Although the antiandrogenic and antiestrogenic properties of TCDD have been associated with the ability of HAHs to induce enzymes involved in androgen and estrogen metabolism, TCDD can interact with androgen-, estrogen- and progestin-modulated pathways in a number of ways, including interference with neuroendocrine development (Petersen *et al.*, 2006). AhR-mediated effects of TCDD can interfere with the biosynthesis of testosterone by a mechanism which alters the regulation of the synthesis and release of LH (Sikka *et al.*, 2005). It has also been shown in cell cultures that TCDD can disrupt testosterone signal transduction pathways (Jana *et al.*, 1999). The liganded AhR/Arnt heterodimer appears to be able to interact with inhibitory DREs (iDREs) in selected tissues to suppress the expression of some genes induced by estrogens (Safe, 2005), as well as be able to actually block the ability of estrogen–ER complexes to bind to their HREs (Kharat and Saatcioglu, 1996; Thomas and Khan, 2005). It is likely that a variety of other means of crosstalk between TCDD- and estrogen-mediated signaling pathways exist, and, in fact, TCDD has actually been shown to have the potential for estrogenic activity through interactions between liganded AhR/Arnt heterodimers and unliganded ERs (both ER$\alpha$ and ER$\beta$) (Ohtake *et al.*, 2003; Bigsby *et al.*, 2005; Thomas and Khan, 2005). Ohtake *et al.* (2003) have reported that these novel interactions resulted in the recruitment of unliganded ERs and p300 co-activator to gene promoters which are responsive to estrogens. Based on the results of the various experiments performed with TCDD, it is important to remember that the effects observed following exposure to HAHs and EDCs, in general, can be dependent on animal species involved, as well as the type of tissue, organ or physiological response being evaluated.

***Epigenetic mechanisms of action of endocrine disrupting chemicals***

In recent years there has been increasing interest in the association between prenatal exposures to some reproductive toxicants and the postnatal development of neoplasia (cancer) involving the reproductive tract, as well as the occurrence of transgenerational or vertically transmitted adverse reproductive effects (Crews and McLachlan, 2006). These two phenomena are not mutually exclusive of one another, and, in fact, there is increasing evidence of vertically transmitted neoplasia involving reproductive organs (McLachlan *et al.*, 2006). Both tumor formation and transgenerational reproductive abnormalities can occur because of "genetic" mutations or alterations in the genotype (i.e. DNA sequence) or as a result of "epigenetic" changes where there are heritable modifications in the properties of a cell which do not represent genetic changes (inherited phenotypic alteration without genotypic change) (Lewin, 1998; McLachlan, 2001; Crews and McLachlan, 2006).

Epigenetic changes are a normal part of development and most likely represent one means for heritable environmental adaptation (Crews and McLachlan, 2006). One of the more common mechanisms of epigenetic modification in mammals is DNA methylation of CpG nucleotides in the promoter regions of genes, which results in methylated genes being "turned off" and unmethylated or demethylated genes being "turned on" (McLachlan, 2001; Anway and Skinner, 2006). Patterns of DNA methylation are generally established during development at the gastrulation stage (i.e. lineage-specific pattern in somatic cells) and after sex determination (i.e. germ line-specific lineage pattern in the gonad) (Anway and Skinner, 2006). DNA methylation can facilitate "genomic imprinting", a form of epigenetic gene regulation resulting in the expression of the allele from only one parent (i.e. monoallelic expression) (McLachlan, 2001; Anway and Skinner, 2006). The ability of developmental exposures to xenobiotics to provide a basis for adult disease, such as neoplasia, might very likely involve epigenetic changes involving methylation or demethylation of the promoters for specific

genes (Newbold *et al.*, 2006). Epigenetic modification by alterations in DNA methylation patterns in the germ line might be one mechanism for observed xenobiotic-induced transgenerational (vertically transmitted) effects associated with infertility and tumor susceptibility in rodents (Anway and Skinner, 2006; Newbold *et al.*, 2006).

#### *Disruption of "non-reproductive" endocrine systems*

Although it can be argued that almost all endocrine systems are "reproductive" to some extent, there are multiple systems with primary functions which are not directly related to reproduction, and several of these systems have also been identified as potential targets of EDCs. In addition, gonadal steroids and xenobiotics which mimic these endogenous hormones can have "non-reproductive" effects. The synthesis of triiodothyronine ($T_3$) and thyroxine ($T_4$) by the thyroid gland can be decreased by chemicals which inhibit the uptake of iodine (e.g. perchlorate and thiocyanate) and also by xenobiotics which inhibit thyroperoxidase (e.g. thiourea, propylthiourea (PTU), some sulfonamides, methimazole, carbimazole, aminotriazole and acetoacetamide) (Capen, 2001). Polybrominated diphenyl ethers (PBDEs) have been shown to have antithyroidal activity (Guillette, 2006), and thyroid hormone secretion can be inhibited by exposure to excessive amounts of iodine or lithium (Capen, 2001). Xenobiotics, such as the *o,p′*-DDD metabolite of dichlorodiphenyltrichloroethane (DDT), can interfere with glucocorticoid metabolism (Guillette, 2006), and there has been increasing interest in the relationship between gestational and neonatal exposures to xenoestrogens and the development of obesity (Cooke and Naz, 2005; Newbold *et al.*, 2005, 2006). Some EDCs (e.g., organotin compounds) have recently been described as "obesogens" because of their ability to affect adipogenesis by several different mechanisms, including interactions involving the isoforms of 11β-hydroxysteroid dehydrogenase (11β-HSD) which play key roles in glucocorticoid metabolism (Grün and Blumberg, 2006). Because of increased societal concerns about obesity, there is likely to be greater future interest in organotins and other EDCs with similar "obesogenic" activities.

## NORMAL ANIMAL REPRODUCTION

Reproduction is a complex and dynamic process involving precise coordination and integration of the functions of multiple organs within the body. The production of viable and functional gametes and their transport and union to form a zygote which develops into a healthy and fertile individual require that many stringent physiological and metabolic needs be met. A thorough understanding of the mechanisms involved in reproduction is absolutely essential in order to recognize which steps in the reproductive process are most susceptible to the adverse effects of potential toxicants. It is critical that one be able to understand the pathophysiological basis for reproductive abnormalities. In addition, it is necessary, from a clinical perspective, to identify what constitutes "normal" reproduction in order to recognize abnormal reproductive behaviors and morphological changes in both domestic and wild animals. Impaired reproductive function in domestic animals, which is associated with exposure to toxic amounts of xenobiotics, necessitates the use of diagnostic, prognostic and therapeutic procedures which require a thorough knowledge of normal reproductive anatomy and physiology.

Normal reproduction will be reviewed in this chapter to provide a basis for discussion concerning specific reproductive toxicants. Although the emphasis will be on mammalian reproduction, many of the principles will be applicable to other classes of vertebrates. If additional information is needed, textbooks are available which provide a comprehensive overview of animal reproduction (Hafez and Hafez, 2000; Senger, 2003), as well as general veterinary anatomy (Dyce *et al.*, 2002). Other references can be consulted for descriptions of various aspects of normal reproduction in species of domestic or laboratory animals, which might be of particular interest to the reader (Johnston *et al.*, 2001; Hedrich and Bullock, 2004; Suckow *et al.*, 2006; Youngquist and Threlfall, 2007).

### Neuroendocrine control of reproduction

In humans and animals alike, visual, olfactory, auditory and other sensory data are integrated within the brain and are reflected in endocrine events. The neuroendocrine functions of the pineal gland, hypothalamus and pituitary gland play an important role in the integration and endocrine regulation of the body's physiological processes and are potential targets for many reproductive toxicants (i.e. dioxins). These structures within the brain and proper function of the hypothalamic–pituitary–gonadal axis facilitate development of the reproductive tract and endocrine regulation of spermatogenesis in the male and the estrous or menstrual cycle in the female. The onset of puberty and sexual behavior in males and females, the ability to achieve erection and ejaculation in males, and the normal progression of gestation, parturition and lactation in females are all affected by the secretions of the hypothalamus and pituitary gland, as well as interactions between these structures and the reproductive tract (Ginther, 1992; Senger, 2003; Evans *et al.*, 2007).

The hormones involved in the neuroendocrine control of reproduction are produced in several regions of the brain. Melatonin is produced in the pineal gland. The major hormones of reproductive interest which are of hypothalamic origin are dopamine, CRF, GnRH and TRH. Oxytocin is released from the posterior pituitary (neurohypophysis), and ACTH, FSH, LH, prolactin and TSH are synthesized

and released from the anterior pituitary (adenohypophysis) (Ginther, 1992; Evans *et al.*, 2007). The production and release of these hormones are regulated by various positive and negative feedback loops, which are potentially susceptible to the effects of hormonally active xenobiotics.

## Puberty

### *The onset of puberty*

Puberty in male and female offspring implies reproductive competence and corresponds to the onset of normal spermatogenesis in the male and reproductive cyclicity in the female. Puberty can be indicated in the female by the age at first estrus or ovulation or even the age at which pregnancy can be maintained safely (Senger, 2003; Evans *et al.*, 2007). In the male, the age at the time of preputial separation and the acquisition of the ability to ejaculate or the age at the first appearance of spermatozoa in the ejaculate or urine, as well as the production of threshold concentrations of fertile sperm in the ejaculate, have all been used as indicators of puberty (Senger, 2003). Species, nutritional status, environmental and social factors, pheromones and photoperiod in short- or long-day breeders can all influence the age of onset of puberty in animal species (Senger, 2003; Evans *et al.*, 2007).

### *The endocrinology of puberty*

From an endocrine perspective, puberty is associated with the ability of the hypothalamus to release enough GnRH to induce gonadotropin production by the anterior pituitary gland (Senger, 2003). This endocrine milestone is brought about by the postnatal developmental changes which allow the hypothalamus to overcome the negative feedback of testicular androgens and estrogens in males and which facilitate the ovary's ability to produce sufficient estrogens to induce the preovulatory surge of GnRH in females (Senger, 2003; Evans *et al.*, 2007). Many of the endocrine changes which come into play with the onset of puberty are also involved in the transition from anestrus to the ovulatory season in seasonally polyestrous female animals (Evans *et al.*, 2007).

### *The susceptibility of the pubertal process to reproductive toxicants*

While puberty is often described simply in terms of a single, initial reproductive event (e.g. first estrus, ovulation or ejaculation), the attainment of reproductive competency is actually a process which is susceptible to the affects of reproductive toxicants. Xenobiotics can interfere with important physiological and morphological transformations necessary for the normal stepwise progression toward reproductive competency. Pre-pubertal follicular development, as well as the onset of the preovulatory LH surge in the female and the transition in testicular estrogen synthesis from the Sertoli cell to the Leydig cell in the males of many species, in addition to the postnatal proliferation of Sertoli cells in some mammals, are all potentially susceptible to the adverse effects of xenobiotics. Pre- or peri-pubertal exposure to hormonally active xenobiotics, such as anabolic steroids and antiandrogens, can interfere with postnatal reproductive development and function and can impair an animal's ability to reach its maximum reproductive potential (Monosson *et al.*, 1999; Evans *et al.*, 2007).

## Normal male reproductive anatomy and physiology

### *Reproductive anatomy of the male*

Anatomical structures associated with reproduction in the male usually include, especially in mammals, paired testes (male gonads) positioned outside the abdominal cavity in most species; an excurrent duct system (i.e. efferent ductules, paired epididymidies, vas deferens and urethra); accessory sex glands (i.e. ampullae, seminal vesicles, prostate and bulbourethral glands); a scrotum and its associated thermoregulatory functions to protect the testes from mechanical and thermal insult and some form of copulatory organ or penis with a mechanism for protrusion, erection, emission of glandular secretions and ejaculation of sperm. The primary functions of the testis (testicle) are spermatogenesis [production of male gametes (sperm or spermatozoa)] and steroidogenesis (production of androgens and estrogens). Unlike the female in which oogonia are no longer replicating and the full complement of potential oocytes are present at birth, spermatogonia are proliferating and differentiating into spermatozoa continuously, and the testis is organized in such a way as to maximize sperm production (Senger, 2003).

#### *Testicular structure*

The parenchyma of the testis is divided into the tubular and interstitial compartments (Senger, 2003) (Figure 14.1a). The structural and functional units of the testis are the seminiferous tubules within the tubular compartment and the Leydig (interstitial cells) within the interstitial compartment. Depending on the species, it is estimated that the seminiferous tubules comprise approximately 80% of the adult testis, with the interstitium comprising most of the remaining 20% (Genuth, 2004b). Seminiferous tubules form highly convoluted loops (tubulus contortus) which begin and end with straight portions (tubulus rectus) that connect to the rete tubules (Thomas and Thomas, 2001; Senger, 2003; Genuth, 2004b). In some species, the rete tubules coalesce in a fibrous region of the testis referred to as the mediastinum, which joins with septal projections of

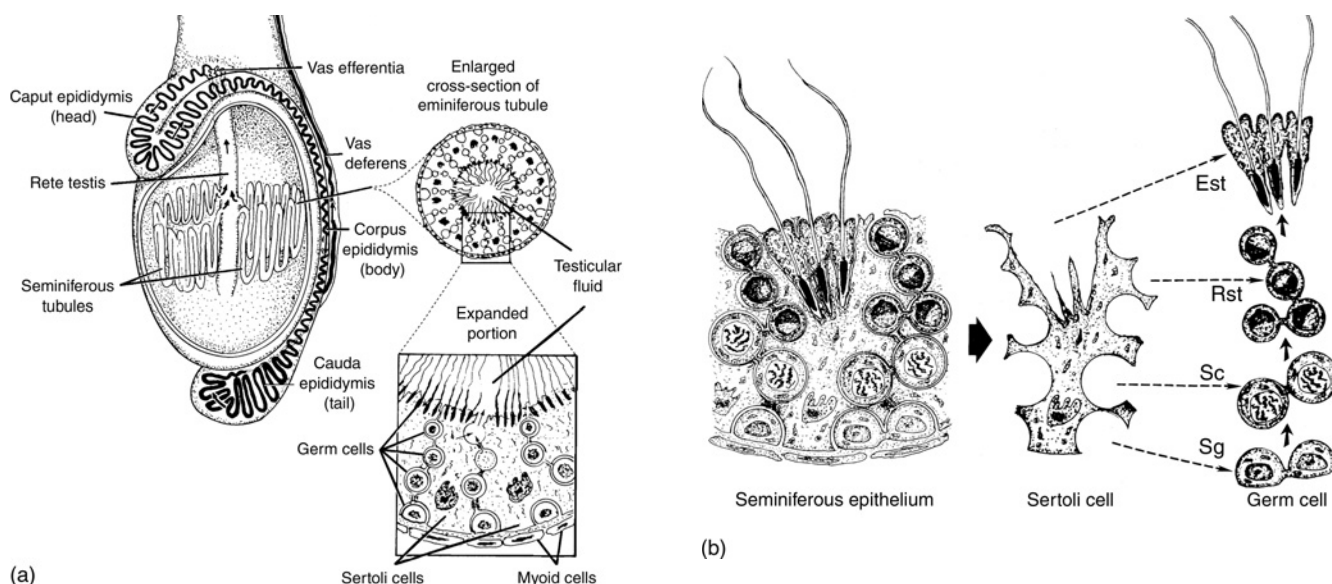


**FIGURE 14.1** Loops of the seminiferous tubules, rete testis and excurrent duct system (i.e. efferent ductules (vas efferentia), epididymidis (epididymis) and ductus deferens (vas deferens), as well as a cross-section of a seminiferous tubule showing the microanatomy of the seminiferous epithelium in a "typical" mammalian testis, are shown in (a). Mature spermatozoa follow the pathway denoted by arrows. Testicular fluid is secreted by the Sertoli cell into the lumen of the seminiferous tubule. The portion of the testicular parenchyma outside of the seminiferous tubules is the interstitium. The predominant cell type within the interstitium is the Leydig or interstitial cell. The complex nature of the association between Sertoli cells and developing germ cells within the seminiferous epithelium in a "typical" mammalian testis is shown in (b). The Sertoli cell and germ cells are shown schematically disassociated to demonstrate how spermatozoal precursors occupy spaces between adjacent Sertoli cells. Spermatogonia, spermatocytes, round spermatids and elongate spermatids are denoted by Sg, Sc, RSt and ESt, respectively. This figure was adapted, with permission, from Garner DL, Hafez ESE (2000) In *Reproduction in Farm Animals*, 7th edn, Hafez ESE Hafez B (eds). Lipincott Williams & Wilkins, Philadelphia, PA (modifications and artwork courtesy of Don Connor and Howard Wilson).

the tunica albuginea, part of the testicular capsule (Senger, 2003). The rete tubules join with the efferent ductules which attach to the epididymidis.

Within the seminiferous tubules are germ cells at various stages of differentiation and Sertoli cells which provide germ cells with structural support and nutrients, as well as regulatory and paracrine factors (Thomas and Thomas, 2001) (Figure 14.1b). Tight junctions (junctional complexes) between adjacent Sertoli cells divide the seminiferous epithelium into basal and adluminal compartments, with Sertoli cells anchored to the basement membrane and surrounding the developing populations of germ cells (Thomas and Thomas, 2001; Senger, 2003; Genuth, 2004b). The seminiferous tubules are surrounded by peritubular myoid cells, which in combination with the junctional complexes, form the "blood–testis barrier" to prevent free exchange of large proteins and some xenobiotics between the blood and the fluid within the seminiferous tubules (Thomas and Thomas, 2001; Senger, 2003).

Within the interstitial compartment are the Leydig (interstitial) cells, as well as capillaries, lymphatic vessels and connective tissue (Senger, 2003). The Leydig cells are homologous to the theca interna cells in the ovary and produce testosterone (also estrogen in some species). There are species differences with respect to the abundance of Leydig cells in the interstitium, and these differences are important to recognize when reporting Leydig or interstitial cell hyperplasia in response to toxicant exposure. It should also be noted that Leydig and, to a lesser extent, Sertoli cells contain enzymes involved in xenobiotic biotransformation, and the synthesis of toxic metabolites can actually occur within the testis, in close proximity to the target cells for a given reproductive toxicant (Thomas and Thomas, 2001; Creasy and Foster, 2002).

*Excurrent duct system*

The excurrent duct system consists of the efferent ductules, the epididymal duct and the ductus deferens. This duct system functions to conduct spermatozoa, rete fluid and some testicular secretory products away from the testis and eventually into the pelvic urethra (Senger, 2003). The reabsorption of fluid by a species-variable number of efferent ductules is essential for normal testicular function (O'Donnell *et al.*, 2001; Hess, 2003), and these tubules terminate by joining a single highly coiled epididymal duct, commonly referred to as the epididymidis or epididymis. Depending on the species, the epididymidis is generally subdivided into the initial segment, head (caput), body (corpus) and tail (cauda), with the various portions sometimes being further subdivided (França *et al.*, 2005). The primary functions of the epididymidis are transport and

sustenance of sperm, reabsorption and secretion of fluid (initial segment and head, respectively); spermatozoal acquisition of motility and fertile potential (i.e. sperm maturation); recognition and elimination of defective spermatozoa; sperm storage prior to ejaculation and secretory contributions to the seminal fluid (Sutovsky *et al.*, 2001). The epididymal transit time varies somewhat with species, but is generally approximately 7–14 days in length, depending on several factors including ejaculation frequency. The ductus deferens conducts spermatozoa matured in the epididymidis to the pelvic urethra which helps to form the penis.

*Accessory sex glands*

There are a number of accessory sex glands (the complement of which varies with species) that contribute to the composition of the seminal fluid in mammals. These glands include the ampullae, seminal vesicles (vesicular glands), prostate and bulbourethral glands (Senger, 2003). Laboratory rodents (i.e. mice and rats) have an additional gland referred to as the preputial gland, which appears to have a role in the production of pheromone (Creasy and Foster, 2002). These accessory sex glands in the male are generally considered to be androgen dependent, with conversion of testosterone to DHT occurring in the prostate and seminal vesicles of many species) (Creasy and Foster, 2002; Senger, 2003). The weights of the accessory sex glands can be used as an indirect measure of testosterone concentrations or exposure to antiandrogens (Thomas and Thomas, 2001; Senger, 2003).

*External genitalia*

The external genitalia of the male consist of the copulatory organ or penis, the prepuce, which protects the penis from environmental and mechanical injury, and the scrotum for testes positioned outside of the abdominal cavity. Penile structure is extremely species variable, with some species even having a special penile bone (i.e. os penis), but the shaft of the penis generally consists of erectile tissue (corpus cavernosum and corpus spongiosum) which surrounds the pelvic urethra. The glans penis is homologous to the female clitoris, and stimulation of the glans is the primary factor involved in the initiation of ejaculation (Senger, 2003). The scrotum protects the testes from mechanical injury and, in conjunction with the tunica dartos, cremaster muscle and pampiniform plexus, plays a major thermoregulatory role with respect to temperature sensitive, testicular spermatogenesis. In some species of wildlife (e.g. elephants and marine mammals), the testes are positioned intra-abdominally. Xenobiotics, which cause hyperthermia (i.e. ergopeptine alkaloids) or which induce fever, have the potential to adversely affect spermatogenesis.

***Spermatogenesis***

Spermatozoa are highly specialized haploid cells equipped with a self-powered flagellum to facilitate motility, as well as an acrosome to mediate penetration of the zona pellucida. Spermatogenesis takes place within the seminiferous tubules and consists of all the changes germ cells undergo in the seminiferous epithelium in order to produce adequate numbers of viable spermatozoa each day and to continuously replace spermatogonial stem cells (Thomas and Thomas, 2001; Senger, 2003). Spermatogenesis provides for genetic diversity and ensures that germ cells are in an immunologically favored site (Senger, 2003). The duration of spermatogenesis varies with species but generally ranges between 4 and 8 weeks (approximately 30–60 days) in domestic and laboratory animals and is approximately 75 days (almost 11 weeks) in humans. It is important to keep in mind the durations of spermatogenesis and epididymal sperm transport in a given species, as well as the normal, species-specific number of spermatozoa produced daily by the testes, when determining the period of toxicant exposure relative to the appearance of abnormal spermatozoa in an ejaculate and when assessing the severity and reversibility of toxicant-induced damage to sperm precursors within the testes.

Spermatogenesis can be subdivided into three phases or stages referred to as "proliferation", "meiosis" and "differentiation". During each of these phases, sperm precursors or male germ cells (spermatogonia, spermatocytes or spermatids) undergo specific, stepwise changes as they develop into spermatozoa which will eventually be released into the excurrent duct system. Each of these phases involves a different type of germ cell undergoing a different developmental process, and, as such, these phases have the potential to differ in their susceptibility to the mechanisms of action of various reproductive toxicants.

*Proliferation (mitosis or spermatocytogenesis)*

The "proliferation" phase of spermatogenesis has also been referred to as "mitosis" or "spermatocytogenesis" and occurs within the basal compartment of the seminiferous tubule. Proliferation denotes all of the mitotic divisions involving spermatogonia (Senger, 2003). A large number of B-spermatogonia result from the mitoses of several generations of spermatogonia (e.g. $A_1$, $A_2$, $A_3$, $A_4$ and I; some species variations in nomenclature) (Senger, 2003; Genuth, 2004b). Stem cell renewal is accomplished during proliferation by the reversion of some spermatogonia to more primitive germ cells (Senger, 2003). Germ cell mitosis during spermatogenesis ends with the transformation of B-spermatogonia into primary spermatocytes, and this process is particularly susceptible to toxicants, such as chemotherapeutic agents and radiation, which target rapidly dividing cells.

### Meiosis

"Meiosis" takes place within the adluminal compartment of the seminiferous tubules and involves the participation of primary and secondary spermatocytes in a total of two meiotic divisions. The chromosomal reduplication, synapsis and crossover, as well as cellular division and separation, which occur during this phase of spermatogenesis, are extremely complex and guarantee genetic diversity (Senger, 2003; Genuth, 2004b). The meiosis phase of spermatogenesis is considered by some to be most susceptible to toxic insult (Thomas and Thomas, 2001) and ends with the production of haploid round spermatids (Senger, 2003).

### Differentiation (spermiogenesis)

Spermatozoa have been aptly characterized as "sophisticated, self-propelled packages of DNA and enzymes" (Senger, 2003). "Differentiation" or "spermiogenesis" involves all the changes occurring within the adluminal compartment, which transform round spermatids into spermatozoa possessing an acrosome for penetration of the zona pellucida and a tail or flagellum to facilitate motility (Genuth, 2004b). Differentiation can be subdivided into the "Golgi", "cap", "acrosomal" and "maturation" phases, which correspond respectively to acrosomal vesicle formation; spreading of the acrosomal vesicle over the nucleus; elongation of the nucleus and cytoplasm and final assembly involving the formation of the post nuclear cap organization of the tail components (Senger, 2003). Following the nuclear and cytoplasmic reorganization which characterizes the changes to germ cells during spermiogenesis, differentiated spermatozoa are released from Sertoli cells into the lumen of the seminiferous tubules by a process referred to as "spermiation". The complex signaling pathways and genomic imprinting involved in regulating the differentiation of round spermatids into spermatozoa are potential targets for EDCs.

### The cycle of the seminiferous epithelium

In most sexually mature mammals, spermatozoa are produced continuously, with the entry of germ cells into the proliferation phase of spermatogenesis occurring in a coordinated cyclic manner (Genuth, 2004b). Spermatogonia A in a given region of the seminiferous tubule commit to proliferate in a synchronous manner, with cohorts of their progeny germ cells (cellular generations) connected by intercellular bridges and developing and differentiating in unison (Thomas and Thomas, 2001; Senger, 2003). Including spermatogonia A, four or five generations or concentric layers of sperm precursors are present in each cross-section of the seminiferous tubules (Figure 14.1) (Thomas and Thomas, 2001; Creasy and Foster, 2002; Senger, 2003). The cycle of the seminiferous epithelium in most mammals is characterized by germ cells in each spermatogenic phase associating with contiguous generations in a repeatable pattern of specific cellular associations or "stages" (Thomas and Thomas, 2001; França *et al.*, 2005). There is generally only one stage per seminiferous tubular cross-section in subprimates (França *et al.*, 2005), and each stage transitions into the next at predictable intervals (Senger, 2003). At any given point along a seminiferous tubule, the entire cycle of the seminiferous epithelium occurs over a set time interval closely associated with the spermatogonial turnover rate for that particular mammalian species (Thomas and Thomas, 2001; Creasy and Foster, 2002). The number and duration of the various stages of the cycle of the somniferous epithelium vary with species (Senger, 2003), and various classification schemes have been used, based on the morphological characteristics of the spermatid nucleus or the development of the acrosomic system (França *et al.*, 2005). In subprimates, sequential stages are arranged along the length of the seminiferous tubule in consecutive order, forming a "spermatogenic wave" (Creasy and Foster, 2002; Senger, 2003). The progeny of one spermatogonium A will progress through approximately 4.5 cycles of the seminiferous epithelium before being released into the lumen of the seminiferous tubule and progressing through the rete testis into the excurrent duct system (Thomas and Thomas, 2001). An understanding of the cycle of the seminiferous epithelium is very useful for the evaluation of the effects of xenobiotics on spermatogenesis and for the determination of populations of germ cells most susceptible to a given toxicant.

## *Male reproductive physiology*

### Gonadal steroid synthesis in the testes

The endocrine events which regulate spermatogenesis and sexual behavior in males are very distinct from those which take place in females (see below). The primary gonadal steroids produced by the testes are androgens [testosterone and DHT (also produced from testosterone in selected non-gonadal tissues)] and estrogens (primarily estradiol in most species), which are now recognized as playing essential roles in male reproductive development and function (O'Donnell *et al.*, 2001; Hess, 2003). Leydig cells in the interstitium synthesize pregnenolone and then progesterone from cholesterol and convert progesterone to testosterone under the influence of LH (Senger, 2003; Genuth, 2004b). The site of estrogen synthesis (i.e. aromatase activity) varies with the age and species of animal. In the male fetus, postnatal immature male and, in some species, the adult male, Sertoli cells within the seminiferous tubules play a major role in the aromatase-mediated conversion of testosterone to estradiol under the influence of FSH (O'Donnell *et al.*, 2001; Senger, 2003). In many mammals, Leydig cells in the fetal testis and, especially, the postnatal immature testis gradually

begin to synthesize estrogens, and, at sexual maturity, a major portion of the estrogens in these species is produced by aromatase activity in the Leydig cells, under the influence of LH rather than FSH (O'Donnell *et al.*, 2001; Hess, 2003). More recently, germ cells have been identified as another potential source of estrogens in the testis, and it is possible that germ cell-derived estrogens play major roles in regulating male reproductive function (Hess, 2003).

*Endocrine regulation of spermatogenesis*

While the female hypothalamus has both fully developed tonic and surge centers for GnRH release (especially prior to ovulation), the hypothalamic GnRH surge center in the male is diminished, and the anterior pituitary gland of the male does not experience surges in GnRH stimulation (Senger, 2003). This gender-specific alteration in the hypothalamus facilitates the normal endocrine milieu which maintains continuous spermatogenesis and stimulates normal sexual behavior (Figure 14.2). The tonic pulsatile release of GnRH induces the anterior pituitary to produce pulses of LH and FSH several times during the day and facilitates adequate LH-dependent testosterone production and, depending on the species, normal FSH-dependent Sertoli function, both of which are essential for spermatogenesis to occur continuously in the seminiferous tubules (Senger, 2003; Genuth, 2004b). In some species, FSH is primarily required for the onset of puberty and the initiation of spermatogenesis, with many of the functions of FSH in the immature male being taken over by testosterone in the sexually mature animal (Creasy and Foster, 2002).

Testosterone stimulates Sertoli cells to produce several androgen-regulated proteins, including androgen-binding protein, which are required for spermatogenesis (Creasy and Foster, 2002; Senger, 2003). Interference with this testosterone-mediated effect by antiandrogens (e.g., vinclozolin) which prevent interactions between testosterone and the androgen receptor or by the inhibition of testosterone synthesis by excessive glucocorticoids (e.g., chronic stress, alterations in endogenous glucocorticoid metabolism or the administration of xenobiotics with glucocorticoid-like activities) has the potential to adversely affect Sertoli cell function, and, therefore, spermatogenesis. Estrogens are required for various aspects of the normal development and function of Sertoli cells and germ cells within the seminiferous tubules (O'Donnell *et al.*, 2001; Hess, 2003). Xenobiotics which mimic or inhibit the actions of estradiol within the testis can disrupt normal spermatogenesis.

*Positive and negative feedback loops involved in male reproduction*

Positive and negative feedback mechanisms help maintain an endocrine environment which if conducive to normal male reproductive function (Figure 14.2). The Sertoli cell can produce activin and inhibin which respectively

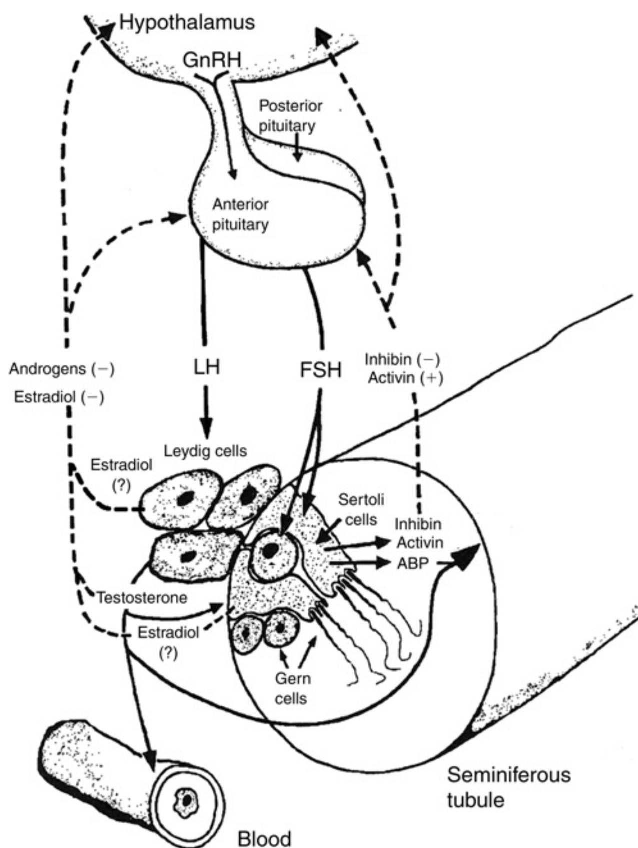


**FIGURE 14.2** The relationship between the tubular and the interstitial compartments of the testicular parenchyma and the endocrine regulation of testicular function in mammalian species is shown. Solid lines indicate positive feedback mechanisms, and dashed lines denote negative feedback pathways. The question mark associated with the production of estradiol by the Sertoli cells and Leydig cells is used to indicate that this hormone, as well as other estrogens, can be produced in the testis by either primarily Sertoli or Leydig cells, depending on the species and stage of development. Although not shown, it should be kept in mind that DHT also provides negative feedback to the hypothalamus and anterior pituitary and germ cells can also aromatize testosterone and produce estradiol. This figure was adapted, with permission, from Garner DL, Hafez ESE (2000) In *Reproduction in Farm Animals*, 7th edn, Hafez ESE, Hafez B (eds). Lipincott, Williams & Wilkins, Philadelphia (modifications courtesy of Don Connor and Howard Wilson).

increase and decrease the secretion of FSH by gonadotropes and, in some species, GnRH release from the hypothalamus (Creasy and Foster, 2002). Testosterone, DHT and estradiol all provide negative feedback to the hypothalamus with respect to GnRH release, and testosterone can also directly inhibit LH secretion by gonadotropes (Creasy and Foster, 2002; Senger, 2003). Xenoestrogens and xenoandrogens have the potential to disturb the hypothalamic–pituitary–gonadal axis (O'Donnell *et al.*, 2001). It is currently thought that antiandrogens and a variety of other xenobiotics can interfere with these feedback loops and possibly other endocrine pathways, resulting in Leydig or interstitial cell hyperplasia (O'Connor *et al.*, 2002; Thomas and Thomas, 2001).

*Epididymal and accessory sex gland function*

Epididymal development and function are dependent on the proper balance of androgenic and estrogenic stimulation and are required for normal male reproductive function and fertility. The accessory sex glands are considered to be primarily androgen dependent, and the secretions of these glands, as well as those of the epididymidis are important components of seminal fluid. Conversion of testosterone to DHT can generally occur in the epididymidis, prostate and seminal vesicles. Hormonally active xenobiotics which alter the normal endocrine events associated with epididymal and accessory gland development and function can have adverse effects on male fertility.

*Sexual behavior, erection, emission and ejaculation*

Sexual behavior is mediated by estradiol in postnatal males and females. The conversion of the steadily produced testosterone in the male to estradiol in the brain (plus the effects of estrogens of testicular origin) results in the male being sexually receptive most of the time (Senger, 2003). Adequate libido and sexual receptivity, as well as adequate concentrations of testosterone, are necessary for erection of the penis, which is required for intromission during copulation (Sikka *et al.*, 2005). Olfactory (detection of pheromones), auditory and visual stimuli play roles in facilitating cholinergic and NANC (non-adrenergic/non-cholinergic) parasympathetic neuron-mediated penile erection, which, depending on the species, involves various degrees of nitric oxide-associated vasodilation and vascular engorgement (Senger, 2003; Sikka *et al.*, 2005). During copulation, the events which lead to emission of the secretions of the accessory sex glands and the ejaculation of spermatozoa generally involve tactile stimuli to the glans penis (Senger, 2003) and stimulation by sympathetic neurons (Sikka *et al.*, 2005).

## Normal female reproductive anatomy and physiology

### *Reproductive anatomy of the female*

Although there are some distinct morphological differences between species (e.g. simplex uterus in primates, duplex cervices in rabbits), the female reproductive tract generally consists of paired ovaries and the "tubular genitalia", which include the paired oviducts (uterine tubes) and uterine horns contiguous with a uterine body and cervix, vagina, vestibule and vulva (Senger, 2003; Evans *et al.*, 2007). The organs involved in female reproductive function are physiologically and morphologically dynamic and function to produce the oocyte, facilitate its fertilization, provide an environment for embryonic and fetal development, and transport the fetus from the maternal to the external environment. Variations in size, appearance, location and function of the female reproductive organs depend on the endocrine milieu dictated by the effects of sexual maturation, stage of the estrous or menstrual cycle, gestational hormone production of maternal, fetal and/or placental origin, exposure to exogenous HAAs and seasonal influences (Ginther, 1992; Senger, 2003; Evans *et al.*, 2007).

The primary functions of the ovary are oogenesis [production of female gametes (oocytes or ova)] and steroidogenesis (production of estrogens and progesterone). The ovaries of most domestic mammals consist of a peripheral parenchymatous zone (cortex), containing various stages of follicular and luteal gland development and a central vascular zone (medulla), comprised of collagenous connective tissue rich in blood vessels (Senger, 2003) (Figure 14.3). The structural and functional unit of the ovary is the follicle. Follicles are classified as primordial, primary (some become atretic), secondary and tertiary (antral) follicles based on their stage of development (Evans *et al.*, 2007).

A primary oocyte surrounded by a single, flattened cell layer is a primordial follicle. A basal lamina separates the single layer of what will become granulosa cells from the adjacent stromal tissue which eventually develops into the theca cells (theca interna and theca externa). The granulosa cells homologous to the Sertoli cells in the testis, and the theca interna cells are the female equivalent of the Leydig cells (Senger, 2003). Following the appropriate endocrine stimulation, primordial follicles are recruited to undergo possible further differentiation into estrogen-producing antral follicles and ultimately ovulation, which results in the release of a secondary oocyte (primary oocyte in dogs) and formation of a corpus luteum (CL) which produces progesterone (Ginther, 1992; Senger, 2003; Evans *et al.*, 2007).

### *Female reproductive physiology*

Females are born with a finite pool of primordial follicles (up to hundreds of thousands), and reproductive cyclicity (i.e. estrous or menstrual cycles) provides females with repeated opportunities for the establishment of pregnancy. The majority of mammalian species (subprimates) have estrous cycles, which reflect the physiological changes occurring between successive ovulations and/or periods of sexual receptivity (estrus) (Senger, 2003). Humans and non-human primates experience menstrual rather than estrous cycles and don't have defined periods of sexual receptivity (i.e. estrus). Unlike the estrous cycles in subprimates, the reproductive cycle in menstruating animals is divided into phases (i.e. menses, proliferative and secretory phases), which are defined based on the physiological state of the uterine endometrium, rather than on the predominant ovarian structures (Senger, 2003; Genuth, 2004b).

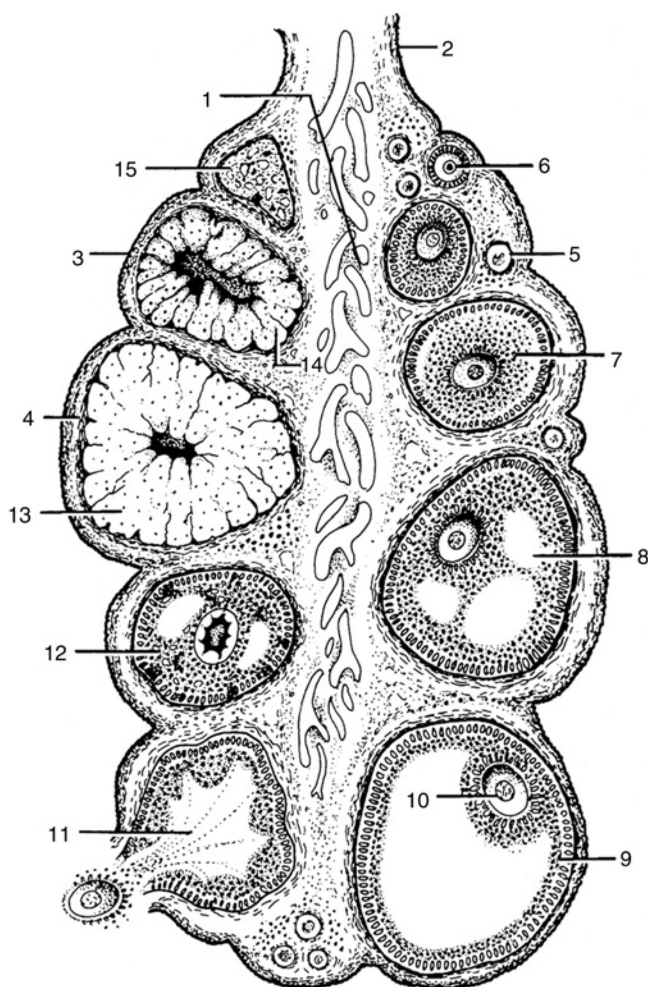


**FIGURE 14.3** Although there is some interspecies variation with respect to ovarian structure, a schematic representation of a "typical" mammalian ovary is shown to demonstrate the major ovarian structures: (1) medulla; (2) mesovarium; (3) surface epithelium; (4) tunica albuginea, which is poorly developed in the ovary as compared to the testis; (5) primordial follicle; (6) primary follicle; (7) secondary follicle; (8) early tertiary or antral follicle; (9) mature antral follicle; (10) oocyte; (11) ruptured follicle and ovulated secondary oocytes (except for the dog); (12) atretic follicle; (13) CL; (14) atretic CL; (15) corpus albicans. This figure was adapted, with permission, from Dyce *et al.* (2002) (modifications courtesy of Don Connor and Howard Wilson).

*The estrous cycle*

The follicular and luteal phases of the estrous cycle describe the predominant ovarian structures and the corresponding gonadal steroid concentrations which result from the follicular secretion of estrogens or the luteal secretion of progesterone, respectively (Ginther, 1992; Senger, 2003; Evans *et al.*, 2007). Both the follicular and luteal phases can generally be further subdivided into two stages each, proestrus and estrus (sexual receptivity) for the follicular phase and metestrus and diestrus (sexual non-receptivity) for the luteal phase (Senger, 2003). Proestrus represents the period of transition from the diestrus dominance of progesterone to the dominance of estrogens during estrus, while metestrus represents the opposite shift in the endocrine milieu (estrogen dominance to progesterone dominance) (Senger, 2003; Evans *et al.*, 2007).

The durations of the various stages of the estrous cycle vary with species and can, depending on the animal in question, either occur throughout the year, multiple times within an ovulatory season that is dependent on photoperiod (long-day or short-day polyestrous animals) or only once a year (Senger, 2003). The domestic bitch does not have a metestrus and, in fact, is actually most receptive to copulation when estradiol is declining and there is a unique, preovulatory surge in progesterone. This endocrine environment predisposes the bitch to cystic endometrial hyperplasia and pyometra following exposure to some xenoestrogens and progestins. Felids, which are induced (reflex) ovulators, like ferrets, mink, camelids and rabbits, the period of time following an estrus in which copulation has not occurred has been described as post-estrus rather than metestrus because there is no increase in progesterone secretion following the end of sexual receptivity (Senger, 2003). Anestrus is the time period during which reproductive cyclicity ceases and can be seasonal (estradiol and progesterone production are at basal levels) or can be associated with various endocrine milieus related to species of animal, pregnancy, lactation, stress and/or pathological conditions, some of which can be induced by xenobiotics.

*Follicular development*

The general sequence of endocrine and morphological changes occurring during the estrous cycle involves a variety of positive and negative feedback loops affecting the hypothalamic–pituitary–gonadal axis and leads to the development of antral follicles, the primary source of estrogens, and, eventually, the formation of corpora lutea, which produce progesterone (Figures 14.3 and 14.4). During the time of year when females are exhibiting reproductive cyclicity, there are cyclic alterations in the pattern of hypothalamic GnRH secretion from the tonic and surge centers, which interact with the anterior pituitary to influence the relative amounts of FSH and LH secreted by anterior pituitary gonadotropes. Over the course of the ovulatory season, many (up to several hundred or more) primordial follicles leave the reserve pool in a cyclic fashion (under the influence of FSH) and enter the active pool of follicles (primary follicles) undergoing growth and differentiation (folliculogenesis) and eventually atresia or ovulation (Senger, 2003; Evans *et al.*, 2007). The oocyte in the developing follicle grows in size, the zona pellucida is formed and the granulosa cells surrounding the oocyte undergo mitosis and further differentiation

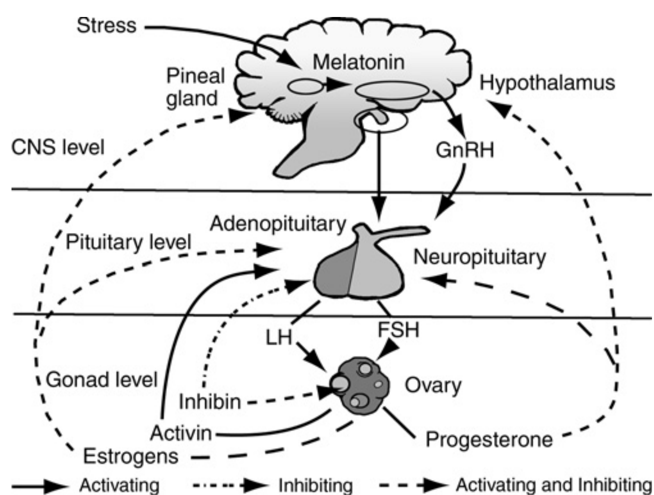


**FIGURE 14.4** The endocrine regulation of ovarian function and the feedback loops for the hypothalamic–pituitary–gonadal (ovarian) axis in the female are depicted. This figure was adapted, with permission, from Wilker and Ellington (2006) (modifications courtesy of Don Connor and Howard Wilson).

(Senger, 2003). A primary follicle is transformed into a secondary follicle when there are several layers of granulosa cells. Preantral follicles (primary and secondary follicles) become antral (tertiary) follicles, when fluid from the granulosa cells of secondary follicles coalesces to form an antrum (Evans *et al.*, 2007).

Cyclic increases in FSH concentrations facilitate recruitment antral follicles. Granulosa cells can produce activin which is thought to provide positive feedback to the anterior pituitary, further increasing gonadotropic FSH secretion (Figure 14.4) (Senger, 2003; Wilker and Ellington, 2006). Recruited antral follicles, which are gonadotropin sensitive, undergo several waves of follicular development beginning in metestrus and ending in proestrus (Ginther, 1992; Senger, 2003; Evans *et al.*, 2007). The final wave of one or more dominant follicles, destined for ovulation, rather than atresia, produces the large amounts of estrogens typical of estrus and required for sexual receptivity and the preovulatory estrous surges in GnRH and LH secretion (Senger, 2003).

*Ovarian follicular synthesis of estrogens*

The production of estrogens (predominantly estradiol) by antral follicles is accomplished by a mechanism termed the "two-cell or two-gonadotropin model", which can vary somewhat between species (Senger, 2003; Evans *et al.*, 2007). Cells from the theca interna and/or granulosa cells (depending on the species) produce progesterone from pregnenolone synthesized from cholesterol and, under the influence of relatively low concentrations of LH, theca interna cells convert this progesterone into androgens and, ultimately, testosterone (Evans *et al.*, 2007). In granulosa cells (reportedly theca interna cells in some species), the release of FSH from the anterior pituitary induces aromatase-mediated conversion of testosterone produced in the theca cells into estradiol (Senger, 2003; Evans *et al.*, 2007). Stimulation of aromatase activity by xenobiotics can have an overall estrogenic effect on exposed animals (increased production of estradiol).

*The effects of estrogenic feedback on the hypothalamic–pituitary–gonadal axis*

Increasing concentrations of estrogens associated with estrus alter the hypothalamic GnRH secretory pattern and decrease pituitary secretion of FSH, while greatly increasing the amount LH produced and released by the anterior pituitary gland (preovulatory LH surge) (Senger, 2003; Evans *et al.*, 2007). Although inhibin produced by granulosa cells further decreases FSH secretion, dominant follicles surviving to estrus do not undergo atresia because of an enhanced sensitivity to basal FSH levels (Senger, 2003; Wilker and Ellington, 2006). Xenoestrogens have the potential to either imitate or inhibit these estradiol feedback mechanisms in sexually mature females, depending on amount estrogenic xenobiotic, the endocrine milieu at the time of the exposure and the relative binding affinity of the xenobiotic for ERs.

*Ovulation*

The granulosa cells in the one or more dominant estrous follicles (Graafian follicles) cease to divide shortly prior to ovulation and undergo further differentiation, with increased numbers (upregulation) of LH receptors which will be responsive to the estrogen-induced preovulatory LH surge (Senger, 2003; Evans *et al.*, 2007). As LH increases, granulosa cells (theca interna cells in some species) continue to convert pregnenolone to progesterone, but estradiol production decreases, resulting in a slight preovulatory decline in estradiol (Evans *et al.*, 2007). The preovulatory LH surge is associated with increased follicular pressure, degeneration of theca cells and weakening of the follicular wall, completion of the first meiotic division within the oocyte (end of meiotic inhibition except in dogs and foxes) and, finally, ovulation of a secondary oocyte arrested in metaphase II (Senger, 2003; Evans *et al.*, 2007). In felids, ferrets, mink, camelids and rabbits, the preovulatory LH surge is induced by copulation (intromission or vaginal stimulation in most induced ovulators; seminal fluid in camelids). Toxicants which interfere with copulation or sexual contact in these species can interfere with the ovulatory process.

*Formation and function of a CL*

Following ovulation, a cascade of endocrine changes takes place in the female subprimate which facilitates the

transition from sexual receptivity to non-receptivity. Once an ovulation occurs, blood concentrations of follicular estradiol and inhibin return to their basal levels, and granulosa cells continue their growth, differentiation and increased production and release of progesterone (luteinization) under the influence of LH (Evans *et al.*, 2007). The functional ovarian structure which eventually develops from each ovulated follicle is a CL, which is comprised of large and small luteal cells derived form the granulosa and theca interna cells (granulosa cells in horses), respectively (Senger, 2003; Evans *et al.*, 2007). In most species, luteal cells are responsive to LH and produce progesterone until, shortly before the usual end diestrus in non-pregnant animals, the CL undergoes luteolysis mediated by oxytocin-stimulated production of prostaglandins $F_{2\alpha}$ ($PGF_{2\alpha}$) (Ginther, 1992; Senger, 2003; Evans *et al.*, 2007). While luteolysis is an intraovarian event in primates, oxytocin-stimulated endometrium produces the luteolysin ($PGF_{2\alpha}$) in subprimates (Senger, 2003). Xenobiotics, which can cause endometritis or mimic the actions of oxytocin or $PGF_{2\alpha}$ [e.g. endotoxin or lipopolysaccharide (LPS)] can be associated with premature luteolysis. Conversely, toxicants with the opposite oxytocin/$PGF_{2\alpha}$-related effects would be expected to disrupt normal reproductive cyclicity by prolonging the lifespan of the CL and causing a prolonged diestrus or pseudopregnancy (e.g. xenoestrogens in swine).

Species of animals can vary in the number of fertile ovulations and, therefore, CLs, which are characteristically associated with each estrous cycle. Monotocous mammalian species usually only ovulate a single secondary oocyte each estrous cycle. The ovaries of litter-bearing (polytocous) mammals generally develop multiple follicles which mature, ovulate and form functional CLs.

#### *Summary of the effects of estrogens and progesterone during the female reproductive cycle*

The endocrine changes which occur during the estrous cycle are reflected in behavior and the size, morphology, position and function of the tubular genitalia. Estrogens have a multiple effects on the female reproductive tract with include: the previously discussed interactions with the hypothalamus and anterior pituitary to alter the patterns GnRH and gonadotropin secretion, which govern follicular development and ovulation; facilitation of sexual receptivity; increased blood flow, genital swelling, leukocytosis, mucosal secretion and myometrial tone; altered tissue electrical conductivity and initiation of the growth of endometrial and mammary glands (Senger, 2003). Like estrogens, progesterone also has several effects on the reproductive tract of the female, but the effects of progesterone generally oppose those of estrogens, favoring pregnancy maintenance and sexual non-receptivity over ovulation and appropriately timed sexual receptivity associated with estrogenic stimulation (Ginther, 1992; Senger, 2003; Evans *et al.*, 2007). Progesterone is generally associated with negative feedback to the hypothalamus and anterior pituitary gland which limits GnRH and gonadotropin secretion (Senger, 2003; Evans *et al.*, 2007). Sexual receptivity and myometrial contractility and tone are diminished in an endocrine environment dominated by progesterone, while mammary and endometrial gland development and secretion are promoted (Senger, 2003). Toxicants which disrupt the communication and coordination between the ovary and the other parts of the reproductive tract (e.g. xenoestrogens, xenoandrogens and antiestrogens) will alter the appearance and function of the reproductive organs and can interfere with survival of the oocyte, embryo and/or fetus.

## Oocyte/sperm transport, normal capacitation of sperm and fertilization

### *Transport of the ovulated oocyte*

The primary reproductive organs involved in the transport of ovulated secondary oocytes (primary oocytes in the bitch) are the oviducts or uterine tubes. Each oviduct consists of an infundibulum, isthmus and ampulla, which have some distinct differences in structure, as well as function (Evans *et al.*, 2007). The ovulated ovum enters the funnel-like opening to the infundibulum and is transported through the isthmus to the ampulla or ampullary–isthmic junction for fertilization. Unlike spermatozoa which can generally survive for several days in the oviduct, secondary oocytes usually, depending on the species, are viable for 12–24 h (Genuth, 2004b). The appropriate endocrine environment is required for adequate oviductal entry and transport of ovulated oocytes to the site of fertilization. Delayed transport of oocytes within the uterine tubes can result in the death of ova before contact can be made with fertile spermatozoa.

### *Transport and capacitation of spermatozoa*

#### *Transport of spermatozoa*

During mammalian copulation, mature sperm stored in the caudae epididymidies travel through the vas deferens and penile urethra to be ejaculated into the anterior vagina, cervix or uterine body of the female reproductive tract, depending on the species. Spermatozoa can be lost from the female reproductive tract by retrograde loss and phagocytosis by leukocytes (Senger, 2003). Contractions of the smooth muscle within the tubular genitalia (muscularis), as well as interactions involving components of the seminal fluid and luminal secretions of the female reproductive tract, facilitate the transport of sperm to the oviducts (uterine tubes) where, depending on the species, fertilization takes place in the ampulla or at the junction

of the ampulla and the isthmus (ampullary–isthmic junction) (Senger, 2003; Genuth, 2004b). While sperm can be rapidly transported to the ampullary–isthmic junction or ampullae of the oviducts within minutes of natural or artificial semination, the relatively slow, sustained transport of motile sperm from reservoirs of spermatozoa in the cervix and uterotubal junctions is the primary mechanism by which the viable sperm that can participate in fertilization actually enter the oviducts (Senger, 2003). Xenobiotics which interfere with the endocrine milieu required for appropriate muscularis contractility and the cervical and uterine mucosal secretions which facilitate sperm transport (e.g. phytoestrogens) can prevent spermatozoa from getting to the site of fertilization in a timely manner.

#### *Capacitation of spermatozoa*

Spermatozoa can generally survive in the oviducts for several days following insemination. Ejaculated sperm are not competent to either bind to the zona pellucida or to undergo the acrosomal (acrosome) reaction, both of which are required for fertilization of ova by mature spermatozoa. Sperm must be capacitated in order interact with the ovum. The capacitation process involves calcium influx and biochemical changes to the sperm plasma membrane which result in the "removal" or modification of epididymal and seminal plasma proteins and the exposure of the surface molecules required for spermatozoal binding to the zona pellucida of the ovulated secondary oocyte (Senger, 2003; Genuth, 2004b). Depending on the species and, to some extent, the site of their deposition, spermatozoa become capacitated within the cervix, uterus and/or the oviduct (Senger, 2003).

### *Fertilization*

Fertilization of secondary oocytes by capacitated sperm is a complex process involving a cascade of events which prevents fertilization of an ovum by more than one sperm (polyspermy) and ends in the fusion of the male and female pronuclei (syngamy) (Senger, 2003). In the oviductal ampulla or at the ampullary–isthmic junction, the motility of capacitated sperm becomes hyperactive, facilitating the precise sequence of events which includes the following in their respective order: (1) sperm binding to the zona pellucida of the oocyte involving interactions between species-specific sperm and oocyte proteins; (2) the sperm acrosomal reaction, which results in the release of acrosomal enzymes and exposure of the equatorial segment of the sperm plasma membrane; (3) acrosomal enzyme-associated penetration of zona pellucida by a single spermatozoon; (4) fusion of the plasma membrane of the sperm at its equatorial segment with the plasma membrane of the oocyte; (5) membrane fusion-associated sperm engulfment and the oocyte cortical reaction, which prevents additional oocyte zona binding and membrane fusion (i.e. polyspermy prevention); female pronucleus formation and completion of meiosis; decondensation within the sperm nucleus and male pronucleus formation and, finally, the fusion of male and female pronuclei or syngamy which produces a zygote ready to undergo embryogenesis (Senger, 2003; Genuth, 2004b). From the complexity of the fertilization process, it be apparent that toxicants which result in subtle aberrations in sperm and oocyte formation and maturation can have profound effects on gamete function.

## The endocrinology of pregnancy and placentation

### *Gestational hormones*

Pregnancy begins with fertilization of the oocyte within the oviduct, followed by the first cleavage of the zygote, and terminates with parturition. Although the endocrine physiology and duration of mammalian pregnancy are very species specific and are characterized by a great deal of interspecies variation, the overall goals during the entire gestation for all pregnant mammals, their embryo(s) and, eventually, the maternal–fetal–placental unit are the same. A uterine environment conducive to embryonic and fetal development must be facilitated and the pregnancy (pregnancies in multitocous animals) must be maintained for the entire normal gestational length. The primary hormones involved in establishing the proper uterine environment and maintaining pregnancy are progesterone secreted by the maternal ovary and/or the placenta, as well as, in some species, a variety of placental progestins. In addition, a variety of other endogenous hormones of maternal, fetal and/or placental origin (depending on the species and gender of the offspring), including androgens, estrogens, prolactin, placental lactogen, equine and human chorionic gonadotropins (eCG and hCG, respectively) and relaxin, also have important gestational functions. Normal embryonic and fetal development require that gestational hormones, especially endogenous androgens and estrogens, be synthesized and secreted in sufficient quantities and at the appropriate time during pregnancy. The proper reproductive development of the female fetus is primarily dependent on exposure to estrogens at specific times during gestation. However, the male fetus must have appropriately timed exposure to normal amounts of both androgens and estrogens for normal development of the reproductive tract and optimal adult reproductive performance (Hess, 2003). Depending on the timing of exposure, EDCs, especially those which function as gonadal steroid receptor agonists and antagonists, can potentially interfere with normal gestational signaling and sexual differentiation.

### *Maternal recognition of pregnancy*

The embryo generally enters the uterus several days after fertilization. One of the first endocrine events which must occur in most mammalian species, other than those for which the timing of luteolysis and duration of pregnancy are very similar to one another (i.e. dogs and cats), is the prevention of luteolysis (i.e. entry into the next estrus or period of sexual receptivity) and the maintenance of luteal phase progesterone concentrations (Senger, 2003; Evans *et al.*, 2007). The mechanism for this embryo–endometrium interaction in subprimates (intraovarian event in primates), also referred to as "maternal recognition of pregnancy", has been elucidated in several species and involves embryonic production of species-specific interferon-$\tau$ in ovine and bovine species (o-IFN-$\tau$ and b-IFN-$\tau$, respectively), estradiol secretion by porcine embryos, intrauterine embryonic migration in equids and placental chorionic gonadotropin (hCG) in humans (Senger, 2003; Genuth, 2004b). The timing of "maternal recognition of pregnancy" is species specific and in subprimates generally corresponds to the time period spanning the normal oxytocin-mediated synthesis and release of $PGF_{2\alpha}$ by the endometrium, as well as transport of the luteolysin to the ovary. Xenobiotics which interfere with embryonic and, in the case of humans, placental development or those toxicants which mimic the actions of the luteolysin (e.g. endotoxin) can terminate early pregnancies. Some species of mammals, such as dogs, cats, camelids, goats, swine and rabbits, depend solely on luteal progesterone secretion for the maintenance of pregnancy (Senger, 2003). The placenta takes over progesterone-associated pregnancy maintenance in sheep at approximately 50 days post-conception and between the 6th and 8th month of gestation in cattle (Senger, 2003). The uterofetoplacental unit of the mare begins to produce a unique assortment of progestins classified as 5$\alpha$-pregnanes, beginning at about day 70 of pregnancy (Ginther, 1992; Evans *et al.*, 2007). A number of toxicants (e.g. ergopeptine alkaloids) have been found to interfere with normal progestin metabolism in the mare (Evans, 2002).

### *Placentation*

Most mammalian species are "eutherian" and, during pregnancy, form a placenta comprised of both fetal and maternal components, which acts as an attachment between the fetal and the maternal systems, functions as a transient endocrine organ and plays essential roles in the exchange of gases, nutrients and metabolic wastes between the maternal and the fetal circulations (Ginther, 1992; Senger, 2003). The yolk sac, chorion, amnion and allantois are the extraembryonic membranes formed by the pre-attachment mammalian embryo (Senger, 2003). While the yolk sac in most mammalian species normally undergoes regression, the allantois and chorion generally fuse to form the allantochorion, and the fluid-filled amnion provides a shock absorbing, aquatic environment to facilitate fetal development and transport (Ginther, 1992; Senger, 2003). The allantochorionic membrane is the fetal contribution to the placenta and the chorionic villi are the structures which interdigitate with the maternal endometrium (Senger, 2003).

#### *Types of placentas*

Mammalian placentation can be classified according to the degree of intimacy between the maternal and the fetal circulations (i.e. the number of tissue layers separating maternal and fetal blood) and by the pattern of distribution of the chorionic villi on the surface of the placenta facing the maternal endometrium (Senger, 2003). Epitheliochorial placentas (placentae) have a total of six layers separating the maternal and fetal circulations and are observed in variety of species, including equids and swine. Ruminant placentation is described as syndesmochorial because of the transient erosion and regrowth of the maternal epithelium, which results in the intermittent exposure of maternal endothelium (capillaries) to chorionic epithelium (Senger, 2003). Canine and feline placentas are classified as endotheliochorial, and the hemochorial placentation observed in rodents and primates has essentially only chorionic epithelium separating the maternal blood from that of the fetus. The placenta of each species is associated with a typical distribution of the chorionic villi, classified as being either diffuse (e.g. equids and swine), cotyledonary (e.g. ruminants), zonary (e.g. dogs and cats) or discoid (e.g. rodents and primates). Some species, such as the rabbit, have variations in their placentation over the course of gestation, and what begins as an epitheliochorial placenta has transformed into an endotheliochorial type of placentation by the end of pregnancy (Rozman and Klaassen, 2001).

#### *Placental function*

In multitocous species, each fetus has its own placenta, with the previously described endocrine functions, which "attaches" the fetus to the endometrium and facilitates the exchange of gases, nutrients, metabolic wastes and xenobiotics between the fetal and the maternal circulations. Although the term "implantation" is frequently used to describe the appropriately timed attachment of the placental membranes to the endometrium, only the conceptuses of rodent and primate species undergo true implantation (Senger, 2003). Placental exchange involves the processes of simple (passive) diffusion, facilitated diffusion and active transport, as well as pinocytosis and phagocytosis of some nutrients (Senger, 2003).

The passage of materials across the placenta has been traditionally thought of as primarily a function of the intimacy (i.e. number of tissues layers) between the maternal and the fetal circulations, especially with respect to maternal immunoglobulins which cross hemo- and endotheliochorial

placentas but not those types placentae having more layers. However, since most xenobiotics cross the placenta by simple diffusion, it is currently thought that molecular size and solubility are the most important determinants of the ability of potential teratogens in the maternal circulation to cross the placenta into the fetal circulation (Rozman and Klaassen, 2001; Senger, 2003). Some toxic xenobiotics can be actively transported by mechanisms intended for structurally similar endogenous molecules (Rozman and Klaassen, 2001), and it is thought that transplacental transport of lead can mimic that of calcium (Evans *et al.*, 2003).

*The "placental barrier"*

Because the placenta "blocks" the ability of very large molecules to cross from the maternal circulation into the fetal circulation, the term "placental barrier" has been used to describe this protective function of the placenta. Given that a large number of potential toxicants diffuse across the placenta and reach the fetus, the term "barrier" might be somewhat of a misnomer. However, multidrug resistance protein and enzymes involved in biotransformation of xenobiotics have been found in the placenta (Rozman and Klaassen, 2001), and regardless of its relative inefficacy as a "barrier" between the maternal and fetal circulations, with respect to at least some xenobiotics, the nutritional and endocrine functions of the placenta are essential for successful completion of the mammalian pregnancies. Placental toxicity will be discussed in much greater detail in Chapter 15.

## Normal embryonic and fetal development

### *Blastocyst formation and differentiation of the germ cell layers*

In order for a zygote to develop into a viable offspring, multiple steps involving cellular division, migration, differentiation and organization must take place. Embryonic and fetal survival requires that these various steps take place in a precise order and at set times during the gestation of each species. Within 24 h following fertilization, the zygote located in the oviduct begins to divide, within the confines of the zona pellucida, into multiple blastomeres, which ultimately form a ball of cells referred to as the morula (Ginther, 1992; Senger, 2003). A fluid-filled cavity (blastocoele) develops, and the newly formed blastocyst, which is divided into cells forming either the inner cell mass (future embryo proper) or the trophoblast (future chorion), enters the uterus (Senger, 2003). The blastocyst undergoes rapid growth and "hatches" from the zona pellucida. The subsequent cellular division and differentiation results in the formation of the three germ layers (i.e. endoderm, mesoderm and ectoderm), which are destined to develop into the embryonic tissues forming the various organs and body systems, as well as the extraembryonic membranes involved in placental formation and attachment (Ginther, 1992; Senger, 2003). Germ layer differentiation leads to organogenesis and the transformation of an embryo into the fetus which continues to grow and develop for the remainder of pregnancy. With respect to reproductive toxicity in non-rodent mammals, the organogenic and other developmental processes occurring during the first trimester of pregnancy are especially susceptible to the teratogenic effects of xenobiotics. The abnormalities induced by a teratogen are dependent on the specific developmental processes or signaling pathways targeted by that toxicant and the timing of the exposure.

### *Sex determination and sexual differentiation of reproductive function*

*Genotypic sex and development of the primitive sex cords*

The genotypic sex of a mammalian conceptus is determined at fertilization by the sex chromosome (X or Y) contributed by the sperm, which, in combination with the X chromosome in the ovum, denotes either a genotypically female (XX) or a male (XY) zygote. During early gestation in most species, the primordial germ cells arise from the epithelium of the embryonic yolk sac and migrate through the developing mesentery to the gonadal (genital) ridge (testicular or ovarian anlage) in its position contiguous with the mesonephros (Senger, 2003; Evans *et al.*, 2007). Germ cells and stimulated somatic cells proliferate and organize into primitive sex cords within undifferentiated (bipotential) gonads, which have the potential to develop into either ovaries or testes (Senger, 2003; Basrur, 2006).

*Gonadal sex determination and phenotypic sexual differentiation*

Development of a phenotypically male or female mammalian fetus occurs during the first trimester of pregnancy in most species and consists of the determination of gonadal sex followed by the further development and differentiation of either the mesonephric or the paramesonephric ducts and regression of the other duct system. The selection of the mesonephric or paramesonephric ducts for retention and further differentiation results in the formation of genitalia (phenotypic sex) appropriate for either the male or female gonads, respectively (Genuth, 2004b). Gonadal sex determination and phenotypic sexual differentiation are dependent on complex and carefully timed signaling events and are extremely susceptible to disruption by xenobiotics. Toxicants which alter epigenetic programming or mimic or inhibit endogenous hormones can have potentially deleterious effects on sexual development (Basrur, 2006). Xenobiotic-induced abnormalities in phenotypic sexual differentiation can arise from defects in testicular formation, defects in androgen production and defects in androgenic action (Basrur, 2006; Hughes *et al.*, 2006). While some toxicant-induced abnormalities in sexual

differentiation can be very obvious [e.g. hermaphroditism (presence of ovotestes), pseudohermaphroditism (differences in gonadal and phenotypic sex), hypospadias (feminized external genitalia; failure of urethral fold fusion) and cryptorchidism (failure of testicular descent)], other, more subtle effects can be related to functional rather than structural abnormalities. In order to identify the steps in gonadal sex determination and phenotypic sexual differentiation most likely to be targeted by the effects of EDCs and other reproductive toxicants, it is important to understand how these processes are initiated within the fetus and how they impact subsequent fetal development.

For the last several decades, the model for gonadal sex determination and phenotypic sexual differentiation has been based on the premise that a "testis determining factor" (TDF) on the Y chromosome dictates that a gonad differentiates into a testis and initiates the cascade of endocrine changes (Figure 14.5) which results in a phenotypically

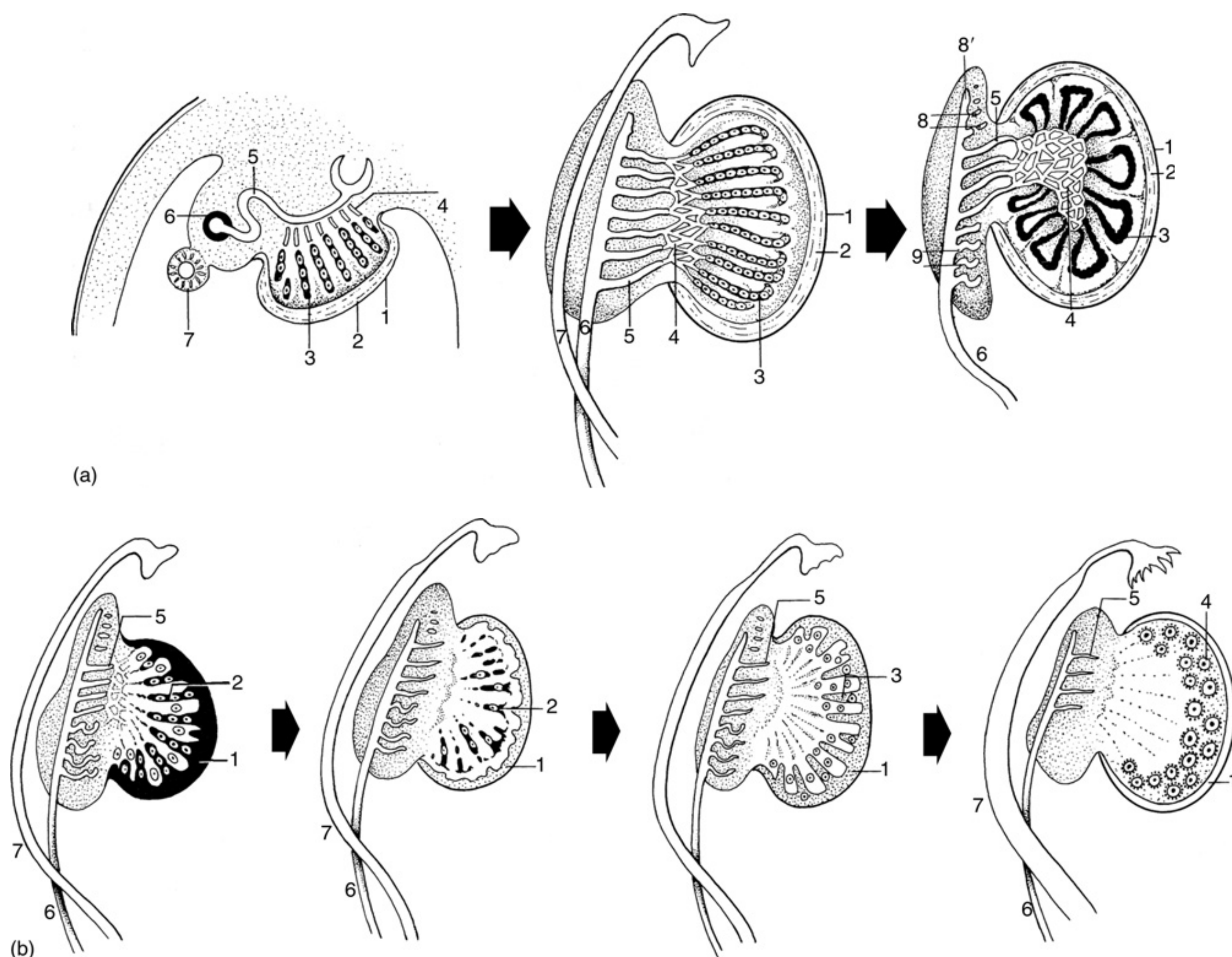


**FIGURE 14.5** The initial stages in the development of the testis and the formation of the excurrent duct system are shown in (a). The initial formation of the tunica albuginea isolates the epithelial cords from the surface epithelium, and the epithelial cords, rete testis and mesonephric tubules (also referred to as the mesonephric ductules or mesonephric duct system) subsequently interconnect. The epithelial cords (sex cords) will eventually become the seminiferous tubules, and the mesonephric ductules will be incorporated into the formation of the excurrent duct system. (1) Celomic epithelium; (2) tunica albuginea; (3) epithelial cords (future seminiferous tubules); (4) rete testis; (5) mesonephric tubules (later efferent ductules); (6) mesonephric duct (future epididymis (proximal portion contiguous with mesonephric tubules and ductus deferens (distal portion)); (7) paramesonephric duct; (8) cranial remnant of mesonephric duct system (aberrant ductules); (8′) remnant of mesonephric duct (appendix of epididymis) and (9) caudal remnant of mesonephric duct (paradidymis). The initial stages in the development of the ovary and the formation of paramesonephric ducts are shown in (b). The epithelial cords (sex cords) penetrate and then regress within the developing ovary, eventually fragmenting and organizing into cell clusters which consist of a single oocyte surrounded by a layer of granulosa cells (primordial follicles). The paramesonephric ducts undergo further development and differentiation, and the mesonephric duct system begins to regress: (1) celomic epithelium; (2) epithelial cords which initially penetrate then regress and fragment; (3) early formation of future cortical region; (4) primordial follicles; (5) regressing mesonephric tubules; (6) mesonephric duct which will eventually regress and (7) paramesonephric duct which will undergo further development and differentiation into the major female tubular genitalia. This figure was adapted, with permission, from Dyce *et al.* (2002) (modifications courtesy of Don Connor and Howard Wilson).

male fetus (developed mesonephric duct system; regressed paramesonephric ducts) (Senger, 2003; Genuth, 2004b; Basrur, 2006). Without the determination that the gonads will develop into testes, the "default" or "constitutive" pathway is followed and ovarian gonads are formed in association with a developed paramesonephric duct system and regressed mesonephric ducts (Senger, 2003; Genuth, 2004b; Basrur, 2006). While this model is useful to explain rather complex developmental processes, it should be kept in mind that other toxicant-susceptible mechanisms might also play a role in gonadal sex determination and sexual differentiation. It is apparent that very precise, sex-specific patterns of germ line epigenetic programming and interactions with somatic cells take place during the early stages of sexual differentiation (Anway and Skinner, 2006). Recent data has suggested that these signaling pathways are susceptible to epigenetic modifications induced by some antiandrogens (Anway *et al.*, 2005; Anway and Skinner, 2006). It has also been suggested that gonadal sex determination involves other genes on both sex and autosomal chromosomes that might be targeted by reproductive toxicants (Genuth, 2004b; Basrur, 2006).

*Development of the male phenotype*

Once previously undifferentiated gonads commit to testes development (TDF present), a coordinated series of endocrine-induced morphological changes take place, resulting in both a genotypically and a phenotypically male fetus (Figure 14.5a). The sequence of signaling and developmental changes, which result in male sexual differentiation, include the following: (1) Sertoli cell development and secretion of anti-Müllerian hormone (AMH) or Müllerian inhibiting substance (MIS); (2) AMH-induced regression of the paramesonephric (Müllerian) ducts and differentiation of Leydig cells capable of producing testosterone; (3) testosterone-facilitated development of the mesonephric or Wolffian ducts; (4) differentiation of the mesonephric ducts into the rete testes, efferent ductules, epididymidies and ducti deferens; (5) development of primordial accessory sex glands and the formation of external genitalia from primordia and, finally, in most species (some exceptions in wildlife species); (6) testicular descent of the intra-abdominal testes into their extra-abdominal position in the scrotum, prior to or very shortly after birth (some species) (Senger, 2003; Genuth, 2004b; Basrur, 2006; Edwards *et al.*, 2006).

*Development of the female phenotype*

If the previously undifferentiated gonads do not commit to testes development (TDF absent), ovaries are formed and a cascade of morphological changes occurs in the absence of AMH and testosterone stimulation, resulting in a genotypically and phenotypically female fetus (Figure 14.5b). This sequence of "default" or "constitutive" morphological and endocrine alterations results in the following sequence of developmental events: (1) regression of mesonephric (Wolffian ducts); (2) differentiation of the paramesonephric (Müllerian) ducts into the oviducts, uterine horns, uterine body, cervix and anterior vagina; (3) remodeling of the ovary into its typical parenchymal and cortical structure; (4) cortical development of primordial follicles, with primary oocytes arrested in meiosis and surrounded by future granulosa and theca interna cells and (5) development of the caudal vagina and vulva from the urogenital sinus (external genitalia primordia) (Senger, 2003; Genuth, 2004b; Basrur, 2006; Edwards *et al.*, 2006; Evans *et al.*, 2007).

*Sexual differentiation of the brain*

Sex-specific endocrine patterns and the resulting gender appropriate sexual behaviors in animals are necessary for fertile copulations to occur and require that the brain also undergo prenatal (postnatal in some species) sexual differentiation. Although large amounts of estradiol defeminize the brain; alpha-fetoprotein prevents most of the endogenous estrogens in the female fetus from crossing the blood–brain barrier (Senger, 2003). The brain remains inherently female under the influence of minimal amounts of estradiol, and both the GnRH tonic and surge centers are maintained within the hypothalamus of the female fetus in this low-estradiol environment (Ford and D'Occhio, 1989; Senger, 2003). Testosterone produced by the fetal testes crosses the blood–brain barrier and is converted to estradiol within the brain, and, as a result of this estradiol synthesis, the hypothalamic GnRH surge center in the male fetus is minimized (Senger, 2003).

While the differentiation of male sexual behavior in large domestic animals generally involves prenatal defeminization, especially in species having longer gestations, it should be noted that postnatal defeminization of the brain is occurs in male swine and rodents (Ford and D'Occhio, 1989). There is also evidence to suggest that the males of some species with prenatal defeminization of the brain might also require postnatal exposure to androgens for maximum masculinization of the brain (Senger, 2003). Depending on the timing of exposure, xenoestrogens and exogenously administered testosterone and, possibly, some xenoandrogens, which cross the placenta and the blood–brain barrier have the potential to have profound effects on sexual differentiation of the brain and future reproductive function.

## Parturition and lactation

### Physiology of parturition

Parturition constitutes transport of the fetus and its associated membranes from the maternal to the external

environment, and represents transition of the fetus to a neonate. Maturation of the fetal hypothalamic–pituitary–adrenal axis plays an important role in the cascade of neural and endocrine events which lead to parturition in most mammals (Senger, 2003; Evans, 2007). As most clearly demonstrated in ruminants, fetal CRF stimulates the release of ACTH from the fetal pituitary, and ACTH, in turn, stimulates fetal secretion of cortisol by the adrenal glands (Senger, 2003). Elevations in fetal cortisol (fetal LH may be involved as well) activate placental steroidogenic enzyme systems, resulting in decreased progestins and elevated estrogens prior to parturition (Ginther, 1992; Evans, 2007). The resulting increase in the estrogen:progestin ratio facilitates several important processes (e.g. cervical softening, upregulation of myometrial oxytocin receptors, uterine synthesis of $PGF_2\alpha$ and increased blood flow to the gravid uterus and placenta) which prepare the uterus for parturition (Evans *et al.*, 2007). Teratogen-induced congenital defects in the fetal pituitary gland can result in prolonged gestation (e.g. *Veratrum californicum*), and any xenobiotic exposure causing maternal and/or fetal stress can be associated with abortion or premature parturition (e.g. nitrates and pine needle abortion).

Normal parturition approaches as neural signals caused by fetal movements and myometrial contractions, along with elevated basal levels of oxytocin and increased secretion of $PGF_2\alpha$, bring about the first stage of labor. A rapid increase in oxytocin and $PGF_2\alpha$ secretion leads to rupture of the allantochorionic membrane and the commencement of the second stage of labor. Strong myometrial contractions result in the delivery of offspring, as well as the expulsion of the fetal membranes during the third stage of labor (Senger, 2003; Evans *et al.*, 2007).

#### *Physiology of lactation*

##### *Lactogenesis*

Appropriately timed lactogenesis is critical for survival of mammalian offspring. Lactogenesis is a two-stage process involving: (1) the enzymatic and cytological differentiation of the alveolar cells within the mammary gland and (2) the copious secretion of milk, which is distinct from the colostral sequestration of antibodies (Tucker, 1994). Growth hormone, aldosterone, prostaglandins, insulin, estrogens, progestins and prolactin are required for the first stage of lactogenesis, which generally occurs during the last trimester of pregnancy (McCue, 1993; Tucker, 1994). Large increases in pulsatile prolactin secretion by lactotropes in the anterior pituitary are necessary for the initiation of the second stage of lactogenesis, which generally occurs in close temporal association with the endocrine milieu of parturition (Evans, 1996). In many species, circulating concentrations of prolactin are elevated above basal levels for a month or two after parturition (McCue, 1993). In some species of animals, a placental lactogen performs many of the same endocrine functions as prolactin.

##### *Control of prolactin secretion*

Lactotropic prolactin secretion is tonically inhibited by dopamine secreted by hypothalamic neurons belonging to either the tuberoinfundibular or tuberohypophysial dopaminergic systems (TIDA and THDA, respectively) (Neill and Nagy, 1994; Evans *et al.*, 2007). Vasoactive intestinal peptide (VIP) and TRH are thought to act as prolactin releasing factors and can interfere with the dopamine-associated tonic inhibition of prolactin release (Evans, 1996). Oxytocin, in conjunction with the suckling reflex, will increase pituitary lactotropic production and secretion of prolactin (Neill and Nagy, 1994). In species strictly dependent on prolactin for lactogenesis, toxicants which mimic dopamine and tonically inhibit prolactin secretion (e.g. ergopeptine alkaloids) pose a risk to fetal survival.

## THE MECHANISMS AND EFFECTS OF REPRODUCTIVE TOXICANTS

### The effects of EDCs on wildlife species, humans and domestic animals

It should be evident from the previous discussion that maximum reproductive efficiency, including normal embryonic and fetal development, is dependent on the structural and functional integrity of multiple organs and tissues, as well as various signaling pathways (Figure 14.6). There is increasing concern within the scientific and regulatory communities regarding the effects of prenatal exposures to EDCs on wildlife species and humans. Domestic animals have been shown to be susceptible to the effects of several naturally occurring reproductive toxicants in clinical settings, and it is possible that low-level environmental exposures to EDCs can also hinder reproductive function.

#### *Endocrine disruption in wildlife species*

There have been many, well-documented instances of reproductive abnormalities in species of wildlife living in environments contaminated by industrial and/or agricultural chemicals (McLachlan, 2001; Hess and Iguchi, 2002; Jobling and Tyler, 2006; McLachlan *et al.*, 2006). The deleterious reproductive effects of DDT on birds reported in Rachel Carson's *Silent Spring* have been shown to be result of eggshell thinning related to abnormalities in prostaglandins synthesis induced by the *p,p*-DDE metabolite of DDT (Lundholm, 1997; Guillette, 2006). Wildlife populations are very likely sentinels for endocrine disruption because of the contamination of the aquatic habitats in which many of them live and the likelihood that predatory

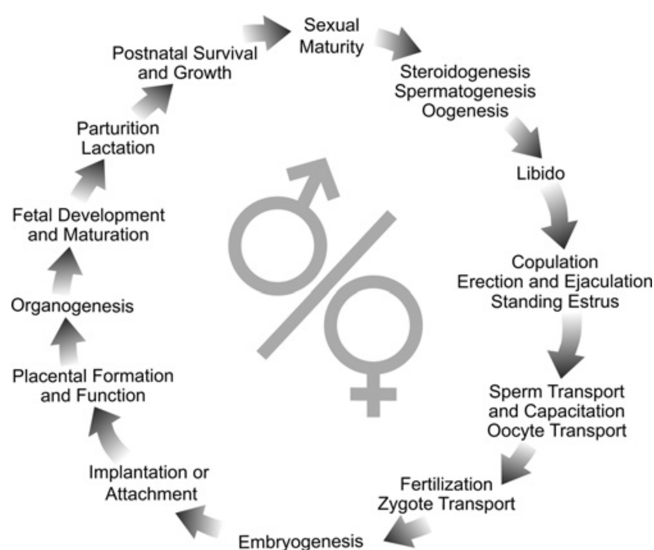


**FIGURE 14.6** The multiple steps involved in reproductive development and function in both males and females are shown schematically to illustrate the complexity of reproduction in mammalian species and to demonstrate the various stages in the reproductive process which can be targeted for toxic insult. With respect to embryonic, fetal and placental development, it should be understood that these events are species-dependent and interrelated, with many of them taking place concurrently. This figure was adapted, with permission, from Ellington and Wilker (2006) (modifications and artwork courtesy of Don Connor and Howard Wilson).

animals will have relatively high exposures to chemicals which bioaccumulate within the environment (Hess and Iguchi, 2002). Lessons learned from instances of endocrine disruption in wildlife species can be applied to EDC exposures involving humans and domestic animals.

### *"Androgenic" and "estrogenic" effects of EDCs on wildlife species*

Prenatal and postnatal exposures to androgenic and estrogenic environmental contaminants, as well as chemicals classified as having the opposite phenotypic effects, have been associated with various reproductive abnormalities in wildlife. Effluents from pulp and paper mills, as well as runoff from cattle feedlots where the synthetic androgen, trenbolone, was used for growth promotion, have been shown to be androgenic and capable of masculinizing female fish (Orlando *et al.*, 2004; Gray *et al.*, 2006). "Androgynization" or a state of indeterminate sexual development encompassing both feminization and demasculinization in males has been observed in populations of fish, amphibians, reptiles, birds and mammals and is thought to be similar to the testicular dysgenesis syndrome described in humans (Edwards *et al.*, 2006). Adult and immature amphibians exposed to the herbicide, atrazine, which has been associated with increased aromatase activity in a number of species, have been reported to exhibit various manifestations of feminization (Hayes *et al.*, 2006). Hatchling, juvenile and adult male alligators (*Alligator mississippiensis*), originating from a Florida lake previously contaminated with DDT and other persistent, bioaccumulated pesticides, as well as ethylene dibromide and DBCP, have demonstrated varying patterns of androgynization, including phallic malformations, which are thought to result from ovo exposure of maternal origin, as well as post-embryonic modifications and/or continuing environmental exposures to EDCs (Milnes *et al.*, 2006).

### *Endocrine disruption in humans*

Based, in part, on the observations of endocrine disruption in wildlife and ongoing concerns about reproductive dysgenesis, as well as the effects of embryonic and/or fetal exposure to diethylstilbestrol (DES), the emphasis with respect to endocrine disruption in humans and one of the bases for the "Theory of Hormone Disrupting Chemicals" (THDC) or the "Environmental Endocrine Hypothesis" (Krimsky, 2000, 2001) has been the enhanced effects of prenatal, as compared to postnatal, exposures to suspected endocrine disruptors. The embryo and fetus, without a developed blood–brain barrier and with only rudimentary DNA repair mechanisms and hepatic detoxifying and metabolizing capabilities, are especially susceptible, as compared to adults, to the adverse effects of low-level exposures to xenobiotics (Newbold *et al.*, 2006). In addition, previous discussions in this chapter have described the important organizational events taking place during gonadal and phenotypic sexual differentiation, which are potentially very sensitive to alterations in the normal endocrine milieu.

Although still extremely controversial, there is a growing body of evidence to support the observation that sperm counts in men within some industrialized regions of the world have been decreasing over the last several decades (Swan *et al.*, 2000; Skakkebæk *et al.*, 2006; Jørgensen *et al.*, 2006a). In conjunction with these alterations in sperm numbers within ejaculates, there appears to have been a concurrent increase in developmental abnormalities within the male reproductive tract consistent with TDS (Skekkebæk *et al.*, 2001). Similar to what has been observed in xenobiotic-exposed wildlife, reproductive dysgenesis in human males (i.e. TDS) is associated with a suite of clinical abnormalities which include reduced semen quality, cryptorchidism, hypospadias, decreased anogenital distance and testicular cancer (Skakkebæk *et al.*, 2001; Edwards *et al.*, 2006). Failure of Sertoli cell proliferation and functional maturation within the seminiferous tubules has been one mechanism proposed for the pathogenesis of TDS (Sharpe *et al.*, 2003). The findings of a recently completely epidemiological study have suggested a relationship between decreased anogenital distance and prenatal phthalate exposure in male infants (Swan *et al.*, 2005), and a possible rodent model for human TDS has been developed using prenatal exposure to dibutyl phthalate [di (*n*-butyl) phthalate] (Fisher *et al.*, 2003; Mahood *et al.*, 2005, 2006).

In addition to phthalates, which are used as plasticizers, a number of other widely used agricultural and industrial chemicals have been associated with adverse reproductive effects in humans and/or rodent models. In epidemiological studies a correlation has been shown between reduced semen quality in men within certain regions of the United States and the metabolites of several economically important herbicides (Swan *et al.*, 2003a, b). Metabolites of the commercially available fungicide, vinclozolin, have been demonstrated to interfere with interactions between androgens and their nuclear receptor, resulting in antiandrogenic effects on exposed rodents (Wong *et al.*, 1995; Monosson *et al.*, 1999; O'Connor *et al.*, 2002; Kubota *et al.*, 2003; Gray *et al.*, 2006). At concentrations well below those routinely found in humans, bisphenol A, which is widely used in the plastics industry and other manufacturing processes, can initiate nongenomic estrogenic responses with plasma membrane receptors and interact with the nuclear estrogen receptor as an SERM (Welshons *et al.*, 2006). With the increased societal awareness of the possible effects of hormonally active xenobiotics on human reproduction, further research is required to make educated decisions, based on "good science", with respect to the continued use and/or regulation of economically important chemicals associated with the potential for reproductive abnormalities in humans and/or documented impairment of reproductive function in laboratory animals.

### *The effects of reproductive toxicants on domestic animals*

#### *Abortion, teratogenesis and impaired fertility in domestic animals*

Animal-based agriculture is dependent on the efficient production of viable and reproductively functional offspring. Toxicant-induced abortions, congenital defects and male or female infertility can have devastating effects on livestock production. Cattle are commonly at increased risk, especially under drought conditions, for adult mortality and abortions in pregnant cows related to the consumption of nitrate-accumulating forages [e.g. *Sorghum* spp., oat hay (*Avena sativa*), cornstalks (*Zea mays*), and many others], which cause nitrite-induced fetal methemoglobinemia, hypoxia and, consequently, fetal stress and, potentially, even death (Casteel and Evans, 2004). Multiple congenital contractures (MCC) ("crooked calf disease") associated with the ingestion of lupines (*Lupinus* spp.) have resulted in the loss of large numbers of calves in the western United States (Panter, 2002), and multiple species of livestock exposed to swainsonine-containing plants (e.g. species of *Astragalus* and *Oxytropis* in North America species of *Swainsona* in Australia) have experienced congenital defects, abortions and/or ovarian and testicular abnormalities (Cheeke, 1998). Cleft palate, cyclops lambs, prolonged gestation and various tracheal and limb deformities have resulted from different periods of exposure of pregnant ewes to *Veratrum californicum* (false hellebore) (Burrows and Tyrl, 2001). Ergot alkaloids produced by the tall fescue endophyte, *Neotyphodium coenophialum*, are responsible for suboptimal reproductive performance in large numbers of cattle and horses, with late-gestational mares being particularly susceptible to endophyte-related prolonged gestation and agalactia (Evans *et al.*, 2004).

#### *Endocrine disruption in domestic animals*

With respect to the adverse reproductive effects of endocrine disruption on domestic animals, there have been many instances of impaired reproductive function involving naturally occurring EDCs of plant and fungal origin. However, there is still much to be learned about the potential adverse effects of pre- as well as postnatal environmental exposures to EDCs in these species where selection of breeding animals is often based on reproductive soundness. Postnatal exposures to phytoestrogens in some leguminous plants, including soybeans, have resulted in reproductive abnormalities and subfertility in multiple species (Cheeke, 1998; Ford *et al.*, 2006). The adverse effects of postnatal exposures to the estrogenic mycotoxin, zearalenone, on swine fertility have also been well documented (Cheeke, 1998). In experimental studies, female swine appear to be more sensitive than rodents to the effects of atrazine on the hypothalamic–pituitary–gonadal axis (Gojmerac *et al.*, 2004), and it is possible that companion and agricultural animals are also more susceptible than laboratory species to other EDCs. As many hormonally active xenobiotics also have important agricultural and industrial uses, it is important to continue research which attempts to accurately predict the effects of environmental exposures to EDCs, as well as other reproductive toxicants, on domestic animals.

## Toxicants affecting the male reproductive function

There have been relatively few documented reports regarding the adverse effects of reproductive toxicants on male fertility in the major animal species of veterinary interest. Realistically the lack of examples is more likely a reflection of the limited number of controlled studies performed using non-rodent mammalian species and/or the number of toxicant-associated reproductive abnormalities which remain undiagnosed, rather than an accurate indication of the scope of the problem (Schrader, 2002). A large number of chemicals are currently thought to have the potential for causing abnormalities in male reproductive function in domestic animals. Based on extrapolations from effects observed in a various mammalian species and the limited scientific and anecdotal reports, a partial listing of these

**TABLE 14.1 Xenobiotics/environmental and physiological factors that affect male fertility**

| Xenobiotic | Observed effect(s) on male |
|---|---|
| *Antimicrobials* | |
| Metronidazole | High doses: ↓ sperm number; ↑ abnormal morphology |
| Nitrofurantoin | High doses: ↓ sperm number |
| Tetracycline | Very high doses: ↓ sperm number; ↓ sperm capacitation; testis atrophy |
| Trimethoprim | 1-month course: ↓ sperm number by 7–88% |
| *Antifungals* | |
| Ketaconazole | Decreased testosterone and libido; ↓ sperm number and motility |
| Miconazole | Interferes with testosterone in male fetus |
| *Immunosuppressants* | |
| Cyclophosphamide | Decreased sperm number; birth defects in offspring |
| *Hormones* | |
| Testosterone | Decreased sperm number; testicular degeneration |
| Anabolic steroids | Decreased sperm number, motility and morphology |
| Trenbolone | Increased sperm abnormalities |
| Estrogens | Decreased sperm number; behavioral feminization |
| Phytoestrogens | Bioaccumulation in cats on soy diets can cause poor fertility |
| Zearanol | Decreased spermatogenesis |
| *Antivirals* | |
| Acyclovir | Dose- and age-dependent testicular degeneration |
| Ganciclovir | Decreased sperm number and quality |
| *Carbonic anhydrase inhibitors* | |
| Acetazolamide | Decreased libido and impotence |
| *Psychoactive drugs* | |
| Buspirone | Decreased libido and impotence |
| Benzodiazepine tranquilizers | Impotence and possible ejaculatory dysfunction |
| Phenothiazine tranquilizers | Priapism and impotence |
| Tricyclic antidepressants | Decreased libido and erectile dysfunction |
| *Antihistamines* | |
| Chlorpheniramine | *In vitro* experiments: ↓ sperm motility |
| *Antineoplastics* | |
| Adrimycin | Dose- and age-dependent testicular toxicity |
| Cisplatin | Decreased sperm number; ↓growth in offspring; pregnancy loss |
| Vincristine | Decreased sperm number; possible reversibility |
| *Antimetabolite* | |
| Cytarabine | Decreased sperm number |
| *Gastrointestinal tract drugs* | |
| Cimetidine | Decreased sperm number |
| Metaclopramide | Impotence |
| *Non-steroidal antiinflammatories* | |
| Naproxen | Decreased seminal prostaglandins; ↓ sperm motility |
| Phenylbutazone | Inhibition of sperm acrosome reaction; unknown effect on fertility |
| Sulfasalazine | Decreased sperm number and motility |
| *Glucocorticoids* | |
| Prednisone | Decreased sperm number and motility; ↓ testosterone |
| *Herbicides* | |
| 2,4-D (dichlorophenoxyacetic acid) | Abnormal sperm quality; testicular degeneration; ↓ fertility |
| Diquat and paraquat | Altered sexual differentiation in the male |
| *Solvents* | |
| Nitrobenzene | Decreased sperm number and motility; testicular degeneration |
| Napptha | Decreased fertility |
| *Phthalic acid esters* | |
| Diethylhexaphthalate (DEHP) | Testicular atrophy |
| *Gasoline additives* | |
| Ethylene dibromide | Testicular degeneration and poor sperm quality |

(*Continued*)

**TABLE 14.1 (Continued)**

| Xenobiotic | Observed effect(s) on male |
|---|---|
| *Insecticides* | |
| Carbamates | Decreased sperm quality |
| Chlorinated hydrocarbons | Testicular degeneration and atrophy |
| Methoxychlor | Testicular degeneration associated with estrogenic activity |
| Kepone | Decreased sperm number |
| Lindane | High doses: testicular toxicant<br>In utero exposure: ↓ sperm number and ↓ testosterone in offspring |
| Organophosphates | No information |
| Pyrethrins | *In vitro*: 40–60% ↓ in testosterone binding to androgen receptor |
| *Fungicides/nematocides* | |
| DBCP (dibromochloropropane) | Decreased sperm number; testicular toxicant |
| *Fungicide* | |
| Vinclozolin | Antiandrogen: disruption of male phenotypic sexual differentiation |
| *Heavy metals* | |
| Cadmium | High doses: ischemic necrosis of the testis |
| Chromium | Decreased testosterone; ↓ sperm number |
| Lead | Decreased testosterone; ↓ sperm number; ↓ fertilization rates |
| Mercury | Decreased sperm quality |
| *Miscellaneous xenobiotics* | |
| Dioxin | Decreased libido; abnormal sperm morphology; ↓ response to GnRH |
| DBP (dibutyl phthalate) | Altered sexual differentiation |
| PCBs | Decreased sperm number; altered hypothalamic–pituitary–adrenal axis |
| Gossypol | Decreased sperm number |
| Ethylene glycol | Decreased sperm number and motility |
| *Environmental factors* | *Observed effect(s) on male* |
| Heat | Damaged sperm chromatin and quality |
| Microwaves | Decreased sperm number |
| Radiation | High doses: death of stem cells and permanent azoospermia |
| Stray voltage (AC and DC) | Decreased sperm number |
| *Physiological factors* | *Observed effect(s) on male* |
| Stress | Decreased sperm motility |
| Fever (hyperthermia) | Damaged sperm chromatin and quality |

This table was adapted, with permission, from Ellington and Wilker (2006).

compounds is presented in Table 14.1 (Ellington and Wilker, 2006). In the following section a few selected male reproductive toxicants and their proposed mechanisms of action will be briefly described in order to familiarize the reader with the various different ways that male fertility can be affected by reproductive toxicants.

### *Selected male reproductive toxicants and mechanisms of action*

#### *Cell-specific reproductive toxicants*

Some reproductive toxicants adversely affect specific cells within the testes. Ethane dimethane sulfonate is specifically cytotoxic to the Leydig cells, and excessive exposure to this compound results in complete loss of this population of cells within the interstitium, and, consequently, the ability of the testes to synthesize testosterone and, in some species, estrogens (Creasy and Foster, 2002). Tri-*o*-cresyl phosphate (TOCP) is an industrial chemical used in lacquers and varnishes, which inhibits LH-induced steroidogenesis in the Leydig cells and, after Leydig cell-mediated conversion to its active metabolite, morphological abnormalities in Sertoli cells (Thomas and Thomas, 2001; Creasy and Foster, 2002).

Sertoli cells are specifically targeted by several toxicants, including diethylhexyl phthalate (DEHP), 1,3-dinitrobenzene (DNB) and 2,5-hexanedione (metabolite of *n*-hexane) (Creasy and Foster, 2002) and the effects of these xenobiotics are age- and species specific (Thomas and Thomas, 2001). With respect to DNB, the parent compound is converted to its toxic metabolites, nitosonitrobenzene and nitroaniline, within the target Sertoli cells, and, similar to other Sertoli cell-specific toxicants, germ cell death and exfoliation occur secondary to toxic insult to the Sertoli cells (Creasy and Foster, 2002). Sertoli cell microtubules

appear to be the intracellular targets of 2,5-hexanedione (Thomas and Thomas, 2001; Creasy and Foster, 2002). The fungicide dibromochloropropane (DBCP) appears to affect the Sertoli cell (Thomas and Thomas, 2001), but its metabolites epichlorhydrin and $\alpha$-chlorhydrin induce capillary permeability and vascular damage within the epididymis (Creasy and Foster, 2002).

There are a number of reproductive toxicants which target-specific populations of germ cells. Spermatogonia, spermatocytes, round spermatids and elongate spermatids are specifically targeted by busulfan, 2-methoxyethanol, ethylmethane sulfonate and dibromacetic acid, respectively (Creasy and Foster, 2002). Ionizing radiation and a variety of chemotherapeutic agents, including cyclophophamide, nitrogen mustard, vincristine and vinblastine, generally target rapidly dividing mitotic or meiotic germ cells in the testes, and TCDD appears to adversely affect several populations of spermatozoal precursors (Thomas and Thomas, 2001). The compound 7,12-dimethylbenz[*a*] anthracene (DMBA) is toxic to spermatogonia but must undergo a stepwise biotransformation in the Leydig cell and, subsequently, the Sertoli cell to produce the ultimately toxic metabolite (Creasy and Foster, 2002).

### *Heavy metals*

Lead and cadmium are ubiquitous heavy metals and have both been associated with testicular toxicity and impaired fertility in a number of species. Excessive cobalt can potentially interfere with normal spermatogenesis, and severe cobalt intoxications have actually resulted in generalized hypoxia related to increased blood viscosity or which affects the testes (Thomas, 1995). Chromium and vanadium have also been associated with adverse reproductive effects (Thomas and Thomas, 2001), and cis-platinum exposure has been associated with the death of spermatocytes and spermatids, as well as disruption of Sertoli cell tight junctions (Thomas, 1995). Although testicular toxicity is generally not observed with excessive parenteral exposure to zinc (other than possibly secondary to hemolytic anemia-related hypoxia) (Thomas, 1995), intratesticular injections with zinc gluconate have been successfully used for chemical castration in several species.

Divalent lead is known to interact with physiological processes involving calcium and generally has an affinity for sulfhydryl groups. Lead is reported to be directly toxic to germ cells and Leydig cells and can suppress anterior pituitary secretion of LH and FSH (Thomas and Thomas, 2001). Lead also appears to be able to adversely affect the ability of spermatozoa to fertilize ova, but, this effect, like others associated with lead exposure, appears to be dependent on age and individual variations in susceptibility, adaptation and reversibility (Sokol, 2006).

Like lead, cadmium is thought to adversely affect male reproduction by several different mechanisms. With respect to spermatogenesis, the stage of the seminiferous epithelium associated with spermiation appears to be specifically inhibited by cadmium (Thomas, 1995). Cadmium has also been shown to have possible interactions with the hypothalamic–pituitary–gonadal axis (Akinloye *et al.*, 2006). The endothelium of the testicular and epididymal vasculature is extremely susceptible to toxic insult by cadmium, potentially resulting in reduced vascular perfusion and testicular necrosis (Creasy and Foster, 2002). Cadmium can also alter the actin filaments in the junctional complexes between adjacent Sertoli cells, thereby disrupting the integrity of the blood–testis barrier (Thomas and Thomas, 2001). Cadmium can interfere with the cellular metabolism of zinc, an essential trace element necessary for normal reproductive function, and diets deficient in zinc can predispose individuals the toxic effects of cadmium (Akinloye *et al.*, 2006). Pretreatment with zinc has been reported to reduce the incidence of cadmium-induced Leydig cell cytotoxicity and neoplasia (Thomas, 1995).

### *Gossypol*

Gossypol is a yellow, polyphenolic pigment, which is contained in most of the parts of plants belonging to the *Gossypium* genus and is concentrated in pigment glands within the seeds (Morgan, 2004; Casteel, 2007). Gossypol exists as two isomers within plants (+and the more toxic −), and these isomeric forms can be non-toxic and bound to plant proteins or toxic and "free" or unbound (Cheeke, 1998). The concentrations of the toxic free form of gossypol vary widely in whole seeds and meals, with the gossypol in direct solvent-extracted cottonseed meal being much more readily bioavailable than the gossypol contained in whole seeds (Cheeke, 1998; Casteel, 2007).

Gossypol can cause systemic and reproductive disease syndromes, depending on the species of exposed animal and the dosage of free gossypol consumed (Randel *et al.*, 1992). The toxic effects of gossypol are cumulative, and systemic disease, characterized by hepatic, renal, cardiovascular and pulmonary abnormalities, is generally observed in monogastric animals (Cheeke, 1998). Mature ruminants are considered to be relatively resistant to the severe systemic effects of free gossypol because of the propensity of this form of the pigment to become bound to proteins in ruminal fluid and, therefore, "detoxified" (Casteel, 2007). Gossypol-induced male subfertility has been observed in monogastrics and, especially, ruminant species and is dependent on the dosage of free gossypol and the duration of gossypol exposure (Randel *et al.*, 1992; Cheeke, 1998).

Exposure of peri-pubertal or sexually mature males, to sufficient dosages of free gossypol, adversely affects the seminiferous epithelium and disrupts normal spermiogenesis, resulting in spermatozoa with aplastic midpieces (i.e. segmental aplasia of the mitochondrial sheath) (Randel *et al.*, 1992; Chenoweth *et al.*, 2000).

Additional sperm abnormalities, possibly associated with gossypol-induced oxidative damage (Velasquez-Pereira *et al.*, 1998), can potentially develop as stressors, related to the acquisition of motility in the epididymidis, alter the structural integrity of already weakened spermatozoa (Chenoweth *et al.*, 2000). The spermatozoal abnormalities induced by exposure of immature bulls to free gossypol are most likely reversible (Hassan *et al.*, 2004) and can be ameliorated by concurrent treatment with vitamin E (Velasquez-Pereira *et al.*, 1998). Total dietary concentrations of free gossypol supplied as cottonseed meal or whole cottonseed should not exceed 150 and 600 ppm, respectively, in young developing bulls, or, similarly, 200 and 900 ppm in sexually mature animals (Morgan, 2004).

*Xenoestrogens and antiestrogens*

Reproductive function in sexually mature males can potentially be adversely affected by exposures to nuclear ER agonists or antagonists, as well as by estrogenic or antiestrogenic EDCs acting independently of receptor-mediated interactions. However, it is clear from the feminizing effects of prenatal exposures to DES (McLachlan, 2001; Newbold *et al.*, 2006) and observations of androgynization in wildlife species (Edwards *et al.*, 2006), that the male fetus is much more sensitive to the adverse effects of endocrine disruptors than male animals during postnatal period (Hess and Iguchi, 2002). It is also important to remember that, even in males, xenobiotics which interfere with estrogenic signaling pathways can adversely affect normal reproductive development and function (O'Donnell *et al.*, 2001; Hess, 2003).

*Xenoandrogens and antiandrogens*

Normal phenotypic sexual differentiation of the male fetus, as well as all of the postnatal events which result in the delivery of fertile spermatozoa to the female reproductive tract, is dependent on appropriately timed androgenic stimulation of the male. It has been well-recognized for quite some time that xenoandrogens (e.g., anabolic steroids and exogenous testosterone and DHT) can interfere with hypothalamic–pituitary–gonadal feedback mechanisms (Figure 14.2), resulting in decreased LH release, sperm abnormalities and testicular atrophy (Ellington and Wilker, 2006). In recent years, there has been increasing interest in xenobiotics which can interfere with interactions between androgens and their receptors or, in some other way, disrupt androgen-dependent signaling pathways. The dicarboximide fungicides, vinclozolin and procymidone and/or their metabolites inhibit the binding of androgens to nuclear androgen receptors and can demasculinize and feminize the prenatally exposed male fetus or induce important alterations in pre- or peri-pubertally exposed offspring (Monosson *et al.*, 1999; Gray *et al.*, 2006). Vinclozolin has also recently been shown to be capable of inducing epigenetic modifications which facilitate the occurrence of transgenerational or vertically transmitted reproductive abnormalities (Anway *et al.*, 2005; Anway and Skinner, 2006). Other EDCs, including linuron, *p,p′*-DDE (another metabolite of DDT) and prochloraz, can also function as androgen receptor antagonists, and PBDEs can act as competitive inhibitors of the androgen receptor as well as androgen-induced gene expression (Gray *et al.*, 2006). The AhR-mediated effects of TCDD can interfere with the biosynthesis of testosterone and disrupt testosterone signal transduction pathways (Jana *et al.*, 1999; Sikka *et al.*, 2005).

*Phthalates*

It is recognized that phthalates, which are used as plasticizers and which are abundant within the environment, share a unique antiandrogenic mechanism, which can result in reproductive dysgenesis in male offspring. Unlike vinclozolin, phthalates are not androgen receptor antagonists, but it is also clear that they are not uterotropic nor are they capable of inducing a persistent estrus, as would be expected with estrogenic EDCs (Gray *et al.*, 2006). Phthalates actually alter fetal Leydig cell function, resulting in decreased testosterone synthesis and downregulated expression of insulin-like peptide-3, which is required for gubernacular cords formation (Foster, 2006; Gray *et al.*, 2006). Appropriately timed fetal exposure to di (*n*-butyl) phthalate can result in an abnormal aggregation of Leydig cells in the fetal rat testis, resulting in a failure of Sertoli cell proliferation and functional maturation, similar to what has been proposed as a possible mechanism the development of TDS in humans (Sharpe *et al.*, 2003; Mahood *et al.*, 2005, 2006).

## Toxicants affecting the female reproductive function

There have been many documented reports of female reproductive abnormalities associated with exposures of domestic animal species to naturally occurring EDCs (i.e. phytoestrogens and zearalenone). Likewise, the reproductive effects of the ergot alkaloids produced by the tall fescue endophyte *Neotyphodium coenophialum* are also very well understood (Evans *et al.*, 2004). Unfortunately, however, there are still instances of toxicant-induced subfertility which very likely go unrecognized. An effort will be made to the review some of the major mechanisms of action for toxic insult to the female reproductive tract in domestic animals, in the hope that the reader might better understand the potential scope of xenobiotic-induced reproductive effects.

### *Selected female reproductive toxicants and mechanisms of action*

*Cell-specific reproductive toxicants*

In general, the effects of toxicants on specific cell types within the female reproductive tract, and especially the ovaries, are not as well understood as they are in the testes (Thomas and Thomas, 2001). Many female reproductive

toxicants do not target particular cell lines, *per se*, but, rather, disrupt the endocrine milieu of the tubular genitalia or cause changes in ovarian structures secondary to alterations in the hypothalamic–pituitary–gonadal axis (Yuan and Foley, 2002). There are, however, a number of xenobiotics considered to "ovotoxic". Phthalates and TCDD can delay or decrease ovulations (Devine and Hoyer, 2005). Ionizing radiation and some of the same chemotherapeutic agents reported to adversely affect rapidly dividing germ cells within the testes (e.g. cyclophosphamide, nitrogen mustard and vinblastine) can also adversely affect primordial follicles within the ovary (Thomas and Thomas, 2001; Devine and Hoyer, 2005). Several PAHs [i.e. BaP, 3-methylcholanthrene (3-MC) and DMBA] and 1,3-butadiene appear to target oocytes in preantral follicles, and DMBA, BaP and 1- and 2-bromopropane, as well as 1,2-dibromopropane, can adversely affect antral follicular development (Devine and Hoyer, 2005). Exposure to free gossypol has been associated with increased numbers of degenerating embryos in heifers (Casteel, 2007).

Like the testes, the ovaries also have some xenobiotic biotransformation capabilities (Thomas and Thomas, 2001; Yuan and Foley, 2002). As in other organs, oxidative damage can adversely affect ovarian structure and function. In the case of 1,3-butadiene and 4-vinylcyclohexene, the adverse effects of these toxicants on small and growing follicles are due, in part, to the toxic actions of the epoxidated metabolites of these xenobiotics (Devine and Hoyer, 2005).

*Heavy metals*

The ovaries do not appear to be as sensitive to the toxic effects heavy metals as do the testes, and those adverse effects which are observed are more subject to variation between species than what was observed in the male gonads (Thomas, 1995). Anterior pituitary release of FSH and LH and ovarian steroidogenesis appear to be inhibited by cadmium in the female (Hoyer, 2006). With respect to lead, the neuroendocrine function of the hypothalamic–pituitary–gonadal axis appears to be targeted by lead in the female, as well as in the male (Thomas, 1995; Hoyer, 2006).

*Phytoestrogens*

Several genera of leguminous plants produce estrogenic compounds collectively referred to as "phytoestrogens", which can be associated with clinically relevant effects in livestock species and companion animals. Species of clover, including subterranean clover (*Trifolium subterraneum*), red clover (*Trifolium pratense*), white clover (*Trifolium repens*) and alsike clover (*Trifolium hybridum*), contain phytoestrogens classified as isoflavones (Cheeke, 1998; Burrows and Tyrl, 2001). It should also be noted that other leguminous plants, such as soybean (*Glycine max*), which is commonly used as a feed source for agricultural animals, can contain varying amounts of isoflavones. Alfalfa (*Medicago sativa*) contains another class of phytoestrogenic compounds referred to as coumestans (Cheeke, 1998).

The relatively inactive, "parent" glycosides for the most clinically relevant isoflavones in clover are formononetin, biochanin A and genestin, and the total concentration of these phytoestrogens can be as high as 2–4% (20,000–40,000 ppm) in clover (Burrows and Tyrl, 2001). Primarily within the gastrointestinal tract and, especially, the rumen, formononetin is converted into an intermediate metabolite, daidzein, and, then subsequently into the more estrogenically active phytoestrogen, equol (Cheeke, 1998; Burrows and Tyrl, 2001). Conversely, biochanin A and genestin are initially converted into genestein and then subsequently into estrogenically inactive phenolic compounds (i.e. phenolic acid and *p*-ethylphenol).

Coumestrol is the primary phytoestrogen in alfalfa. This compound has significantly greater estrogenic activity than many of the isoflavones present in other legumes, and this activity generally decreases during the drying process involved in hay production (Cheeke, 1998; Burrows and Tyrl, 2001). Coumestrol is usually only present at very low concentrations (10–20ppm) in the vegetative stages of alfalfa growth, but its estrogenic activity can vary with plant maturity and from year to year (Burrows and Tyrl, 2001).

A number of clinical syndromes have been associated with phytoestrogen exposure in domestic animals. "Clover disease" in sheep and, to a lesser extent, cattle is associated with the consumption of the isoflavones in clover, resulting in infertility associated with abnormal estrous cycles and structural and functional changes in the cervix (Cheeke, 1998; Burrows and Tyrl, 2001). Phytoestrogen-induced alterations in ovine cervical mucus interfere with the slow, sustained transport of motile spermatozoa from their cervical reservoirs (Cheeke, 1998). Genestein can induce structural changes and, possibly, irreversible organizational abnormalities in the cervix and uterus of exposed gilts (Ford *et al.*, 2006). Coumestan exposure in cattle can be associated with various and, sometimes, seemingly conflicting clinical presentations (e.g. hyperestrogenism, nymphomania, swelling of the external genitalia, estrus suppression, inhibition of ovulation and cystic ovarian disease) (Cheeke, 1998; Casteel, 2007). It is important to remember that phytoestrogens, like other xenoestrogens, can function also as antiestrogens through the inhibition of LH and FSH release from the anterior pituitary and by competing with endogenous estrogens for receptor sites within the tubular genitalia (Cheeke, 1998).

*Zearalenone*

The estrogenic mycotoxin zearalenone is produced by *Fusarium graminearum* (formerly *Fusarium roseum*), under certain environmental and storage conditions and, sometimes, in conjunction with vomitoxin or deoxynivalenol (DON). Cereal grains associated with zearalenone production include corn, wheat, barley and oats, and some grasses

in New Zealand have also been reported to have been contaminated with zearalenone (Cheeke, 1998). Swine have been shown to be particularly susceptible to the adverse effects of zearalenone, with pre-pubertal gilts being affected by concentrations of zearalenone in the feed as low as 1–3 ppm (Cheeke, 1998; Casteel, 2007). The increased sensitivity of pigs to the estrogenic effects of zearalenone is most likely related to the slow metabolism and enhanced enterohepatic recirculation of zearalenone noted in this particular species (Cheeke, 1998). Cattle and other ruminants can be affected by zearalenone but only at dietary concentrations much higher than those associated with clinical signs in swine (Casteel, 2007). Hyperestrogenism in pre-pubertal gilts is characterized swelling of the vulva and mammary glands, uterine enlargement and ovarian atrophy, and testicular atrophy and preputial swelling have been observed in immature male swine (Cheeke, 1998). As with other xenoestrogens, interference with estrogenic feedback mechanisms and various ovarian abnormalities, including follicular cysts, have been observed with excessive exposure to zearalenone. Since estrogens are luteotrophic in swine, zearalenone can be associated with prolonged luteal phases (pseudopregnancy), as well as nymphomania in cycling gilts and sows, depending on the phase of the estrous cycle at the time of exposure.

*Synthetic xenoestrogens and antiestrogens*

A wide range of agricultural and industrial chemicals, as well as pharmaceuticals used in birth control preparations, have estrogenic and/or antiestrogenic activities, depending on the endocrine environment, presence of endogenous estrogens and stage of development at the time of exposure. The type of tissue and physiological response being discussed, as well as the relative distribution of ER$\alpha$ and ER$\beta$ receptors will also affect the types of endocrine effects observed in a given circumstance. As has been emphasized previously, the developing fetus is particularly susceptible to the adverse effects of estrogenic and antiestrogenic endocrine disruptors (Hess and Iguchi, 2002).

Some of the synthetic xenobiotics most commonly discussed with respect to their estrogenic and/or, in some instances, their antiestrogenic activity include DES, DDT, PCBs, bisphenol A, nonylphenol, kepone and TCDD (MacLachlan, 2001). While the overall adverse effects of xenoestrogens have already been discussed in this chapter with respect to reproductive development and endocrine disruption, there are several unique clinical aspects of exposures to these types of xenobiotics which should be addressed for completeness. Prenatal human exposures to the synthetic, non-steroidal xenoestrogen, DES, have been associated with feminization of the male fetus, as well the increased occurrence of clear cell adenocarcinoma of the vagina in young women (McLachlan 2001; Rogers and Kavlock, 2001; McLachlan *et al.*, 2006; Newbold *et al.*, 2006). The use of DES for mismating or pregnancy prevention in dogs has been, in some instances with an increased incidence of cystic endometrial hyperplasia and pyometra. The apparent ability of the widely distributed xenoestrogen, bisphenol A, to cause adverse developmental effects at very low, environmental concentrations and in a manner characterized by a non-monotonic (inverted U) dose response remains controversial but is worthy of further, detailed discussions because of its scientific ramifications and societal relevance (Welshons *et al.*, 2006).

*Xenoandrogens*

While the emphasis in the area of endocrine disruption has traditionally been on the adverse effects of xenoestrogens reproductive development and function, there is increasing evidence that there are also instances of endocrine disruptors having androgenic activity. The effluents from pulp and paper mills have recently been shown to be able to masculinize female fish (Gray *et al.*, 2006). In addition, the runoff from cattle feedlots where the synthetic androgen, trenbolone, was used for the promotion of growth, has also been shown to have androgenic activity and is also suspected of being associated with masculinization of females in wildlife species (Orlando *et al.*, 2004).

## Teratogenesis and abortion

### *Mechanisms of actions of teratogenesis and abortion*

A large number of xenobiotics have been classified with respect to their teratogenic potential, and these are listed in Table 14.2. The ability of EDCs to interfere with phenotypic sexual differentiation in the fetus has already been extensively reviewed in this chapter. Teratogenesis can be associated with each of the following mechanisms of action: (1) excessive cell death; (2) interference with apoptosis; (3) reduced cellular proliferation rate; (4) failed interactions between cells; (5) impaired morphogenetic movements; (6) reduced synthesis of components essential for growth and development; (7) mechanical disruption; (8) and alterations in pH (Hood *et al.*, 2002; Hood, 2006). Some teratogens are capable of more than one mechanism of action. Premature parturition or abortion can be induced by any xenobiotics which cause fetal or, potentially, maternal stress and initiate the cascade of endocrine and neural signaling events which would normally lead to parturition. Any intoxication in a pregnant animal has the potential to threaten fetal survival. Toxicants which cause sudden fetal death or complete cessation of placental function or disruption of the gestational source of progestins can also induce abortion in animals.

*Heavy metals*

Several heavy metals have been identified as teratogens and possible abortifacients in humans and animals. The

**TABLE 14.2 Safety of drugs in pregnancy**

| Drug | Recommendation | Comments |
|---|---|---|
| *Antimicrobial drugs* | | |
| Amikacin | C | Aminoglycoside antibiotics easily cross the placenta and may cause 8th nerve toxicity or nephrotoxicity. |
| Ampicillin | A | Crosses the placenta but has not been shown to be harmful to fetus. |
| Amoxicillin | A | Crosses the placenta but has not been shown to be harmful to fetus. |
| Carbenicillin | A | Crosses the placenta but has not been shown to be harmful to fetus. |
| Cephalosporins | A | Crosses the placenta but has not been shown to be harmful to fetus. |
| Chloramphenicol | C | May decrease protein synthesis in fetus, particularly in bone marrow. |
| Ciprofloxacin | D | Do not use during pregnancy; quinolones have been associated with articular cartilage defects. |
| Clavulanic acid–amoxicillin (Clavamox, Beecham) | A | Crosses the placenta but has not been shown to be harmful to fetus. |
| Clindamycin | A | Crosses the placenta but has not been shown to be harmful to fetus. |
| Cloxacillin | A | Crosses the placenta but has not been shown to be harmful to fetus. |
| Dicloxacillin | A | Crosses the placenta but has not been shown to be harmful to fetus. |
| Doxycycline | D | Tetracyclines can cause bone and teeth malformations in fetus and may cause toxicity in mother. |
| Enrofloxacin | D | See ciprofloxacin. |
| Erythromycin | A | Appears to be safe except for erythromycin estolate, which has been shown to increase the risk of hepatotoxicity in women. |
| Gentamicin | C | Aminoglycoside antibiotics easily cross the placenta and may cause 8th nerve toxicity or nephrotoxicity. However, specific toxicities from gentamicin have not been reported, and it may be used for a serious infection in place of a suitable alternative. |
| Hetacillin | A | Crosses the placenta but has not been shown to be harmful to fetus. |
| Kanamycin | C | Aminoglycoside antibiotics easily cross the placenta and may cause 8th nerve toxicity of nephrotoxicity. |
| Lincomycin | A | Crosses the placenta but has not been shown to cause problems in fetus. |
| Metronidazole | C | Teratogenic in laboratory animals, but there is no information for dogs and cats. It should be avoided during the first 3 weeks of pregnancy. |
| Neomycin | A | Not obsorbed sufficiently to cause systemic effects after oral administration. |
| Oxacillin | A | Crosses the placenta but has not been shown to be harmful to fetus. |
| Oxytetracycline | D | Toxic to fetus and may increase risk of hepatitis in mother (see tetracycline). |
| Penicillin G (benzyl penicillin) | A | Crosses the placenta but has not been shown to be harmful to fetus. |
| Streptomycin | D | See gentamicin. Streptomycin is associated with higher incidence of 8th nerve toxicity than other aminoglycosides. |
| Sulfonamides | B | Sulfonamides cross the placenta and have produced congenital malformations in rats and mice, but problems have not been reported in dogs or cats; in people, they have caused neonatal icterus when administered near term. Avoid long-acting sulfonamides. |
| Tetracycline | D | Tetracyclines can cause bone and teeth malformations in fetus and may cause toxicity in mother. |
| Trimethoprim–sulfadiazine (Tribrissen, Coopers) | B | Manufacturer states that it is safe during pregnancy in dogs; see also trimethoprim and sulfonamides. |
| Trimethoprim | B | Teratogenic in rats but probably safe in other species. Folate antagonism and bone marrow depression are possible with prolonged use. |
| Ticarcillin | A | Crosses the placenta but has not been shown to be harmful to fetus. |
| Tobramycin | C | Aminoglycoside antibiotics easily cross the placenta and may cause 8th nerve toxicity or nephrotoxicity. |
| Tylosin | B | No information is available. |
| *Antifungal drugs* | | |
| Amphotericin-B | C | There are no known teratogenic effects, but amphotericin is extremely toxic. Use only if the disease is life threatening, in absence of a suitable alternative. |
| Griseofulvin | D | Teratogenic in rats; causes multiple skeletal and brain malformations in cats. |
| Ketoconazole | B | Teratogenic and embryotoxic in rats; antiandrogenic; stillbirths have been reported in dogs. |
| Miconazole | A | Apparently safe if applied topically. |

(*Continued*)

**TABLE 14.2 (Continued)**

| Drug | Recommendation | Comments |
|---|---|---|
| *Antiparasitic drugs* | | |
| Amitraz | C | Manufacturer states that reproduction studies have not been done; no information available. |
| Diethylcarbamazine | A | Manufacturer states that the drug may be given to dogs throughout gestation. |
| Dithiazanine iodide (Dizan, TechAmerica) | B | No information is available; iodide salts may cause congenital goiter if administered for prolonged periods during pregnancy. |
| Fenbendazole | A | Safe. Has been administered to pregnant bitches without producing adverse effects. |
| Dichlorvos (Task, Solvay) | B | Caution is advised when administering cholinesterase inhibitors to pregnant animals; it should not be administered to puppies or kittens, but studies in pregnant dogs and cats suggest that there are no adverse effects during pregnancy. |
| Ivermectin | A | Safe. Reproduction studies in dogs, cattle, horses and pigs have not shown adverse effects. |
| Levamisole | C | No information available. |
| Mebendazole | A | Safe. In reproduction studies in dogs, it was not teratogenic or embryotoxic. |
| Piperazine | A | Safe. No known contraindications for the use of piperazine. |
| Praziquantel | A | Safe. No adverse effects were seen when tested in pregnant dogs and cats. |
| Thiacetarsamide (Caparsolate sodium, CEVA) | C | No specific information regarding toxicity to fetus is available. It can be hepatotoxic and nephrotoxic, and heartworm adulticide should be postponed until after parturition. |
| Bunamidine | A | Has been administered to pregnant bitches without problems and is safe in pregnant cats. Slight interference with spermatogenesis has been seen in male dogs. |
| Pyrantel | A | Safe. Toxicity studies have not shown any adverse effects. |
| Thenium | A | Safe. Manufacturer states that except in young puppies, there are no known contraindications. |
| Thiabendazole | B | Thiabendazole is not teratogenic in laboratory animals, but high doses have produced toxemia in ewes. |
| Trichlorfon | C | Caution is advised when administering organophosphates to pregnant animals. Congenital toxicoses have been reported following administration to pregnant sows. Manufacturer states that trichlorfon should not be administered to pregnant mares, but there are no recommendations for dogs and cats. |
| *Anticancer drugs* | | |
| Doxorubicin hydrochloride (Adriamycin, Adria) | C | May produce malformations in newborn or embryotoxicity. |
| Azathioprine | C | May produce congenital malformations but has been used in pregnant women safely. It may be a suitable alternative to other drugs when immunosuppressive therapy is required. |
| Chlorambucil | C | May produce malformations in newborn or embryotoxicity. |
| Cisplatin | C | May produce congenital malformations, embryotoxicity or nephrotoxicity. |
| Cyclophosphamide | C | May produce malformations in newborn or embryotoxicity. |
| Methotrexate | C | May produce malformations in newborn or embryotoxicity. |
| Vincristine | C | May produce malformations in newborn or embryotoxicity. |
| *Analgesic drugs* | | |
| Acetaminophen | C | Safety not established in dogs; toxic in cats. |
| Aspirin | C | Embryotoxicity has been seen in laboratory animals but not in other species. Late in pregnancy, it may produce pulmonary hypertension and bleeding problems (see text). |
| Flunixin meglumine | C | Safety in pregnancy has not been determined. |
| Gold (aurothioglucose) | D | Laboratory animal studies clearly show increased congenital malformations. |
| Ibuprofen | C | Safety in dogs and cats not established. |
| Indomethacin administered near term. | C | Can be toxic in adult dogs; can cause premature closure of ductus arteriosus if |
| Phenylbutazone | C | Safety has not been established. Long-term use can depress bone marrow. |
| Salicylates | | Embryotoxicity has been seen in laboratory animals but not in other species. Late in pregnancy, it may produce pulmonary hypertension and bleeding disorders. |
| *Anesthetic and preanesthetic drugs* | | |
| Acepromazine | B | Phenothiazines should be avoided near term; they may produce neonatal CNS depression. |
| Atropine | B | Crosses the placenta and has been used safely but may cause fetal tachycardia. |
| Butorphanol | B | Safe for short-term use. Neonatal depression can be treated with naloxone. |
| Codeine | B | Safe for short-term use. Neonatal depression can be treated with naloxone. |
| Diazepam | C | See anticonvulsants. |
| Fentanyl | B | Safe for short-term use. Neonatal depression can be treated with naloxone. |

(*Continued*)

**TABLE 14.2 (Continued)**

| Drug | Recommendation | Comments |
|---|---|---|
| Glycopyrrolate | B | Safe. Does not cross placenta as readily as astropine. Studies in rats and rabbits have not revealed teratogenic effects. |
| Halothane | C | Decreased learning ability has been reported in rats after *in utero* exposure; depression may be seen in neonates after cesarean section; excessive uterine bleeding may be seen when administered during cesarean section. |
| Isoflurane | B | Probably safe. Depression may be seen in neonates after cesarean section. |
| Ketamine | B | Probably safe. Depression may be seen in puppies delivered by cesarean section; may increase intrauterine pressure and induce premature labor. |
| Lidocaine | A | All local anesthetics appear to be safe when used for a local nerve block or epidural anesthesia. |
| Meperidine | B | Opiates can produce neonatal sedation and respiratory depression, but the effects can be reversed with the administration of naloxone. |
| Methoxyflurane | C | Neonatal depression is seen when used for cesarean section. |
| Morphine | B | Opiates can produce neonatal sedation and respiratory depression, but the effects can be reversed with the administration of naloxone. |
| Naloxone | A | Has been shown to be safe when administered to newborns within a few minutes after birth. |
| Nitrous oxide | B | Probably safe. Used frequently for cesarean section without adverse effects. |
| Oxymorphone | B | Opiates can produce neonatal sedation and respiratory depression, but the effects can be reversed with the administration of naloxone. |
| Pentobarbital | D | Associated with high incidence of neonatal mortality. |
| Thiamylal | C | Easily crosses the placenta; all barbiturates produce respiratory depression in fetus; however, thiobarbiturates are not as toxic as pentobarbital. |
| Thiopental | C | Easily crosses the placenta. All barbiturates produce respiratory depression in fetus; however, thiobarbiturates are not as toxic as pentobarbital. |
| *Gastrointestinal drugs* | | |
| Antacids | A | Safe. Not absorbed systemically. |
| Antiemetics | B | Probably safe if administered short term. |
| Cimetidine | B | Safety has not been established, but no reports of toxicity in humans. |
| Dimenhydrinate | B | Safe if used short term. |
| Diphenhydramine | B | Safe if used short term. |
| Diphenoxylate | C | Studies have reported adverse effects in laboratory animals, but no adverse effects have been reported in pregnant dogs, cats and humans. |
| Laxatives | B | All laxatives, except castor oil (Squibb), are considered safe if they are used short term. Castor oil causes premature uterine contractions. |
| Loperamide | C | Same comment as diphenoxylate. |
| Metoclopramide | B | Safe in laboratory animals, but no studies available for cats or dogs. |
| Methscopolamine | C | Safety not established. |
| Misoprostol | D | Synthetic prostaglandin, causes a termination of pregnancy. |
| Prochlorperazine | B | No reports of toxicity when administered short term. |
| Ranitidine | B | Safety has not been established, but no reports of toxicity were reported in humans. |
| Sucralfate | A | Probably safe. Not absorbed systemically. |
| Sulfasalazine | B | Salicylate component is not absorbed enough to produce adverse effects; sulfonamide may produce neonatal icterus when used near term (see text). |
| *Cardiovascular drugs* | | |
| Atropine | B | Probably safe but may produce fetal tachycardia. |
| Captopril | C | Has been shown to be embryotoxic in laboratory animals and goats. |
| Digitalis | A | Probably safe. No adverse effects seen in humans and laboratory animals (see text). |
| Furosemide | B | No adverse effects have been reported. |
| Dopamine | B | Probably safe at therapeutic doses. |
| Heparin | B | Does not appear to cross placenta. |
| Hydralazine | B | Probably safe. There have been reports of minor toxicity in rats, but it has been administered safely to pregnant women. |
| Isoproterenol | C | May cause fetal tachycardia; beta-adrenergic drugs inhibit uterine contractions. |
| Lidocaine | B | Probably safe. May cause fetal bradycardia. |
| Nitroglycerin | C | No information available. |
| Nitroprusside | C | There is a risk of fetal cyanide toxicity with prolonged use. |
| Procainamide | B | Probably safe. May cause fetal bradycardia. |
| Propranolol | C | May cause fetal bradycardia, respiratory depression and neonatal hypoglycemia; avoid use near term. |

(*Continued*)

**TABLE 14.2 (Continued)**

| Drug | Recommendation | Comments |
|---|---|---|
| *Cardiovascular drugs* (Cont.) | | |
| Quinidine | B | Probably safe. May cause fetal bradycardia. |
| Theophylline | B | No reports of adverse effects. |
| Thiazide diuretics | C | May cause increased incidence of perinatal mortality. |
| Warfarin | D | Causes embryotoxicity and congenital malformations, neural tube defects in laboratory animals and humans. |
| *Anticonvulsant drugs* | | |
| Diazepam | C | Has been associated with congenital defects in mice, rats and people. |
| Phenobarbital | B | Has been associated with rare congenital defects and bleeding tendencies in newborn but may be safer than other anticonvulsants (see text). |
| Phenytoin | C | Teratogenic in rats, mice and people. |
| Primidone | C | Same risks as phenobarbital and has been associated with increased incidence of hepatitis in adult dogs. |
| Valproic acid | C | May cause congenital malformations. |
| *Muscle relaxants* | | |
| Dantrolene | C | Safety not established. |
| Dimethyltubocurarine | B | Quarternary base with negligible placental transfer; it does not affect the fetus unless administered in large doses. |
| Gallamine | B | Quarternary base with negligible placental transfer; it does not affect the fetus unless administered in large doses. |
| Methocarbamol | C | Safety not established; manufacturer states that it should not be administered during pregnancy. |
| Pancuronium | B | Quarternary base with negligible placental transfer; it does not affect the fetus unless administered in large doses. |
| Succinylcholine | B | Quarternary base with negligible placental transfer; it does not affect the fetus unless administered in large doses. |
| *Endocrine drugs* | | |
| Betamethasone | C | Corticosteroids have been associated with increased incidence of cleft palate and other congenital malformations, and they may induce premature labor and abortion in dogs (see text). |
| Cortisone | C | Corticosteroids have been associated with increased incidence of cleft palate and other congenital malformations, and they may induce premature labor and abortion in dogs (see text). |
| Dexamethasone | C | Corticosteroids have been associated with increased incidence of cleft palate and other congenital malformations, and they may induce premature labor (see text). Dexamethasone has caused abortion and fetal death in dogs. |
| Diethylstilberstrol (DES) | D | Malformation of male and female genitourinary systems. |
| Estradiolcypionate (ECP) | D | Malformation of male and female genital tracts and bone marrow depression. |
| Flumethasone | C | Corticosteroids have been associated with increased incidence of cleft palate and other congenital malformations, and they may induce premature labor and abortion in dogs (see text). |
| Mitotane (*o,p′*-DDD) | D | Adrenocortical necrosis. |
| Prednisolone | C | Although prednisolone has been administered to pregnant women without adverse effects, caution is advised (see dexamethasone). Prednisolone may be used in serious diseases in absence of a suitable alternative. |
| Stanozolol | D | Manufacturer states that it should not be administered to pregnant dogs and cats. |
| Testosterone | D | Causes masculinization of female fetus. |
| Thyroxine | B | Does not cross placenta easily and has not been associated with any problems. |
| *Miscellaneous drugs* | | |
| Ammonium chloride | B | May cause fetal acidosis; discontinue use during pregnancy. |
| Aspartame (Nutra Sweet) | A | No risk. |
| Dimethylsulfoxide (DMSO) | C | Teratogenic in laboratory animals; manufacturers state that it should not be applied to breeding animals. |

A: Probably safe. Although specific studies may not have proved the safety of all drugs in dogs and cats, there are no reports of adverse effects in laboratory animals or in women.
B: Safe for use if used cautiously. Studies in laboratory animals may have uncovered some risk, but these drugs appear to be safe in dogs and cats or these drugs are safe if they are not administered when the animal is near term.
C: These drugs may have potential risks. Studies in people or laboratory animals have uncovered risks, and these drugs should be used cautiously, as a last resort when the benefit of therapy clearly outweighs the risks.
D: Contraindicated. These drugs have been shown to cause congenital malformations or embryotoxicity.

adverse affects of *in utero* lead exposure, on the developing nervous systems both human and laboratory animal species, have been well documented (Evans *et al*., 2003; Rogers and Kavlock, 2001). Prenatal exposure to organotins has been associated with pregnancy loss and impaired ossification in rodents (Ema and Hirose, 2006), and organic mercury is a known developmental neurotoxicant (Golub, 2006b). Other heavy metals, including cadmium and mercury, have been associated with placental toxicity, and this topic is covered in greater detail in Chapter 15.

***Selected plant-associated teratogens and abortifacients***

Many potentially toxic plants have been found to induce teratogenesis and/or abortion in mammals. While it is not possible to extensively review all of these plants, there are several well-documented examples of plant-induced birth defects and abortion which clearly illustrate the basic principles regarding the pathogenesis of teratogenesis and abortion in domestic animals.

*Veratrum californicum*

Jervanine alkaloids (e.g. cyclopamine, cycloposine and jervine) in *Veratrum californicum* (false hellebore) and, potentially, some closely related plants have been associated with the occurrence of cyclops lambs and other developmental abnormalities in sheep (Burrows and Tyrl, 2001). The mechanism of action of the teratogenic alkaloids in species of *Veratrum* involves the inhibition of neuroepithelial cell mitosis and migration during neurulation and decreased proliferation of chondrocytes (Cheeke, 1998; Burrows and Tyrl, 2001). The specific developmental abnormalities and the precise timing of maternal exposure to *Veratrum californicum*, which results in these defects is as follows: cyclops lambs and prolonged gestation (associated with the absence of pituitary gland) from maternal exposure on days 12–14 of gestation; embryonic death from maternal exposure on gestational days 19–21; cleft palate from maternal exposure on gestational days 24–30 and metacarpal, metatarsal and tracheal cartilage defects from maternal exposure on gestational days 27–36 (Burrows and Tyrl, 2001). No abnormal effects were observed with maternal exposure to *Veratrum californicum* before day 10 or after day 36 of pregnancy, and *Veratrum*-associated birth defects can generally be prevented by avoiding exposure until at 5 weeks after breeding.

*Multiple congenital contractures*

Species of tobacco (*Nicotiana* spp.), poison hemlock (*Conium maculatum*) and lupines (*Lupinus* spp.) have all been shown to be able to induce MCC or "crooked calf disease" in cattle and, potentially, other species exposed to their toxic principles at the appropriate stage of gestation. Pyridine alkaloids (e.g. anabasine) in tobacco, piperidine alkaloids in poison hemlock (e.g. coniine and $\gamma$-coniceine) and piperidine-derived and quinolizidine alkaloids in lupines (e.g. ammodendrine and anagyrine, respectively) cause stimulation then depression (depolarizing neuromuscular blockade) of nicotinic receptors in the fetus (Cheeke, 1998; Burrows and Tyrl, 2001; Panter, 2002). This stimulation followed by neuromuscular blockade of fetal nicotinic receptors during the critical period of joint development (i.e. gestational days 40–100 in cattle; days 30–60 in sheep; days 30–60 in swine) results in decreased fetal movement and the failure of joints to form in a normal fashion (Panter, 2002).

*Pine needle abortion*

Ingestion by cattle of pine needles (*Pinus ponderosa*) containing isocupressic acid (also possibly present in the vegetation of some junipers and cypresses) results in late-term abortion (Cheeke, 1998). Inhibition of the catechol estrogen-induced blockade of potential-sensitive $Ca^{2+}$ channels, by isocupressic acid, leads to enhanced entry of divalent calcium into arterial smooth muscle cells supplying the gravid uterus and results in profound vasoconstriction (Casteel, 1997; Cheeke, 1998; Burrows and Tyrl, 2001). This isocupressic-acid-induced vasoconstriction can lead to a 50% reduction in the blood flow reaching the fetal circulation and causes fetal stress, leading to the induc-tion of premature parturition or abortion (Cheeke, 1998; Burrows and Tyrl, 2001). Late-term abortion occurs 2–14 days following heavy consumption of green or dry pine needles and results in up to 75% of exposed cows aborting or delivering dead or weak calves (Cheeke, 1998; Burrows and Tyrl, 2001; Casteel, 2007). Dams are frequently dull and depressed, with weak uterine contractions, incomplete cervical dilation, retained placenta and frequent post-partum metritis (Cheeke, 1998).

## CONCLUSIONS

Reproduction is a critical biological process, required for financially viable livestock production, as well as species survival. Toxicant-induced abortions, congenital defects, infertility can have devastating effects on both domestic animals and wildlife species. There is growing concern within the scientific and government regulatory communities about the effects of exposures to reproductive toxicants on human fertility. The information presented in this chapter was intended to familiarize the reader with terminology and concepts pertinent to reproductive toxicity and endocrine disruption, as well as to provide an overview of normal reproductive development, anatomy and physiology and the various aspects of these processes most susceptible to toxic insult by naturally occurring and synthetic xenobiotics. It is hoped that the information and references provided in this chapter will assist readers in

making informed decisions in the course of their future clinical investigations, experimental designs and interpretations of scientific literature and/or regulatory policies.

## REFERENCES


Akinloye O, Arowojulu AO, Shittu OB, Anetor JI (2006) Cadmium toxicity: a possible cause of male infertility in Nigeria. *Reprod Biol* **6**(1): 17–30.

Anway MD, Skinner MK (2006) Epigenetic transgenerational actions of endocrine disruptors. *Endocrinology* **147**(6) (Suppl.): S43–9.

Anway MD, Cupp AS, Uzumcu M, Skinner MK (2005) Epigenetic transgenerational actions of endocrine disruptor and male fertilitys. *Science* **308**: 1466–9.

Basrur PK (2006) Disrupted sex differentiation and feminization of man and domestic animals. *Environ Res* **100**(1): 18–38.

Bigsby RM, Mercado-Feliciano M, Mubiru J (2005) Molecular mechanisms of estrogen dependent processes. In *Endocrine Disruptors: Effects on Male and Female Reproductive Systems*, 2nd edn, Naz RK (ed.). CRC Press and Taylor and Francis Group, LLC, Boca Raton, FL, pp. 217–47.

Brayman MJ, Julian J, Biserka, Mulac-Jericevic B, Conneely OM, Edwards DP, Carson DD (2006) Progesterone receptor isoforms A and B differentially regulate MUC1 expression in uterine epithelial cells. *Mol Endocrinol* (Epub ahead of print).

Britt KL, Findlay JK (2002) Estrogen actions in the ovary revisited. *J Endocrinol* **175**: 269–76.

Burrows GE, Tyrl RJ (2001) *Toxic Plants of North America*. Iowa State University Press, Ames, IA, pp. 1–1342.

Capen CC (2001) Toxic responses of the endocrine system. In *Casarett and Doull's Toxicology: The Basic Science of Poisons*, 6th edn, Klaassen CD (ed.). McGraw-Hill, New York, pp. 711–59.

Casteel SW, Evans TJ (2004) Nitrate. In *Clinical Veterinary Toxicology,* Plumlee KH (ed.). Mosby, Inc., St. Louis, MO, pp. 127–30.

Casteel SW (2007) Reproductive toxicants. In *Current Therapy in Large Animal Theriogenology*, Youngquist RS (ed.), 2nd edn. Saunders Elsevier, St. Louis, pp. 420–427.

Cheeke PR (1998) *Natural Toxicants in Feeds*, 2nd edn. Interstate Publishers, Inc., Danville, IL, pp. 1–479.

Chenoweth PJ, Chase CC, Risco CA, Larsen RE (2000) Characterization of gossypol-induced sperm abnormalities in bulls. *Theriogenology* **53**: 1193–203.

Cooke PS, Naz A (2005) Effects of estrogens and the phytoestrogen genistein on adipogenesis and lipogenesis in males and females. *Birth Defects Res A Clin Mol Teratol* **73**: 472–3.

Creasy DM, Foster PM (2002) Male reproductive system. In *Handbook of Toxicologic Pathology*, vol. 2, Haschek WM, Rousseaux CG, Wallig MA (eds). Academic Press, San Diego, CA, pp. 785–846.

Crews C, McLachlan JA (2006) Epigenetics, evolution, endocrine disruption, health and disease. *Endocrinology* **147**(6) (Suppl.): S4–10.

Devine PJ, Hoyer PB (2005) Ovotoxic environmental chemicals: indirect endocrine disruptors. In *Endocrine Disruptors: Effects on Male and Female Reproductive Systems*, 2nd edn, Naz RK (ed.). CRC Press and Taylor and Francis Group, LLC, Boca Raton, FL, pp. 67–100.

Dutertre M, Smith CL (2000) Molecular mechanisms of selective estrogen receptor modulator (SERM) action. *J Pharmacol Exper Ther* **295**(2): 431–7.

Dyce KM, Sack WO, Wensing CJG (2002) *Textbook of Veterinary Anatomy*, 3rd edn. Saunders, Philadelphia, PA, pp. 1–840.

Eaton DL, Klaassen CD (2001) Principles of toxicology. In *Casarett and Doull's Toxicology: The Basic Science of Poisons*, 6th edn, Klaassen CD (ed.). McGraw-Hill, New York, pp. 11–34.

Edwards TM, Moore BC, Guillette Jr LJ. (2006) Reproductive dysgenesis in wildlife: a comparative view. Environment, reproductive health and fertility. *Internat J Androl* **29(1)**: 109–19.

Ellington JE, Wilker CE (2006) Reproductive toxicology in the male companion animal. In *Small Animal Toxicology*, 2nd edn, Peterson ME, Talcott PA (eds). Elsevier Saunders, St. Louis, MO, pp. 500–18.

Ema M, Hirose A (2006) Reproductive and developmental toxicity of organotin compounds. In *Metals, Fertility and Reproductive Toxicity*. CRC Press and Taylor and Francis Group, LLC, Boca Raton, FL, pp. 23–64.

Evans TJ (1996) The effects of bromocriptine, domperidone, and reserpine on circulating, maternal levels of progestins, estrogens, and prolactin in pregnant pony mares. Masters Thesis, University of Missouri-Columbia, MO.

Evans TJ (2002) Endocrine alterations associated with ergopeptine alkaloid exposure during equine pregnancy. *Vet Clin Equine* **18**: 371–8.

Evans TJ, James-Kracke MR, Kleiboeker SB, Casteel SW (2003) Lead enters Rcho-1 trophoblastic cells by calcium transport mechanisms and complexes with calcium-binding proteins. *Toxicol Appl Pharmacol* **186**: 77–89.

Evans TJ, Rottinghaus GE, Casteel SW (2004) Fescue. In *Clinical Veterinary Toxicology*, Plumlee KH (ed.). Mosby, Inc., St. Louis, MO, pp. 243–50.

Evans TJ, Constantinescu GM, Ganjam VK (2007) Clinical reproductive anatomy and physiology of the mare. In *Current Therapy in Large Animal Theriogenology*, Younquist RS, Threlfall WR (eds), 2nd edn. Saunders Elsevier, St. Louis, pp. 47–67.

Fisher JS, MacPherson S, Marchetti N, Sharpe RM (2003) Human "testicular dysgenesis syndrome": a possible model using in-utero exposure to dibutyl phthalate. *Hum Reprod* **18**(7): 1383–94.

Ford JJ, D'Occhio (1989) Differentiation of sexual behavior in cattle, sheep and swine. *J Anim Sci* **67**(7): 1816–23.

Ford Jr JA, Clark SG, Walters EM, Wheeler MB, Hurley WL (2006) Estrogenic effects of genestein on reproductive tissues of ovariectomized gilts. *J Anim Sci* **84**: 834–42.

Foster PMD (2006) Disruption of reproductive development in male rat offspring following in utero exposure to phthalate esters. *Internat J Androl* **29**(1): 140–7.

França LR, Avelar GF, Almeida FFL (2005) Spermatogenesis and sperm transit through the epididymis in mammals with emphasis on pigs. *Theriogenology* **63**: 300–18.

Genuth SM (2004a) General principles of endocrine physiology. In *Physiology*, Berne RM, Levy MN, Koeppen BM, Stanton BA (eds), 5th edn. Mosby, Inc., St. Louis, MO, pp. 719–42.

Genuth SM (2004b) The reproductive glands. In *Physiology*, 5th edn, Berne RM, Levy MN, Koeppen BM, Stanton BA (eds). Mosby, Inc., St. Louis, MO, pp. 920–78.

Ginther OJ (1992) *Reproductive Biology of the Mare: Basic and Applied Aspects*, 2nd edn. Equiservices, Cross Plains, WI.

Gojmerac T, Pleadin J, Zuric M, Rajkovic-Janje R, Korsic M (2004) Serum luteinizing hormone response to administration of gonadotropin-releasing hormones to atrazine-treated gilts. *Vet Hum Toxicol* **46**(5): 245–7.

Golub MS (ed.) (2006a) *Metals, Fertility and Reproductive Toxicity.* CRC Press and Taylor and Francis Group, LLC, Boca Raton, FL, pp. 920–78.

Golub MS (2006b) Reproductive toxicity of mercury, arsenic and cadmium. In *Metals, Fertility and Reproductive Toxicity*. CRC Press and Taylor and Francis Group, LLC, Boca Raton, FL, pp. 6–22.

Gray Jr LE, Wilson VS, Stoker T, Lambright C, Furr J, Noriega N, Howdeshell K, Ankley GT, Luillette L (2006) Adverse effects of environmental antiandrogens and androgens on reproductive development in mammals. Environment, reproductive health and fertility. *Internat J Androl* **29**(1): 96–104.

Gregus Z, Klaassen CD (2001) Mechanisms of toxicity. In *Casarett and Doull's Toxicology: The Basic Science of Poisons*, 6th edn, Klaassen CD (ed.). McGraw-Hill, New York, pp. 35–81.

Grün F, Blumberg B (2006) Environmental obesogens: organotins and endocrine disruption nuclear receptor signaling. *Endocrinology* **147**(6) (Suppl.): S50–5.

Guillette Jr LJ (2006) Environmental disrupting contaminants-beyond the dogma. *Environ Health Perspect* **114**(S-1): 9–12.

Hafez B, Hafez ESE (eds) (2000) *Reproduction in Farm Animals*, 7th edn. Lipincott Williams & Wilkins, Philadelphia, PA, pp. 1–509.

Hassan ME, Smith GW, Ott RS, Faulkner DB, Firkins LD, Ehrhardt EJ, Schaffer DJ (2004) Reversibility of the reproductive toxicity of gossypol in peripubertal bulls. *Theriogenology* **16**(6): 1171–9.

Hayes TB, Stuart AA, Mendoza M, Collins A, Noriega N, Vonk A, Johnston G, Liu R, Kpodzo D (2006) Characterization of atrazine-induced gonadal malformations in African clawed frogs (*Xenopus laevis*) and comparisons with effects of an androgen antagonist (cypterone acetate) and exogenous estrogen (17-estradiol): support for the demasculinization/feminization hypothesis. *Environ Health Perspect* **114**(S-1): 134–41.

Hedrich HJ, Bullock G (eds) (2004) *The Laboratory Mouse*. Elsevier Academic Press, Boston, MA, pp. 1–600.

Hess RA (2003) Estrogen in the adult male reproductive tract: a review. *Reprod Biol Endocrinol* **1**: 52–65.

Hess RA, Iguchi T (2002) Role of herbicides and pesticides on endocrine disruption. In *Proceedings of Annual Conference of the Society for Theriogenology and American College of Theriogenologists*. Colorado Springs, CO, pp. 443–52.

Hodgson E, Mailman RB, Chambers JE, Dow RE (eds) (2000) *Dictionary of Toxicology*, 2nd edn. Grove's Dictionaries Inc., New York, pp. 1–504.

Hood RD (ed.) (2006) *Developmental and Reproductive Toxicology: A Practical Approach*, 2nd edn. CRC Press and Taylor and Francis Group, LLC, Boca Raton, FL, pp. 1–1149.

Hood RD, Rousseaux CG, Blakely PM (2002) Embryo and fetus. In *Handbook of Toxicologic Pathology*, vol. 2, Haschek WM, Rousseaux CG, Wallig MA (eds). Academic Press, San Diego, CA, pp. 895–936.

Hoyer PB (2006) Impact of metals on ovarian function. In *Metals, Fertility and Reproductive Toxicity*, Golub MS (ed.). CRC Press and Taylor and Francis Group, LLC, Boca Raton, FL, pp. 155–73.

Hughes IA, Martin H, Jääskeläinen J (2006) Genetic mechanisms of fetal male undermasculinization: a background to the role of endocrine disruptors. *Environ Res* **100**: 44–9.

Jana NR, Sarkar S, Ishizuka M, Yonemoto J, Tohyama C, Stone H (1999) Cross-talk between 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and testosterone signal transduction pathways in LNCaP prostate cancer cells. *Biochem Biophys Res Commun* **256**: 462–6.

Jobling S, Tyler CR (2006) The ecological relevance of chemically induced endocrine disruption in wildlife. *Environ Health Perspect* **114**(S-1): 1–160.

Johnston SD, Root MV, Olson PNS (eds) (2002) *Canine and Feline Theriogenology*. W.B. Saunders, Philadelphia, PA, pp. 1–592.

Jørgensen N, Asklund C, Carlsen E, Skakkebæk NE (2006a) Coordinated European investigations of semen quality: results from studies of Scandinavian young men is a matter of concern. *Internat J Androl* **29**(1): 51–9.

Jørgensen N, McGrigor K, Toppari J, Skakkebæk NE (eds) (2006b) Environment, reproductive health and fertility. *Internat J Androl* **29**(1): 1–312.

Katzenellenbogen BS, Katzenellenbogen JA (2000) Estrogen receptor transcription and transactivation: estrogen receptor alpha and estrogen receptor beta: regulation by selective estrogen receptor modulators and importance in breast cancer. *Breast Cancer Res* **2**: 335–44.

Keith LH (1997) *Environmental Endocrine Disruptors: A Handbook of Property Data*. John Wiley & Sons, Inc., New York, pp. 1–1232.

Kharat I, Saatcioglu F (1996) Antiestrogenic effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin are mediated by direct transcriptional interference with the liganded estrogen receptor. *J Biol Chem* **271**(18): 10533–7.

Knobil E (1999) Chair, Committee on Hormonally Active Agents in the Environment. *Hormonally Active Agents in the Environment*. National Academy Press, Washington, DC, pp. 1–430.

Krimsky S (2000) *Hormonal Chaos: The Scientific and Social Origins of the Environmental Endocrine Hypothesis*. Johns Hopkins University Press, Baltimore, MD, pp. 1–284.

Krimsky S (2001) An epistemological inquiry into the endocrine disruptor thesis. In *Environmental Hormones: The Scientific Basis of Endocrine Disruption*, McLachlan JA, Guillette LJ, Iguchi T, Toscano Jr WA (eds). *Annals NY Acad Sci* **948**: 130–42.

Kubota K, Ohsako S, Kurosawa S, Takeda K, Qing W, Sakaue M, Kawakami T, Ishimura R, Tohyama C (2003) Effects of vinclozolin administration on sperm production and testosterone biosynthetic pathway in adult male rat. *J Reprod Develop* **49**: 403–12.

Lewin B (1998) *Genes*, 6th edn. Oxford University Press, New York, pp. 1–1260.

Lundholm CD (1997) DDE-induced eggshell thinning in birds; effects of p,p-DDE on the calcium and prostaglandin metabolism of the eggshell gland. *Comp Biochem Physiol C Pharmacol Toxicol Endocrinol* **118**(2): 113–28.

Mahood IK, Hallmark N, McKinnell C, Walker M, Fisher JS, Sharpe RM (2005) Abnormal Leydig cell aggregation in the fetal testis of rats exposed to di (*n*-butyl) phthalate and its possible role in testicular dysgenesis. *Endocrinology* **146**(2): 613–23.

Mahood IK, McKinnell C, Walker M, Hallmark N, Scott H, Fisher JS, Rivas A, Hartung S, Ivell R, Mason JI, Sharpe RM (2006) Cellular origins of testicular dysgenesis in rats exposed in utero to di(*n*-butyl) phthalate. *Internat J Androl* **29**(1): 148–54.

McCue PM (1993) Lactation. In *EquineReproduction*, McKinnon AO, Voss JL (eds), Lea & Febiger, Philadelphia, pp. 588–95.

McLachlan JA (2001) Environmental signaling: what embryos and evolution teach us about endocrine disrupting chemicals. *Endocrine Rev* **22**(3): 319–41.

McLachlan JA, Simpson E, Martin M (2006) Endocrine disrupters and female reproductive health. *Best Pract Res Clin Endocrinol Metab* **20**(1): 63–75.

Milnes MR, Bermudez DS, Bryan TA, Edwards TM, Gunderson MP, Larkin ILV, Moore BC, Guillette LJ Jr (2006) Contaminant-induced feminization and demasculinization of nonmammalian vertebrate males in aquatic environments. *Environ Res* **100**(1), 3–17.

Monosson E, Kelce WR, Lambright C, Ostby J, Gray Jr LE (1999) Peripubertal exposure to the antoandrogenic fungicide, vinclozolin, delays puberty, inhibits the development of androgen-dependent tissues, and alters androgen receptor function in the male rat. *Toxicol Ind Health* **15**: 65–79.

Morgan S (2004) Gossypol. In *Clinical Veterinary Toxicology*, Plumlee KH (ed.). Mosby, Inc., St. Louis, MO, pp. 119–20.

Mukerjee D (ed.) (2006) Endocrine disruptors. *Environ Res* **100**(1): 1–99.

Naz RK (ed.) (2005) *Endocrine Disruptors: Effects on Male and Female Reproductive Systems*, 2nd edn. CRC Press and Taylor and Francis Group, LLC, Boca Raton, FL, pp. 1–444.

Neill JD, Nagy GM (1994) Prolactin secretion and its control. In *The Physiology of Reproduction*, 2nd edn, Knobil E, Neill JD (eds). Raven Press, New York, pp. 1833–60.

Newbold RR, Padilla-Banks E, Snyder RJ, Jefferson WN (2005) Developmental exposure to estrogenic compounds and obesity. *Birth Defects Res A Clin Mol Teratol* **73**: 478–80.

Newbold RR, Padilla-Banks E, Jefferson WN (2006) Adverse effects of the model environmental estrogen diethylstilbestrol are transmitted to subsequent generations. *Endocrinology* **147**(6) (Suppl.): S11–17.

O'Connor JC, Frame SR, Ladics GS (2002) Evaluation of a 15-day screening assay using intact male rats for identifying antiandrogens. *Toxicol Sci* **69**(1): 92–108.

O'Donnell L, Robertson KM, Jones ME, Simpson ER (2001) Estrogen and spermatogenesis. *Endocrine Rev* **22**(3): 229–318.

Ohtake F, Takeyama K, Matsumoto T, Kitagawa H, Yamamoto Y, Nohara K, Tohyama C, Krust A, Mimura J, Chambon P, Yanaglsawa J, Fuji-Kuriyama Y, Kato S (2003) Modulation of oestrogen receptor signalling by association with the activated dioxin receptor. *Nature* **423**: 545–50.

Orlando EF, Kolok A, Binzcik GA, Gates JL, Horton MK, Lambright CS, Gray Jr LE, Soto AM, Guillette Jr LJ (2004) Endocrine-disrupting effects of cattle feedlot effluent on an aquatic sentinel species, the fathead minnow. *Environ Health Perspect* **112**: 353–8.

Panter KE (2002) Plant and chemical teratogens. In *Proceedings of Annual Conference of the Society for Theriogenology and American College of Theriogenologists*. Colorado Springs, CO, pp. 463–72.

Parkinson A (2001) Biotransformation of xenobiotics. In *Casarett and Doull's Toxicology: The Basic Science of Poisons*, 6th edn, Klaassen CD (ed.). McGraw-Hill, New York, pp. 133–224.

Petersen SL, Krishnan S, Hudgens ED (2006) The aryl hydrocarbon receptor pathway and sexual differentiation of neuroendocrine functions. *Endocrinology* **147**(6)(Suppl.): S33–42.

Plumlee KH (ed.) (2004) *Clinical Veterinary Toxicology*. Mosby, Inc., St. Louis, MO, pp. 1–477.

Pratt WB, Toft DO (1997) Steroid receptor interactions with heat shock proteins and immunophilin chaperones. *Endocrine Rev* **18**(3): 306–60.

Randel RD, Chase Jr CC, Wyse SJ (1992) Effects of gossypol and cottonseed products on reproduction of mammals. *J Anim Sci* **70**(5): 1628–38.

Razandi M, Pedram A, Greene GL, Levin ER (1999) Cell membrane and nuclear estrogen receptors (ERs) originate from a single transcript: studies of ER$\alpha$ and ER$\beta$ expressed in Chinese hamster ovary cells. *Mol Endocrinol* **13**(2): 307–19.

Rogers JM, Kavlock RJ (2001) Developmental toxicology. In *Casarett and Doull's Toxicology: The Basic Science of Poisons*, 6th edn, Klaassen CD (ed.). McGraw-Hill, New York, pp. 351–86.

Rozman KK, Klaassen CD (2001) Absorption, distribution and excretion of toxicants. In *Casarett and Doull's Toxicology: The Basic Science of Poisons*, 6th edn, Klaassen CD (ed.). McGraw-Hill, New York, pp. 107–132.

Safe S (2005) 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and related environmental antiandrogens: characterization and mechanism of action. In *Endocrine Disruptors: Effects on Male and Female Reproductive Systems*, 2nd edn, Naz RK (ed.). CRC Press and Taylor and Francis Group, LLC, Boca Raton, FL, pp. 249–87.

Schrader SM (2002) Sites of toxicant action of male reproductive toxicants. In *Proceedings of Annual Conference of the Society for Theriogenology and American College of Theriogenologists*. Colorado Springs, CO, pp. 437–42.

Senger PL (2003) *Pathways to Pregnancy and Parturition*, 2nd edn. Current Conceptions, Inc., Moscow, ID, pp. 1–368.

Sharpe RM, McKinnell C, Kivlin C, Fisher JS (2003) Proliferation and functional maturation of sertoli cells, and their relevance to disorders of testis function in adulthood. *Reproduction* **125**: 769–84.

Sikka SC, Kendirci M, Naz R (2005) Endoctine disruptors and male infertility. In *Endocrine Disruptors: Effects on Male and Female Reproductive Systems*, 2nd edn, Naz RK (ed.). CRC Press and Taylor and Francis Group, LLC, Boca Raton, FL, pp. 291–312.

Skakkebæk NE, Rajpert-de Meyts E, Main KM (2001) Testicular dysgenesis syndrome: an increasingly common developmental disorder with environmental aspects. *Hum Reprod* **16**(5): 972–8.

Skakkebæk NE, Jørgensen N, Main KM, Rajpert-de Meyts E, Leffers H, Andersson A-M, Juul A, Carlsen E, Krog Mortensen G, Kold Jensen T, Toppari J (2006) Is human fecundity declining? *Internat J Androl* **29**(1): 2–11.

Sokol RZ (2006) Lead exposure and its effects on the reproductive system. In *Metals, Fertility and Reproductive Toxicity*, Golub MS (ed.). CRC Press and Taylor and Francis Group, LLC, Boca Raton, FL, pp. 117–54.

Suckow MA, Weisbroth SH, Frankin CL (eds) (2006) *The Laboratory Rat*, 2nd edn. Elsevier Academic Press, Burlington, MA, pp. 1–912.

Sutovsky P, Moreno R, Ramahlho-Santos J, Dominko T, Thompson W (2001) A putative, ubiquitin-dependent mechanism for the recognition and elimination of defective spermatozoa in the mammalian epididymis. *J Cell Sci* **114**(9): 1665–75.

Swan SH, Elkin EP, Fenster L (2000) The question of declining sperm density revisited: an analysis of 101 studies published 1934–1996. *Environ Health Perspect* **108**(10): 961–6.

Swan SH, Brazil C, Drobnis EZ, Liu F, Kruse RL, Hatch M, Redmon JB, Wang C, Overstreet JW (2003a) Geographical differences in semen quality of fertile U.S. males. *Environ Health Perspect* **111**(4): 414–20.

Swan SH, Kruse RL, Liu F, Barr DB, Drobnis EZ, Redmon JB, Wang C, Brazil C, Overstreet JW (2003b) Semen quality in relation to biomarkers of pesticide exposure. *Environ Health Perspect* **111**(12): 1478–84.

Swan SH, Main KM, Liu F, Stewart SL, Kruse RL, Calafat AM, Mao CS, Redmon JB, Ternand CL, Sullivan S, Teague JL (2005) Decrease in anogenital distance among male infants with prenatal phthalate exposure. *Environ Health Perspect* **113**(8): 1056–61.

Thomas JA (1995) Gonadal-specific metal toxicology. In *Metal Toxicology*, Goyer RA, Klaassen CD, Waalkes MP (eds). Academic Press, Inc., San Diego, CA, pp. 413–36.

Thomas MJ, Thomas JA (2001) Toxic responses of the reproductive system. In *Casarett and Doull's Toxicology: The Basic Science of Poisons*, 6th edn, Klaassen CD (ed.). McGraw-Hill, New York, pp. 673–709.

Thomas P, Khan IA (2005) Disruption of nongenomic steroid actions on gametes and serotonergic pathways controlling reproductive neuroendocrine function by environmental chemicals. In *Endocrine Disruptors: Effects on Male and Female Reproductive Systems*, 2nd edn, Naz RK (ed.). CRC Press and Taylor and Francis Group, LLC, Boca Raton, FL, pp. 3–45.

Tsai M-J, O'Malley BW (1994) Molecular mechanisms of action of steroid/thyroid receptor superfamily members. *Annu Rev Biochem* **63**: 451–86.

Tucker A (1994) Lactation and its hormonal control. In *The Physiology of Reproduction*, 2nd edn, Knobil E, Neill JD (eds). Raven Press, New York, pp. 1065–98.

Velasquez-Pereira J, Chenoweth PJ, McDowell LR, Risco CA, Williams SN, Wilkinson NS (1998) Reproductive effects of feeding gossypol and vitamin E to bulls. *J Anim Sci* **76**: 2894–904.

Warner M, Gustafsson J-A (2006) Nongenomic effects of estrogen: why all the uncertainty? *Steroids* **71**: 91–5.

Welshons WV, Nagel SC, vom Saal FS (2006) Large effects from small exposures. III. Endocrine mechanisms mediating effects of bisphenol A at levels of human exposure. *Endocrinology* **147**(6) (Suppl.): S56–69.

Wilker CE, Ellington JE (2006) Reproductive toxicology in the female companion animal. In *Small Animal Toxicology*, 2nd edn, Peterson ME, Talcott PA (eds). Elsevier Saunders, St. Louis, MO, pp. 475–99.

Wilson JG (1977) Current status of teratology: general principles and mechanisms derived from animal studies. In *Handbook of Teratology*, vol. 1, Wilson JG, Clarke Foster F (eds). Plenum Press, New York, pp. 47–74.

Wong C, Kelce WR, Sar M, Wilson EM (1995) Androgen receptor antagonist versus agonist activities of the fungicide vinclozolin relative to hydroxyflutamide. *J Biol Chem* **270**: 19998–20003.

Youngquist RS, Threlfall (eds) (2007). *Current Therapy in Large Animal Theriogenology*, 2nd edn, Saunders Elsevier, St. Louis, pp. 1–1061.

YuanY-D, Foley GL (2002) Female reproductive system. In *Handbook of Toxicologic Pathology*, vol. 2, Haschek WM, Rousseaux CG, Wallig MA (eds). Academic Press, San Diego, CA, pp. 847–94.

C H A P T E R

# 15

# Placental toxicity

*Ramesh C. Gupta*

## INTRODUCTION

Placental toxicology embraces the knowledge of the responses of placenta and fetus to chemical insult. It is a fascinating subject, as it deals with three components: mother, placenta, and fetus. The subject includes structural and functional changes in the placenta, placentation, implantation, embryotoxicity, fetal death, structural malformation, growth retardation, and functional deficits. Placental toxicology has blossomed and matured in the past several decades from the contributions of scientists around the world. It has received an enormous attention from the pharmacologists, toxicologists, teratologists, biologists, and regulatory agencies, since the incidence of thalidomide in the 1960s.

The placenta is a rapidly growing organ, with a limited lifespan, which interfaces two separate genomes (mother and fetus). During the pregnancy, placenta serves many functions and undergoes numerous changes, such as the production and release of hormones and enzymes, transport of nutrients and waste products, chemical information flows between mother and fetus, implantation, cellular growth and maturation, and at the terminal phase of placental life, participation in delivery (Pelkonen *et al*., 2006). In other words, the placenta plays many roles in the development of the fetus by serving as the lung, kidney, gut, and exocrine/endocrine glands. Its role in providing nutrients to the developing fetus through maternal circulation has been established for a long time. Unfortunately, some of the toxic metals, such as cadmium and lead, are also delivered to the fetus by the same mechanism as that of the nutrients. In fact, many foreign substances, including pesticides, plant alkaloids, and drugs reach the fetus with a very little or no restrictions.

The health of the placenta is a prerequisite for the health of the fetus. In the toxicant-exposed mother, the chemical encounters the placenta before it reaches the fetus. Therefore, the chemical-induced damage in the placenta is likely to reflect in the fetus. In essence, the health of the placenta seems as important as the health of the mother or the fetus. By now, it is well established that the placenta is a target organ for toxicity of a wide range of poisons. Vulnerability of this organ to toxic effects is great, since it has a considerable amount of metabolic activity, and as a result the placenta can enhance the toxicity by forming metabolites of greater toxicity than the parent compounds.

Literature abounds with ample evidence that the poisons of many classes adversely affect the mother and the fetus, however it is sparse for the placenta. Many toxicants adversely affect fetal growth or survival at doses that do not produce any toxic effects in the mother. In other words, the absence of toxic effects in the mother does not preclude toxicity in the fetus. The evidence also suggests that malformations result from specific action of chemicals, rather than as a general response to maternal illness. Another important concern that needs to be mentioned is that the mechanism involved in maternal toxicity most often differs from the mechanism involved in fetotoxicity and teratogenicity. Thus, the placenta appears to be a complex organ, as its anatomical, physiological, and metabolic characteristics vary depending on the age of the placenta. This would make it important to study placentas from different stages of pregnancy. It is beyond the scope of this chapter to discuss the placental toxicity of every single chemical, instead this chapter is focused on common poisons, such as metals, pesticides, mycotoxins, tobacco, etc. that adversely affect pregnant animals, placenta, and fetus.

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## ROLE OF THE PLACENTA

The placenta serves pivotal roles in fetal nutrition, respiration, and excretion. Placenta also provides protection to the fetus. This organ has a limited lifespan, as the umbilical cord, placenta, amnion, and chorion are expelled soon after the birth of the fetus. For the decades, the view of the pharmacologists, toxicologists, teratologists, and biologists has been that the placenta is a rejected tissue and may not be suitable for bioassay or biocomparative assays of drugs and evaluation of the cellular/molecular mechanisms of actions of drugs and toxic chemicals. But, due to substantial progress in placental pharmacology/toxicology research, the view has been changed. Currently, several methods are available to use different parts of the placenta in screening drugs and chemicals and to study the molecular mechanisms for their pharmacological and toxicological effects. Use of pregnant animals is considered the preferred model to assess the toxicological actions of chemicals. In such circumstances, it is important to study placentas from different stages of pregnancy. However, obtaining material for such studies is difficult for practical reasons. Furthermore, in assessing placental toxicity of chemicals, all physiological and anatomical variables should be taken into consideration. In addition, there are several *in vitro* pharmacological/toxicological preparations from the placenta, which include: (1) perfused single placental cotyledon; (2) villus preparation; (3) segments of umbilical, chorionic plate and villus stem arteries and veins; (4) trophoblast plasma membrane; (5) isolated receptors and transporters; and (6) stem cells from umbilical blood. For further details, refer to Schneider (1995), Sastry (1997), and Pelkonen *et al*. (2006).

## TYPES OF PLACENTA

The placentas of different species vary in shape, internal architecture, and nature of the interhemal barrier. Anatomically, the placenta is a complex organ and placentas are classified into four types: (1) hemochorial (rat, rabbit, human); (2) endotheliochorial (cat, dog); (3) syndesmochorial (ruminants); and (4) epitheliochorial (pig, horse). The placenta has also been described as zonary in the dog, bidiscoid in the monkey, and multicotyledonary in the sheep. The placental thickness depends on the number of fetal and maternal cell layers. For example, the rat and rabbit have a single layer of cells, primates and humans have three layers, and pigs and horses have six layers. In addition to anatomical, there are physiological and functional differences among different types of placenta. Because of technical and practical advantages, animal placentas have been used to study placental function, and pharmacology and toxicology. However, the hemochorial placenta has been studied more extensively compared to other types. In hemochorial placenta, the membrane separating the maternal and fetal compartments are consist of three layers (syncytiotrophoblast, connective tissue, and vascular fetal endothelium). These anatomical and physiological similarities and differences in placentas of different species should be taken into consideration when studying and evaluating placental toxicity.

## METABOLISM IN PLACENTA

Both phase I reactions (oxidation, reduction, and hydrolysis) and phase II reactions (conjugation of a chemical with endogenous moiety, such as glucuronic acid or sulfate) of metabolism exist in the placenta as well as in fetus. Placenta can also be active in intermediary metabolism, such as gluconeogenesis, urea, and fatty acid synthesis. The capacity for metabolic processes appears to vary with species, gender, and gestational stage. Carbonic anhydrase activity, a marker of placental metabolism and transfer, is present much greater in pig, rat, and mink than in horse, cow, and human. Xenobiotic metabolism in the placenta seems to be similar to that in maternal tissues, but the extent of the metabolic activity is usually less. However, the metabolic activity is still enough that the placental–fetal toxicity of chemicals can be significantly modified. The placenta has an abundance of drug- and xenobiotic-metabolizing enzyme system, i.e., cytochrome P450. Cyt. P450 exists in multiple forms with distinct, but generally overlapping, substrate specificities and many of the isoforms are inducible by exposure to exogenous agents (Pasanen and Pelkonen, 1994; Juchau, 1995). In the placenta, several steroid hormones are formed and metabolized by Cyt. P450 systems, which may participate in xenobiotic metabolism (Gonzalez, 1989). In general, the metabolites produced by biotransformation are usually less toxic or inactive compared to the parent compound, and thereby the xenobiotic-metabolizing enzymes in the placenta protect the fetus from potentially fetotoxic drugs and chemical toxicants. It is noteworthy, that the same enzyme system can also form metabolites which are more toxic than their parent compounds. For example, oxon metabolites of organophosphorus insecticides and epoxides of cyclodiene chlorinated hydrocarbons have a greater potential for fetotoxicity/teratogenicity than their parental forms. In addition, placental metabolism has the capacity to form carcinogen-DNA adducts. For further details, see comprehensive reviews (Pelkonen, 1984; Pasanen and Pelkonen, 1994; Juchau, 1995). Compared to phase I, phase II reactions

in the placenta have not been studied in detail because the placenta contains very low activities of these enzymes (Juchau, 1980, 1995).

## PLACENTAL SUSCEPTIBILITY TO CHEMICAL TOXICANTS

Placenta is a unique tissue that interfaces two separate genomes, i.e., mother and fetus. Since the placenta serves as an exchanger between mother and fetus for nutrients, the health of the fetus seems to be dependent on the health of the mother and placenta. There appear to be several mechanisms by which toxicants are concentrated in the placenta and fetal tissues in greater quantities than in the maternal tissues. The large placental surface area comes in contact with a relatively large volume of maternal blood, which is required for normal placental function, and that makes the placenta vulnerable to toxicants (Eisenmann and Miller, 1996). In addition, the placenta has several biomolecules, such as proteins, lipids, carbohydrates, nucleic acids/nucleotides, and drug metabolizing and many other enzymes. The placenta, being rich in proteins, bioconcentrates chemical residues by means of protein binding and release them into the placental circulation and ultimately into the fetus. In essence, the structure and function of the placenta are so unique that its susceptibility to chemical toxicity seems far greater than that of the mother. Thus, any damage to the placenta caused by a toxic chemical is likely to reflect in the fetus.

## PLACENTAL BARRIER FOR CHEMICAL TOXICANTS

The placenta is the entry through which the fetus is exposed to most chemical poisons, and the term placental barrier appears to be a false notion. The placenta has been characterized as a lipid membrane that permits bidirectional transfer of substances between maternal and fetal compartments rather than as a barrier. In general, the placental barrier consists of the trophoblastic epithelium covering the villi, the chorionic connective tissue, and the fetal capillary endothelium. Foreign chemicals are transferred through placental membranes by passive diffusion. The two most common factors that are involved in transplacental transfer of common toxicants are: (1) physicochemical properties of the chemical and (2) type of placenta. In general, any chemical with a molecular weight $<1000$ readily crosses the placenta, and therefore the majority of the chemical toxicants are not restricted from reaching the fetus. It is important to mention that chemical properties, such as lipophilicity, polarity, and degree of ionization, can influence the placental barrier (Pacifici and Nottoli, 1995). The second factor that predominately influences the transplacental transfer of chemical toxicants is the type of placenta. For instance, the complex multilayered placenta of higher mammals can make it more difficult for chemicals to gain access to the fetus compared to the simpler choriovitelline or chorioallantoic type of placenta (Welsch, 1982; Juchau, 1995; Ala-Kokko *et al*., 2000). A number of other factors that can influence the rate and extent of transplacental transfer of chemical toxicants include: (1) maternal–fetal chemical gradient, (2) uterine and umbilical blood flow, (3) molecular weight of the chemical, (4) protein binding, and (5) lipid solubility. These factors can also determine the time required for maternal–fetal equilibrium of a toxicant. Direct or indirect evidence for placental transfer of chemical toxicants is determined based on residue analysis of these compounds and/or their metabolites in the placenta, umbilical cord, and embryo/fetus.

## FACTORS THAT MAY INFLUENCE THE PLACENTAL TOXICITY

Anatomical, physiological, and metabolic characteristics vary depending on the stage of the placenta. There are three major factors, which can modify the overall toxicity of a chemical during pregnancy: (1) maternal toxicity, (2) placental transfer of a chemical, and (3) placental–fetal metabolism.

### Maternal toxicity

Maternal toxicity can be defined as the transitory or permanent state of alteration in maternal physiology or behavior with a potential to cause adverse effect in the offspring during embryo/fetal or postnatal development. The common factors related to maternal toxicity are as follows:

1 route of drug or chemical exposure;
2 maternal drug or chemical distribution;
3 maternal drug or chemical metabolism;
4 uterine blood flow;
5 pH of the blood.

Although the precise mechanism by which maternal toxicity factors are responsible for fetal toxicity or teratogenesis has remained unexplained, alterations in placental function appear to be important. It seems that maternal

toxicity plays a major role in adverse fetal outcome by modifying placental function.

### Placental transfer of toxicants

The factors related to placental morphology, physiology, and metabolism seem to be interrelated, and with continuing change they become more complex as gestation advances. The important placental transfer factors include:

1. placental blood flow;
2. pH of the blood;
3. placental permeability (passive or active transport system);
4. placental maturity over gestation period (size, surface area, and thickness);
5. interspecies variation in placental morphology;
6. lipid–protein content of the membranes;
7. placental metabolism;
8. plasma protein binding;
9. physicochemical properties of toxicants.

Plasma protein (mainly albumin) binding appears to be the important factor that can modify placental toxicity. A chemical can cross the placenta only if the chemical is in free form, but a protein-bound form can be released into free form, since the protein binding is a reversible process. Once the chemical has reached the fetus, either it remains free to produce toxic effects, or once again it can bind to proteins of blood or fetal tissues, a phenomenon described as the "sink effect."

### Placental–fetal metabolism

Placental–fetal metabolism appears to be one of the three major factors that can alter the placental toxicity of poisonous substances. Metabolism occurs in all placental and fetal tissues, as it occurs in maternal tissues, but the capacity differs at all stages of gestation. During pregnancy, metabolism of the toxicant is complicated by two major factors: (1) the pregnancy itself, since the general metabolic activity is significantly low, which may lower the degradation of toxicants and thereby increase the toxicity and (2) pre-exposure or simultaneous exposure to other chemicals or environmental pollutants generally results in either reduced or enhanced metabolism, and consequently, alters the placental toxicity. For example, previous exposure to drug-metabolizing enzyme inducers, such as enhanced monooxygenase activity by polychlorinated biphenyls (PCBs) or similar environmental pollutants, can potentiate the toxicity of the "thioate" type of organophosphorus insecticides.

## PLACENTAL TOXICITY OF METALS

Trace minerals and heavy metals are common contaminants of the environment due to their ubiquitous presence, and thereby pose a serious threat to the environment as well as human and animal health. In excessive concentrations, these minerals and metals are known to adversely affect pregnancy and the development of the conceptus, in addition to pregnant mother. Placental toxicity of some important metals is described here in brief. It should be noted that much of the pertinent literature mentioned here is from laboratory animals.

### Aluminum

Aluminum (Al) commonly contaminates food and water, as it is one of the three most abundant metals in the earth's crust. Al can be absorbed through oral, dermal, inhalation, and parenteral routes, and concentrated in the liver, lungs, bone, kidney, spleen, heart, brain, and muscles. Al also transplacentally traverses and accumulates in the fetal tissues in amounts that adversely influence fetal development (Yokel and McNamara, 1985). Studies conducted in mice and rabbits revealed that the placenta contained 4–5 times greater Al levels than the fetal or maternal tissues (Yokel and McNamara, 1985; Cranmer *et al.*, 1986). However, the placenta of the guinea pig does not accumulate this metal. It is important to mention that the placental accumulation of Al in mice and rabbits does not preclude its accumulation in the fetal tissues.

For the detailed toxicokinetics of Al, readers are referred to the review of Yokel (1997).

Pregnancy in general enhances susceptibility to Al toxicity. Following oral administration, only a small fraction of Al is absorbed, and therefore the degree of severity of developmental toxicity is often minimized. However, based on some animal and human studies, it is likely that the oral absorption of aluminum can vary 10-fold based on chemical form alone, ranging from approximately 0.1% for relatively non-bioavailable water insoluble forms such as aluminum hydroxide to relatively bioavailable water soluble forms such as aluminum citrate. Animal studies suggest that Al accumulates in the brain and is preferentially distributed to the hippocampus, the region which is most relevant to memory and Alzheimer's disease in humans. Hippocampus can have approximately 32 times higher Al than that in the cortex and cerebellum. Animal studies also indicate that Al has the potential to cross the placenta and accumulate in the fetus.

Oral administration of Al is known to cause a developmental syndrome, which includes *in utero* death, delayed ossification of skeleton, malformations, and growth retardation, at doses that also influence maternal weight gain.

An increased incidence of resorptions occurred in female BALB/c mice treated with 41 mg/kg/day as Al chloride by gavage on GD 7–16 (Cranmer *et al.*, 1986). Gestational exposure of mice to Al lactate (83 mg/kg/day) caused increased incidences of cleft palate, dorsal hyperkyphosis, and delayed parietal ossification. Skeletal changes, such as delayed ossification and hypoplastic deformed ribs, were induced in rats exposed to 38–77 mg Al/kg/day by gavage on GD 6–14 (Patermain *et al.*, 1988). The severity of the effects is highly dependent on the form of Al administered. Studies using high Al exposures obtained by intravenous (i.v.) or intraperitoneal (i.p.) administration report a developmental toxicity syndrome consisting of death and resorptions, skeletal and soft tissue abnormalities, low birth weights, and growth retardation in rats, mice, and rabbits (Wide, 1984; Yokel, 1997). The high incidence of resorptions was found to be significantly higher in pregnant rats treated with Al chloride at dose level of 75 mg/kg on GD 9–13, 100 mg/kg on GD 14–18, and 200 mg/kg on GD 9–13 (Benett *et al.*, 1975). A high incidence of dead offspring was recovered from mothers treated with Al chloride at a dose level of 200 mg/kg on GD 9–13. These studies indicate that Al can cause delays in skeletal development in pups. Gross fetal abnormalities include abnormal digits, wavy ribs, missing ribs, absence of xiphoid, and poor ossification (particularly in the cranial bones, lower part of the vertebral column, and the long bones of the limbs). The high incidence of skeletal defects, and also poor ossification in fetuses of mothers treated with Al chloride suggest that Al has an adverse influence on fetal bone formation. This is due to the fact that Al binds with phosphate, thereby reducing the amount of phosphate available for bone formation. Depletion of phosphate can also result in fetal internal hemorrhage, due to failure of the blood clotting mechanism. Interestingly, neurotoxicity and neurobehavioral changes have been noted in offspring following Al exposure of rats, mice, and rabbits at doses of Al that did not produce maternal toxicity (Wide, 1984; Domingo, 1994; Yokel, 1997). Other forms of Al, such as Al citrate and Al nitrate, can also produce similar developmental effects. Overall, developing conceptuses are more sensitive than adults to Al toxicity.

## Arsenic

Arsenic (As) occurs in many forms and commonly found in high concentrations in water and food. Both animal and human studies have shown that As crosses the placenta. Inorganic As crosses the placental barrier and selectively accumulates in the neuroepithelium of the developing animal embryo. Uptake, distribution, and metabolism of sodium arsenite (2.5 μg/kg, po) and sodium arsenate (40 μg/kg, po) were determined in pregnant mice on GD 18 (Hood *et al.*, 1987). Maximum concentrations of As in the placenta were found at 4 and 2 h after administration of arsenite and arsenate, respectively. Corresponding concentrations in the fetal tissues appeared at 24 and 6 h. In the fetal tissues, inorganic As is converted to the organic form and methylation appears to be the major detoxification mechanism. As is completely eliminated from the placental/fetal tissues within 24 h of exposure. Unlike inorganic As, organic As does not cross the placenta, instead it is stored in the placenta. In other studies conducted on hamsters, rats, and mice, As ingested at higher doses has been found to be fetotoxic and teratogenic. There is evidence that sodium arsenate and sodium arsenite are embryotoxic (Chaineau *et al.*, 1990). Common deformities include hypoplasia of the prosencephalon, somite abnormalities, and failure of development of limb buds and sensory placodes. In hamsters, a single i.v. injection of sodium arsenate (20 mg/kg) given on GD 8 resulted in 49% malformed and 84% either malformed or resorbed embryos on GD 13 (Holmberg and Ferm, 1969). Common malformations included exencephaly, encephalocele, cleft lip/palate, micro/anophthalmia, and ear malformations. In rats, the spectrum of malformations induced by As is similar to that described for hamsters. Placental/fetal toxic effects of As have been more extensively described in mice than in rats and hamsters. Exposure of female mice with arsenite/arsenate on GD 9 produces the highest incidence of malformed live fetuses, whereas exposure on GD 11 or 12 produces the highest rate of resorptions. The common external malformations include exencephaly, micrognathia, exophthalmia, anophthalmia, cleft lip, hydrocephalus, micromelia, ectrodactyly, open eyes, rib defects, and vertebral defects. Rats treated with a single gavage dose of 23 mg As/kg as As trioxide on day 9 of gestation had a significant increase in post-implantation loss and a decrease in viable fetuses per litter, while those treated with 15 mg/kg showed no effects (Stump *et al.*, 1999). Rats and mice are much more sensitive than hamsters to organoarsenicals, in terms of placental toxicity. But the most sensitive species is the rabbit, in which 1.5 mg As/kg/day can increase resorptions and decrease viable fetus.

Although the exact mechanism involved in placental toxicity of As is not yet explained, As is known to interact with protein sulfhydryl groups, thereby adversely affecting the activity of many enzymes, including glutamic–oxaloacetic transaminase, pyruvate oxidase, monoamine oxidase, choline oxidase, glucose oxidase, urease, oxidoreductases, and kinases. As has also been shown to adversely affect a number of enzymes that are involved in mitochondrial respiration (Rogers, 1996). In addition, As inhibits succinic dehydrogenase activity and thereby uncouples oxidative phosphorylation and causes fall of ATP levels affecting virtually all cellular functions ($Na^+/K^+$ balance, protein synthesis, etc.). Since As is known to affect vasculature and altered placental and/or embryonal vasculature has been suggested as a mechanism leading to

neural tube defects, the embryo may be sensitive to this manifestation of As toxicity. At high dose, As impairs assembly and disassembly of microtubules, thus interfering with mitotic spindle formation and embryonal cell division. As compounds also cause chromosomal aberrations, which may disrupt cell cycling. The direct toxic effects of high levels of As in the developing embryo result not from a difference in the mechanism of toxicity during development, but rather from the existence of a unique target tissue, the neuroepithelium (ATSDR, 2005). Induction of stress proteins or heat shock proteins synthesis in the embryo has been explained as a common mechanism of teratogenesis. Animal studies have also presented evidence that inorganic As may be a transplacental carcinogen.

In essence, inorganic arsenicals are much more toxic than organic arsenicals. Trivalent As is developmentally more toxic than pentavalent As. In fact, pentavalent As compounds exert their toxic actions only after their conversion to the trivalent form. Placental/fetal toxicity of As is of serious concern, especially at the levels that are maternotoxic.

## Cadmium

Cadmium (Cd) is a naturally occurring metal found in the earth's crust. Most of the Cd is produced as a byproduct during the production of other metals, such as copper, lead, and zinc. The major source of Cd exposure to animals can be from industrial pollution and environmental contamination, especially water. Much of the information obtained on developmental toxicity of Cd is from laboratory animals. By now, it is well established that the placenta itself is a target organ for Cd toxicity. In low to moderate doses, Cd is sufficiently sequestered in the placenta, whereas in high doses, Cd accumulates in the placenta, which perturbs the placental transport of essential elements (such as calcium and zinc), and causes placental necrosis and fetal toxicity. Maternal exposure to Cd is known to cause ultrastructural changes in the placenta, especially trophoblast cell layer II (DiSaint' Agnese *et al.*, 1983). Common changes include lysosomal vesiculation, nuclear chromatin clumping, nucleolar alterations, and apparent mitochondrial calcification. Cd-induced placental necrosis occurs initially in trophoblast cell layer II, and that follows rapidly in the remaining trophoblasts. Subcutaneous administration of Cd chloride, Cd acetate, or Cd lactate to pregnant rats at a dosage of 0.04 mmol/kg between the 17th and 21st day of gestation resulted in rapid, progressive placental damage, especially in the pars fetalis (Parizek, 1964). Cd-induced necrotic changes could occur as early as within 6 h.

Maternal exposure to Cd during pregnancy can result in a variety of adverse reproductive outcomes, such as maternal toxicity, placental damage/hemorrhage, impaired implantation, increased resorptions, reduced litter size, fetal growth retardation, congenital malformation in the fetuses, and embryonic/fetal death. Exposure to Cd during mid-to-late gestation can result in both placental toxicity (reduced blood flow and necrosis) and diminished nutrient transport across the placenta. Exposure during the late gestation can also result in fetal death, despite low levels of Cd entering the fetus (Levin and Miller, 1980; Rogers, 1996). Many studies conducted on rats and mice indicate that Cd can be fetotoxic from oral exposures prior and during gestation. The fetotoxicity is most often manifested as reduced fetal or pup weight, but malformations, primarily of the skeleton, have been reported in some studies. Common malformations or skeletal effects include sirenomelia (fused lower limbs), amelia (absence of one or more limbs), and delayed ossification of the sternum and ribs, dysplasia of facial bones and rear limbs, edema, exenteration, cryptorchism, and palatoschisis.

Cd has also been shown to induce teratogenesis in rats, mice, and hamsters. Cd sulfate administered to pregnant hamsters (2 mg/kg, i.v.) caused high incidence of resorptions. Live fetuses showed a high rate of malformations, including facial clefts, exencephaly, anophthalmia, limb defects, and rib fusions (Ferm and Carpenter, 1967). In rats and mice, the common malformations are club foot, dysplasia of facial bones and rear limbs, cleft palate, micrognathia, sirenomelia/amelia, delayed ossification of the sternum and ribs, microphthalmia/anophthalmia, cryptorchism, gastroschisis, and palatoschisis.

The placenta serves as a remarkable barrier to Cd, and thereby it minimizes fetal toxicity. Cd is known to induce the synthesis of metallothionein (MT), which is a small protein rich in sulfur-containing amino acids and commonly synthesized in maternal tissues and the placenta. MTs retain Cd in maternal tissues and the placenta, and thereby reduce Cd transport to the conceptus. The mechanism involved in developmental toxicity of Cd can be explained based on the interaction between Cd and Zn. In brief, Cd substitutes for Zn in metalloenzymes, thereby Cd interferes with Zn transfer across the placenta. Because of the high affinity of MT for Zn, MT sequesters Zn in the placenta, impeding transfer to the conceptus. Reduced uteroplacental blood flow, reduced nutrient transport, and placental toxicity by Cd seem to be the major contributing factors for low fetal birth weight, fetal toxicity, malformations, and death. Cd-induced impaired fetal growth is partly due to Zn deprivation, since maternal tissue and the placenta retain Zn. In addition, Cd accumulates in the fetus and produces a variety of adverse effects directly on the fetus. These adverse effects include: (1) embryotoxicity (Fein *et al.*, 1997); (2) inhibition of embryonic DNA and protein synthesis (Holt and Webb, 1987); and (3) reduced calcium handling ability of trophoblasts, which is a consequence of alterations in subcellular cytosolic calcium binding properties.

## Lead

The common sources of lead (Pb) exposure include Pb-based paints, batteries, crank-case oil, ceramics, shots and sinkers, calking and roofing materials, and ammunition. Plants and animals appear to bioconcentrate lead, but lead is not biomagnified in the aquatic or terrestrial food chain. Pb exposure in the pregnant animals usually occurs through oral route. It is also known that absorption of Pb increases during pregnancy. After oral absorption, Pb is distributed to most of the tissues, but it deposits mainly in the skeleton, kidney, and brain (primarily gray matter). Pb is known to cross the placenta and it accumulates in the fetus. Accumulation of Pb occurs in the fetal brain owing to lack of blood–brain barrier. Lead also accumulates in the placenta in times of fetal stress. In fact, a large maternal–fetal concentration gradient exists and the placenta poses a limited transplacental barrier. In addition, a number of adverse maternal health conditions can affect the transfer of lead to the fetus and/or the retention of Pb by the mother or the fetus.

Lead produces toxicity in general by interfering with protein/hemoprotein biosynthesis and by inhibiting membrane and mitochondrial enzymes (Eisenmann and Miller, 1996). Pb is also known to cause deficits in cholinergic, dopaminergic, and glutamatergic functions, although the exact mechanism involved in placental toxicity of Pb is yet to be elucidated. Following *in utero* exposure Pb can have direct effects on the developing conceptus. In fact, developing nervous system is the most sensitive target of lead toxicity. Developmental toxic effects of Pb have been determined in experimental animals, including rats, mice, hamsters, and chicks. *In utero* exposure to Pb can cause reduction in fertility and growth retardation. Embryotoxic and fetotoxic effects of Pb seem to be dependent on the exposure occurs on GD 9, teratogenic effects are observed with few resorptions, compared with exposure on GD 16, with hydrocephalus and CNS hemorrhage (McClain and Becker, 1975). At maternotoxic doses, Pb can cause retarded skeletal development. In mice, Pb can cause post-implantation mortality and skeletal malformations in fetuses. The common skeletal anomaly observed is the fusion of two or more cervical vertebrae (Jacquet and Gerber, 1979). Malformations in hamsters due to Pb exposure occur in the tail, ranging from stunting to complete absence of the tail (Ferm and Carpenter, 1967). In experimental animals, common malformations include brain defects, neural tube defects, urogenital system, and tail defects. It is important to mention that Pb has a greater potential for neurotoxicity than for placental and developmental toxicity.

## Mercury

Mercury (Hg) occurs naturally in the environment and exists in several forms. Common sources of Hg include mining, industrial, adhesives, inc, fungicides, and interior/exterior paints.

As a result, Hg is a widespread environmental contaminant that threatens the health of animals in general, and aquatic life and wildlife in particular. It is noteworthy that Hg is toxic in all forms. In most foodstuffs, Hg is found in the inorganic form. Fish, marine animals, and some microorganisms have capability to convert elemental Hg to organic Hg, which accumulates in the food chain. In most of the poisoning incidents, MeHg was involved because of its popular use as a fungicide. During the outbreak of MeHg poisoning in Japan and Iraq, it was proven that there was placental transfer of MeHg through the placenta into the fetus. Following *in utero* exposure to MeHg, the target organ is the fetal brain. This is partly due to accumulation of Hg in the fetal brain because of its high requirement for protein synthesis. MeHg is readily absorbed and distributed throughout the body with various concentrations in different tissues. In humans, brain MeHg levels can be as high as 6-fold, compared with blood levels. This is in contrast to rats, which have a brain to blood ratio of 0.06, and mice with a ratio of 1.20. There is evidence that the placenta presents some barrier to Hg. By using the Gray PBPK model for MeHg, the placenta is modeled as four compartments with separate transfer constants for placental barrier and placental tissue transport. Generally, organic and metallic Hg crosses the placenta more readily than inorganic Hg. As a result, MeHg and metallic Hg accumulate in the fetus, whereas inorganic Hg concentrates in the placenta. Metallic Hg, after crossing the placenta, can be oxidized to $Hg^{2+}$ in fetal tissues. $Hg^{2+}$ accumulates in the placenta, where it inhibits the fetal uptake of certain essential metabolites or analogs of these metabolites.

MeHg has been found to produce embryotoxicity, fetotoxicity, and teratogenicity in rats, mice, cats, guinea pigs, and hamsters. In general, resorptions, dead fetuses, and cleft palate are the most common findings. Some other common developmental effects include generalized edema, brain lesions, wavy ribs, asymmetric sternebrae, and decreased ossification of parietal and occipital bones (Domingo, 1994). In essence, prenatal exposure to sufficient amounts of Hg results in developmental toxicity. In addition, Hg has a strong potential for neurotoxicity and neurobehavioral toxicity.

# PLACENTAL TOXICITY OF INSECTICIDES

Depending on the duration, frequency, and level of exposure, the insecticides of various classes (organophosphates (OPs), carbamates (CMs), organochlorines, and pyrethroids) can adversely affect one or all three

components of the maternal–placental–fetal unit. Numerous studies demonstrate that the insecticide residue is present in the exposed mother, placenta, cord blood, embryo, and fetus, thus suggesting that the placenta and fetus are potentially exposed to these compounds. In this chapter, OPs and CMs are discussed together because their effects are quite similar (Gupta, 1995; Gupta and Sastry, 2000; Pelkonen *et al*., 2006).

## OPs and CMs

In general, both OPs and CMs exert their overt toxicity by inhibiting the activity of acetylcholinesterase (AChE). Inactivation of AChE occurs owing to phosphorylation by OPs and carbamylation by CMs. Inhibition of AChE results in accumulation of acetylcholine (ACh), which overstimulates the muscarinic and nicotinic ACh receptors. There is evidence from a rat study that placenta may be a target of direct toxic effect by OP pesticides (Levario-Carrillo *et al*., 2004). By having active metabolic activity, the placenta can modulate the potency of OPs and CMs. The placenta can probably convert certain OPs of the "thioate" or "dithioate" group to their "oxon" analogs, which are many times more potent AChE inhibitors and thus more toxic than their parent compounds. In such circumstances, the placenta can be one of the several determining factors for fetal toxicity.

It is well established that an active cholinergic system exists in the placenta of some species and not in others, and there is evidence that a cholinergic system may have more than one function in placenta. In the placenta, ACh-like activity is highest in membranes, medium in the cotyledons, and minimal in the cord, and it varies in the placenta as a function of gestational stage. The concentration of ACh in the placenta (a non-innervated tissue) is found to be greater than in various regions of the brain, and a marked decline occurs soon after parturition and expulsion of the placenta. The existence of cholinergic system (using choline acetyltransferase activity, ChAT) has been studied in the term placenta of several species (monkey, mongoose, lemur, horse, cow, sheep, goat, pig, hamster, cat, rabbit, guinea pig, and rat) (Hebb and Ratkovic, 1962). Literature abounds showing that the two species in which the placental cholinergic system has definitely been found are the two higher primates, man and monkey. In general, ACh has many functions in the placenta: (1) acts as a local hormone related to uterine contractions and mechanisms associated with birth; (2) the control of permeability and transport; (3) regulation of blood flow and fluid volume in placental vessels; (4) involvement in the mechanism of parturition; and (5) acts as a local messenger molecule. ACh also seems to play a vital role in the maturation of the placenta. In addition, ACh may regulate trophoblastic channels, fluid balance, and osmotic pressure, and therefore may influence contractile properties of myofibroblasts in the placenta. For further details on the roles of ACh, refer to King *et al*. (1991), and Sastry (2000).

The placenta and fetus, by having AChE and other cholinergic elements (muscarinic and nicotinic ACh receptors), remain susceptible to OPs and CMs (Koshakji *et al*., 1974; Cambon *et al*., 1979; Simone *et al*., 1994; Sastry, 2000). Inhibition of AChE and BuChE activities can be used as a marker of exposure to an OP or CM, while AChE can also be used as a marker of effects. OPs and CMs readily cross the placental barrier and can act on the cholinergic and non-cholinergic components of the developing nervous system and other vital organs (Gupta *et al*., 1984, 1985; Gupta and Sastry, 2000; Pelkonen *et al*., 2006). Significant inhibition of AChE and/or BuChE activities in maternal, placental, and fetal tissues of rats and mice following prenatal exposure to several OPs (quinalphos, dicrotophos, methyl parathion, diazinon, etc.) has been demonstrated (Bus and Gibson, 1974; Srivastava *et al*., 1992; Gupta, 1995; Gupta and Sastry, 2000; Abu-Qare and Abou-Donia, 2001). Similar findings have been reported for CMs, including aldicarb, carbaryl, carbofuran, and pirimicarb (Declume and Derache, 1977; Cambon *et al*., 1979, 1980). From all these studies, AChE inhibition appears to be the major mechanism of toxicity. With either type of insecticides, the developing organism is much more susceptible to the induction of functional neural deficits. Furthermore, subchronic prenatal exposure to methyl parathion in rats resulted in altered postnatal development of brain AChE and ChAT activities, in addition to selected subtle alterations in behavior (Gupta *et al*., 1985).

Anticholinesterase compounds have strong potential for embryotoxicity, embryolethality, fetotoxicity, and teratogenesis. These effects vary depending on the particular OP and CM involved. With these compounds, embryolethality is encountered so often that the expression of teratogenesis is rarely seen. In some experimental studies conducted in rats, mice, rabbits, and hamsters, OPs at maternotoxic doses have been shown to produce developmental alterations, including growth retardation and embryotoxicity. Khera (1979) discovered polydactyly in fetuses of cats treated with dimethoate (12 mg/kg/day) during the 14th to 22nd day of pregnancy (Figure 15.1). Methamidophos administration at a no-maternal toxicity dose (1 mg/kg, po) to female rats during GD 6–15 produced neither lethal effect on embryos nor caused congenital malformations at term, however the embryo–fetal maturation process was significantly altered (De Castro *et al*., 2000).

Wide species variability exists in sensitivity to OP-induced placental toxicity. It needs to be mentioned that the mechanisms involved in embryonic/fetal developmental malformation appear to be different from those involved in general toxicity. Alkylation of nicotinamide adenine dinucleotide ($NAD^+$) coenzymes by OPs is the

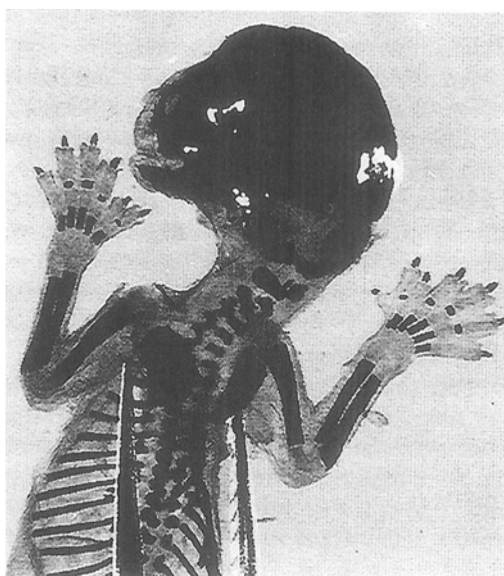

**FIGURE 15.1** Fetal cat showing heptadactyly (right forepaw) and hexadactyly (left forepaw), from a cat exposed to dimethoate (Khera KS (1979) *J Environ Pathol Toxicol* **2**: 1283–8).

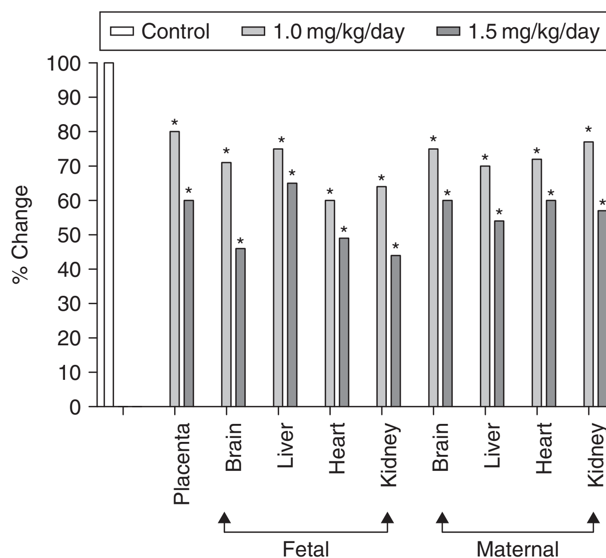


**FIGURE 15.2** Methyl parathion-induced inhibition of *in vivo* protein synthesis in placental, fetal, and maternal tissues. An asterisk indicates a significant difference between methyl parathion-treated and control rats.

major mechanism involved in the induction of teratogenesis (Schoental, 1977). In recent studies, diisopropyl fluorophosphate (DFP) and carbofuran have been shown to cause marked depletion of $NAD^+$ in rats (Gupta *et al.*, 2001a, b). OPs are also known to cause alterations in the levels of RNA, glycogen, sulfated mucopolysaccharides, and calcium in the developing tibiotarsus bones (Ho and Gibson, 1972). Prenatal exposure to OPs has been shown to affect protein synthesis both *in vivo* (Clouet and Waelsch, 1963; Gupta *et al.*, 1984) and *in vitro* (Welsch and Dettbarn, 1971; Marinovich *et al.*, 1996). The inhibitory effect on *in vivo* protein synthesis of methyl parathion (daily oral exposure throughout the period of organogenesis) was shown to be dose-dependent, greater on day 19 than day 15 of gestation, and more pronounced in fetal than in placental or maternal tissues of rats (Gupta *et al.*, 1984). The inhibitory effect on protein synthesis was more pronounced at a dose causing overt maternal toxicity (Figure 15.2). A mixture of OPs, including diazinon, dimethoate, and azinphos, has been found to be more toxic to protein synthesis than any of the single OP compound alone (Marinovich *et al.*, 1996).

Many placental toxicity studies conducted in mice, rats, hamsters, and rabbits revealed no teratogenic responses to OPs. Among CMs, carbaryl is the only insecticide that has been studied in detail for placental toxicity. Like OPs, CMs have a greater potential for embryolethality and fetotoxicity and that precludes an expression of teratogenicity. Beagle dogs receiving carbaryl gave birth of fetuses with terata in 21 of a total of 181 pups. Fetal abnormalities included abdominal–thoracic fissures with varying degrees of intestinal agenesis and displacement, brachygnathia, ecaudate pups, failure of skeletal formation, and superfluous phalanges (Smalley *et al.*, 1968). Carbaryl exposure during organogenesis produced terata in guinea pigs, but not in hamsters and rabbits (Robens, 1969). Other CMs, such as carbofuran and propoxur, have not been found to be teratogenic.

## Organochlorines

Organochlorine insecticides are classified into three groups: (1) dichlorodiphenylethanes (DDTs) (dichlorodiphenyltrichloroethane, dicofol, methoxychlor, and perthane); (2) hexachlorocyclohexanes (benzene hexachloride, chlordane, lindane, mirex, and toxaphene); and (3) chlorinated cyclodienes (aldrin, dieldrin, endrin, chlordane, endosulfan, and heptachlor).

The mechanism of action of organochlorines is not yet fully explained. The DDT-type compounds alter the transport of sodium and potassium ions across axonal membranes, resulting in an increased negative after-potential and followed by prolonged action potentials. As a result, repetitive firing and a spontaneous train of action potentials occur. Specifically, DDT inhibits the activation of sodium channels and the activation of potassium conductance. The mechanism involved in cyclodienes induced hyperactivity of the CNS and convulsions can be explained based on their structural resemblance to the cyclic $\gamma$-aminobutyric acid (GABA) receptor antagonist picrotoxin. The mammalian GABA receptor is coupled to an intrinsic chloride ion channel and is the primary mediator of neuronal inhibition in

the brain. Like picrotoxin, cyclodienes block the inhibitory action of GABA. Similar to other organochlorines, mirex and kepone cause stimulation of the CNS, hepatotoxicity, and induction of the mixed-function oxidase system. In the CNS, symptoms observed in animals by cyclodiene organochlorines include tremors, convulsions, ataxia, and changes in EEG patterns. The CNS symptoms could be due either to (1) inhibition of the $Na^+/K^+$ − ATPase or the $Ca^{2+}/Mg^{2+}$ − ATPase activity, which can then interfere with nerve action or release of neurotransmitters, and/or (2) inhibition of the GABA receptor function.

The acute toxic signs associated with DDT and chlorinated benzene types of insecticides include paresthesia of the tongue, lips and face, apprehension, tremors, and convulsions. Stimulation of the CNS is the most prominent effect. The acute signs and symptoms produced by cyclodienes include dizziness, nausea, vomiting, myoclonic jerking, motor hyperexcitability, and convulsive seizures. Usually, epoxide metabolites of cyclodiene type of insecticides are much more toxic than their parent compounds. It is important to mention that the developing nervous system is the most sensitive target of organochlorines toxicity.

There are only few placental toxicity studies have been conducted with organochlorine compounds. In experimental studies, organochlorines, including DDT, DDE, DDD, dieldrin, endosulfan, methoxychlor, and toxaphene, have been associated with estrogen-like effects in the reproductive system of laboratory animals. Exposure of female rats to methoxychlor before and during pregnancy can cause blockade of implantation, suppression of uterine decasualization, lack of corpora lutea, and atresia of ovarian follicles. Exposure during the preimplantation blocks implantation, whereas exposure during the post-implantation causes fetal resorptions. Interference in the requisite hormonal milieu seems to be the major effect. Other effects of methoxychlor include estrogenic effect on uterine preimplantation differentiation, ovum transport rate, luteal regression, and post-implantation decidual growth. In an experimental study, exposure of Swiss mice to lindane at different stages of pregnancy produced various toxicological effects, such as fetotoxicity and reproductive failure (Sircar and Lahiri, 1989). Lindane exposure during early pregnancy (days 1–4) caused total absence of any implantation; during mid-pregnancy (days 6–12) caused total resorptions of fetuses; and during late-pregnancy (days 14–19) caused the death of all pups within 12 h to 5 days after parturition. Lindane can also cause reproductive failure by inducing a deficiency of steroid hormones (estrogen and progesterone).

Some studies in animals suggest that young animals exposed during gestation and infancy may be very sensitive to heptachlor and heptachlor epoxide. Changes in nervous system and immune function were found in these animals. Exposure to higher doses of heptachlor in animals can also result in decreases in body weight and death in animal newborn babies. A decrease in fertility and an increase in resorptions were observed in female rats acutely exposed to 1.8 mg/kg/day. Reduced fertility has also been observed in mice exposed to 8.4 mg/kg/day. A number of animal studies have demonstrated that exposure to heptachlor can result in decreased fertility and pregnancy losses. Impaired fertility was reported in female rats administered via gavage of 0.6 mg/kg/day heptachlor in groundnut oil for 14 days prior to mating (Amita Rani and Krishnakumari, 1995). In essence, though the exact mechanism in the placental toxicity has yet to be elucidated, impaired fertility and pregnancy losses, in addition to the impaired development of the nervous system and immune system have been found with organochlorine.

There are reports that dieldrin produced teratogenic effects, such as supernumerary ribs, with concomitant decrease in ossification centers in fetal hamsters (Chernoff *et al.*, 1975, 1979). In rats, exposure to mirex during pregnancy has been associated with perinatal deaths due to persistent cardiovascular problems, such as first- to third-degree fetal heart blockade (Grabowski, 1983). In addition, mirex causes altered lens growth and cataracts, along with other biochemical, physiological, and histological changes (Rogers and Grabowski, 1983).

## Pyrethrins and pyrethroids

In recent years, the use of synthetic pyrethroids over the other classes of insecticides has increased tremendously because of their selectively high toxicity to insects and low toxicity to mammals. Still, the risks to animal health exist because the products containing pyrethrins and pyrethroids are commonly used as ectoparasiticides. Exceeding the recommended levels of these insecticides or time of exposure often results in poisoning and deaths, especially in cats and dogs. Pyrethroids are of two types. Type I pyrethroids are those which lack $\alpha$-cyano moiety and give rise to the tremor syndrome (T syndrome). The syndrome includes the signs of whole body tremors, incoordination, prostration, tonic–clonic convulsions, and death. Few common examples of type I are pyrethrin I, allethrin, tetramethrin, resmethrin, and permethrin. Type II pyrethroids are those which contain $\alpha$-cyano moiety and cause the choreoathetosis/salivation (CS) syndrome. The CS syndrome is characterized by hyperactive behavior, profuse salivation, tremors, motor incoordination, and hunch-backed posture. Few common examples of type II pyrethroids include cyphenothrin, cypermethrin, deltamethrin, and fenvalerate.

Intoxication by pyrethroids results primarily from hyperexcitation of the nervous system. Type II syndrome involves primarily an action in the CNS, whereas with type I syndrome, peripheral nerves are also involved. Hyperexcitation of the nervous system is caused by repetitive firing and

depolarization in nerve axons and synapses. Pyrethroids act directly through interaction with the sodium channel gating mechanism, thereby interfering with the generation and conduction of nerve impulses and inducing marked repetitive activity in various parts of the brain. Type I pyrethroids affect sodium channels in nerve membranes, causing repetitive neuronal discharge and a prolonged negative after-potential, the effect being similar to those produced by DDT type of insecticides. Type II pyrethroids produce an even longer delay in sodium channel inactivation, leading to a persistent depolarization of the nerve membrane without repetitive discharge, a reduction in the amplitude of the action potential and eventually failure of axonal conduction and a blockade of impulses.

Prenatal or early postnatal exposure to pyrethroids (cypermethrin, fenvalerate, and others) has been linked with significant neurochemical alterations in neonatal rats (Husain *et al.*, 1991, 1992; Malaviya *et al.*, 1993). Delayed maturation of the cerebral cortex occurs due to alterations in key enzymes of the neurotransmission process (e.g., monoamine oxidase, acetylcholinesterase, and $Na^+/K^+ -$ ATPase). Prenatal exposure to these insecticides significantly delays differential responses in the levels of brain regional polyamines and ontogeny of sensory and motor reflexes in offspring. Other biochemical and neurochemical effects of these insecticides include impairment at the neurotransmitter receptors, including dopaminergic, cholinergic, and catecholaminergic.

Compared to other classes of insecticides, pyrethroids are not well studied for placental toxicity because they are relatively less toxic to mammalian species. For example, permethrin at concentrations of 2000–4000 ppm showed only a week to moderate influence on *in utero* fetal development. Female rats dermally exposed to cyhalothrin throughout gestation period had offspring with delayed fur development, delayed ear and eye opening, and delayed descent of the testes, but with no change in the age of vaginal opening. In adulthood, however, the sexual behavior of both male and female rats exposed to cyhalothrin prenatally is no different from that of control animals (Gomes *et al.*, 1991a, b). Prenatal exposure of rats to deltamethrin caused increase in early embryonic deaths and fetuses with retarded growth, hyperplasia of the lungs, dilatation of the renal pelvis, and increase in placental weight (Abdel-Khalik *et al.*, 1993).

## MYCOTOXINS

Mycotoxins are secondary metabolites of fungi and several of them are found to be toxic *in utero* in both man and animals. In general, mycotoxins can produce placental toxicity by one or more of three common mechanisms. In brief, mycotoxins can interfere with hormonal activity, which can damage parental gametes, producing infertility or abnormal offspring if successful fertilization occurs. Mycotoxins can cause fetal malformations, especially during organogenesis, by interfering with fetal nucleic acid and protein biosynthesis. This interrupts normal cell differentiation and organogenesis, resulting in fetal malformations. Mycotoxins can also affect the fetus indirectly by affecting vital maternal organs, such as the liver, reducing nutrient transfer to the fetus, or increasing transfer of toxicants (Hayes, 1981; Lebepe-Mazur *et al.*, 1995). Some of the common mycotoxins that cause placental toxicity are briefly discussed below.

### Aflatoxins

Aflatoxins ($B_1$, $B_2$, $G_1$, and $G_2$) are mainly produced by *Aspergillus flavus and Aspergillus parasiticus*, which commonly contaminate a variety of animal food. Aflatoxins are proven to be mutagens, carcinogens, and teratogens. From a limited studies conducted on laboratory animals, aflatoxin $B_1$ and $G_1$ appears to have strong potential for embryocidal, fetotoxic, and teratogenic effects. Aflatoxin $B_1$, and $G_1$, with varying doses administered orally or i.p. in mice on gestation day (GD) 12 and 13, produced moderate retardation in fetal development, cleft palate, and changes in diaphragm. Aflatoxin $G_1$ also produced malformations in the kidneys (Roll *et al.*, 1990). Pregnant rats exposed to aflatoxin $B_1$ via the oral route has been shown to result in significant anti-implantational activity and loss of viability among the litters (Choudhary *et al.*, 1992).

### Fumonisins

Fumonisins ($FB_1$, $FB_2$, $FB_3$, $FB_4$, $FA_1$, $FA_2$, and $C_1$) are produced by *Fusarium* fungi, primarily *Fusarium moniliforme* and *Fusarium proliferatum*. These mycotoxins are found in the corn and therefore contaminate corn-based food/feed worldwide. It is well established that $FB_1$, and $FB_2$ are most abundant and most toxic mycotoxins of this group. Fumonisins-induced toxic effects appear to be species specific. Consumption of fumonisins-contaminated food/feed has been associated with many organ-specific diseases. For example, esophageal cancer in man in the Transkie region in South Africa and Linxian County in China, hepatopathy and hepatocarcinoma in laboratory rats, porcine pulmonary edema (PPE) in swine, and equine leukoencephalomalacia (ELEM) or moldy corn poisoning in equidae. In addition, fumonisins produce mild to fatal toxicity in the liver, kidney, and heart of rats and horses. Among all species, equidae is the most sensitive because fumonisins affect the brain of this species.

Experimental animal studies and anecdotal field-case findings revealed that fumonisins have a strong potential for developmental toxicity and teratogenicity. Purified $FB_1$ and *F. proliferatum* culture extract have been shown to be embryotoxic when inoculated into incubated eggs (Javed *et al.*, 1993a, b). In addition to mortality, which was dependent on the dose and time of administration (i.e., day 1 or 10), embryonic changes occurred, including hydrocephalus, enlarged beaks, and elongated necks. In hamsters, $FB_1$ caused increased incidence of prenatal losses, including deaths and resorptions. At a dose of 12 mg/kg, all litters were affected and all the fetuses were dead and resorbed, without any clinicopathological evidence of maternal toxicity. Fetal resorptions were associated with a greater severity of placental necrosis. Placental tissues showed signs of degeneration and involution, focal distortion, and necrosis of the trophoblastic layer, especially at the periphery, and hemorrhage and necrosis at the base of the placenta. Placentas had extensive necrosis and fetal capillaries in the hemochorial portion, with concomitant collapse of the stroma. Such changes were consistent with the embryolethal effects of $FB_1$. External malformations include hooked or curled tail, ectrodactyly of the front/rear limbs, and cleft palate. Developmental effects of aqueous culture extract of *F. moniliforme* in CD1 mice are reported to be due to $FB_1$. The effects include maternal deaths, reduced maternal body weight gain, increased embryonic resorptions, reduced pup weights, and fetal malformations, such as cleft palate, hydrocephalus, and ossification deficits (Gross *et al.*, 1994; Reddy *et al.*, 1995).

$FB_1$ is found to be fetotoxic in F344/N rats. Pregnant rats receiving $FB_1$ at a dose of 30 or 60 mg/kg, po (GD 8–12) developed significant impairment of ossification of the sternebrae and vertebral bodies in their fetuses (Lebepe-Mazur *et al.*, 1995). It appears that $FB_1$ produces fetotoxicity in rats by suppressing growth and fetal bone development (Figure 15.3). However, in Sprague–Dawley rats, there is little or no evidence of embryotoxicity or fetotoxicity by $FB_1$, especially in the absence of maternal toxicity (Ferguson *et al.*, 1997). Pregnant New Zealand white rabbits receiving $FB_1$ showed no evidence of embryotoxicity or teratogenicity, despite the fact that the pregnant rabbits were very sensitive to $FB_1$ (LaBorde *et al.*, 1997).

In pregnant mares, late-term abortions have been observed by consuming fumonisins contaminated corn/corn-based feed. The surviving colts are usually slow learners and behaviorally abnormal. Suppressed growth, bone development, and immune system appeared to be the major contributing factors. Abortions have also been observed in pregnant sows by consuming fumonisins contaminated corn that caused hypoxia resulting from maternal respiratory distress (Harrison *et al.*, 1990).

Although the exact mechanism of action involved in fumonisins toxicity is yet to be established, it has been demonstrated that fumonisins inhibit sphinganine-sphingosine *N*-acetyl transferase (ceramide synthase) activity, and consequently increase the concentrations of sphinganine to sphingosine in target organs of many species (Wang *et al.*, 1992; Riley *et al.*, 1993). Accumulation of sphinganine may be responsible for cell death because long-chain bases such as sphinganine and sphingosine are known protein kinase C inhibitors and can be cytotoxic. It is suggested that an abnormal sphinganine/sphingosine ratio in the fetus, while normal ration in the dam, may attest to the unique sensitivity of the conceptus to fumonisins (Riley *et al.*, 1993; Floss *et al.*, 1994a, b).

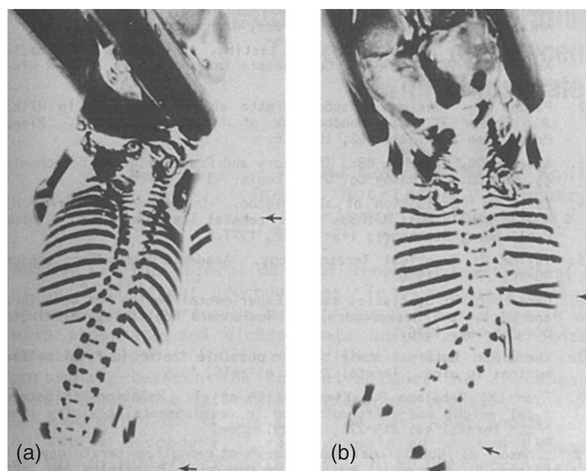


**FIGURE 15.3** Skeletal development of control (a) and fumonisin (b) treated rat pup. (a) Pup from a control dam, with normally shaped ribs and normal ossification of the phalanges and vertebrae bodies. (b) Pup from a dam treated with 60 mg/kg fumonisin $B_1$, having under-ossification of the vertebral bodies of the cervical and lumbar regions as well as the phalanges (Lebepe-Mazur *et al.* (1995) *Vet Hum Toxicol* **37**: 126–30).

## Ochratoxin A

Ochratoxin A (OTA) is produced by several *Aspergillus* and *Penicillium* species. This mycotoxin is known to contaminate food/feed and thereby cause a variety of adverse health effects (nephrotoxicity, carcinogenicity, teratogenicity, and immunotoxicity), nephrocarcinogenicity being the most prominent. OTA has been demonstrated to cause carcinogenesis, immunotoxicity, and teratogenesis in rats and rabbits (Mayura *et al.*, 1982a; Wangikar *et al.*, 2004). In general, the underlying mechanisms involved in the toxicity of OTA include: (1) inhibition of protein synthesis, (2) increased lipid peroxidation, and (3) partial inhibition of ATP-dependent calcium uptake and inhibition of the cell-mediated immune response. In addition, OTA inhibits the phosphorylase enzyme system, possibly by competing with 3′, 5′-cyclic AMP for phosphorylase b kinase activity.

In the kidney, OTA specifically affects organic anion transport, but not organic cation transport, in both the brush border and basolateral membranes, which explains its entry into the renal cells and the development of toxicity. Gene expression study indicates that OTA-induced renal toxicity involves the impairment of defense potential causing oxidative damage that eventually lead to cell proliferation and cancer. Epigenetic mechanism appears to be highly probable (Marin-Kuan *et al.*, 2006).

It has been established that OTA transplacentally traverses in rodents and other species (Galtier, 1991), and causes fetal death in laboratory and domestic animals (Still *et al.*, 1971). Pregnant rats receiving a culture of *Aspergillus ochraceus* on GD 10 showed embryo resorptions and fetal deaths. Subsequent studies in rats further demonstrated that a high dose of purified OTA administered on GD 10 also caused a large number of fetal deaths and embryo resorptions. However in sheep, placental transfer of OTA was found to be in trace amounts resulting in very little or no evidence of fetal toxicity or teratogenesis. Rabbits receiving a combination of OTA and aflatoxin $B_1$ showed antagonistic interaction, yet revealed a characteristic cardiac anomaly with a valvular defect at auriculo-ventricular junction (Wangikar *et al.*, 2005).

## Rubratoxins

Both rubratoxin A and B are produced by *Penicillium rubrum* and *Penicillium perpurogenum*. Rubratoxin B is the primary mycotoxin that causes hepatotoxicity, nephrotoxicity, and splenotoxicity in several animal species. In both laboratory and domestic animals, typical signs of poisoning are hepatic failure, coagulopathy, hemorrhage of the gut mucosa, bloody feces, and death. Rubratoxin $B_1$ has been demonstrated to be a potent mutagen and teratogen in laboratory animals (Wilson and Harbison, 1973). Although, the underlying mechanism in rubratoxin-induced placental toxicity is yet to be explained, rubratoxin B is known to produce gross malformations, internal anomalies, skeletal malformations, embryo lethality, intrauterine growth retardation, increased skeletal variations, and delayed ossification in the mouse fetuses. The teratogenic effects in near-term fetuses include exophthalmos, missing ears, spina bifida, microphthalmia, anophthalmia, short tail, exencephaly, and hydramnion.

## Secalonic acid D

Secalonic acid D (SAD) is produced by *Penicillium oxalicum*. In rats and mice, SAD is known to cause embryotoxicity, embryolethality, fetotoxicity, and teratogenicity. Pregnant CD1 mice receiving SAD (>5 mg/kg/day, ip, GD 7–15) showed a reduction in maternal body weight gain, and an increase in resorptions of implanted embryos (Reddy *et al.*, 1981). The resorptions rate was found to be 100% at 15 mg/kg dose. Multiple gross, skeletal, and visceral anomalies were noted in fetuses born to mothers receiving 10 mg/kg or more. Major malformations included cleft palate, cleft lip, open eyelids, missing phalangeal ossification centers, and shortened mandibles. In CD1 mice, SAD is proved to be an embryocidal, fetotoxic, and teratogenic. Pregnant rats receiving SAD as a single sc dose (25 mg/kg) on one of GD 6–10, 12, 14; or 15 mg/kg on GD 10, produced fetotoxic and teratogenic effects, although the effects were less marked with the lower dose (Mayura *et al.*, 1982b). The highest number of resorptions, greatest depression of fetal body weight, and largest number of malformations occurred when SAD was injected on GD 10. The major gross malformations were anophthalmia (GD 9 and 10), exencephaly (GD 9), and defects in limbs, digits, and tail (GD 10). The major skeletal defects involved the vertebrae and ribs. The major internal soft tissue defects were hydronephrosis (GD 9 and 10), tracheo-esophageal fistula, and renal agenesis (GD 10). Thus, it can be concluded that pregnant rats are most sensitive to SAD exposure on GD 10 for fetotoxicity and teratogenicity.

## Trichothecenes

The trichothecenes are a group of structurally related mycotoxins with varying degrees of cytotoxic potency. Diacetoxyscirpenol (DAS), T-2 toxin, and deoxynivalenol (DON or vomitoxin) are the three major mycotoxins of this group that are commonly encountered in animal poisonings. T-2 and DAS are produced by *Fusarium sporotrichiodes* and vomitoxin is produced by *Fusarium roseum*. There is strong evidence that trichothecenes cross the placental barrier and can cause both embryonic death and structural malformations when administered to laboratory animals during pregnancy (Francis, 1989). Since domestic and avian species are exposed to trichothecenes through consumption of contaminated feed, and trichothecenes in general cause feed refusal, it is expected that the induced maternal/fetal toxicity to be minimal. Furthermore, *in vivo* studies with different animal species indicate that orally or parenterally administered trichothecenes do not bioaccumulate due to their short elimination half-lives: <30 min for T-2 toxin in swine, cattle, and dogs (Yagen and Bialer, 1993); and 3–5 h for DON in swine and cattle (Rotter *et al.*, 1996).

## DON

Feeding of a DON (vomitoxin)-contaminated diet at 5 ppm to rats throughout gestation does not adversely affect pregnancy or increase birth defects (Morrissey, 1984). Vomitoxin

does not produce teratogenesis even at a 20-ppm level. However, at this dose it can decrease the rate of pregnancy. Mice intubated with vomitoxin (2.5 and 5 mg/kg) on GD 8–11 produced embryolethality, and at doses of 5 mg/kg or greater produced embryolethality (Khera *et al.*, 1982). In mice, vomitoxin at a dose of 10 mg/kg caused maternal mortality. Fetal mortality by vomitoxin is dose-dependent (100% at 10 mg/kg, 80% at 5 mg/kg, and 72% at 2.5 mg/kg). However, none of the dose of vomitoxin induced damage to the placenta or teratogenesis in mice.

## DAS

There is very little known about placental toxicity of DAS. In rats, DAS at a dose rate of 2, 3, or 6 mg/kg i.p. given on one of GD 7–11 caused no maternal toxicity (Mayura *et al.*, 1985). The highest dose of DAS caused 100% resorptions, while with the lowest dose, the surviving pups showed a variety of malformations, including hydrocephaly and exencephaly.

## T-2 toxin

T-2 toxin is the most potent mycotoxin of the trichothecenes group, and has been involved in mass poisoning in animals (Hsu *et al.*, 1972). It has been well established that T-2 toxin crosses the placenta (Hayes, 1981). In rats, T-2 toxin produced behavioral teratogenesis, when the toxin was given prenatally (Francis, 1989). Mice exposed to a single dose of T-2 toxin (3 mg/kg) on GD 7, 8, 10, 11, or 12, showed maternal toxicity with 17% mortality and whole litter resorptions (Roussoux *et al.*, 1985). Maternal death was typically due to placental hemorrhage, and surviving pups showed retardation defects of the skeletal system, exencephaly, abdominal defects, and cleft palate. Findings of a comprehensive study in mice treated with T-2 toxin (0.5, 1.0, or 1.5 mg/kg i.p.) on GD 7, 9, 10, or 11 are briefly described here (Stanford *et al.*, 1975). Treatment on GD 9 reduced the survival rate to 85% at 1 mg/kg and to 8% at 1.5 mg/kg, without any evidence of teratogenesis in surviving pups. Among the surviving pups of dams treated at 1.0 or 1.5 mg/kg on GD 10, 37% grossly malformed and 42% had skeletal malformations. Commonly observed malformations were exencephaly, congenitally open eyelids, and retarded jaws. Treatment on GD 11 resulted in greater embryotoxicity (75% deaths at 1 mg/kg and 100% deaths at 1.5 mg/kg), but the malformation rate among surviving pups was not greater than 13% at any dosage. In another study, mice given T-2 toxin in feed at 5 and 10 ppm throughout gestation resulted in whole litter abortions in 67% and 100% of the dams, respectively (Francis, 1989). Delayed ossifications of the coccyx and growth retardation were the prominent effects. Overall, the observed effects were cumulative and dose-dependent.

It appears that the trichothecenes induce embryotoxicity at maternotoxic doses in laboratory animals. Death of embryo/fetus is the most common finding. The surviving pups rarely show frank malformations of nervous system and skeletal system.

## Zearalenone

Zearalenone is produced by the fungi *Fusarium graminearum* and *Fusarium culmorum*, and other *Fusarium* species. It is important to study the placental toxicity of zearalenone and its two major metabolites ($\alpha$- and $\beta$-zearalenol) as they bind to estrogen receptor and produce a variety of reproductive problems. Zearalenone has been found to cross the placenta and reach fetus of mice and rats (Appelgren *et al.*, 1982; Bernhoft *et al.*, 2001). In maternal tissues, zearalenone and its metabolites accumulate in the liver, but in fetal tissues it is yet to be established. Due to its estrogenic activity, zearalenone is known to perturb the ovulation cycle and reduce litter size in domestic animals, particularly swine. Similar findings have been noted in experimental studies conducted in swine, mice, and rats, where exposure to zearalenone during pregnancy reduces fetal survival (Kuiper-Goodman *et al.*, 1987). The structurally related compound $\alpha$ -zearalanol, which is used for growth promotion in cattle, has been shown to accelerate testicular development and exert alterations in Leydig cells in mice after *in utero* exposure (Perez-Martinez *et al.*, 1997).

# TOBACCO

Tobacco plants and their products contain many toxic alkaloids, but nicotine is believed to be the major component causing alterations in embryo and fetal development that leads to teratogenesis. Pets are exposed to tobacco by ingesting commercial products (e.g., cigarettes, chewing tobacco, etc.), whereas livestock by consuming discarded tobacco stalks or contaminated hay with tobacco plant dripping in the barn. Most often, poisoning encountered in animals with tobacco is of acute in nature exhibiting the signs of neurologic and muscular disturbances.

The pharmacological and toxicological effects of nicotine are dose-dependent and primarily occur in the CNS, cardiovascular system, skeletal muscles, and gastrointestinal (GI) tract. Nicotine is a rapidly acting sympathetic and parasympathetic ganglionic depolarizer. In small doses, nicotine stimulates the autonomic ganglia, while in larger doses it blocks the ganglia. Similarly, in skeletal

muscles small doses of nicotine initially stimulate the nicotinic receptors of the motor end plate, while larger doses block these receptors. Cardiovascular signs, such as tachycardia and hypertension, result from stimulation of sympathetic ganglia and the adrenal medulla. Death occurs from paralysis of respiratory muscles and cardiac arrest.

Some tobacco alkaloids, especially nicotine, have been studied for their distribution, metabolism, and elimination in animals. It is also established that some of these alkaloids cross the placenta and produce the teratogenic effects (Suzuki *et al.*, 1974; Sastry and Janson, 1995; Panter *et al.*, 1999; Czekaj *et al.*, 2002). In some studies, tobacco components have been shown to produce deleterious effects on embryonic development in the early stages of pregnancy, before the placenta is fully formed. In mice, nicotine increases $[Ca^{2+}]_i$ and reactive oxygen species levels, which play a role in nicotine-induced embryonic apoptosis and malformations, and that eventually leads to teratogenesis (Zhao and Reece, 2005). Swine appears to be the most sensitive species in the context of teratogenesis. The fetal arthrogryposis occurs without signs of intoxication in the dams and without fetal deaths or abortions, but the deformed piglets usually die shortly after birth. With *Nicotiana glauca* fetal deformities are preceded by the appearance of signs of acute toxicity in the dam (Keeler *et al.*, 1981). Deformities in the newborn following *in utero* exposure include severe flexure and lateral rotation of the carpal joints, moderate rotation of the fetlocks, and less commonly spinal malformations resulting in lordosis or scoliosis, and cleft palate (Burrows and Tyrl, 2001). Exactly, which component(s) of the tobacco is involved in inducing teratogenesis is yet to be confirmed.

## CONCLUSIONS AND FUTURE DIRECTIONS

Until recently, placental toxicity has received very little attention compared to fetal or maternal toxicity. This is due to the fact, that placenta is discarded at the termination of pregnancy. However, healthy placenta with normal structure and function is required for proper supplies of nutrition to the fetus and its protection from adverse conditions. It is important to mention that the placenta is also a source of several growth factors that are required by the fetus. Placenta by having active metabolism appears to influence fetal toxicity of chemicals. Many chemicals cross the placenta and produce deleterious effects on fetus, while other chemicals cause damage to the placenta and thereby adversely affect the fetus. Detailed studies need to be done to understand exactly how the toxicants from various classes interfere with the structure, function, and development of the placenta. Special emphasis should be given to explore the molecular mechanism(s) involved in deleterious effects of chemicals in placenta, as much as given to the fetus or mother.

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## REFERENCES

Abdel-Khalik MM, Handfy MS, Abdel-Aziz MI (1993) Studies on the teratogenic effects of deltamethrin in rats. *Dtsch Tierarztl Wochenschr* **100**: 142–3.

Abu-Qare AW, Abou-Donia MB (2001) Inhibition and recovery of maternal and fetal cholinesterase enzyme activity following a single cutaneous dose of methyl parathion and diazinon, alone and in combination, in pregnant rats. *J Appl Toxicol* **21**: 307–16.

Ala-Kokko TI, Myllynen P, Vähäkangas K (2000) *Ex vivo* perfusion of the human placental cotyledon: implications for anesthetic pharmacology. *Int J Obstet Anesth* **9**: 26–38.

Amita Rani BS, Krishnakumari MK (1995) Prenatal toxicity of heptachlor in albino rats. *Pharmacol Toxicol* **76**: 112–14.

Appelgren LE, Arora RG, Larson P (1982) Autoradiographic studies of $[^3H]$zearalenone in mice. *Toxicology* **25**: 243–53.

ATSDR (2005) *Toxicological profile for arsenic. Agency for Toxic Substances and Disease Registry*, Atlanta, GA, pp. 201–9.

Benett RW, Persaud TVN, Moore KL (1975) Experimental studies on the effects of aluminum on pregnancy and fetal development. *Anat Anz* **138**: 365–78.

Bernhoft A, Behrens GHG, Ingebrigtsen K, *et al.* (2001) Placental transfer of the estrogenic mycotoxin zearalenone in rats. *Reprod Toxicol* **15**: 545–50.

Burrows GE, Tyrl RJ (2001) *Toxic Plants of North America*. Iowa State University Press, Ames, IA, pp. 1122–5.

Bus JS, Gibson JE (1974) Bidrin: perinatal toxicity and effects on the development of brain acetylcholinesterase and choline acetyltransferase in mice. *Food Cosmet Toxicol* **12**: 313–22.

Cambon C, Declume C, Derache R (1979) Effect of the insecticidal carbamate derivatives (carbofuran, pirimicarb, aldicarb) on the activity of acetylcholinesterase in tissues from pregnant rats and fetuses. *Toxicol Appl Pharmacol* **49**: 203–8.

Cambon C, Declume C, Derache R (1980) Fetal and maternal rat brain acetylcholinesterase isoenzymes changes following insecticidal carbamate derivatives poisoning. *Arch Toxicol* **45**: 257–62.

Chaineau E, Binet S, Pol D (1990) Embryotoxic effects of sodium arsenite and sodium arsenate on mouse embryo in culture. *Teratology* **41**: 105–12.

Chernoff N, Kavlock RJ, Katherin JR, *et al.* (1975) Prenatal effects of dieldrin and photodieldrin in mice and rats. *Toxicol Appl Pharmacol* **31**: 302–8.

Chernoff N, Kavlock RJ, Hanisch RC, *et al.* (1979) Perinatal toxicity of endrin in rodents. Fetotoxic effects of prenatal exposure in hamsters. *Toxicology* **13**: 155–65.

Choudhary DN, Sahay GR, Singh GN (1992) Effect of some mycotoxins on reproduction in pregnant albino rats. *J Food Sci Technol* **29**: 264–5.

Clouet D, Waelsch H (1963) Amino acid and protein metabolism in the brain. The effect of an organophosphorus inhibitor on the incorporation of $^{14}$C-lysine into the proteins of rat brain. *J Neurochem* **10**: 51–63.

Cranmer JM, Wilkins JD, Cannon DJ, *et al.* (1986) Fetal–placental–maternal uptake of aluminum in mice following gestational exposure: effect of dose and route of administration. *Neurotoxicology* **7**: 601–8.

Czekaj P, Palasz A, Lebda-Wyborny T, *et al*. (2002) Morphological changes in lungs, placenta, liver and kidneys of pregnant rats exposed to cigarette smoke. *Int Arch Occup Environ Health* **75**(Suppl): S27–35.

De Castro VL, Chiorato SH, Pinto NF (2000) Relevance of developmental testing of exposure to methamidophos during gestation to its toxicology evaluation. *Toxicol Lett* **118**: 93–102.

Declume C, Derache R (1977) Placental passage of an anticholinesterase carbamate on the effectivity of carbaryl insecticide. *Chemosphere* **6**: 141–6.

DiSaint' Agnese PA, Jensen K, Levin AA, Miller RK (1983) Placental toxicity of cadmium in the rat: an ultrastructural study. *Placenta* **4**: 149–63.

Domingo JL (1994) Metal-induced developmental toxicity in mammals: a review. *J Toxicol Environ Health* **42**: 123–41.

Eisenmann CJ, Miller RK (1996) Placental transport, metabolism, and toxicity of metals. In *Toxicology of Metals*, Chang LW (ed.). CRC Lewis Publishers, Boca Raton, FL, pp. 1003–26.

Fein A, Torchinsky A, Pinchasov M, *et al.* (1997) Cadmium embryotoxicity: evidence of a direct effect of cadmium on early rat embryos. *Bull Environ Contam Toxicol* **59**: 520–4.

Ferm VH, Carpenter JS (1967) Teratogenic effects of cadmium and its inhibition by zinc. *Nature* **216**: 1123.

Ferguson SA, St. Omer VEV, Kwon OS, *et al*. (1997) Prenatal fumonisin ($FB_1$) treatment in rats results in minimal maternal or offspring toxicity. *Neurotoxicology* **18**: 561–70.

Floss JL, Casteel SW, Johnson GC, *et al.* (1994a) Developmental toxicity of fumonisin in Syrian hamsters. *Mycopathologia* **128**: 33–38.

Floss JL, Casteel SW, Johnson GC, *et al.* (1994b) Developmental toxicity in hamsters of an aqueous extract of *Fusarium moniliforme* culture material containing known quantities of fumonisin $B_1$. *Vet Hum Toxicol* **36**: 5–10.

Francis BM (1989) Reproductive toxicology of trichothecenes. In *Trichothecene Mycotoxicosis: Pathophysiologic Effects*, Beasley VR (ed.). CRC Press, Boca Raton, FL, pp. 143–59.

Galtier P (1991) Pharmacokinetics of ochratoxin A in animals. *IARC Sci Publ* **115**: 187–200.

Gomes MDS, Bernardi MM, Spinosa HDS (1991a) Pyrethroid insecticides and pregnancy: effect on physical and behavioral development of rats. *Vet Hum Toxicol* **33**: 315–17.

Gomes MDS, Bernardi MM, Spinosa HDS (1991b) Effect of prenatal pyrethroid insecticide exposure on the sexual development of rats. *Vet Hum Toxicol* **33**: 427–8.

Gonzalez FJ (1989) The molecular biology of cytochrome P450s. *Pharmacol Rev* **40**: 243–88.

Grabowski CT (1983) Persistent cardiovascular problems in newborn rats prenatally exposed to sub-teratogenic doses of the pesticide, mirex. *Dev Toxicol Environ Sci* **11**: 537–40.

Gross SM, Reddy RV, Rottinghaus GE (1994) Developmental toxicity of fumonisin $B_1$-containing *Fusarium moniliforme* culture extract in CD1 mice. *Mycopathologia* **128**: 11–20.

Gupta RC (1995) Environmental agents and placental toxicity: anticholinesterases and other insecticides. In *Placental Toxicology*, Sastry BVR (ed.). CRC Press, Boca Raton, FL, pp. 257–78.

Gupta RC, Milatovic D, Dettbarn W-D (2001a) Depletion of energy metabolites following acetylcholinesterase inhibitor-induced status epilepticus: protection by antioxidants. *Neurotoxicology* **22**: 271–82.

Gupta RC, Milatovic D, Dettbarn W-D (2001b) Nitric oxide modulates high-energy phosphates in brain regions of rats intoxicated with diisopropylphosphorofluoridate or carbofuran: prevention by *N-tert*-butyl-$\alpha$ -phenylnitrone or vitamin E. *Arch Toxicol* **75**: 346–56.

Gupta RC, Rech RH, Lovell KL, Welsch F, Thornberg JE (1985) Brain cholinergic, behavioral, and morphological development in rats exposed *in utero* to methyl parathion. *Toxicol Appl Pharmacol* **77**: 405–13.

Gupta RC, Sastry BVR (2000) Toxicology of the placenta. In *General and Applied Toxicology*, vol. 2, Ballantyne B, Marrs TC, Syversen T (eds). Macmillan Reference Ltd., London, pp. 1233–63.

Gupta RC, Thornberg JE, Stedman DB, Welsch F (1984) Effect of subchronic administration of methyl parathion on *in vivo* protein synthesis in pregnant rats and their conceptuses. *Toxicol Appl Pharmacol* **72**: 457–68.

Harrison LR, Colvin MB, Green JT, *et al.* (1990) Pulmonary edema and hydrothorax in swine produced by fumonisin $B_1$, a toxic metabolite of *Fusarium moniliforme*. *J Vet Diagn Invest* **2**: 217–21.

Hayes AW (1981) *Mycotoxin Teratogenicity and Mutagenicity*, CRC Press, Boca Raton, FL, pp. 41–66.

Hebb CO, Ratkovic D (1962) Choline acetylase in the placenta of man and other species. *J Physiol (London)* **216**: 307–13.

Ho M, Gibson MA (1972) A histochemical study of the developing tibiotarsus in malathion-treated chick embryos. *Can J Zool* **5**: 1293–8.

Holt D, Webb M (1987) Teratogenicity of ionic cadmium in the Wistar rat. *Arch Toxicol* **59**: 443–7.

Holmberg RE, Ferm VH (1969) Interrelationship of selenium, cadmium and arsenic in mammalian teratogenesis. *Arch Environ Health* **18**: 873–7.

Hood RD, Vedel GC, Zaworotko M, *et al*. (1987) Distribution, metabolism and fetal uptake of pentavalent arsenic in pregnant mice following oral or intraperitoneal administration. *Teratology* **35**: 19–25.

Hsu IC, Smalley EB, Strong FM, Ribelin WE (1972) Identification of T-2 toxin in moldy corn, associated with a lethal Toxicosis in dairy cattle. *Appl Microbiol* **24**: 684–90.

Husain R, Gupta A, Khanna VK, *et al*. (1991) Neurotoxicological effects of a pyrethroids formulation fenvalerate in rat. *Commun Chem Pathol Pharmacol* **73**: 111–14.

Husain R, Malaviya M, Seth PK, Husain R (1992) Differential responses of regional brain polyamines following *in utero* exposure to synthetic pyrethroids insecticides: a preliminary report. *Bull Environ Contam Toxicol* **49**: 402–9.

Jacquet P, Gerber GB (1979) Teratogenic effects of lead in the mouse. *Biomedicine* **30**: 223–9.

Javed T, Bennett GA, Richard JL, *et al.* (1993a) Mortality in broiler chicks on feed amended with a *Fusarium proliferatum* culture or with purified fumonisin $B_1$ and moniliformin. *Mycopathologia* **123**: 171–84.

Javed T, Richard JL, Bennett GA, *et al.* (1993b) Embryopathic and embryocidal effects of purified fumonisin $B_1$ or *Fusarium proliferatum* culture material extract on chicken embryos. *Mycopathologia*, **123**: 185–93.

Juchau MR (1980) Drug biotransformation in the placenta. *Pharmacol Ther* **8**: 501–24.

Juchau MR (1995) Placenta enzymes: cytochrome P450s and their significance. In *Placental Toxicology*, Sastry BVR (ed.) CRC Press, Boca Raton, FL, pp. 197–212.

Keeler RF, Balls LD, Panter KE (1981) Teratogenic effects of *Nicotiana glauca* and concentration of anabasine, the suspect teratogen in plant parts. *Cornell Vet* **71**: 47–53.

Khera KS (1979) Evaluation of dimethoate (Cygon 4E) for teratogenic activity in the cat. *J. Environ Pathol Toxicol* **2**, 1283–8.

Khera KS, Whalen C, Angers G, *et al*. (1982) Embryotoxicity of 4-deoxynivalenol (vomitoxin) in mice. *Bull Environ Contam Toxicol* **29**: 487–91.

King RG, Gude NM, Krishna BR, Chen S, Brennecke SP, Boura ALA, Rook TJ (1991) Human placental acetylcholine. *Reprod Fertil Dev* **3**: 405–11.

Koshakji RP, Sastry BVR, Harbison RD (1974) Studies on the levels and nature of cholinesterases in humans and mouse placenta. *Res Commun Chem Pathol Pharmacol* **9**: 181–4.

Kuiper-Goodman T, Scott PM, Watanabe M (1987) Risk assessment of the mycotoxin Zearalenone. *Regul Toxicol Pharmacol* **7**: 253–306.

LaBorde JB, Terry KK, Howard PC, *et al*. (1997) Lack of embryotoxicity of fumonisin $B_1$ in New Zealand White rabbits. *Fundam Appl Toxicol* **40**: 120–28.

Lebepe-Mazur S, Bal H, Hopmans E, *et al.* (1995) Fumonisin $B_1$ is fetotoxic in rats. *Vet Hum Toxicol* **37**: 126–30.

Levario-Carrillo M, Olave ME, Corral DC, Alderete JG, Gagioti SM, Bevilacqua E (2004) Placental morphology of rats prenatally exposed to methyl parathion. *Exp Toxicol Pathol* **55**: 489–96.

Levin AA, Miller RK (1980) Fetal toxicity of cadmium in the rat: maternal vs. fetal injections. *Teratology* **22**: 1–5.

Malaviya M, Husain R, Seth PK, Husain R (1993) Perinatal effects of two pyrethroid insecticides on brain neurotransmitter function in the neonate rat. *Vet Hum Toxicol* **35**: 119–22.

Marin-Kuan M, Nestler S, Verguet C, *et al.* (2006) A toxicogenomics approach to identify new plausible epigenetic mechanisms of ochratoxin A carcinogenicity in rat. *Toxicol Sci* **89**: 120–34.

Marinovich M, Ghilardi F, Galli C (1996) Effect of pesticide mixtures on *in vitro* nervous cells: comparison with single pesticides. *Toxicology* **108**: 201–06.

Mayura K, Reddy RV, Hayes AW, Berndt WO (1982a) Embryocidal, fetotoxic and teratogenic effects of Ochratoxin A in rats. *Toxicology* **25**: 175–85.

Mayura K, Hayes AW, Berndt WO (1982b) Teratogenicity of Secalonic acid D in rats. *Toxicologist* **25**: 311–12.

Mayura K, Smith E, Heidelbaugh N, Philips TD (1985) Diacetoxyscirpenol induced prenatal dysmorphogenesis in the mouse. *Toxicologist* **5**: 187.

McClain RM, Becker BA (1975) Teratogenicity, fetal toxicity, and placental transfer of lead nitrate in rats. *Toxicol Appl Pharmacol* **31**: 72–82.

Morrissey RE (1984) Teratological study in Fischer rats fed diet containing added vomitoxin. *Food Chem Toxicol* **22**: 453–7.

Pacifici GM, Nottoli R (1995) Placental transfer of drugs administered to the mother. *Clin Pharmacokinet* **28**: 235–69.

Panter KE, James LE, Gardner DR (1999) Lupines, poison-hemlock, and Nicotiana spp: toxicity and teratogenicity in livestock. *J Nat Toxins* **8**: 117–33.

Parizek J (1964) Vascular changes at sites of estrogen biosynthesis produced by parenteral injection of cadmium salts: the destruction of placenta by cadmium salts. *J Reprod Fertil* **7**: 263–5.

Pasanen M, Pelkonen O (1994) The expression and environmental regulation of P450 enzymes in human placenta. *Crit Rev Toxicol* **24**: 211–29.

Patermain JL, Domingo JL, Llobet JM, *et al*. (1988) Embryotoxic and teratogenic effects of aluminum nitrate in rats upon oral administration. *Teratology* **38**: 253–7.

Pelkonen O (1984) Xenobiotic metabolism in the maternal–placental–fetal unit: implications for fetal toxicity. *Dev Pharmacol Ther* **7**(Suppl. 1): 11–17.

Pelkonen O, Vähäkangas K, Gupta RC (2006) Placental toxicity of organophosphate and carbamate pesticides. In *Toxicology of Organophosphate and Carbamate Compounds*, Gupta RC (ed.). Academic Press/Elsevier, Amsterdam, pp. 463–79.

Perez-Martinez C, Ferreras-Estrada MC, Garcia-Iglesias MJ, *et al.* (1997) Effects of *in utero* exposure to nonsteroidal estrogens in mouse testis. *Can J Vet Res* **61**: 94–98.

Reddy CS, Reddy RV, Hayes AW, Ciegler A (1981) Teratogenicity of secalonic acid D in mice. *J Toxicol Environ Health* **7**: 445–55.

Reddy RV, Reddy CS, Johnson GC, *et al*. (1995) Developmental effects of pure fumonisin $B_1$ in CD1 mice. *Toxicologist* **15**: 157.

Riley RT, Showker NK, Yoo H-S, *et al*. (1993) Alterations of tissue and serum sphinganine to sphingosine ratio: an early biomarker of exposure to fumonisin-containing feeds in pigs. *Toxicol Appl Pharmacol* **118**: 105–12.

Robens JF (1969) Teratologic studies of carbaryl, diazinon, norea, disulfiram, and thiram in small laboratory animals. *Toxicol Appl Pharmacol* **15**: 152–63.

Rogers JM (1996) The developmental toxicology of cadmium and arsenic with notes on lead. In *Toxicology of Metals*, Chang LW (ed.). CRC Lewis Publishers, Boca Raton, FL, pp. 1027–45.

Rogers JM, Grabowski CT (1983) Mirex-induced fetal cataracts: lens growth histology and cation balance, and relationship to edema. *Teratology* **27**: 343–9.

Roll R, Matthiaschk G, Konte A (1990) Embryotoxicity and mutagenicity of mycotoxins. *J Environ Pathol Toxicol Oncol* **10**: 1–7.

Rotter BA, Preluski DB, Pestka JJ (1996) Toxicology of deoxynivalenol (vomitoxin). *J Toxicol Environ Health* **48**: 1–34.

Roussoux CG, Nicholson S, Schieffer HB (1985) Fetal placental hemorrhage in pregnant CD1 mice following one oral dose of T-2 toxin. *Can J Comp Med* **49**: 95–8.

Sastry BVR (1997) Human placental cholinergic system. *Biochem Pharmacol* **53**: 1577–86.

Sastry BVR (2000) Placental acetylcholine. In *Molecular Aspects of Placental and Fetal Membrane Autacoids*, Rice GE, Brenecke SP (eds). CRC Press, Boca Raton, FL, pp. 157–93.

Sastry BVR, Janson VE (1995) Smoking, placental function, and fetal growth. In *Placental Toxicology*, Sastry BVR (ed.). CRC Press, Boca Raton, FL, pp 45–81.

Schneider H (1995) Techniques: *in vitro* perfusion of human placenta. In *Placental Toxicology*, Sastry BVR (ed.). CRC Press, Boca Raton, FL, pp. 1–25.

Schoental R (1977) Depletion of coenzymes at the site of rapidly growing tissues due to alkylation: the biochemical basis of the teratogenic effects of alkylating agents, including organophosphorus and certain other compounds. *Biochem Soc Trans* **5**: 1016–17.

Simone C, Derewlany LO, Oskamp M, *et al*. (1994) Acetylcholinesterase and butyrylcholinesterase activity in the human term placenta: implications for fetal cocaine exposure. *J Lab Clin Med* **123**: 400–6.

Sircar S, Lahiri P (1989) Lindane (gamma-HCH) causes reproductive failure and fetotoxicity in mice. *Toxicology* **59**: 171–7.

Smalley HE, Curtis JM, Earl FL (1968) Teratogenic action of carbaryl in beagle dogs. *Toxicol Appl Pharmacol* **13**: 392–403.

Srivastava MK, Raizada RB, Dikshith TS (1992) Fetotoxic response to technical quinalphos in rats. *Vet Hum Toxicol* **34**: 131–3.

Stanford GK, Hood RD, Hayes AW (1975) Effect of prenatal administration of T-2 toxin in mice. *Res Commun Chem Pathol Pharmacol* **10**: 743–8.

Still PE, Macklin AW, Ribelin WE, Smalley EB (1971) Relationship of ochratoxin A to fetal death in laboratory and domestic animals. *Nature* **234**: 563–4.

Stump D G, Holson JF, Fleeman TL (1999) Comparative effects of single intraperitoneal or oral doses of sodium arsenate or arsenic trioxide during *in utero* development. *Teratology* **60**: 283–91.

Suzuki K, Horiguchi T, Comas-Urrutia AC, *et al.* (1974) Placental transfer and distribution of nicotine in the pregnant rhesus monkey. *Am J Obstet Gynecol* **119**: 253–62.

Wang E, Ross PF, Wilson TM, *et al*. (1992) Alteration of serum sphingolipid upon dietary exposure of ponies to fumonisins, mycotoxins produced by *F moniliforme J Nutr* **122**: 1706–16.

Wangikar PB, Dwivedi P, Sinha N (2004) Teratogenic effects of Ochratoxin A in rabbits. *World Rabbit Sci* **12**: 159–171.

Wangikar PB, Dwivedi P, Sinha N, Sharma AK, Telang AG (2005) Teratogenic effects in rabbits of simultaneous exposure to ochratoxin A and aflatoxin $B_1$ with special reference to microscopic effects. *Toxicology* **215**: 37–47.

Welsch F (1982) Placental transfer and fetal uptake of drugs. *J Vet Pharmacol Ther* **5**: 91–104.

Welsch F, Dettbarn W-D (1971) Protein synthesis in lobster walking leg nerves. *Comp Biochem Physiol* **38B**: 393.

Wide M (1984) Effect of short-term exposure to five industrial metals on the embryonic and fetal development of the mouse. *Environ Res* **33**: 47–53.

Wilson BJ, Harbison RD (1973) Rubratoxin. *J Am Vet Med Assoc* **163**: 1274–6.

Yagen B, Bialer M (1993) Metabolism and pharmacokinetics of T-2 toxin and related trichothecenes. *Drug Metab Rev* **25**: 281–323.

Yokel RA, McNamara PJ (1985) Aluminum bioavailability and disposition in adult and immature rabbits. *Toxicol Appl Pharmacol* **77**: 344–52.

Yokel RA (1997) The metabolism and xenobiotics of aluminum relevant to neurotoxicity. In *Metal and Mineral Neurotoxicity*, Yasui M, Strong MJ, Ota K, Verity MA (eds). CRC Press, Boca Raton, FL, pp. 81–9.

Zhao Z, Reece EA (2005) Nicotine-induced embryonic malformations mediated by apoptosis from increasing intracellular calcium and oxidative stress. *Birth Defects Res (Part B)* **74**: 383–91.

CHAPTER

# 16

# Dermal toxicity

*Faqir Muhammad and Jim E. Riviere*

## INTRODUCTION

Skin is an essential and dynamic organ. In addition to performing important functions including thermoregulation and preventing insensible water loss, it also has important metabolic, immunological and neurosensory properties. However, the predominant function of skin is to protect the body against a variety of toxicological insults. Animals are relatively less protective against such insults as compared to humans due to lack of clothing, inferior housing and different social interactions. In most instances, the skin of animals directly contacts environmental, chemical and other pollutant exposure without the benefit of manmade protection. Although the largest organ of the body can often face these insults to a certain threshold, animals exhibit symptoms of dermal toxicity when this limit is passed.

Knowledge of the basic structure of skin is necessary to understand the mechanisms of dermal absorption and toxicity of topically applied toxicants. Skin is composed of three distinct layers namely, epidermis, dermis and hypodermis. Out of these layers, the epidermis (consists of kerationocytes and non-keratinocytes) is considered the most important barrier to dermal penetration of most chemicals. The epidermal layers can further be classified from external surface inward as stratum corneum, stratum lucidum, stratum granulosum, stratum spinosum and stratum basale. The stratum basale consists of single layer of cuboidal or columnar cells resting on basal lamina and is the viable layer of cells in the epidermis. These cells are mitotically active, thus keeping the epidermis replenished as the stratum corneum (SC) cells are sloughed from the surface epidermis (Lavker and Sun, 1983; Monteiro-Riviere, 2006). The approximate cell turnover and self-replacement time in normal human skin is 28 days. This differs widely across species. Mechanical or chemical injuries can increase the mitotic rate of basal cells. Stratum spinosum consists of several layers of irregular polyhedral cells. The cells of this layer have numerous tonofilaments and small membrane bound organelles (lamellar granules). Stratum granulosum consists of several layers of flattened cells containing irregularly shaped, non-membrane bound and electron-dense keratohyalin granules. These granules have a role in keratinization and maintaining the barrier functions of the skin. The lamellar granules contain several types of lipids (ceramides, cholesterol, fatty acids) and hydrolytic enzymes including proteases, acid phosphatases, lipases and glycosidases (Downing, 1992; Monteiro-Riviere, 2006). Stratum lucidum is a thin layer only present in very thick areas of skin such as palmer and plantar surfaces.

The outermost layer of epidermis is the SC that consists of several layers of completely keratinized dead cells (corneocytes) embedded in an extracellular lipid matrix. This has been depicted as the "brick and mortar model" where keratinized cells, the bricks, are embedded in the lipid mortar. The dead keratinized cells are highly water absorbent and keep the skin moist and soft. The water holding capacity of the epidermis is further maintained by sebum (natural oil covering the skin) secretion from glandular structures of the skin. A number of non-keratinocyte cell types are found in skin. The melanocytes are involved in skin pigmentation; Merkel cells act as mechanoreceptors for touch, while Langerhans cells play a major role in the skin immune response. These specialized cells are not involved in barrier functions of skin (Monteiro-Riviere, 2006).

Other specialized regions of the epidermis are the skin appendages which include hair, sweat and sebaceous glands, hoof, claw, nail, feathers and horn. The dermis consists of dense irregular connective tissue with collagen, elastic and reticular fibers in a mucopolysaccharides ground

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substance. Fibroblasts, mast cells and macrophages are the predominant cells of this layer. In addition, sweat glands, sebaceous glands, hair follicles and erector pili muscles are located in the dermis. The hypodermis is a layer of loose connective tissues beneath the dermis. This layer helps to anchor the dermis to the underlying tissues such as muscle or bone (Monteiro-Riviere, 2006).

SC is considered the primary barrier of skin against the penetration of foreign substances as this layer provides up to 1000 times the resistance to exogenous compounds as compared to the layers beneath it. Disruption of this layer either by physical (tape striping) or by chemical means will adversely affect the barrier properties. Extraction of these epidermal lipids with organic solvents (Monteiro-Riviere *et al.*, 2001) and jet fuel hydrocarbons (Muhammad *et al.*, 2005b) can reduce the barrier functions of the skin. The other two deeper layers of skin (dermis and hypodermis) offer no resistance to penetration of most compounds. Once a substance has penetrated the epidermis, it will easily traverse the other layers. The potential exceptions are very lipophilic compounds (log lipid to water partition coefficient, greater than four) that may tend to stay in the lipid environment of skin.

## ABSORPTION

Toxicants traverse biological membranes either by passive diffusion or by active transport. A passive diffusion process implies that the solute flux is linearly dependent on the solute concentration gradient; while, active transport processes typically involve a saturable mechanism (Friedman, 1986). Dermal absorption is a passive process. Generally, topical absorption involves a sequence of events that include partitioning of the molecule into the SC from the applied vehicle phase, diffusion through the SC, partitioning from the SC into the viable epidermis, diffusion through the epidermis and upper dermis and finally capillary uptake and systemic exposure. Hence, the movement of toxic molecules across the skin involves transport through a series of resistances. In general, for polar toxicants, the diffusional resistance of the SC is large compared to that presented by the viable epidermis and dermis. For more lipophilic molecules, the resistance of the SC is smaller. However, the SC maintains a rate-controlling role since a highly lipophilic molecule does not favorably partition out of the SC into the more aqueous viable epidermis. From the description of SC components and structure, one can envisage that a diffusing molecule can adopt one or more of the following penetration pathways:

1 The intercellular/para-cellular path, via the tortuous but continuous intercellular lipids.
2 The transcellular path, indicating that the toxicants transfer sequentially and repeatedly through the "bricks" and "mortar".
3 The transappendageal path via hair follicles, sweat pores, etc.

Most molecules follow the first penetration pathway, yet the absorption of certain compounds can take place via transfollicular path or sweat pores, often resulting in skin penetration (residing within skin) rather than true absorption (systemic exposure). Both absorption and penetration are important determinants of direct chemical toxicity to the skin. Diffusion occurs through the intercellular lipids hence partitioning into these lipids is often the primary determinant of absorption. Species differences in lipid makeup thus also translate into species differences in absorption.

Percutaneous absorption through the intercellular pathway of the SC is driven by passive diffusion down a concentration gradient described at steady state by Fick's law of diffusion (Riviere, 1999):

$$\text{Flux} = [(D \bullet PC \bullet SA)/H](\Delta x)$$

where $D$ is the diffusion coefficient and $PC$ is the partition coefficient, $SA$ is the applied surface area, $H$ is membrane thickness (or more precisely the intercellular path length) and $\Delta x$ is the concentration gradient across the membrane. Since *in vivo* blood concentrations after absorption are negligible compared to applied surface concentration, $\Delta x$ reduces to the concentration ($C$). It is this relationship that allows the prediction of compound flux across the skin to be correlated to factors predictive of $D$ and $PC$ (e.g. octanol/water partition coefficients reflecting chemical partitioning into the SC lipids). Flux is expressed in terms if applied surface area, often normalized to $cm^2$.

The term ($D \bullet \mathrm{PC/H}$) is compound dependent and is termed the permeability coefficient ($K_p$), reducing the determination of flux to $K_p \bullet \Delta X$ or $K_p \bullet C$, a first-order pharmacokinetic equation ($dx/dt = kX$). Rearrangement of this equation yields the primary method used to experimentally determine $K_p$:

$$K_p = \text{steady state flux/concentration}$$

It must be stressed that both transdermal flux and $K_p$ are not only chemical dependent, but also tightly constrained by the membrane system studied as well as the method of topical application (neat compound, vehicle, length of experiment, etc.). The PC that is integral to $K_p$ is the PC between the surface or applied vehicle and the SC. Different vehicles will thus result in different PCs. Similarly, skin from different species may result in different PC due to differences in the SC lipids and intercellular path lengths.

Cutaneous biotransformation of drugs is also a barrier to absorption of certain compounds, and has been used to promote the absorption of certain chemicals (pro drugs) across the skin. This aspect is important in skin toxicology as non-toxic parent compounds may be bioactivated within the epidermis, such as benzo[*a*]pyrene to an epoxide (Riviere, 1999).

There are numerous factors that can affect the dermal toxicity in animals. These include the species, breed, age, health status, skin condition (dryness, hairiness or thickness) and local environment (weather, humidity, temperature). Some of these factors including epidermal thickness, epidermal cell size, number of cell layers and blood flow patterns can vary between species as well as within the species (Monteiro-Riviere *et al*., 1990). As a general rule, young and emaciated animals are more prone to dermal intoxication than are adults or healthy animals. In this chapter, the agents involved in dermal toxicity directly or indirectly are described. There are a variety of compounds including pesticides, polycyclic aromatic hydrocarbons (PAHs), volatile organic compounds (VOCs), heavy metals, photosensitizing agents and toxic plants that can affect the skin of animals by multiple mechanisms.

## PLANTS CAUSING DERMAL TOXICITY IN ANIMALS

Exposure to plant toxins may be the most common cause of dermal toxicity in animals, since the majority of production animals are reared on pastures where they come in contact with various types of vegetation. Some plants have compounds that directly affect the skin after contact, while others have thorns/spines that can cause mechanical injury to the skin. Many compounds may induce allergic dermatitis, similar to poison ivy in humans. A discussion of contact sensitization is beyond the scope of the present chapter. A text in immunology or allergy medicine should be consulted. Many plants have pigments or compounds that once absorbed from the gastrointestinal tract, induce a direct effect on non-pigmented skin when exposed to light. Other plants can result in a secondary photosensitization by virtue of toxic alkaloids that cause irreversible liver disease.

### Photosensitization

Photosensitization is a severe dermatitis resulting from a complex reaction induced by plant pigments exposed to ultraviolet (UV) sunlight in the skin of animals that have eaten certain plants (Rowe, 1989). UV radiation can induce acute or chronic photobiologic reactions in the absence of exogenous chromophores such as nuclear DNA (Horio *et al*., 2005). Non-pigmented skin gives the most severe reaction where these reactive compounds are directly exposed to UV light, most likely secondary to light-enhanced photooxidation. The amino acids susceptible to oxidation (histidine, tyrosine and tryptophan) once oxidized evoke an intense inflammatory response in the blood vessels and surrounding cells resulting in tissue necrosis. In addition to plant pigments, fungal toxins and certain chemicals can induce photosensitization. Quite frequently horses and cattle develop photosensitization during grazing on pasture may be due to oxidative damage in photodynamic therapy (Clare, 1955; Babilas *et al*., 2005). Similarly outbreaks of facial eczema (pithomycotoxicosis), a hepatogenous photosensitization caused by the mycotoxin sporidesmin, have been reported in ruminants in the Azores Islands of Portugal (Pinto *et al*., 2005).

Photosensitization may be classified into primary and secondary types (Table 16.1). Primary photosensitization is associated with photodynamic compounds in certain plants, which react in the non-pigmented skin with UV light to cause a severe dermatitis. Secondary photosensitization, results when an animal's liver is sufficiently diseased to be unable to remove plant by-products that can react with UV light to cause photosensitization. Phylloerythrin, a bacterial breakdown product of chlorophyll, is a potent photosensitizing compound. Normally phylloerythrin is removed by the liver and is excreted in the bile, but if the liver is severely diseased, it accumulates in the blood to cause photosensitization if a white skinned animal is exposed to UV light. Secondary photosensitization is much more common in livestock than primary photosensitization.

Primary photosensitization develops when animals eat plants having polyphenolic pigments. Historically, two plants buckwheat (Kingsbury, 1964) and St. John's wort (Marsh, 1930; Araya and Ford, 1981) are associated with primary photosensitization. Bishop's weed (*Ammi majus*), Rain lily (*Cooperia pedunculata*), Spring parsley (*Cymopterus watsonii*), Buckwheat (*Fagopyrum esculentum*), Giant hogweed (*Heracleum mantegazzianum*), St. John's wort (*Hypericum perforatum*) and Dutchman's britches (*Thamnosma texana*) are reported as primary photosensitizing plants (Knight and Walter, 2003). These plant species (Table 16.1) contain photodynamic furanocoumarin compounds that have been associated with photosensitivity through ingestion and direct contact with the skin (Dollahite *et al*., 1978; Oertli *et al*., 1983). A seasonal photosensitivity of cattle, associated with the consumption of the dead leaves of rain lily has been reported in southeast Texas (Rowe *et al*., 1987; Casteel *et al*., 1988). Similarly, giant hogweed (*Heracleum mantegazzianum*) and Cow parsnip (*Heracleum* spp.) are also known to cause photosensitivity in Europe and North America, respectively (Knight and Walter, 2003).

**TABLE 16.1 Important plants causing photosensitization in animals**

| Common name | Botanical name | Type of photo-sensitivity |
|---|---|---|
| St. John's wort | *Hypericum perforatum* | Primary |
| Spring parsley | *Cymopterus watsonii* | Primary |
| Bishop's weed | *Ammi majus* | Primary |
| Dutchman's britches | *Thamnosma texana* | Primary |
| Rain lily | *Cooperia pedunculata* | Primary |
| Groundsels, Senecio | *Senecio* spp. | Secondary |
| Fiddle neck | *Amsinckia* spp. | Secondary |
| Rattle box | *Crotolaria* spp. | Secondary |
| Hound's tongue | *Cynoglossum officinale* | Secondary |
| Blue weed | *Echium vulgare* | Secondary |
| Lantana | *Lantana camara* | Secondary |
| Panic grasses | *Panicum* spp. | Secondary |
| Horse brush | *Tetradymia* spp. | Secondary |
| Alsike clover | *Trifolium hybridum* | Secondary |
| Black sage | *Artemisia nigra* | Secondary |
| Puncture vine | *Tribulus terrestris* | Secondary |

Secondary photosensitization is primarily caused by a variety of compounds toxic to the liver that are found in plants, the most important of which are the pyrrolizidine alkaloids (PAs). There are four important plant genera namely, *Senecio*, *Crotolaria*, *Cynoglossum* and *Amsinckia* that cause liver disease and secondary photosensitization in North America (Table 16.1). Most PA poisoning of livestock in the Western United States is attributable to three species of *Senecio*: tansy ragwort (*S. jacobaea*), threadleaf or wooly groundsel (*S. douglasii* var. *longilobus*), (Johnson and Molyneux, 1984; Johnson *et al*., 1989) and Riddell's groundsel (*S. riddellii*) (Johnson *et al*., 1985; Molyneux *et al*., 1991). *Senecio* species are also the most common cause of PA poisoning throughout the world (Lombardo de Barros *et al*., 1992; Odriozola *et al*., 1994). Fiddleneck (*Amsinckia intermedia*), and hound's tongue (*Cynoglossum officinale*), members of the Boraginaceae, have significant quantities of PA capable of producing secondary photosensitization in cattle and horses (John *et al*., 1974; Stegelmeier *et al*., 1994). The PA content of plants varies considerably, generally increasing with maturation of the plant and reaching a maximum just before the flower buds open (Candrian *et al*., 1984). Flowers have the greatest amount of the alkaloid, while seeds of *Crotolaria* and *Amsinckia* concentrate high levels of PA (Johnson and Smart, 1983; Nobre *et al*., 1994). Pigs are the most susceptible to PA poisoning, followed by poultry, cattle, horses, goats and sheep (Mattocks, 1968; Craig *et al*., 1992).

A variety of plant toxins other than PAs are also involved in secondary photosensitization. Amongst these, horse brush (*Tetradymia glabrata* and *T. canescens*) has been attributed to cause secondary photosensitivity in sheep in North America (Johnson, 1974; Flemming *et al*., 1922). However, sheep are much more susceptible to horse brush photosensitivity if they concurrently browse on black sage (*Artemisia nigra*), big sage (*A. tridentata*) or both (Flemming *et al*., 1922; Johnson, 1974). These plants frequently grow in the same locations in western rangelands, and when eaten together have a synergistic effect in causing photosensitivity. Certain plant species like *Lantana camara*, *Agave lecheguilla*, *Tribulus terrestris* and *Panicum* grass species cause secondary photosensitization through inflammation and obstruction of the biliary system (Knight and Walter, 2003). These photosensitizing plants contain saponins that cause inflammation and obstruction of the bile ducts. When the liver cannot excrete bile normally, photosensitizing compounds will accumulate in the blood of animals and will result in photosensitization (Radostits *et al*., 1994). Sheep eating puncture vine (*Tribulus terrestris*) develop photosensitivity secondary to biliary obstruction that was initially thought to be caused by a mycotoxin, but later found to be the result of steroidal saponins in the plant (Glasonbury and Doughty, 1984; Kellerman *et al*., 1994). An outbreak of photosensitization in sheep in Great Britain and Western Europe due to bog asphodel (*Narthecium ossifragum*) was caused by plant saponin that occluded the biliary system (Pass, 1987; Burrows, 1990). Alsike clover (*Trifolium hybridum*) is a perennial legume that is commonly grown for livestock consumption in northern parts of North America. This plant can cause secondary photosensitivity in horses that can be attributed to the accumulation of phylloerythrin in the horse's circulation as a result of liver failure (Nation, 1991; Colon *et al*., 1996).

Animals can also develop photosensitivity while eating moldy grains containing hepatotoxic mycotoxins (aflatoxins) produced by fungi belonging to the genera of *Aspergillus* and *Penicillium*. Moldy straw and water-damaged alfalfa hay may also cause photosensitivity in cattle as a result of mycotoxins that induce hepatitis and cholangitis (Richard, 1973; Putnam *et al*., 1986; Scruggs and Blue, 1994). Feeding moldy alfalfa hay and silage to cattle has been associated with a secondary photosensitivity due to liver toxins produced by a variety of fungi cultured from the hay (House *et al*., 1996). However, cattle appear to be more susceptible to these unidentified toxins as compared to sheep, goats and horses (Casteel *et al*., 1994).

Blue-green algae or cyanobacteria is reported to cause poisoning in North America include members of the genera *Microcystis*, *Anabaena* and *Aphanizomenon*. These organisms release potent toxins into the water that can cause severe poisoning in animals drinking the contaminated water. Some of these toxins can act on the liver to cause liver failure and photosensitization (Beasley *et al*., 1989a, b; Osweiler *et al*., 1985a; Hoover and Smith, 1995). Southdown sheep may also develop photosensitivity due to a congenital defect in the liver's ability to excrete the photoreactive compound phylloerythrin (Hancock, 1950). In addition to plants, certain drugs and chemicals such as phenothiazine sulfoxide, some tetracycline antibiotics and psoralen, which

employs this toxic property as a mechanism of its therapeutic effect to treat conditions such as psoriasis, also produce photosensitivity in animals.

Some molds are not only photosensitizing, but may be toxic to the skin of animals directly or indirectly by other mechanisms. Ergot alkaloids can induce gangrene in all species of animals if ingested over a period of several days or weeks. The most commonly involved fungus is *Claviceps purpurea.* The distal extremities, tail, ears and nose may slough away during this disease. Other mold toxins like serotoxins (trichothecene toxin T-2) have the ability to irritate the skin and mucous membranes of animals on contact. Stomatitis, oral ulcers and necrosis of areas of skin that are in direct contact to moldy plant may be seen in the affected animals. Another trichothecene toxin produced by *Fusarium tricinctum* is diacetoxyscirpenol. It may cause dermal necrosis and gangrene in cattle fed on moldy corn. Acute fescue (a common pasture grass in United States) toxicosis in cattle resembles gangrenous ergotism. In this condition, animals can develop dry gangrene of the extremities. The clavicipetaceous fungi are mostly present in fescue pasture. Fescue hay and seed infected with this mold are reported to be toxic to cattle (Jackson *et al.*, 1984).

## TOPICAL CHEMICALS CAUSING DERMAL TOXICITY IN ANIMALS

Skin is the second most frequent route by which chemicals enter the body of animals. Liquid chemicals are generally absorbed well through the skin if they can partition into the SC lipids. Chemicals in the forms of solids, gases and vapors are only absorbed through the skin if they are first dissolved in the moist layer at the surface of the skin. The skin is a major target for gaseous and liquid pollutants. Various allergic or inflammatory conditions of skin including eczema, atopic dermatitis or acne are often observed on exposure to various chemicals (Baudouin *et al.*, 2002). The topical chemicals that react most specifically with the skin are PAHs, VOCs, pesticides and heavy metals. A great deal of research has been conducted on experimental exposures to the common laboratory animals as components of mechanistic carcinogenesis studies or as a component of human risk assessments. Unfortunately, less is known about mechanisms of actions of compounds of veterinary importance.

### PAHs

PAHs including benz[*a*]anthracene, benzo[*b*]fluoran-thene, benzo[*j*]fluoranthene, benzo[*k*]fluoranthene, benzo-[*a*]-pyrene, dibenz[*a,j*]acridine, dibenz[*a,h*]acridine, dibenz[*a,h*]-anthracene, -dibenzo[*c,g*]carbazole, dibenzo[*a,e*]pyrene, dibenzo[*a,h*]pyrene, dibenzo[*a,i*]pyrene, dibenzo[*a,l*]pyrene, indeno[1,2,3-*cd*]pyrene and 5-methylchrysene are reported to cause toxicity in experimental animals (IARC, 1987). Most of these PAHs have been studied in biomedical, biochemical and other laboratory-based experiments. Out of these 15 PAHs, at least 8 are present in coal tar that is used as a fuel in the steel industry and blast furnaces. Coal tar is also used in the treatment of skin diseases like dermatitis, psoriasis and eczema. Coal tar can cause allergic or irritant contact dermatitis, erythema, stinging, precipitation of exfoliative dermatitis or generalized pustular psoriasis, tar folliculitis, atrophy, telangiectasia, pigmentation, cutaneous horns, keratoacanthomas and tar warts (Andrew and Moses, 1985; Burden *et al.*, 1994). Coal tar pitch is used for roofing, surface coatings and a variety of other applications (IARC, 1983); 3 out of these 15 PAHs are found in bitumen and asphalt that are used for paving roads, water proofing and coating pipes. Therefore, considerable risk exists for the animals to come in contact with these toxic PAHs in these localities/applications. Animals are exposed to PAHs through inhalation, eating fodders grown on contaminated soils or through skin when in contact with PAH contaminated soil or products such as heavy oils, coal tar and roofing tar or creosote (a wood preservative). The toxic levels of PAH has been measured in various tissues of fish that are supposed to be absorbed through the skin (Oliveira-Ribeiro *et al.*, 2005).

When administered topically, benz[*a*]anthracene and benzo[*j*]fluoranthene induced-skin papillomas in mice while benzo[*k*]fluoranthene was active as an initiator of skin tumors in female mice (IARC, 1983). Benzo[*a*]pyrene-induced skin papillomas and carcinomas in mice, rats, guinea pigs and rabbits. Dibenz[*a,j*]acridine has also been reported to cause skin tumors in mice. Whereas dibenzo-[*a,e*]pyrene, dibenzo[*a,h*]pyrene, dibenzo[*a,i*]pyrene and 5-methylchrysene produced skin tumors in mice when applied topically (IARC, 1973). To date, dibenzo[*a,i*]pyrene has been found to be the strongest tumor-initiating PAH ever tested in rodent skin and mammary tumor-models (Mahadevan *et al.*, 2005). However, the tumor promoting potential of benzopyrene can be increased many folds in combination with other toxic chemicals. For example the combinations of benzo[*a*]pyrene and UV-A light (Wang *et al.*, 2005), or benzo[*a*]pyrene and arsenic (Fischer *et al.*, 2005) treatment synergistically increased tumor incidence and multiplicity in hairless mouse skin. It has been reported that cytochrome P450 1B1 determines susceptibility to dibenzo[*a*]pyrene-induced tumor formation (Buters *et al.*, 2002). However, the organic extracts of black raspberries can inhibit benzo[a]pyrene-induced cell transformation *in vitro* possibly by inhibiting the transactivation of activated protein-1 and nuclear factor kappa-B (Huang *et al.*, 2002).

Chlorinated PAHs such as polychlorodibenzodioxines and polychlorofuranes are found in impurities in certain pesticides or as secondary residues in industrial processes.

These halogenated PAHs are very toxic for the skin and can cause chloride acne (Baudouin *et al*., 2002). In a recent incident, former Ukraine Prime Minister and Presidential Candidate Victor Yushchenko developed chloracne from dioxin poisoning. This mysterious poisoning had left his face pockmarked and ashen.

Skin can act as a storage organ for certain halogenated PAHs like hexachlorobiphenyl (Di Francesco *et al*., 1988). The halogenated PAHs are oily compounds, which can obstruct the pores of the skin, thus resulting in accumulation of sebum and keratin, leading to the formation of cysts. This hypothesis was supported with hyperkeratinization observed around the follicles along with involution of the sebaceous glands by topically applied 2,3,7,8-tetrachlorodibenzo-*p*-dioxin to mouse skin (Panteleyev *et al*., 1997).

The skin is particularly active in the metabolism of PAHs. Some PAHs are activated by epidermal cytochrome P450 and hydrolases to generate epoxides and diols, active metabolites able to bind to DNA and initiate the processes of carcinogenesis. Other PAHs cause the skin toxicity by different mechanisms. For example, topical application of 7H-dibenzo[*c,g*]carbazole caused the appearance of DNA adducts in mouse skin (Schurdak and Randerath, 1989). Another study exhibited the potentiating effect of iron on 7, 12-dimethylbenz[*a*]anthracene in the mouse skin. In addition to genotoxic effects of this PAH, the resulting oxidative stress makes this association tumorigenic in the skin tissue (Rezazadeh and Athar, 1997). The photoactivation of certain PAHs like 2-nitrofluorene by UV radiations in rats, can generate intermediates that can bind covalently to RNA and to the skin proteins. This association of 2-nitrofluorene and UV-A is mutagenic and could cause skin cancers (Wierckx *et al*., 1992).

## VOCs

VOCs such as hydrocarbons, ketones, aldehydes, solvents (benzene, fluorocarbons) and gases (methane) are generated from automobiles and industries. Certain VOCs such as benzene are highly genotoxic, inducing cancers in various tissues. Other VOCs can cause precancerous lesions in the lungs and in the skin (Michielsen *et al*., 1999). Some VOCs can produce inflammatory mediators in cultured epidermal keratinocytes. These mediators such as interleukin-8 and -1β are significantly increased that could favor the allergic/inflammatory reactions as in atopic dermatitis or eczema (Ushio *et al*., 1999).

Certain VOCs present in jet fuel like ethyl benzene, o-xylene and naphthalene did not produce direct skin irritation in pigskin (Muhammad *et al*., 2005a). Interestingly, these three hydrocarbons had higher absorption through pigskin compared to other aromatic and aliphatic hydrocarbons studied. Transmission electron microscope and Fourier

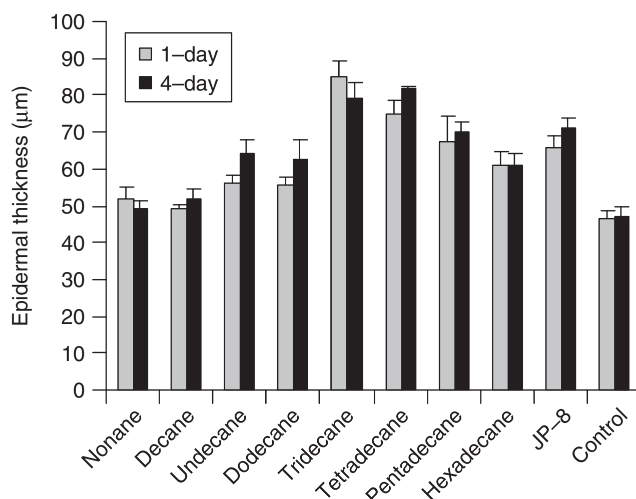


**FIGURE 16.1** Epidermal thickness (mean + SEM) after 1 and 4 days of exposure to *in vivo* JP-8 or constituent aliphatic hydrocarbons in pigs. Note the maximum toxic *in vivo* response with tridecane (C-13) and tetradecane (C-14) hydrocarbons.

transform infrared spectroscopy indicated that these PAHs have the potential to extract the lipids out of the SC of skin thus affecting the skin's permeability barrier and increasing the absorption of exogenous substances (Muhammad *et al*., 2005b). On the other hand, less volatile hydrocarbons of jet fuels such as tridecane, tetradecane and pentadecane produced significant direct dermal irritation and microscopic changes in pigskin (Figure 16.1).

Polychlorinated naphthalenes are present in lubrication greases, oils, fuels and in wood preservatives. Chlorinated naphthalenes can cause severe hyperkeratosis in cattle. The most prominent lesions were observed on the neck and wither but may affect the entire integument. This dermal toxicity in cattle needs to be differentiated from avitaminosis A. Chlorinated naphthalenes did not produce hyperkeratosis in sheep and swine (Clarke and Clarke, 1975). Ethylene glycol monomethyl ether (EGME) and its acetate ester (EGMEA) are moderately volatile liquids with very good solubility properties. They are used in paints, lacquers, stains, inks and surface coatings and as an antiicing additive in hydraulic fluids and jet fuel. EGME and EGMEA are efficiently absorbed via dermal penetration. Skin absorption in animals may contribute substantially to the total uptake following skin contact with liquids or vapors containing EGME or EGMEA (Johanson, 2000).

## Pesticides

The word "pesticide" in this chapter will encompass various relevant chemicals including insecticide for controlling termites or fleas, herbicides for ridding the crops/lawn of dandelions or fungicides that protect plants during fungal

diseases. Despite oral ingestion, dermal absorption is the major source of pesticide exposure in occupational, agricultural and veterinary settings, a topic recently reviewed (Riviere, 2006). Since most of the pesticides are highly lipophilic, their penetration and accumulation in skin with resulting local dermal irritation, and/or mild to severe systemic toxic effects in the exposed animals cannot be overlooked.

Livestock and pet animal exposure to pesticides is usually accidental. The most common source of dermal toxicity induced with insecticides in both large and small animals is the miscalculation of concentrations for spraying and dipping procedures. A misplaced decimal point can result in hundred-or thousand-fold increase in exposure. A common source of non-recommended exposure to animals is the inadvertent mixing of powdered pesticides, mistaken for salt or mineral preparations, into animal feeds. A low level contamination of animals by insecticides can also occur with the use of persistent chlorinated hydrocarbons such as dichlorodiphenyltrichloroethane (DDT), aldrin, dieldrin and heptachlor, etc. on crops which may be used for animal feeds. These insecticides may persist in soil for several years. Thus, forage crops grown on such soils could be sufficiently contaminated to produce toxic effects in animals. Other sources of insecticide exposure in animals includes carelessness in leaving insecticides accessible to animals, leaving insecticide preparations on sills of sheds or barns, inadequate covers on back rubbers containing insecticide concentrates, or using pesticide containers to water and feed animals without proper decontamination (Osweiler *et al*., 1985b). Thus, animals can exhibit signs of dermal toxicity after oral or systemic exposure to pesticides. On the other hand, washing the skin of humans or animals after exposure to a pesticide or other chemical may leave a major portion of the dose on/in the washed skin. This skin residue can contribute to the toxicity of a pesticide by continued post-wash absorption (Zendzian, 2003). According to a recent statistics on occupational dermatosis among private farmers, pesticides accounts for 18% as the causative factor for various skin disorders (Spiewak, 2003). The other most frequently identified causative factors for occupational dermatosis were plant dusts (38%), animal allergens (36%), metals (29%) and rubber chemicals (15%).

Certain organophosphorus insecticides such as chlorpyriphos have been shown to exert its systemic effects in the form of cholinesterase inhibition after dermal application to rats (Abu-Qure *et al*., 2001). The topical application of chlorpyriphos and cypermethrin to rats showed an inhibition of acetylcholinesterase and butyrylcholinesterase activity in the brain (Latuszynska *et al*., 2001). Another study suggested that skin could act as a reservoir and may release chlorpyriphos over a prolonged period (Griffin *et al*., 2000). Pyrethrins are insecticides that are derived from the extract of chrysanthemum flowers. The synthetic forms of pyrethrins are called pyrethroids. The initial plant extract (crude pyrethrum) contains about 30–35% pyrethrins. Allergic contact dermatitis has been observed in sensitized and non-sensitized guinea pigs by topical exposure to various extracts from pyrethrum flowers (Rickett *et al*., 1972). Occupationally, skin is the main route of pyrethroid absorption. The major toxic effect of dermal exposure is paraesthesiae, presumably due to hyperactivity of cutaneous sensory nerve fibers (Bradberry *et al*., 2005). The face is most commonly affected and the paraesthesiae are exacerbated by sensory stimulation such as sunlight, scratching, heat, sweating or water application. The specific treatment is not generally required, as paraesthesiae usually resolve in 12–24 h. Topical application of dl-$\alpha$ tocopherol acetate (vitamin E) may reduce the severity (Bradberry *et al*., 2005). Similarly, cypermethrin (a pyrethroid) can penetrate through the skin of animals and can exert its systemic effects (Latuszynska *et al*., 2001). Dermal exposure to permethrin (another pyrethroid) has often been considered a mitigating factor in Gulf War Syndrome (Riviere *et al*., 2002). Studies have shown that systemic exposure of certain chemicals such as diisopropyl fluorophosphate (DFP) or pyridostigmine bromide and topical application of DFP or *N*,*N*-diethyl-*m*-toluamide (DEET) can alter the disposition of [14C]permethrin in skin and possibly its bioavailability in soldiers simultaneously exposed to these chemicals (Baynes *et al*., 2002a).

A number of chemicals are used as fumigants for control of insects, nematodes, fungi and weeds in soils, stored grains, feedstuffs, storage spaces in mills and common carrier vehicles. Most of the toxicology of such chemicals is studied in laboratory animals and need to be extrapolated for the domestic animals. One such chemical (ethylene dichloride) is reported as irritating to the skin and mucous membranes. It can be absorbed through the skin although large doses are required to cause toxicosis in animals. Ethylene dibromide and metham-sodium are other skin irritants that also have the potential to absorb through the skin. Fluoroacetate was developed for insecticide and rodent control applications in early 1940s. This chemical has been reported to absorb through the abraded skin but not through the intact skin (Osweiler *et al*., 1985b). Paraquat is a non-volatile bipyridyl herbicide and is insoluble in water. Low-to-moderate amounts of bipyridyl herbicides are absorbed from the skin of animals. Direct contact of animals with paraquat results in irritation to skin (Longstaffe *et al*., 1981). Experimental studies in pigskin demonstrated epidermal toxicity in the absence of appreciable transdermal absorption (Srikrishna *et al*., 1992).

Many chemicals and solvents are dermal penetration enhancers. Such chemicals may increase dermal penetration by damaging the skin's barrier, by modifying the SC, or by promoting the partitioning of co-penetrating chemicals (Moser *et al*., 2001; Medi *et al*., 2006). This scenario may serve useful purposes in transdermal drug delivery but at

the same time, penetration enhancers may also increase skin penetration of harmful chemicals (Baynes *et al.*, 2002a). The percutaneous penetration of five pesticides having different solubilities was studied through a slightly damaged skin. It was observed that the percutaneous penetration of the most hydrophilic compounds will be affected most, and may significantly affect the rate, lag-time as well as total penetration of chemicals (Nielsen, 2005). A combination of two solvents (pyridostigmine bromide and DFP) increased the dermal absorption of DEET through isolated perfused porcine skin (Riviere *et al.*, 2003). Earlier studies by Baynes *et al.* (1997) reported that the extent of DEET absorption was greater with dimethyl sulfoxide (DMSO) and acetone than with ethanol in rat and mouse skin. These studies support that DEET can have sufficient systemic exposure to potentially cause signs of toxicity when simultaneously applied with pesticides such as permethrin or carbaryl. Thus, penetration enhancement can have undesirable consequences. In this context, commercial sunscreen formulations are found to significantly increase the transdermal penetration of a herbicide 2,4-dichlorophenoxyacetic acid in *in vitro* rat skin (Pont *et al.*, 2004). In addition to the active ingredients, "inert" ingredients in sunscreen or pesticide formulations may also be responsible for controlling the transdermal penetration of herbicide 2,4-dichlorophenoxyacetic acid (Pont *et al.*, 2003) and carbamate insecticide carbaryl (Baynes and Riviere, 1998), respectively.

Pentachlorophenol (PCP) has been widely used as a pesticide. The persistence of PCP in soil and water and apparent widespread use has resulted in significant exposure to animals. Young swine have died following dermal exposure to freshly PCP treated wood used in farrowing crates or farrowing houses. *In vivo* studies in swine demonstrated that exposure to PCP-contaminated soil can result in significant dermal absorption of the pesticide (Wester *et al.*, 1993; Qiao *et al.*, 1997). It has been demonstrated that the presence of solvent and/or surfactant mixtures can influence PCP absorption in the isolated, perfused porcine skin flap (IPPSF) (Riviere *et al.*, 2001). Baynes *et al.* (2002b) demonstrated that PCP is fairly well absorbed across porcine skin. PCP absorption in skin was greater in water or water-based mixtures than in 100% ethanol. PCP is barely soluble in water, but this pesticide is more likely to partition from water than an organic solvent vehicle such as ethanol into the SC. This situation warrants the dermal uptake of lipophilic pesticides through the skin of animals as the later have free access to water all the times. Both PCP and its major metabolite tetrachlorohydroquinone (TCHQ) could induce mice skin epidermal hyperplasia and proliferating cell nuclear antigen (PCNA) labeling index in the epidermis. However, TCHQ caused a more significant induction of epidermal hyperplasia and PCNA positive cells than PCP. Furthermore, topical application of PCP-induced significant organ enlargement and lymphoma in mice (Chang *et al.*, 2003).

The insecticide D-limonene toxicosis has been rarely described in canine species. One study has reported skin lesions consisted of coalescing erythematous patches in the cat groomed previously with a D-limonene-based insecticidal shampoo. Dermatohistopathologic changes included multifocal areas of acute coagulative epidermal necrosis (Lee *et al.*, 2002). Epichlorohydrin (ECH) is one of the more commercially important aliphatic epoxides used extensively as an insecticide, an industrial intermediate and as a laboratory reagent. It is a volatile, colorless liquid and behaves as an alkylating agent. Reports have shown that it causes the dermal toxicity in animals (Giri, 1997). Dodecen-1-ol (an insect attractant) acted as a primary skin irritant, since it caused superficial chemical burns (Beroza *et al.*, 1975). Ortho-phenylphenol (OPP) and its sodium (SOPP) and potassium (POPP) salts are used as fungicides and disinfectants. OPP has been reported as irritating, and SOPP and POPP as corrosive for the skin and mucous membranes (Bomhard *et al.*, 2002).

White phosphorus had been used to prepare rodenticides that are generally mixed with a greasy or oily base. Skin irritation or burning may occur from dermal exposure to high concentrations of such rodenticides but absorption is not observed with dermal exposure. Thallium is another rodenticide that is primarily used by government agencies. In subacute thallium poisoning in dogs and cats, reddening and pustule formation in the skin starting from the ears and nose and progressing to the axilla, abdomen and the rest of the body; associated with loss of hair was reported. In chronic thallium poisoning, skin hyperkeratosis, parakeratosis, hyperemia and vivid discoloration of skin has been observed in the cat (Osweiler *et al.*, 1985b).

## Detergents, solvents, corrosives and other household preparations

Several daily use products (soaps, detergents, drain cleaners; acids, alkalies, etc.) available in homes and other work places may cause dermal irritation. There is scarce information in literature regarding toxicosis due to these daily use products in animals. We will discuss briefly the possibilities of dermal irritation in animals based on well-known scientific principles and published reports.

The evaluation of cleansing products depends upon their cleansing properties (detergent or adsorptive), on the rinsability of the product, on the amount and nature of the additives and on the general and specifically epidermal toxicity of the components (Raab, 1990). In this context, one such preparation is denture cleaner in which the major toxic component is sodium perborate which decomposes to form hydrogen peroxide and sodium borate. These products are strongly alkaline and very irritating to the skin and mucous membranes thus can produce dermal toxicity in exposed animals. Similarly, constant or repeated exposure of skin to

anionic detergents (sulfomated or phosphorylated hydrocarbons) causes irritation with the removal of natural oils and can result in thickening of skin along with weeping, cracking, scaling and blistering (Dreisbach, 1983). This is evident that detergent substances are important components of cleansing materials. But their detergent cleansing action may also result in skin toxicity (Marks and Dykes, 1990). Non-ionic detergents such as alkyl and aryl polyether sulfates, alcohols or sulfonates are comparatively less irritating than ionic ones. The acute skin irritation potential of various detergent formulations can be assessed with patch test (Robinson *et al.*, 2005). In this test, the time of exposure required for 50% of subjects to show a positive skin reaction ($TR_{50}$ value) is calculated for each product. Using this approach, 24 detergent preparations were tested in 7 individual studies. The dermal irritation profiles could be categorized as follows (by decreasing irritancy): mold/mildew removers (average $TR_{50} = 0.37$ h) > disinfectants/sanitizers (0.64 h) > fabric softener concentrate (1.09 h) = aluminum wash (1.20 h) > 20% SDS (1.81 h) > liquid laundry detergents (3.48 h) > liquid dish detergents (4.16 h) = liquid fabric softeners (4.56 h) = liquid hand soaps (4.58 h) = shampoos (5.40 h) = hard surface cleaners (6.34 h) > powder automatic dish detergents (>16 h) = powder laundry detergents (>16 h) (Robinson *et al.*, 2005). Cleansing can also be accomplished with non-detergent containing cleaners (Marks and Dykes, 1990). Drain cleaners consisting of high concentrations (25–36%) of sodium hydroxide and sodium hypochlorite are extremely toxic and caustic to the skin. Direct skin contact to these agents causes coagulative to liquefaction necrosis by dissolving proteins and saponifying lipids. Soaps have been used for thousands of years as part of daily life. Soaps are limited by their irritancy to the skin due to their alkaline nature and their tendency to form insoluble and inactive salts when combined with either hard water or sea water (Kirsner and Froelich, 1998).

Calcium cyanide, which is a special constituent of fertilizers, may act as contact irritant causing skin ulcers. Chlorobromomethane (a fire extinguisher liquid) causes intense skin irritation from exposure to either liquid or its vapors, although this chemical has low dermal absorption. Skin contact to another chemical methyl bromide (used in fire extinguishers, refrigerants or fumigants) can result in irritation and vesiculation. Clinical poisoning may occur from its skin absorption (Osweiler *et al.*, 1985b). Liquid soldering fluxes mainly contain zinc chloride and a high proportion of hydrochloric acid. These components are caustic or corrosive and acute exposure results in direct irritation to skin. Glues and adhesives are often supplied in hydrocarbon solvents. Dermal exposure to these preparations may also produce irritation. The corrosive activity of hypochlorite (a common component of laundry bleaches) on skin is due to oxidizing potency or available chlorine. Alkalinity of some preparations may contribute to tissue injury. Metal cleaners and oven cleaners contain components like soda/potash and KOH/NaOH in high concentrations, respectively, in addition to petroleum-based solvents. All these components would be expected to cause dermal irritation. Ethylene glycol and propylene glycol (PG) are used in most of the topical pharmaceutical and cosmetic preparations as solvents. The localized dermal effects from both solvents are mild, while the published data suggested that PG might have a skin contact sensitization potential (Lakind *et al.*, 1999).

The alkalies present in oven cleaners may cause severe necrotic lesions similar to drain cleaners. Domestic animals, especially pets, may be exposed dermally to paints and varnish removers containing mixtures of benzene, methanol, acetone and toluene. These solvents can absorb through the skin in considerable amounts. Certain perfumes can cause local irritation of skin. This is probably due to alcohol serving as the primary vehicle. Rubbing alcohol (ethyl alcohol) may sometimes cause cutaneous hyperemia. Rust removers whose major toxic components are hydrochloric acid, phosphoric acid, hydrofluoric acid, etc. can have direct corrosive and necrotizing action like other acids. Most shampoos would not be expected to cause severe dermal irritation. Generally, skin exposures to acids, alkalies and phenols may lead to lesions that can vary from mild dermatitis to severe corrosion of the skin.

## SYSTEMIC COMPOUNDS CAUSING DERMAL TOXICITY IN ANIMALS

### Heavy metals

Metals are intrinsic in nature and are utilized by animals for many essential functions. As a rule, metals are required for life and health in small quantities but are toxic in excessive amount. The toxic heavy metals in animals include arsenic, lead, cadmium, chromium and nickel. These metals contaminate the environment from industrial, automobile and pesticide activities. The moderately toxic metals are iron, zinc, selenium, mercury and copper. Redistribution of metals in the environment is mainly responsible for access of animals to toxic metals not normally assessable. Metal toxicosis in animals results from the systemic administration of metals. The mechanisms involved in the toxicity of certain metals like cadmium, chromium and nickel have indicated that these metals act directly or indirectly on intracellular proteins in the skin (Carlisle *et al.*, 2001). Inorganic arsenic exposure in drinking water is linked to skin, bladder and lung cancer. The mechanism of arsenic-induced cancer is not clear, but this is proposed that arsenic can generate reactive oxygen species, suggesting that oxidation of DNA may play a role in carcinogenesis (Fischer *et al.*, 2005).

Heavy metals can form reversible complexes with organic ligands during the chelation process. For example, lead may be concentrated as a complex in bile. Proteins such as metallothionein serve as transport molecules for metals (Hammond and Beliles, 1980). This scenario may alter the availability and delivery of metals to specific organs. On the other hand, certain toxic metals can accumulate in definite tissue locations. For instance, bone which can store high concentrations of lead, can result in distribution to plasma protein or skin and hair. Susceptibility to dermal absorption of metals may be increased when the metal is organically bound, such as methyl mercury or tetraethyl lead. One such study indicated that the dermal symptoms of inorganic, aryl and methoxy ethyl forms of mercury in cattle include skin pustules, ulcers, depilation (starting at the root of tail) and skin keratinization. Animals with severe skin damage are severely ill. The febrile response and skin color changes occurring in mercury poisoning can easily be confused with hog cholera and erysipelas. In the start of clinical syndrome, mercury poisoning in pigs also resemble the early skin reddening and abnormal gait resulted in organic arsenic poisoning (Osweiler *et al*., 1985b).

Hair of animals may be the most common site of metal depositions since hair has a chemical affinity for heavy metals like arsenic, lead, thallium, selenium, bismuth and mercury. These metals have the potential to react with sulfhydryl groups of amino acids particularly cysteine, in the follicular proteins and incorporated into the keratin. Because of the relative ease of getting hair samples, concentrations of metals in hair may be used as a biomarker for risk assessment surveys to determine the chronic heavy metal exposure in animals.

Forage crops grown on seleniferous soils contain sufficient amounts of selenium to cause poisoning in domestic animals. Selenium may cause acute and chronic poisoning in all animals, especially cattle, sheep, horses, swine, chicken and dogs. A loss of hair/wool, deformation and sloughing of the hoof is seen in cattle, sheep and horses that were poisoned chronically with selenium. This condition is commonly known as alkali disease, a misnomer (Osweiler *et al*., 1985b). Chronic selenium (Se) toxicosis has been diagnosed in pigs with loss of hair, necrotic areas in the skin, lesions of the coronary band and hooves. The cause of this intoxication was the addition of the calculated amounts of sodium selenite directly to the feedstuff instead to mineral premix (Mihailovic *et al*., 1992). In one occurrence of arsenic poisoning in cattle, arsenic in the hair of affected survivors was assayed at 0.8–3.40 ppm versus 0.09–0.10 ppm in randomly selected control samples (Riviere *et al*., 1981). Inorganic tin salts are poorly absorbed and rapidly excreted in the feces; as a result they have a low toxicity. The main results of inorganic tin toxicity are skin and eye irritation; cholangitis of the lower biliary tract, and later hepatotoxicity; and neurotoxicity (Winship, 1988). Similarly, considerable skin changes in the form of scaliness on the dorsum of the neck and sloughing of the epidermis over neck, withers, ears, briskets, shoulders, back and tail was observed in beef calves after systemic administration of iodine. A slight degree of hair loss was also reported (Osweiler *et al*., 1985b).

Chromium is an essential trace element required for normal protein, fat and carbohydrate metabolism. It also helps in energy production and increasing lean body mass. A unique form of bioavailable chromium is niacin-bound chromium (NBC) that is used to promote a healthy lipid profile. The primary skin irritation test with NBC on New Zealand Albino rabbits indicated that NBC was slightly irritating (Shara *et al*., 2005).

Compounds other than metals can also produce dermal irritation on systemic administration. Oral administration of benzo[*a*]pyrene (PAH) caused the formation of DNA adducts in the skin (Baudouin, *et al*., 2002). The ingestion of hexachlorobenzene (VOC) in the rat leads to the formation of precancerous lesions in the skin and lungs (Michielsen *et al*., 1999). The high doses of pyrethrins (insecticides) fed to rats can induce benign skin lesions (USEPA, 1995). Polybrominated biphenyls were used as fire retardant in many parts of the world. On accidental ingestion of this chemical with contaminated feed or by licking the contaminated wood, animals with alopecia and thickened skin over the thorax, neck and shoulder had been noted. Systemic exposure of ruminants and sheep to thiocarbamate (herbicide) can induce alopecia (Osweiler *et al*., 1985b). Severe dermatitis over the ventral portion of the abdomen and inner surfaces of the limbs was developed in pigs that were given adriamycin (a systemic antibiotic) intravenously (Van Vleet *et al*., 1979).

## ASSESSMENT METHODS

*In vivo* testing of certain chemicals for their irritating or sensitizing potentials and assessment of their dermal uptake are other sources of skin toxicity in experimental animals. Animal welfare regulations mandate the careful monitoring of dermal irritation and toxicity studies due to the potential for causing pain and skin damage resulting from cutaneously applied materials. In the USA, the care and use of rabbits, guinea pigs, minipigs, dogs and non-human primates are regulated by the US Department of Agriculture under the Animal Welfare Act (AWA, amended, 1985). Rats and mice are not included in this act, yet most institutions use these animals under similar internal standards for these species. According to this act skin irritancy testing is a procedure which can cause slight pain in the animal. Severe erythema, erosions, ulcers, abscesses and necrosis are the painful skin reactions produced in response to topical application of chemicals. The degree of skin toxicity in experimental animals varies with response to acute, subchronic or chronic dermal irritation studies.

Amongst assessment methods capable of causing dermal toxicity, cutaneous microdialysis, tape stripping and skin surface biopsies are the major *in vivo* models used to assess the dermal uptake of topically applied toxicants. Microdialysis is a promising technique for determination of *in vivo* dermal absorption (Simonsen *et al*., 2004). This technique consists of a semipermeable membrane forming a thin hollow tube that functionally resembles a blood vessel. The microdialysis probe is implanted in the dermis of the skin via a guide cannula. This technique is minimally invasive, and produces a minor trauma during insertion of the guide cannula for the implantation of the microdialysis probe; although this trauma is reversible (Groth and Serup, 1998) yet this can damage the skin considerably in sensitive animals. After the insertion of probe, an equilibration period of minimum 90 and 30 min in human and rat skin, respectively, is necessary to allow the effects of trauma to diminish (Groth, 1996). Tape stripping is suitable for studying the penetration of topically applied chemicals into the SC. This is composed of serial stripping of SC with the help of common adhesive tapes. This technique is invasive to the minimal extent and frequently used to study the reservoir effect of the SC for topically applied constituents and the assessment of their *in vivo* percutaneous penetration effects (Muhammad and Riviere, 2006). Another minimal invasive technique is skin surface biopsies. With the help of this technique, it is possible to track the permeation of a compound through the SC by taking consecutive biopsies in the same area (Dykes *et al*., 1997).

## REFERENCES


Abu-Qure AW, Abdel-Rahman A, Brownie C, Kishk AM, Abou-Donia MB (2001) Inhibition of cholinesterase enzymes following a single dermal dose of chlorpyrifos and methyl parathion, alone and in combination in pregnant rats. *J Toxicol Environ Health A* **63**: 173–89.

Andrew NL, Moses K (1985) Tar revisited. *Int J Dermatol* **24**: 216–18.

Araya OS, Ford EJH (1981) An investigation of the type of photosensitization caused by the ingestion of St John's wort (*Hypericum perforatum*) by calves. *J Comp Pathol* **91**: 135–41.

Babilas P, Karrer S, Sidoroff A, Landthaler M, Szeimies RM (2005) Photodynamic therapy in dermatology – an update. *Photodermatol Photoimmunol Photomed* **21**: 142–9.

Baudouin C, Charveron M, Tarroux R, Gall Y (2002) Environmental pollutants and skin cancer. *Cell Biol Toxicol* **18**: 341–8.

Baynes RE, Riviere JE (1998) Influence of inert ingredients in pesticide formulations on dermal absorption of carbaryl. *Am J Vet Res* **59**: 168–75.

Baynes RE, Halling KB, Riviere JE (1997) The Influence of diethyl-*m*-toluamide (DEET) on the percutaneous absorption of permethrin and carbaryl. *Toxicol Appl Pharmacol* **144**: 332–9.

Baynes RE, Monteiro-Riviere NA, Riviere JE (2002a) Pyridostigmine bromide modulates the dermal disposition of (14C) permethrin. *Toxicol Appl Pharmacol* **181**: 164–73.

Baynes RE, Brooks JD, Mumtaz M, Riviere JE (2002b) Effect of chemical interactions in pentachlorophenol mixtures on skin and membrane transport. *Toxicol Sci* **69**: 295–305.

Beasley VR, Cook WO, Dahlem AM (1989a) Algae intoxication in livestock and waterfowl. *Vet Clin North Am Food Anim Pract* **5**: 345–61.

Beasley VR, Dahlem AM, Cook WO (1989b) Diagnostic and clinically important aspects of cyanobacterial (blue-green algae) toxicoses. *J Vet Diagn Invest* **1**: 359–65.

Beroza M, Inscoe MN, Schwartz PH, Keplinger ML, Mastri CW (1975) Acute toxicity studies with insect attractants. *Toxicol Appl Pharmacol* **31**: 421–9.

Bomhard EM, Brendler-Schwaab SY, Freyberger A, Herbold BA, Leser KH, Richter M (2002) *O*-phenylphenol and its sodium and potassium salts: a toxicological assessment. *Crit Rev Toxicol* **32**: 551–625.

Bradberry SM, Cage SA, Proudfoot AT, Allister Vale J (2005) Poisoning due to pyrethroids. *Toxicol Rev* **24**: 93–106.

Burden AD, Muston H, Beck MH (1994) Intolerance and contact allergy to tar and dithranol in psoriasis. *Contact Dermat* **31**: 185–6.

Burrows G (1990) Apparent *Agave lecheguilla* poisoning in Angora goats. *Vet Hum Toxicol* **32**: 259–60.

Buters JT, Mahadevan B, Quintanilla-Martinez L, Gonzalez FJ, Greim H, Baird WM, Luch A (2002) Cytochrome P450 1B1 determines susceptibility to dibenzo[a,l]pyrene-induced tumor formation. *Chem Res Toxicol* **15**: 1127–35.

Candrian U, Luthy J, Schlatter C (1984) Stability of pyrrolizidine alkaloids in hay and silage. *J Agric Food Chem* **32**: 935–7.

Carlisle DL, Pritchard DE, Singh J, Patierno SR (2001) Chromium-VI induces p53-dependent apoptosis in diploid human lung and mouse dermal fibroblasts. *Mol Carcinog* **28**: 111–8.

Casteel SW, Rottinghaus GE, Johnson GE (1994) Hepatotoxicosis in cattle induced by consumption of alfalfa-grass hay. In *Plant-Associated Toxins*, Colegate SM, Dorling PR (eds). CAB International, Wallingford, Oxon, UK, pp. 307–12.

Casteel SW, Rowe LD, Bailey EM (1988) Experimentally induced photosensitization in cattle with *Cooperia pedunculata*. *Vet Hum Toxicol* **30**: 101–4.

Clare NT (1955) Photosensitization in animals. *Adv Vet Sci* **2**: 182–211.

Chang WC, Jeng JH, Shieh CC, Tsai YC, Ho YS, Guo HR, Liu HI, Lee CC, Ho SY, Wang YJ (2003) Skin tumor-promoting potential and systemic effects of pentachlorophenol and its major metabolite tetrachlorohydroquinone in CD-1 Mice. *Mol Carcinog* **36**: 161–70.

Clarke EGC, Clarke ML (1975) *Veterinary Toxicology.* Williams and Wilkins, Baltimore, MD.

Colon JL, Jackson CA, Del Piero F (1996) Hepatic dysfunction and photodermatitis secondary to alsike clover poisoning. *Comp Cont Edu* **18**: 1022–9.

Craig AM, Latham CJ, Blythe LL (1992) Metabolism of toxic pyrrolizidine alkaloids from tansy ragwort (*Senecio jacobaea*) in ovine ruminal fluid under anaerobic conditions. *Appl Environ Microbiol* **58**: 2730–6.

Di Francesco C, Gerber HA, Bickel MH (1988) Autoradiographic study of the localization of 2.2′,4,4′,5,5′-hexachlorobiphenyl in liver and skin tissue after *in vitro* uptake. *Eur J Drug Metab Pharmacokinet* **13**: 241–5.

Dollahite JW, Younger RL, Hoffman GO (1978) Photosensitization in cattle and sheep caused by feeding *Ammi majus* (Greater Ammi, Bishop's weed). *Am J Vet Res* **39**: 193–7.

Downing DT (1992) Lipid and protein structures in the permeability barrier of mammalian epidermis. *J Lipid Res* **33**: 301–13.

Dreisbach RH (1983) *Handbook of Poisoning: Prevention, Diagnosis and Treatment*, 11th edn. Lang Medical Publications, Los Altos, CA.

Dykes PJ, Hill S, Marks R (1997) Pharmacokinetics of topically applied metronidazol in two different formulations. *Skin Pharmacol* **10**: 28–33.

Fischer JM, Robbins SB, Al-Zoughool M, Kannamkumarath SS, Stringer SL, Larson JS, Caruso JA, Talaska G, Stambrook PJ, Stringer JR (2005) Co-mutagenic activity of arsenic and benzo[a]pyrene in mouse skin. *Mutat Res* **588**: 35–46.

Flemming CE, Miller MR, Vawter LR (1922) The spring rabbit-brush. *Nevada Agri Exp Stat Bull* **104**: 1–29.

Friedman MH (1986) *Principles and Models of Biological Transport*. Springer Verlag, Berlin, p. 74.

Giri AK (1997) Genetic toxicology of epichlorohydrin: a review. *Mutation Res/Rev Mutation Res* **386**: 25–38.

Glasonbury JRW, Doughty FR (1984) A syndrome of hepatogenous photosensitization resembling geeldikkop in sheep grazing *Tribulus terrestris*. *Aust Vet J* **61**: 314–16.

Griffin P, Payne M, Mason H, Freedlander E, Curran AD, Cocker J (2000) The *in vitro* percutaneous penetration of chlorpyrifos. *Hum Exp Toxicol* **19**: 104–7.

Groth L, Serup J (1998) Cutaneous microdialysis in man: effects of needle insertion trauma and anaesthesia on skin perfusion, erythema and skin thickness. *Acta Dermatol Venereol* **78**: 5–9.

Groth L (1996) Cutaneous microdialysis. Methodology and validation. *Acta Derm Venereol Suppl* **197**: 1–61.

Hammond PB, Beliles RP (1980) Metal. In *Casarett and Doull's Toxicology: The Basic Science of Poisons*, 2nd edn, Doull J, Klaassen CD, Amdur MO (eds). MacMillan, New York, pp. 409–468.

Hancock JJ (1950) Congenital photosensitivity in Southdown sheep. *NZ J Sci Tech Net* **32**: 16–24.

House JK, George LW, Oslund KL (1996) Primary photosensitization related to ingestion of alfalfa silage by cattle. *J Am Vet Med Assoc* **209**: 1604–7.

Hoover JP, Smith TA (1995) Investigating a case of suspected cyanobacteria (blue-green algae) intoxication in a dog. *Vet Med* **90**: 1028–32.

Horio T, Miyauchi-Hashimoto H, Okamoto H (2005) DNA damage initiates photobiologic reactions in the skin. *Photochem Photobiol Sci* **4**: 709–14.

Huang C, Huang Y, Li J, Hu W, Aziz R, Tang MS, Sun N, Cassady J, Stoner GD (2002) Inhibition of benzo(a)pyrene diol-epoxide-induced transactivation of activated protein 1 and nuclear factor kappaB by black raspberry extracts. *Cancer Res* **62**: 6857–63.

IARC (1973) Some polycyclic aromatic hydrocarbons and heterocyclic compounds. *IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Humans*, vol. 3. International Agency for Research on Cancer, Lyon, France, 271 pp.

IARC (1983) Polynuclear aromatic compounds. Part 1. Chemical, environmental and experimental data. *IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Humans*, vol. 32. International Agency for Research on Cancer, Lyon, France.

IARC (1987) Overall evaluation of carcinogenicity. *IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Humans*, Suppl. 7. International Agency for Research on Cancer, Lyon, France, 440 pp.

Jackson Jr JA, Hemken RW, Boling JA, Harmon RJ, Buckner RC, Bush LP (1984) Summer fescue toxicity in dairy steers fed tall fescue seed. *J Anim Sci* **58**: 1057–61.

Johanson G (2000) Toxicity review of ethylene glycol monomethyl ether and its acetate ester. *Crit Rev Toxicol* **30**: 307–45.

John CP, Sangster LT, Jones OH (1974) *Crotolaria spectablis* poisoning in swine. *J Am Vet Med Assoc* **165**: 633–8.

Johnson AE (1974) Predisposing influence of range plants on Tetradymia-related photosensitization in sheep: work of Drs AB Clawson and WT Huffman. *Am J Vet Res* **35**: 1583–5.

Johnson AE, Molyneux RJ (1984) Toxicity of threadleaf groundsel (*Senecio douglasii* var *longilobus*) to cattle. *Am J Vet Res* **45**: 26–31.

Johnson AE, Smart RA (1983) Effects on cattle and their calves of tansy ragwort (*Senecio jacobaea*) fed in early gestation. *Am J Vet Res* **44**: 1215–19.

Johnson AE, Molyneux RJ, Stuart LD (1985) Toxicity of Riddell's groundsel (*Senecio riddellii*) to cattle. *Am J Vet Res* **46**: 577–82.

Johnson AE, Molyneux RJ, Ralphs MH (1989) Senecio: a dangerous plant for man and beast. *Rangelands* **11**: 261–4.

Kellerman TS, Miles CO, Erasmus GL (1994) The possible role of steroidal saponins in the pathogenisis of geeldikkop, a major hepatogenous photosensitization of small stock in South Africa. In *Plant-Associated Toxins*, Colegate SM, Dorling PR (eds). Cab International, Wallingford, pp. 287–92.

Kingsbury JM (1964) *Poisonous Plants of the United States and Canada*. Prentice-Hall, Englewood Cliffs, NJ, pp. 52–7.

Kirsner RS, Froelich CW (1998) Soaps and detergents: understanding their composition and effect. *Ostomy/Wound Manag* **44**(3A Suppl.): 62S–69S.

Knight AP, Walter RG (2003) Plants affecting the skin and liver. In *A Guide to Plant Poisoning of Animals in North America*, Knight AP, Walter RG (eds). International Veterinary Information Service, Ithaca, NY.

Lakind JS, Mckenna EA, Hubner RP, Tardiff RG (1999) A review of the comparative mammalian toxicity of ethylene glycol and propylene glycol. *Crit Rev Toxicol* **29**: 331–65.

Latuszynska J, Luty S, Raszewski G, Tokarska-Rodak M, Przebirowska D, Przylepa E, Haratym-Maj A (2001) Neurotoxic effect of dermally-applied chlorphyrifos and cypermethrin in wister rats. *Ann Agric Environ Med* **8**: 163–70.

Lavker RM, Sun TT (1983) Epidermal stem cells. *J Invest Dermatol* **81**: 121–7.

Lee JA, Budgin JB, Mauldin EA (2002) Acute necrotizing dermatitis and septicemia after application of a D-limonene-based insecticidal shampoo in a cat. *J Am Vet Med Assoc* **221**: 258–62, 239–40.

Lombardo de Barros CS, Driemeier D, Pilati C (1992) *Senecio* spp. poisoning in cattle in southern Brazil. *Vet Hum Toxicol* **34**: 241–5.

Longstaffe JA, Humphreys DJ, Hayward AHS, Stodulski JBJ (1981) Paraquat poisoning in dogs and cats: differences between accidental and malicious poisoning. *Small Anim Pract* **22**: 153–6.

Mahadevan B, Luch A, Bravo CF, Atkin J, Steppan LB, Pereira C, Kerkvliet NI, Baird WM (2005) Dibenzo[a,l]pyrene induced DNA adduct formation in lung tissue in vivo. *Cancer Lett* **227**: 25–32.

Marks R, Dykes PJ (1990) The effects of the detergent action of cleansing agents on the skin. *Wien Medizin Wochensch* **140**: 16–8.

Marsh CD (1930) Toxic effect of St John's wort (*Hypericum perforatum*) on cattle and sheep. *USDA Bull* **202**: 1–23.

Mattocks AR (1968) Toxicity of pyrrolizidine alkaloids. *Nature* **217**: 723–28.

Medi BM, Singh S, Singh J (2006) Assessing efficacy of penetration enhancers. In *Dermal Absorption Models in Toxicology and Pharmacology*, Riviere JE (ed.). CRC Taylor and Francis, Boca Raton, FL, pp. 213–50.

Michielsen CC, van Loveren H, Vos JG (1999) The role of the immune system in hexachlorobenzene-induced toxicity. *Environ Health Perspect* **107**: 783–92.

Mihailovic M, Matic G, Lindberg P, Zigic B (1992) Accidental selenium poisoning of growing pigs. *Biol Trace Elem Res* **33**: 63–9.

Molyneux RJ, Johnson AE, Olsen JD (1991) Toxicity of pyrrolizidine alkaloids from riddell's groundsel (*Senecio riddellii*) to cattle. *Am J Vet Res* **52**: 146–51.

Monteiro-Riviere NA (2006) The integument, Chapter 16. In *Dellmann's Textbook of Veterinary Histology*, 6th edn, Eurell JA, Frappier B (eds). Blackwell Press, Ames, IA, pp. 320–49.

Monteiro-Riviere NA, Bristol DG, Manning TO, Rogers RA, Riviere JE (1990) Interspecies and interregional analysis of the comparative histologic thickness and laser Doppler blood flow measurements at five cutaneous sites in nine species. *J Invest Dermatol* **95**: 582–6.

Monteiro-Riviere NA, Inman AO, Mak V, Wertz P, Riviere JE (2001) Effects of selective lipid extraction from different body regions on epidermal barrier function. *Pharm Res* **18**: 992–8.

Moser K, Kriwet K, Naik A, Kalia YN, Guy RH (2001) Passive skin penetration enhancement and its quantification *in vitro. Europ J Pharmaceut Biopharmaceut* **52**: 103–12.

Muhammad F, Riviere JE (2006) In vivo models. In: *Dermal Absorption Models in Toxicology and Pharmacology*, Riviere JE (ed.) CRC Taylor and Francis, Boca Raton, FL, pp. 49–70.

Muhammad F, Monteiro-Riviere NA, Riviere JE (2005a) Comparative *in vivo* toxicity of topical JP-8 jet fuel and its individual hydrocarbon components: identification of tridecane and tetradecane as key constituents responsible for dermal irritation. *Toxicol Path* **33**: 258–66.

Muhammad F, Monteiro-Riviere NA, Baynes RE, Riviere JE (2005b) Effect of *in vivo* jet fuel exposure on subsequent *in vitro* dermal absorption of individual aromatic and aliphatic hydrocarbon fuel constituents. *J Toxicol Environ Health A* **68**: 719–37.

Nation PN (1991) Hepatic disease in Alberta horses: a retrospective study of "alsike clover poisoning" (1973–1988). *Can Vet J* **32**: 602–7.

Nielsen JB (2005) Percutaneous penetration through slightly damaged skin. *Arch Dermatol Res* **296**: 560–7.

Nobre D, Dagii MLZ, Haraguchi M (1994) *Crotolaria juncea* intoxication in horses. *Vet Hum Toxicol* **36**: 445–8.

Odriozola E, Campero C, Casaro A (1994) Pyrrolizidine alkaloidosis in Argentinian cattle caused by *Senecio selloi. Vet Hum Toxicol* **36**: 205–8.

Oertli EH, Rowe LD, Lovering SL (1983) Phototoxic effect of *Thamnosa texana* (Dutchman's breeches) in sheep. *Am J Vet Res* **44**: 1126–9.

Oliveira-Ribeiro CA, Vollaira Y, Sanchez-Chardi A, Roche H (2005) Bioaccumulation and the effects of organochlorine pesticides, PAH and heavy metals in the Eel (*Anguilla anguilla*) at the Camargue Nature Reserve, France. *Aquatic Toxicol* **74**: 53–69.

Osweiler GD, Carson TL, Buck WB, Van Gelder GA (1985a) Toxic blue-green algae. In *Clinical and Diagnostic Veterinary Toxicology*, 3rd edn. Kendall/Hunt Publishing Co., Dubuque, IA, pp. 451–2.

Osweiler GD, Carson TL, Buck WB, Van Gelder GA (1985b) Fungicide, herbicides, and insecticides. In *Clinical and Diagnostic Veterinary Toxicology*, 3rd edn. Kendall/Hunt Publishing Co., Dubuque, IA, pp. 189–340.

Panteleyev AA, Thiel R, Wanner R (1997) 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) affects keratin 1 and keratin 17 gene expression and differentially induces keratinisation in hairless mouse skin. *J Invest Dermatol* **108**: 330–5.

Pass MA (1987) The mechanism and treatment of lantana poisoning. *Vet Clin Toxicol Proc* **103**: 19–22.

Pinto C, Santos VM, Dinis J, Peleteiro MC, Fitzgerald JM, Hawkes AD, Smith BL (2005) Pithomycotoxicosis (facial eczema) in ruminants in the Azores, Portugal. *Vet Rec* **157**: 805.

Pont AR, Charron AR, Wilson RM, Brand RM (2003) Effects of active sunscreen ingredient combinations on the topical penetration of the herbicide 2: 4-dichlorophenoxyacetic acid. *Toxicol Indust Health* **19**: 1–8.

Pont AR, Charron AR, Brand RM (2004) Active ingredients in sunscreen act as topical penetration enhancers for the herbicide 2,4-dichlorophenoxyacetic acid. *Toxicol Appl Pharmacol* **195**: 348–54.

Putnam MR, Qualls CW, Rice LE (1986) Hepatic enzyme changes in bovine hepatogenous photosensitivity caused by water-damaged alfalfa hay. *J Am Vet Med Assoc* **189**: 77–82.

Qiao GL, Brooks JD, Riviere JE (1997) Pentachlorophenol dermal absorption and disposition from soil in swine: effects of occlusion and skin microorganism inhibition. *Toxicol Appl Pharmacol* **147**: 234–46.

Raab W (1990) Skin cleansing in health and disease. *Wien Medizin Wochensch* **140**: 4–10.

Radostits OM, Blood DC, Gay CC (1994) *Veterinary Medicine*, 8th edn. Baillière Tindall, London, pp. 1600–2.

Rezazadeh H, Athar M (1997) Evidence that iron-overload promotes 7,12-dimethylbenz(a)anthracene-induced skin turmorigenesis in mice. *Redox Rep* **3**: 303–9.

Richard JL (1973) Mycotoxin photosensitivity. *J Am Vet Med Assoc* **163**: 1298–9.

Rickett FE, Tyszkiewicz K, Brown NC (1972) Pyrethrum dermatitis I. The allergic properties of various extracts of pyrethrum flowers. *Pestic Sci* **3**: 56–66.

Riviere JE (1999) Absorption. In *Comparative Pharmacokinetics. Principles, Techniques and Applications*, Riviere JE (ed.). Iowa State Press, Ames, IA, pp. 34–5.

Riviere JE (2006) Dermal absorption/toxicity of organophosphates and carbamates. In *Toxicology of Organophosphates and Carbamate Compounds*, Gupta RC (ed.). Elsevier, New York, pp. 411–22.

Riviere JE, Boosinger TR, Everson RJ (1981) Inorganic arsenic toxicosis in cattle. *Mod Vet Pract* **62**: 209–11.

Riviere JE, Qiao G, Baynes RE, Brooks JD, Mumtaz M (2001) Mixture component effects on the *in vitro* dermal absorption of pentachlorophenol. *Arch Toxicol* **75**: 329–34.

Riviere JE, Monteiro-Riviere NA, Baynes RE (2002) Gulf War related exposure factors influencing topical absorption of 14C-permethrin. *Toxicol Lett* **135**: 61–71.

Riviere JE, Baynes RE, Brooks JD, Yeatts JL, Monteiro-Riviere NA (2003) Percutaneous absorption of topical *N*,*N*-diethyl-*m*-toluamide (DEET): effects of exposure variables and coadministered toxicants. *J Toxicol Environ Health A* **66**: 133–51.

Robinson MK, Kruszewski FH, Al-Atrash J, Blazka ME, Gingell R, Heitfeld FA, Mallon D, Snyder NK, Swanson JE, Casterton PL (2005) Comparative assessment of the acute skin irritation potential of detergent formulations using a novel human 4-h patch test method. *Food Chem Toxicol* **43**: 1703–12.

Rowe LD (1989) Photosensitization problems in livestock. *Vet Clin North Am Food Anim Pract* **5**: 301–23.

Rowe LD, Norman JO, Corrier DE (1987) Photosensitization of cattle in southeast Texas: identification of phototoxic activity associated with *Cooperia pedunculata*. *Am J Vet Res* **48**: 1658–61.

Schurdak ME, Randerath K (1989) Effects of route of administration on tissue distribution of DNA adducts in mice: comparison of 7H-dibenzo(*c*,*g*)carbazole, benzo(*a*)pyrene, and 2-acetyl-laminofluorene. *Cancer Res* **49**: 2633–8.

Scruggs DW, Blue GK (1994) Toxic hepatopathy and photosensitization in cattle fed moldy alfalfa hay. *J Am Vet Med Assoc* **204**: 264–6.

Shara M, Yasmin T, Kincaid AE, Limpach AL, Bartz J, Brenneman KA, Chatterjee A, Bagchi M, Stohs SJ, Bagchi D (2005) Safety and toxicological evaluation of a novel niacin-bound chromium (III) complex. *J Organ Biochem* **99**: 2161–83.

Simonsen L, Jorgensen A, Benfeldt E, Groth L (2004) Differentiated *in vivo* skin penetration of salicylic compounds in hairless rats measured by cutaneous micro dialysis. *Eur J Pharm Sci* **21**: 379–88.

Srikrishna V, Riviere JE, Monteiro-Riviere NA (1992) Cutaneous toxicity and absorption of paraquat in porcine skin. *Toxicol Appl Pharmacol* **115**: 89–97.

Spiewak R (2003) Occupational dermatoses among Polish private farmers, 1991–1999. *Am J Indust Med* **43**: 647–55.

Stegelmeier BL, Gardner DR, Molyneux RJ (1994) The clinicopathologic changes of *Cynoglossum officinale* (houndstongue) intoxication in horses. In *Plant-Associated Toxins*, Colegate SM, Dorling PR (eds). CAB International, Wallingford, pp. 297–302.

United States Environmental Protection Agency (USEPA) (1995) Office of prevention, pesticides and toxic substances. Carcinogenicity peer review of pyrethrins. February 22, Washington, DC.

United States Department of Agriculture: Animal Welfare Act AWA Sections 13(a)3), 13(a)(7), 13(e)(2,3), 9 CFR, Part 2, Sections 2.31 (d)(l)(I,II,III,IV), 2.31 (e)(4), 2.33(b)(4), 9 CFR, Part 3, Section 3.6(b)(5,6,7), amended 1985.

Ushio H, Nohara K, Fujimaki H (1999) Effect of environmental pollutants on the production of pro-inflammatory cytokines by normal human dermal keratinocytes. *Toxicol Lett* **105**: 17–24.

Van Vleet JF, Greenwood LA, Ferrans VJ (1979) Pathologic features of adriamycin toxicosis in young pigs: nonskeletal lesions. *Am J Vet Res* **40**: 1537–52.

Wang Y, Gao D, Atencio DP, Perez E, Saladi R, Moore J, Guevara D, Rosenstein BS, Lebwohl M, Wei H (2005) Combined subcarcinogenic benzo[a]pyrene and UVA synergistically caused high tumor incidence and mutations in H-ras gene, but not p53, in SKH-1 hairless mouse skin. *Int J Cancer* **116**: 193–99.

Wester RC, Maibach HI, Sedik L, Melendres J, Wade M, DiZio S (1993) Percutaneous absorption of pentachlorphenol from soil. *Fundam Appl Toxicol* **20**: 68–71.

Wierckx FCJ, Beijersbergen van Henegouwen GMJ, Van den Broeke LT, De Vries H, Meerman JHN, Mulder GJ (1992) Photoactivation of 2-nitrofluorene in vitro and in the rat in vivo. UVA-induced formation of reactive intermediates that bind covalently to RNA and protein. *Carcinogenesis* **13**: 1759–62.

Winship KA (1988) Toxicity of tin and its compounds. *Adv Drug React Acute Poison Rev* **7**: 19–38.

Zendzian RP (2003) Pesticide residue on/in the washed skin and its potential contribution to dermal toxicity. *J Appl Toxicol* **23**: 121–36.

C H A P T E R

# 17

# Blood and bone marrow toxicity

*Shashi K. Ramaiah and Mary B. Nabity*

## INTRODUCTION

Hematotoxicology is the study of adverse effects of drugs, non-therapeutic chemicals, and other agents in our environment on blood and blood forming tissues such as bone marrow. Assessment of blood and bone marrow in veterinary toxicology is important since chemicals and drugs can significantly affect the cellular and humoral components of the blood. The hematopoietic system is unique and serves as a "cornerstone" to one's understanding of the toxicology of blood and bone marrow. Hematopoietic cells are the most dynamic, proliferative, and mitotically active cells in an animal. Over the course of a mammal's lifetime, the average basal production rate of these cells are 2.5 billion red cells, 2.5 billion platelets, and 1 billion granulocytes per kilogram per day (Gasper, 2000). Therefore, toxicity to circulating blood cells or their precursors may be very serious and can result in life-threatening anemia, hemorrhage, or profound infection.

Bone marrow is a common target for a variety of chemotherapeutic agents in animals and humans, some of which can result in total marrow aplasia (Lund, 2000). Many other drugs can also negatively impact blood components; therefore, the evaluation of blood and bone marrow in preclinical drug development is required by many regulatory agencies when assessing the safety and efficacy of test compounds (Bolliger, 2004). Because blood and bone marrow can be relatively easily assessed, sequential monitoring of blood and bone marrow has become a standard component of toxicity screening by the pharmaceutical industry as well as in the clinics. This chapter is intended to provide a basic introduction to blood and bone marrow as well as illustrate some toxicant-induced changes. Information provided in this chapter should serve as an important and accessible tool for understanding basic concepts, and for monitoring and characterizing toxic responses of the blood and bone marrow.

## ANATOMY

### Bone marrow

Hematopoiesis normally occurs in the bone marrow of both flat bones and long bones, and in mice, it can also occur normally in the spleen (Gasper, 2000). Blood supply to the marrow is mainly provided by nutrient arteries that penetrate cortical bone, particularly at the mid-shaft of long bones. These arteries extend along the longitudinal axis of the bone and send off radial branches throughout the marrow. These branches terminate at the periphery of the marrow cavity and connect with venous vessels. Venous or vascular sinuses of marrow are thin-walled vessels with abundant anastomoses, and they carry blood back to central veins. Marrow lacks lymphatic vessels and nerves in the marrow are vasomotor and found in association with the vasculature.

The hematopoietic compartment of the marrow consists of irregular and anastomosing cords that lie between vascular sinuses (Bloom, 1997). Blood cells are produced in the hematopoietic compartment of the marrow and reach the circulation by migrating through the vascular sinus endothelium. Marrow fat cells represent a mechanical buffer that occupy or release space within the medullary cavity in response to changing demands for hematopoiesis. Adventitial reticular cells provide structural support for hemopoietic colonies. The extent of marrow fat and adventitial coverage of vascular sinuses varies inversely with the degree of hematopoiesis and the rate of cell delivery to the

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bloodstream. Thus, the amount of fat in the marrow increases with age (Bloom, 1997).

## HEMATOPOIETIC STEM CELLS

A pluripotent stem cell that can produce all blood cell types, including lymphocytes, is said to be totipotent. Most totipotent stem cells in adults are not in the cell cycle, but are quiescent. Totipotent stem cells account for less than 0.2% of the total hematopoietic cells in the marrow. In adult mammals, most of these cells are lodged in the bone marrow, but small numbers enter, circulate, and return from blood. Low numbers are also present in the spleen. Totipotent hematopoietic stem cells produce pluripotent myeloid and lymphoid stem cells. Pluripotent stem cells give rise to progenitor cells which are capable of forming colonies in marrow culture, like stem cells, but lack long-term self-renewal capacity and are generally more restricted in their differentiation (Meyer and Harvey, 1998). Many progenitor cells are in the active cell cycle. They are capable of producing progeny (blast cells) that can be recognized morphologically. These resultant blast cells are classified as "precursor cells."

The pluripotent myeloid stem cells give rise to a series of progressively more differentiated progenitor cells that support the production of all nonlymphoid cells. When grown in an *in vitro* cell culture assay, each colony of cells represents one progenitor cell and is referred to as a colony-forming unit (CFU). With time, few large colonies may appear in culture, originating from a more immature progenitor cell and referred to as burst-forming units (BFUs) in the literature (Meyer and Harvey, 1998). Both the local marrow microenvironment and systemic humoral factors influence hematopoiesis to stimulate or suppress the proliferation and differentiation of single or multiple cell lineages. In general, multi-colony growth factors such as interleukin 3 (IL-3) stimulate early progenitor cells, whereas single colony factors such as erythropoietin (EPO) and granulocyte–macrophage colony stimulating factor (GM-CSF) act on more differentiated progenitor cells such as erythroid and granulocyte–macrophage progenitor cells, respectively.

## BLOOD COMPONENTS

Blood consists of cells and protein-rich plasma (Figure 17.1). Blood cells include erythrocytes (red blood cells), platelets (usually called thrombocytes in nonmammalian species), and leukocytes (white blood cells). The leukocytes in most vertebrates are classified as, granulocytes (neutrophils, eosinophils, and basophils) and mononuclear cells (monocytes and lymphocytes). Freshly drawn blood rapidly clots, but if clotting is prevented by the addition of an anticoagulant, the blood cells settle. This allows for separation of the plasma and cellular components into three distinct layers after centrifugation of anticoagulated blood (Andrews, 1998; Meyer and Harvey, 1998). The lowest layer is red since it consists mostly of erythrocytes, and the percent volume of this layer with respect to the total blood volume (normally approximately 35–45%) determines the packed cell volume (PCV) or hematocrit (HCT). The thin, gray–white middle layer immediately above the erythrocytes is the buffy coat, and it accounts for approximately 1% of the blood volume. The buffy coat contains platelets and leukocytes, but immature erythrocytes may also be present in this layer. The top (fluid) layer is the plasma. Plasma contains 91–92% water and 8–9% solutes such as proteins, lipids, and electrolytes (Figure 17.1). A variety of homeostatic mechanisms ensure that plasma volume, pH and constituents are tightly regulated. When clotting of blood occurs, fibrinogen and many coagulation factors are depleted, and the resulting fluid is called serum (Andrews, 1998; Meyer and Harvey, 1998).

### Erythrocyte, leukocyte, and platelet formation

Immature blood cell precursors are generally larger than mature forms, and they have a large euchromatic nucleus resulting in a high nuclear to cytoplasmic (N:C) ratio. As blood cells mature, their size and N:C ratio gradually decrease.

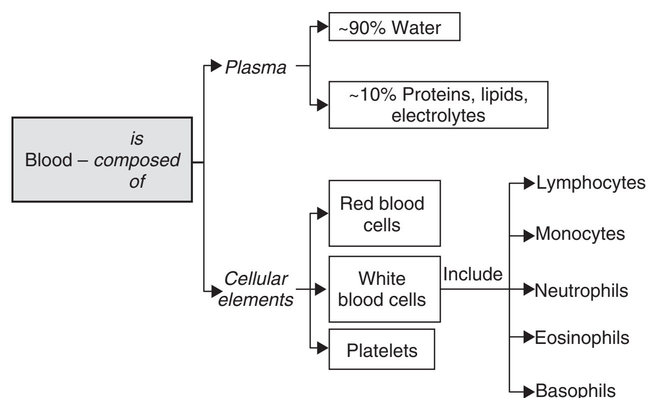


**FIGURE 17.1** Blood components. The blood consists of the fluid-rich plasma component and the cellular component consisting of red and white blood cells. The white blood cells are composed of neutrophils, basophils, eosinophils, monocytes, and lymphocytes. See text for details on morphology of these cells.

### *Erythropoiesis*

Erythropoiesis is the process by which committed hemopoietic progenitor cells develop into anucleate, hemoglobin-containing, biconcave discs called erythrocytes. EPO regulates this process. Rubriblasts, the first recognizable erythroid precursor, undergo approximately four cell divisions to generate the last nucleated stage, the metarubricyte (Figure 17.2a). However, intracellular hemoglobin concentration is an important factor in determining the number of cell divisions undergone to produce this stage (Car, 2000). With the extrusion of the nucleus, the resulting immature erythrocyte is termed a reticulocyte. The reticulocyte persists for about 2 days after release into the circulation before maturation into a mature erythrocyte. Under normal situations, few reticulocytes are seen in peripheral blood, but some strains of rodents may normally have up to 10% circulating reticulocytes (Fernandez and Grindem, 2000). The lifespan of mature erythrocytes ranges from 43 to 145 days depending on the species (Christian, 2000).

### *Granulopoiesis*

Granulopoiesis is the process by which committed hemopoietic progenitor cells develop into granulocytes under the influence of various growth factors and cytokines. Neutrophils and monocytes are derived from a common progenitor, the colony-forming unit granulocyte–macrophage (CFU-GM), whereas eosinophils and basophils are derived from different progenitor cells (CFU-Eo and CFU-Ba, respectively). The first recognizable granulocytic precursor is the myeloblast. Early granulocyte precursors (myeloblast and promyelocyte) appear similar between the various granulocytic cell lines until the myelocyte stage, which is the final stage capable of cell division. At this stage, they develop characteristic secondary lineage-specific granules (neutrophilic, eosinophilic, or basophilic). During the next stage (the metamyelocyte), the nucleus of the granulocyte indents and lobulation begins, eventually producing the characteristic polymorphonuclear appearance (Figure 17.2a, b) (Gasper, 2000; Smith, 2000).

### *Platelet formation*

The megakaryocyte is a polyploidy cell with cytoplasm that becomes increasingly granular with maturity (Figure 17.2). Platelets are derived from the megakaryocyte cytoplasm. The mechanism by which platelets are released from their parent cell is unclear, but it appears to be by cytoplasmic fragmentation. This process may take place in an extramedullary location (i.e. lung, spleen). The cytokine thrombopoietin is the major stimulus for megakaryocyte proliferation, platelet production, and differentiation from a common progenitor cell, the CFU-GEMM (Andrews, 1998). If unconsumed, the platelet lifespan ranges from 3 to 10 days depending on the species (Weiss, 2000).

## Erythrocyte, leukocyte, and platelet biology

### *Erythrocyte*

#### *Erythrocyte function*

The function of red blood cells is to transport oxygen to tissues. Decreased hemoglobin lowers oxygen delivery to tissues. This results in increased EPO secretion from the kidney and subsequent stimulation of erythrocyte production.

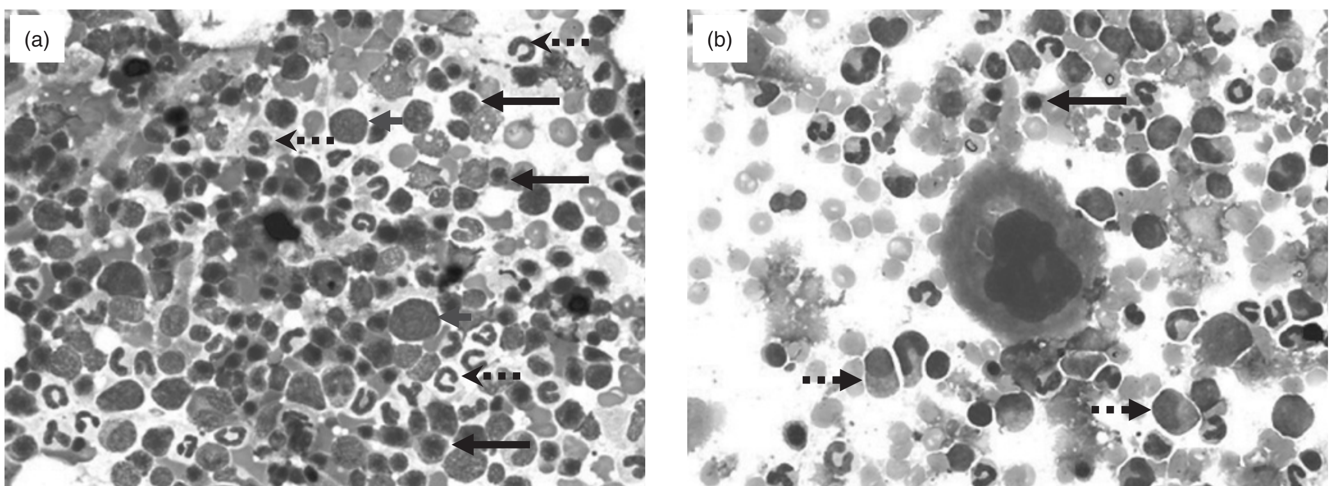


FIGURE 17.2 (a) Examples of erythroid precursors and granulocytic precursors (arrowheads) in the bone marrow of a dog. 60×, Diff-quik stain. Early erythroid precursors (arrow heads), late erythroid precursors (solid arrows) and granulocytic precursors (dotted arrows) are shown. (b) A mature megakaryocyte in the bone marrow of a dog is shown in the center of the picture. Erythroid and myeloid precursors are also noted (arrows). 60×, Modified Wrights stain. This figure is reproduced in color in the color plate section.

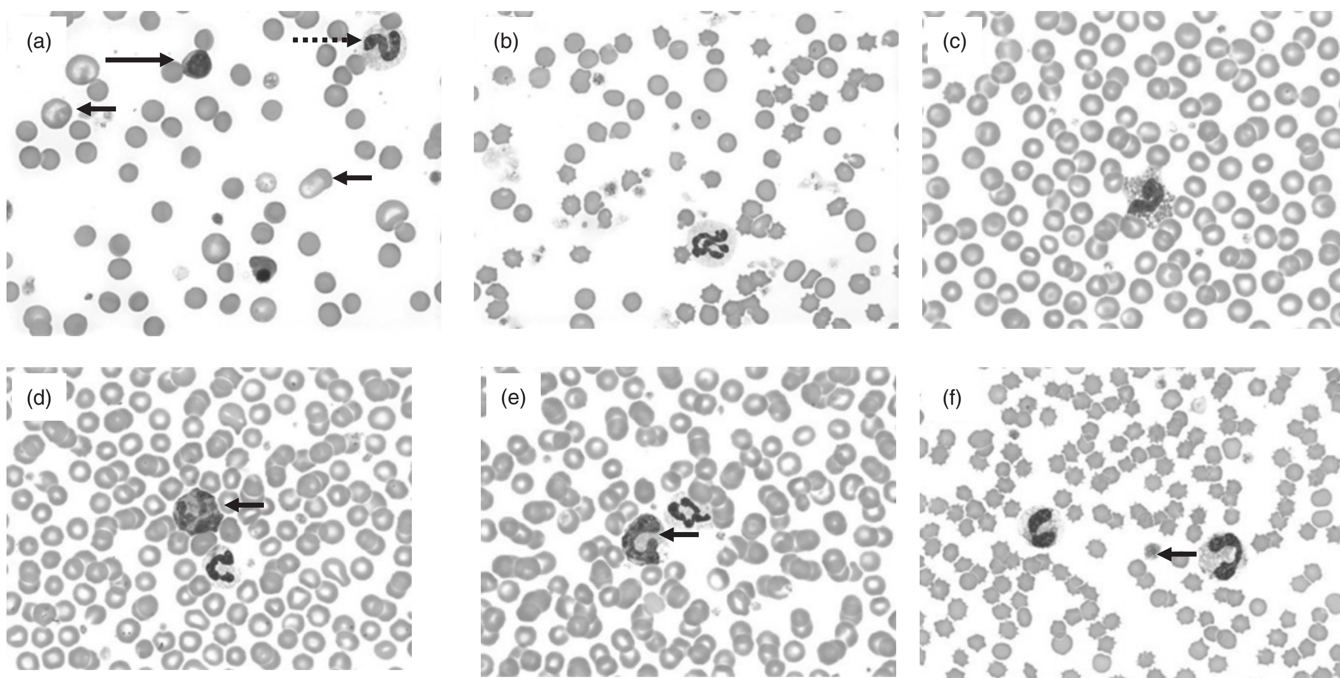


**FIGURE 17.3** Normal and few abnormal erythrocyte and leukocyte morphology. (a) Two large basophilic polychromatophilic erythrocytes or reticulocytes (small solid arrow) are present in the blood from a dog with IMHA. A normal neutrophil (dotted arrow), a metarubricyte and a lymphocyte (long arrow) are also present. 100×, Modified Wrights Stain. (b) Heinz bodies in the blood from a cat appearing as pale "spots" within erythrocytes with a modified Wrights stain, 100×. (c) Eosinophil with round granules in the blood from a dog. 100×, Modified Wrights stain. (d) Basophil (arrow) and neutrophil in the blood from a dog. 100×, Modified Wrights stain. (e) Monocyte (arrow) and neutrophil in the blood from a dog. 100×, Modified Wrights stain. (f) Toxic neutrophils in the blood from a cat demonstrating Döhle bodies, cytoplasmic vacuolation, and basophilia. A platelet (arrow) is also identified in the field 100×, Modified Wrights stain. This figure is reproduced in color in the color plate section.

*Morphology of erythrocytes*

Mature erythrocytes are anucleate biconcave discs. The degree of concavity varies among the domestic species, and typical biconcave erythrocytes, as indicated by central pallor, are present in dogs, cows, and sheep. The concavity and resulting pallor are not prominent in horses and cats. In most species, an increase in central pallor indicates decreased hemoglobin content. Slight anisocytosis (variation in size) of erythrocytes is common in most animal species, and poikilocytosis (variation in shape) is normally present on smears made from the blood of goats and deer. Erythrocytes may adhere to each other and form long chains resembling stacks of coins. This phenomenon, called rouleaux formation, is normally prominent in horses and cats, intermediate in dogs and pigs, and rare in ruminants. Rouleaux formation is often increased in inflammatory states. Reticulocytes are immature anucleate erythrocytes that appear polychromatic (pinkish blue color) and large on Wright's stained blood films (Figure 17.3a). Reticulocytes contain residual ribonucleic acid (RNA) (ribosomes and polyribosomes) and mitochondria, which aggregate into a reticular mesh when stained with vital stains (e.g. new methylene blue) but not with Romanowsky-type stains. Quantitation of reticulocytes in circulation is used as an index of bone marrow erythropoietic response since circulating reticulocytes are numerous when the marrow is responding to an increased demand for erythrocytes. Heinz bodies result from the oxidation and aggregation of hemoglobin, and they appear as pale areas within or along the edge of the cytoplasm (Figure 17.3b). They may be seen in low numbers in normal cats but are an indication of increased oxidation when present in higher numbers (Meyer and Harvey, 1998; Harvey, 2001; Figure 17.3b).

*Heme synthesis*

The synthesis of heme involves a series of reactions that starts in the cytoplasm and mitochondria of the rubriblast, the earliest identifiable precursor of erythrocytes. Heme synthesis begins with the vitamin $B_6$ dependent condensation of succinyl-CoA and glycine to form delta aminolevulinic acid (ALA). ALA then condenses to form a pyrrole, and this step is inhibited by lead. Porphyrin rings are then generated and a final step incorporates an iron atom. Ferrochelatase also inhibited by lead catalyzes this incorporation of the ferrous iron into the tetrapyrrole protoporphyrin IX in the mitochondria (Meyer and Harvey, 1998; Harvey, 2000; Kaneko, 2000).

*Iron metabolism*

Iron is primarily obtained from dietary sources where it is absorbed from the gut and transported as a complex with

transferrin to the marrow. Iron is transferred to red blood cell precursors by receptor-mediated endocytosis. Iron stores are usually assessed by staining the marrow with Prussian blue or by measuring serum ferritin (Jain, 1993; Harvey, 1997). Cats normally lack stainable iron in their marrow (Nadrew *et al*., 1994).

*Red blood cell energy requirements and hemoglobin breakdown*

The energy required by erythrocytes is especially important for the maintenance of a reduction potential to prevent hemoglobin oxidation. In mature red blood cells there are no mitochondria, therefore, the glycolytic pathway and pentose phosphate pathway are the main sources of ATP and NADPH that provide energy to prevent oxidative damage, e.g. via reduced glutathione (GSH). Aged or damaged red blood cells are removed from circulation by macrophages of the reticuloendothelial system in the spleen, liver, and marrow. In the macrophage, hemoglobin is catabolized to globin and heme. The amino acids from globin reenter the protein synthesis pool. Heme is degraded to biliverdin then to bilirubin and excreted in the urine and bile (Jain, 1993).

### Leukocyte

*Leukocyte biology*

Neutrophils primarily function in inflammatory responses and bacterial killing. With an increased demand for neutrophils, neutrophil production is upregulated by growth factors and proinflammatory cytokines. Mature circulating neutrophils are either present in the circulating pool or the marginal pool. Neutrophils in the marginal neutrophil pool are loosely bound to capillary endothelial cells. Several physiological factors such as epinephrine and corticosteroids due to excitement and stress cause demargination of neutrophils and result in neutrophilia (Babior and Golde, 1995).

*Morphology of leukocytes*

Total leukocyte count refers to the total absolute count of circulating neutrophils, eosinophils, basophils, monocytes, and lymphocytes, and this number varies among different animal species. The differential leukocyte count refers to the percentage of each leukocyte population in peripheral blood and should be performed by counting and differentiating a minimum of 200 cells on a stained blood film. Granulocytes are classified according to the staining characteristics of their specific (or secondary) cytoplasmic granules. Eosinophils have pronounced acidophilic granules, basophils possess distinct basophilic granules, and neutrophils have neutral, usually indistinct granules. The proportion of different populations of leukocytes also varies among animal species. For example, neutrophils predominate in dogs and cats, whereas they only slightly outnumber lymphocytes in horses. In ruminants and laboratory animals (rats and mice), lymphocytes predominate. The morphology of the various populations of leukocytes is fairly similar among animal species (Duncan *et al*., 1994).

*Neutrophils*

Once released from the bone marrow into the peripheral blood, neutrophils circulate for 6–14 h before migrating into tissues, where they survive for 1–4 days (Smith, 2000). Neutrophils account for approximately 40–70% of the total leukocyte count in most animal species. Mature neutrophils have a segmented nucleus with three to five lobes joined by thin strands, and they contain heterochromatic (clumped) chromatin (Figure 17.3). Occasionally a nuclear appendage, an extra chromatin lobe resembling a drumstick, is present in neutrophils of female animals. This nuclear appendage is commonly known as the Barr body. The neutrophil cytoplasm is pale and contains a moderate number of fine pink or pale granules, depending on the animal species. Neutrophils contain two types of cytoplasmic granules: azurophilic (primary) and specific (secondary) granules. In birds, reptiles, and fish, the equivalent of a neutrophil is called a heterophil, and it contains large, reddish cytoplasmic granules. The neutrophils in rabbits, guinea pigs, and hamsters also contain similar-appearing granules and are therefore often referred to as heterophils. Rat and mouse granulocytes are unique in that their granulocytic precursors can develop "ring forms" which appear as a small "hole" in the center of the nucleus. These ring forms are also called "stab cells." Ring forms are seen in neutrophil and eosinophil precursors but not in basophil precursors (Bolliger, 2004).

In health, only mature neutrophils are present in circulation. Immature neutrophils, as indicated by a decrease in nuclear segmentation, are normally restricted to the bone marrow but may be released into the blood during a granulocytic response to a disease process. Neutrophil toxicity is seen secondary to a toxic environment in the blood or bone marrow, resulting in maturational defects. Neutrophil toxicity is characterized by increased cytoplasmic basophilia, foamy vacuolation, and Döhle bodies (amorphous blue-grey cytoplasmic inclusions) (Figure 17.3f) (Jain, 1993; Meyer and Harvey, 1998).

*Eosinophils*

Eosinophils are seen in low numbers, and they contain a polymorphic nucleus that is less condensed and less segmented than that of neutrophils. As eosinophils mature in the bone marrow, most primary azurophilic granules disappear or transform into specific granules. In mature cells, the pale blue cytoplasm contains mostly specific eosinophilic granules that are loosely packed in the cell (Figure 17.3c). The size, shape, number, and staining characteristics of eosinophils vary among different animal species (Young, 2000).

*Basophils*

Few basophils are present in normal blood. They have a segmented or irregularly shaped, often string-like, heterochromatic nucleus. The nuclear segmentation, as in eosinophils, is less pronounced in basophils. Cytoplasmic granules are metachromatic and prominent, and they stain reddish violet (Figure 17.3d). The size, number, and staining reaction of granules vary among animal species. Basophils have some morphologic resemblance to mast cells, which are widely distributed in connective tissues and only rarely observed in blood. Compared to basophils, mast cells are bigger with a round nucleus and more abundant cytoplasmic granules (Scott and Stockham, 2000).

*Monocytes*

The monocyte is generally the largest blood leukocyte and accounts for 3–8% of the total leukocyte count. Monocytes are precursors of tissue macrophages, and their nucleus can vary from bean shaped to convoluted. Their cytoplasm is abundant, blue-gray, and often appears foamy or vacuolated with a few large vacuoles (Figure 17.3e). Monocytes often have many fine or indistinct azurophilic cytoplasmic granules (Bienzle, 2000).

*Lymphocytes*

Lymphocytes account for 20–40% of the total leukocyte count in dogs, cats, and horses; 60–70% in ruminants, mice, and rats; and 50–60% in pigs. Morphologically, lymphocytes are classified as small, medium, and large depending on their size. The size, shape, and staining characteristics of lymphocytes vary among and within animal species. Dogs and cats have mostly small circulating lymphocytes (Figure 17.3a). Both small and large lymphocytes are present in cows, sheep, goats, and rodents. In cows, large lymphocytes occasionally may have vacuolated cytoplasm and a few large azurophilic granules (Meyer and Harvey, 1998).

### *Platelet biology*

*Platelet function*

Platelets function largely in primary hemostasis. Initially, platelets adhere to the endothelium of damaged blood vessels, undergo shape changes, and release proteins that cause the aggregation of more platelets to form a platelet plug and help stimulate the coagulation pathway (Figure 17.4). Platelets play a major role in secondary hemostasis as well. For example, platelets provide a surface for the assembly of several enzyme complexes that form during the process of coagulation, provide coagulation factor V, and participate in the activation of factor X. Collagen stimulates platelet aggregation and platelet adhesion. Adhesion occurs between the platelet membrane and subendothelial components (collagen and fibronectin) via von Willebrand factor and glycoprotein receptors. ADP and thromboxane A2 are secreted by adherent platelets and released from damaged tissue and erythrocytes, and these contribute to early platelet aggregation. Fibrinogen plays a major role in the coagulation cascade as well as in platelet aggregation (Bauer and Rosenberg, 1995).

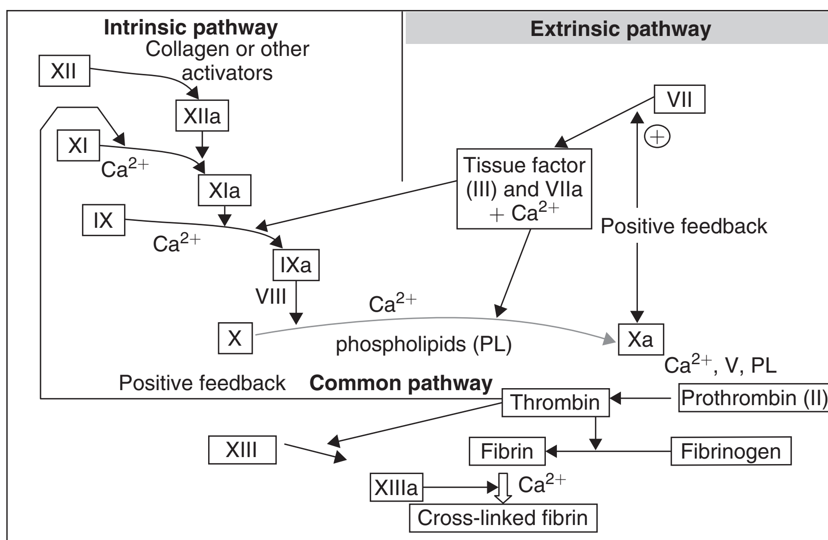


FIGURE 17.4 A diagram of the coagulation cascade consisting of intrinsic, extrinsic, and common pathways. Coagulation is an enzymatic process involving the conversion of inactive enzymes to active forms resulting in amplification of the process. The final product of these pathways is the formation of cross-linked fibrin. PL is phospholipid of platelets. Roman numerals refer to the coagulation factors with these numbers. "a" following a Roman numeral indicates "active enzyme." For details, see section on platelet biology.

*Morphology of platelets*

Platelets generally vary in size and shape and are smaller than red blood cells. In stained blood films, platelets are discoid, spherical, or elongated and may appear individually (Figure 17.3f) or in small to large aggregates. A direct platelet count is performed on blood samples using a hematology analyzer. A subjective screening of platelet numbers (normal, increased, or decreased) can also be made from their relative number on stained blood films. The terms platelet and thrombocyte are used interchangeably, although the term thrombocyte is preferentially used to describe nucleated platelets in fish, reptiles, amphibians, and birds (Boudreaux, 1996; Meyer and Harvey, 1998).

*Hemostasis*

Hemostasis occurs when there is a balance between continuous activation of the coagulation cascade and removal of the end product of coagulation, fibrin, by fibrinolysis. As mentioned previously, hemostasis can be categorized into primary hemostasis, with the formation of a platelet plug, and secondary hemostasis, culminating in a fibrin clot. There are three major pathways involved in secondary hemostasis: the extrinsic, intrinsic, and common pathways (Figure 17.4). In the extrinsic pathway, the initiating stimulus is the release of tissue factor from damaged endothelium and other tissues. The intrinsic pathway can be initiated by either the extrinsic pathway or by contact with negatively charged surfaces such as damaged endothelium. Both the extrinsic and intrinsic pathways stimulate a series of steps, many of which are shared in the common pathway, resulting in the production of thrombin and eventually a stable fibrin clot. Calcium is a requirement in many of these steps, and phospholipids (provided by platelets) are also required for enzyme complex activity. The majority of the coagulation factors are produced by the liver. Some factors such as Factors II, VII, IX, and X are dependent on vitamin K, which is obtained from the diet and produced by intestinal bacteria. Breakdown of the fibrin clot is achieved by activating plasminogen to plasmin, which degrades fibrin. Tests commonly used to measure secondary hemostasis include prothrombin time (PT), partial thromboplastic time (PTT), and activated coagulation time (ACT) (Table 17.1) (Bauer and Rosenberg, 1995; Boudreaux *et al.*, 1996).

## BIOCHEMICAL BASIS OF HEMATOPOIETIC TOXICITY

In toxicology, it is a well-known fact that both the parent compound and its metabolites may mediate toxicity. Although there are several reports of drug-mediated hematopoietic toxicity in clinical cases, the precise mechanisms for such toxicity are not established in the majority

**TABLE 17.1 Commonly used tests to detect toxic effects on blood and bone marrow in clinical and preclinical toxicology**

| Name of test | Usefulness | Comments |
|---|---|---|
| *Complete blood count* (CBC): PCV, MCV, MCHC, WBC count, Differential leukocyte count, platelet count | Routine blood analysis for screening effect of toxic chemicals | Modern blood analyzers report these erythrocyte indices as part of a full blood count, and manual evaluation may be necessary to confirm findings and detect unusual red cell abnormalities |
| *Hemolysis testing* (e.g. osmotic fragility test and Heinz bodies) | To confirm if hemolysis is responsible for lowered red blood cell count | Look for Heinz bodies, methemoglobinemia, and eccentrocytes to detect oxidant-induced changes |
| *Hemostatic tests* (e.g. platelet numbers, PT, PTT, ACT, bleeding time) | To detect abnormalities of coagulation in the extrinsic (PT) and intrinsic (PTT, ACT) path ways and platelet function (bleeding time) | Special tests like D-dimer assay, antithrombin, platelet function tests and individual factor assays are available in some commercial research laboratories |
| *Special blood tests* Reticulocyte count, Coombs, Platelet associated immunoglobulin G (PAIgG) | These tests are based on the routine CBC findings and are considered "problem driven" tests | Reticulocyte evaluation will determine bone marrow response to anemia; Coombs and PAIgG tests determine the immune mediated component in anemia and thrombocytopenia |
| *Bone marrow cytology (evaluate myeloid: erythroid ratio)* | Confirm blood findings and to evaluate marrow function | This is an elaborate and time-consuming procedure. Cytochemical staining can be performed to differentiate abnormal cells in cases of leukemia |
| *In vitro* stem cell assays using clonogenic (CFU-E, CFU-GM, CFU-GEMM) assays | Possible to examine effects on the myeloid, erythroid and megakaryocytic lineages in a fashion where concentration of the chemical and duration of exposure are tightly controlled | *In vitro* clonogenic assays have proven useful in understanding mechanisms of toxicity and in formulating strategies for treatment |

of cases. However, *in vitro* studies have investigated the effect of compounds on the direct lysis of erythrocytes and the inhibition of growth of hemopoietic colonies in culture systems. Although mechanistically important, these results may only be extrapolated to the *in vivo* situation where the compound under test is not modified by host metabolic processes. Classic examples of discrepancy between *in vitro* and *in vivo* studies include chloramphenicol-induced myelosuppression and 2-butoxyethanol-induced hemolysis (Turton *et al*., 2002a, b; Corley *et al*., 2005). The myelosuppressive activity of chloramphenicol is usually ascribed to the parent compound, but the nitroso metabolite of chloramphenicol shows a far greater inhibitory effect in mouse hematopoietic culture assays. In addition, there is considerable variation in the susceptibility of cells from different mouse strains to the nitroso metabolite. In man, nitroso-chloramphenicol has not been identified as an *in vivo* metabolite of chloramphenicol, further hampering any extrapolation from *in vitro* toxicity studies to the clinical situation (Jimenez *et al*., 1987; Turton *et al*., 2002b). The cleaning agent 2-butoxyethanol (2-BE) is reported to be inactive in the *in vitro* hemolysis test in rats, while oral administration in this species produces a hemolytic anemia. However, the major metabolite of 2-BE, butoxyacetic acid, produces marked swelling and lysis of erythrocytes *in vitro* (Corley *et al*., 2005).

Another major mechanism of toxicity seen with many compounds involves the formation of reactive oxygen species (ROS), including superoxide anion, hydrogen peroxide, and hydroxyl radical. ROS are capable of reacting with cellular proteins, lipids, and other molecules leading to changes in their structure and cellular damage. Despite numerous cellular defense mechanisms such as antioxidant enzymes and low molecular weight antioxidants such as GSH, hemolysis may result if these mechanisms are overwhelmed in red blood cells. Many oxidants cause hemolysis by this mechanism (e.g. acetanilide, naphthalene, phenol; Table 17.2). One such compound that has been extensively tested is microcystin-LR, which significantly increases oxidative stress causing lipid peroxidation, changes in membrane fluidity, transformations of cell shape, and hemolysis. A significant dose-and time-dependent increase in lipid peroxidation products was found in red blood cells treated with microcystin-LR when compared with untreated controls (Sicinska *et al*., 2006). Recently, we reported a clinical case in a horse with massive intravascular hemolysis associated with likely pyrrolizidine alkaloid toxicity. In this study, the red blood cell GSH was significantly decreased during the hemolytic crisis and returned to normal when the hemolytic episode subsided, further confirming the probable role of oxidative stress during hemolysis (Ramaiah *et al*., 2003).

The mechanism of leukemia induced by toxic agents, particularly alkylating agents, is likely linked to cytogenetic abnormalities, especially with the loss of part or all of a chromosome. The frequency of cytogenetic abnormalities in human patients who develop dysplastic syndrome or acute leukemia after treatment with alkylating or other antineoplastic agents ranges from 67% to 95%. However, there are other forms of leukemia that have weak correlations with exposure to alkylating agents (Bloom and Brandt, 1997).

## TOXICITY-INDUCED ALTERATIONS OF HEMATOPOIETIC ELEMENTS

An incomplete list of drugs/chemicals shown to have toxic effects on blood and bone marrow is included in Table 17.2.

**TABLE 17.2 Few examples of drugs and chemicals associated with a variety of toxic effects and their likely mechanism of action**

| Drug/chemical | Effect | Mechanism of action |
|---|---|---|
| Chloramphenicol, benzene, sulfonamide, diclofenac | Bone marrow aplasia | Unknown |
| Trimethoprim-sulfadiazine, cephalosporin, phenobarbital | Pancytopenia | Possibly immune-mediated destruction of stem cells |
| Benzene | Idiosyncratic marrow aplasia | Stem cell defect |
| Estrogen | Anemia; bone marrow suppression | Stem cell damage and decreased EPO |
| Amphotericin B, insulin, isoniazid, cisplatin, rifampicin, naproxen, sulfonamide | Immune-mediated hemolytic anemia (IMHA) | Antibody-mediated destruction of erythrocytes |
| Aniline, acetanilide, napthalene, phenol, nitrobenzene, sulfanilamide | Oxidative stress-induced anemia | Oxidative damage to erythrocytes |
| Nitrites, nitrobenzenes, phenacetin, methylene blue | Toxic methemoglobinemia | Oxidation of red blood cell hemoglobin |
| Warfarin, cephalosporins | Bleeding | Inhibition of the carboxylation of vitamin K dependent coagulation factors |
| Heparin, gentamycin, aspirin, acetazolamide, cephalexin, gold salts | Thrombocytopenia | Immune-mediated platelet destruction |

## Myelosuppression

Bone marrow suppression is a widely recognized side effect of chemotherapeutic/antineoplastic agents, primarily due to the high mitotic rate of this tissue. All cell lines may be affected resulting in granulocytopenia, thrombocytopenia and/or anemia. The changes are usually dose related and reversible after withdrawal of the drug/test compound (Turton *et al.*, 2006). The mechanisms involved in such dose-dependent myelosuppression are related to inhibitory effects on primitive cells in the marrow such as BFU-E and CFU-GM (Deldar, 1994). The inhibition of these primitive cells will manifest as erythroid hypoplasia and neutropenia, respectively, and may be due to direct cytotoxicity, inhibition or blockage of specific growth factors, or interference with some fundamental process such as DNA replication. For example, EPO production has reportedly been suppressed by excess estrogen either due to exogenous administration or endogenous release as occurs with estrogen-secreting tumors (Thrall *et al.*, 2004). Other known bone marrow suppressants whose actions are dose dependent and reversible include aminopterin, a folic acid antagonist, and benzene (Table 17.2). Azidothymidine (AZT) used in the treatment of feline immunodeficiency virus and feline leukemia virus is another example of a drug that causes anemia primarily by bone marrow suppression. Ingestion of trichloroethylene is known to cause aplastic anemia in cattle. Other chemicals/drugs known to cause bone marrow suppression include, chloramphenicol, meclofenamic acid, phenylbutazone, quinidine, trimethoprim-sulfadiazine, albendazole, and fenbendazole (Manyan *et al.*, 1972). Chronic treatment with recombinant human erythropoietin (rhEPO) has also been shown to cause erythroid hypoplasia and nonregenerative anemia due to the production of anti-EPO antibodies. This effect is reversible over the course of 2–12 months after discontinuation of the drug (Langston *et al.*, 2003).

## Anemia

A mild anemia is a common feature in preclinical toxicology studies in which high doses of a drug or a chemical are administered. The anemia is usually secondary to toxicity affecting other organ systems and is thought to be similar to anemia of inflammatory disease often seen in clinical cases. This finding is usually nonspecific and insignificant. The other common reason for mild anemia in toxicology studies is attributed to the combination of frequent blood sampling for toxicokinetic studies and the stress of repeated handling, especially in primates (Hall, 1992; Gossett, 2000).

### *Immune-mediated hemolytic anemia*

Drug-induced immune-mediated hemolytic anemia (IMHA) in humans is thought to occur as a result of the following three mechanisms: formation of antibodies (IgG, IgM, or IgA) to drug adhered to red cell membranes (hapten or neoantigen formation), binding of preformed drug-antibody complexes to the red cell membrane, or autoantibody formation. However, documented cases of drug-induced IMHA in animals are infrequent and most are probably related to hapten formation. Examples of IMHA caused by drugs in animals include penicillins and cephalosporins, which are known to cause anemia by a combination of immune-mediated hemolysis and bone marrow suppression. Levamisole is another drug suspected of inducing IMHA in dogs that are being treated for heartworm disease. Vaccination has also been implicated in cases who present with IMHA within short periods of time after vaccination. However, the precise mechanism for such a response due to vaccination is not known at present (Andrews, 1998; Bloom *et al.*, 1988; Gossett, 2000).

### *Oxidative or nonimmune hemolysis*

Exposure to oxidants results in the production of large aggregates of oxidized, precipitated hemoglobin that are attached to the internal surface of erythrocyte membranes (Heinz bodies). Eccentrocytes are also often formed with oxidative damage resulting from altered cytoplasmic-membrane adhesions. The attachment of Heinz bodies to the plasma membrane causes membrane rigidity with subsequent red blood cell lysis and anemia. Several oxidants are implicated in oxidative hemolysis caused by Heinz bodies. Few examples include methylene blue, phenazopyridine, acetaminophen, and propylene glycol in cats, and phenothiazine in horses. Several reports of zinc-induced hemolytic anemia occurred due to ingestion of metallic hardware items, pennies, and topical formulations containing zinc oxide. Sudden release of copper from accumulations in the liver of several species has been reported to cause Heinz bodies and an acute hemolytic crisis. Vitamin K and DL-methionine induce methemoglobinemia in multiple species although Heinz bodies and anemia are not frequent (Houston and Myers, 1993; Schlesinger, 1995).

### *Basophilic stippling*

Basophilic stippling is usually seen in mature erythrocytes and is thought to be due to the presence of rough endoplasmic reticulum aggregates. This condition is seen in lead poisoning and is due to the failure of RNA degradation. Basophilic stippling usually represents a regenerative response to anemia in ruminants and less commonly in other species. Siderotic inclusions (Pappenheimer bodies) in the red blood cells resemble basophilic stippling. Siderotic inclusions contain iron and may be seen after isoniazid therapy, lead toxicity, chloramphenicol, and zinc toxicity.

### *Neutrophilia*

An elevated neutrophil count is often present in cases of bacterial infection and many inflammatory conditions. In addition, chemicals such as lead, mercury, phenacetin, and pyridine may induce neutrophilia as a secondary response to tissue damage or hypersensitivity reactions. Neutrophilia is often seen in laboratory animals as a secondary event in neoplastic conditions or following hemorrhage. Increased neutrophil production and delivery to the circulation in response to inflammation may be the result of multiple signals including endotoxin, interleukins, interferons, and cytokines (Anderson, 1993). Approximately 3–5 days are required for a significant increase in neutrophils to be observed peripherally due to increased bone marrow production (Duncan *et al.*, 1994).

### *Eosinophilia, monocytosis, and lymphoctyosis*

Eosinophilia is seen in parasitic infections, dermatitis, and occasionally in allergic reactions to drugs such as salicylates. Increased eosinophils may be seen in the peripheral blood and wall of the rat gut following the use of dietary expanders such as modified starches and sugars (sorbital, mannitol, lactose, and polyethylene glycol). Eosinophilia has also been reported with excessive fluoride ingestion in rats (Andrews, 1998).

Monocytosis is often seen concomitant with neutrophilia and is usually a nonspecific finding of inflammation or stress. It is also seen acutely after trauma such as accidental gavage of the lung in rodents. Monocytosis has been described resulting from the administration of the antipsychotic drug chlorpromazine (Andrews, 1998).

Increased lymphocyte numbers may be present peripherally following immunization and with physiological excitement due to epinephrine (Meyer and Harvey, 1998).

### *Erythrocytosis and thrombocytosis*

Increased numbers of erythrocytes (erythrocytosis) seen in peripheral blood is usually a relative versus absolute increase, and it is frequently associated with food intake as well as hemoconcentration/dehydration due to fluid loss such as diarrhea and vomiting. The increased erythrocyte counts in these situations may therefore only reflect the change in the plasma compartment rather than alterations in the production or fate of red blood cells. A sudden release of erythrocytes due to splenic contraction is another cause of a relative increase in erythrocytosis (Duncan *et al.*, 1994).

Increased platelets may be seen following acute blood loss or following trauma. The cytotoxic drug 5-fluorouracil causes an immediate fall and then a sustained increase in platelet numbers. Other compounds causing thrombocytosis are the drugs AZT and vincristine and the food additive butylated hydroxytoluene. The mechanisms involved in drug-related thrombocytosis are unclear, although cytokines including stem cell factor and interleukin-6 have been implicated. Thrombocytosis is often synchronous with reticulocytosis, supporting the hypothesis that EPO has a stimulatory effect on megakaryocytes (Boudreaux, 1996).

### *Thrombocytopenia/blood loss/coagulation inhibition*

Thrombocytopenia caused by drugs and chemicals may be directly caused by their cytotoxic effect on bone marrow progenitor cells, as noted with chemotherapeutics agents, or by peripheral destruction due to immune-mediated mechanisms. Other than chemotherapeutic agents, chloramphenicol has been associated with thrombocytopenia in dogs and cats. Similarly, estrogen and interferons can cause destruction of stem cells resulting in marrow suppression and thrombocytopenia. Agents that can specifically destroy megakaryocytes include, anagrelide, ethanol, estrogens, interferons, and thiazide diuretics (Anthony *et al.*, 1994; Okamura *et al.*, 1994; Rodman *et al.*, 1997). Drugs associated with peripheral destruction of platelets include, methyldopa, levadopa, and gold therapy. Toxins that induce thrombocytopenia include, aflatoxin B1, brackenfern, and trichloroethylene (Weiss, 2000). Xenobiotic-induced platelet destruction is due to either immune- mediated or nonimmunologic destruction (Bloom *et al.*, 1988; Shebuski, 1993). Hapten (neoantigen/compound epitope formation), induction of antibodies, and immune complex formation are all possible mechanisms of immune-mediated platelet destruction.

The two main toxicologic consequences of platelet modulation are predisposition to hemorrhage and thrombosis. Nonsteroidal anti-inflammatory drugs have been known to cause blood loss anemia, which is thought to be due to gastrointestinal damage or ulceration secondary to the local inhibition of prostaglandin synthesis (George and Shattil, 1991). Aspirin decreases platelet function and aggregation and can also cause gastrointestinal ulceration resulting in hematemesis and melena in humans and animals.

Coumarin-like compounds, such as warfarin, inhibit the carboxylation of vitamin K dependent coagulation factors II, VII, IX, and X resulting in prolonged clotting times (Bloom, 1997; Bloom and Brandt, 1997). Cephalosporins are also responsible for prolonged clotting times by the same mechanism. Several antibiotics that cause eradication of gut microflora and thus lower the levels of available vitamin K can prolong coagulation and cause bleeding. Any toxic compound that results in liver failure (such as acetaminophen) will cause decreased synthesis of the coagulation factors needed for clotting and may result in bleeding.

### *Toxic leukemogenesis*

A few examples of leukemogenic agents include aromatic hydrocarbons (benzene), alkylating chemotherapeutic agents, and ionizing radiation. The development of

leukemia is not highly predictable or dose related in humans (Irons, 1997). Exposure to chemical clastogens that cause chromosomal damage (benzene and its metabolites) results in genetic damage. In many cases the damage is reversible by DNA repair mechanisms. For example the folic acid antagonist methotrexate causes transient *in vitro* DNA damage, but therapeutic use of the drug is not associated with the development of leukemia. However, a secondary insult may overcome natural DNA repair capability resulting in permanent chromosomal damage. Acute myeloid leukemia can be induced in rats by very small quantities of dimethylbenzanthracene or with ionizing radiation (Levine and Bloomfield, 1992; Andrews, 1998).

## PERSPECTIVES

The hematopoietic system is very susceptible to a variety of toxic chemicals and drugs, and such toxic effects may result in financial liability in cases of preclinical drug development and mortality in clinical practice. Because of this, systematic examination of blood and bone marrow is a major requirement of clinical and preclinical toxicity studies, and it has become a routine part of diagnostic evaluation in the clinics when screening for toxic effects of drugs, especially chemotherapeutic agents. In most cases, bone marrow toxicity can be assessed by evaluating complete blood cell counts and peripheral blood smears. Finding toxic effects of a test compound in a dose-related fashion typically halts further development of the drug.

Similar to other areas of toxicology, there has been significant advancement in an understanding of the growth and differentiation of hematopoietic stem cells as well as early diagnosis of leukemia. The recent "functional genomics" and "proteomics" approaches have not only led to understanding of the molecular basis of toxicity of a variety of test compounds, but they also have revolutionized bone marrow disease classification and treatment strategy.

In conclusion, it is possible for a toxicologist to systematically evaluate blood and bone marrow toxicity in laboratory settings and to devise a hypothesis for development of such toxicity. Because of this, it is imperative for toxicologists to understand the basic concepts of the hematopoietic system in order to interpret the results of toxicological studies.

## REFERENCES

Anderson TD (1993) Cytokine-induced changes in the leukon. *Toxicol Pathol* **21**: 147–57.

Andrews MC (1998) The hematopoietic system. In *Target Organ Pathology: A Basic Text*, Torton J, Hooson J (eds). Taylor and Francis, UK, pp. 177–205.

Anthony A, Dhillon AP, Sim R, Nygard G, PounderRE, Wakefield AJ (1994) Ulceration fibrosis and diaphragm-like lesions in the caecum of rats treated with indomethacin. *Aliment Pharmacol Ther* **8**: 417–24.

Babior BM, Golde DW (1995) Production, distribution and fate of neutrophils. In *Williams Hematology*, Beutler E, Lichtman MA, Coller BS, Kipps TJ (eds). McGraw-Hill, NewYork, p. 773.

Bauer KA, Rosenberg RD (1995) Control of coagulation reactions. In *Williams Hematology*, Beutler E, Lichtman MA, Coller BS, Kipps TJ (eds). McGraw-Hill, NewYork, p. 1239.

Bienzle D (2000) Monocytes and macrophages. In *Veterinary Hematology*, Feldman BF, Zinkl JG, Jain NC (eds). Lippincott Williams and Wilkins, USA, pp. 318–25.

Bloom JC, Thiem PA, Sellers TS, Deldar A, Lewis HB (1988) Cephalosporin-induced immune cytopenia in the dog: demonstration of erythrocyte-, neutrophil- and platelet-associated IgG following treatment with cefazedone. *Am J Hematol* **28**: 71–8.

Bloom JC (1997) Introduction to hematotoxicology. In *Comprehensive Toxicology*, vol. 4, Sipes IG, McQueen CA, Gandolfi AJ (eds). Pergamon Press, Oxford, pp. 263–83.

Bloom JC, Brandt JT (1997) Toxic responses of blood. In *Casarett and Doull's Toxicology – The Basic Science of Poisons*, Klassen C (ed.). McGraw-Hill, USA, pp.389–411.

Bolliger AP (2004) Cytologic evaluation of bone marrow in rats: indications, methods and normal morphology. *Vet Clin Pathol* **33**: 58–67.

Boudreaux MK, Kvam K, Dillon AR, *et al.* (1996) Type I Glanzmann's thrombasthenia in a Great Pyrenees dog. *Vet Pathol* **33**: 503.

Car BD (2000) Erythropoiesis and erythrokinetics. In *Veterinary Hematology*, Feldman BF, Zinkl JG, Jain NC (eds). Lippincott Williams and Wilkins, USA, pp. 105–9.

Christian JA (2000) Red blood cell survival and destruction. In *Veterinary Hematology*, Feldman BF, Zinkl JG, Jain NC (eds). Lippincott Williams and Wilkins, USA, pp. 117–25.

Corley RA, Grant DM, Farris E, Weitz KK, Soelberg JJ, Thrall KD, Poet TS (2005) Determination of age and gender differences in biochemical processes affecting the disposition of 2-butoxyethanol and its metabolites in mice and rats to improve PBPK modeling. *Toxicol Lett* **156**: 127–61.

Deldar A (1994) Drug-induced blood disorders: review of pathogenetic mechanisms and utilization of bone marrow cell culture technology as an investigative approach. *Curr Topics Vet Res* **1**: 83–101.

Duncan JR, Prasse KW, Mahaffey EA (1994) *Veterinary Laboratory Medicine. Clinical Pathology*, Iowa State University Press, Ames, IA.

Fernandez FR, Grindem CB (2000) Reticulocyte response. In *Veterinary Hematology*, Feldman BF, Zinkl JG, Jain NC (eds). Lippincott Williams and Wilkins, USA, pp. 110–16.

Gasper PW (2000) The hemopoietic system. In *Veterinary Hematology*, Feldman BF, Zinkl JG, Jain NC (eds). Lippincott Williams and Wilkins, USA, pp. 63–78.

George JN, Shattil SJ (1991) The clinical importance of acquired abnormalities of platelet function. *N Engl J Med* **324**: 27–39.

Gossett KA (2000) Anemias associated with drugs and chemicals. In *Veterinary Hematology*, Feldman BF, Zinkl JG, Jain NC (eds). Lippincott Williams and Wilkins, USA, pp. 185–9.

Hall RL (1992) Clinical pathology for preclinical safety assessment: current global guidelines. *Toxicol Pathol* **20**: 472–6.

Harvey JW (1997) The erythrocyte physiology, metabolism and biochemical disorders. In *Clinical Biochemistry of Domestic Animals*, Kaneko JJ, Harvey JW, Bruss ML (eds). Academic Press, San Diego, pp. 157–203.

Harvey JW (2000) Erythrocyte metabolism. In *Veterinary Hematology*, Feldman JG, Zinkl JG, Jain NC (eds). Lippincott Williams and Wilkins, USA, pp. 125–8.

Harvey JW (2001) *Atlas of Veterinary Hematology*. W.B. Saunders Co., Philadelphia, PA.

Houston DM, Myers SL (1993) A review of Heinz-body anemia in the dog induced by toxins. *Vet Hum Toxicol* **35**: 158–61.

Irons RD (1997) Leukemogenesis as a toxic response. In *Comprehensive Toxicology,* vol. 4, Sipes IG, McQueen CA, Gandolfi AJ (eds). Pergamon Press, Oxford, pp. 175–99.

Jain NC (1993) *Essentials of Veterinary Hematology,* Jain NC (ed.). Lea and Febiger, USA.

Jimenez JJ, Arimura GK, Abou-Khalil WH, Isildar M, Yunis AA (1987) Chloramphenicol-induced bone marrow injury: possible role of bacterial metabolites of chloramphenicol. *Blood* **70**: 1180–5.

Kaneko JJ (2000) Hemoglobin synthesis and destruction. In *Veterinary Hematology,* Feldman BF, Zinkl JG, Jain NC (eds). Lippincott Williams and Wilkins, USA, pp. 135–9.

Langston CE, Reine NJ, Kittrell D (2003) The use of erythropoietin. *Vet Clin Small Anim* **33**: 1245–60.

Levine EG, Bloomfield CD (1992) Leukemias and myelodysplastic syndromes secondary to drug, radiation, and environmental exposure. *Semin Oncol* **19**: 47–84.

Lund JE (2000) Toxicological effects on blood and bone marrow. In *Veterinary Hematology*, Feldman BF, Zinkl BF, Jain NC (eds). Lippincott Williams and Wilkins, USA, pp. 44–9.

Meyer DJ, Harvey JW (1998) *Veterinary Laboratory Medicine. Interpretation and Diagnosis.* W.B. Saunders Co., Philadelphia.

Manyan DR, Arimura GK, Yunis AA (1972) Chloramphenol-induced erythroid suppression and bone marrow ferrochelatase activity in dogs. *J Lab Clin Med* **79**: 137–44.

Nadrew GA, Chavey PS, Smith JE (1994) Enzyme-linked immunosorbent assay to measure serum ferritin and the relationship between serum ferritin and nonheme iron stores in cats. *Vet Pathol* **31**: 674.

Okamura T, Garland EM, Cohen SM (1994) Glandular stomach hemorrhage induced by high dose saccharin in young rodents. *Toxicol Lett* **74**: 129–40.

Ramaiah SK, Harvey J, Gigure S, Franklin R (2003) Intravascular hemolysis associated with liver disease in a horse with marked neutrophil hypersegmentation. *J Vet Int Med* **17**: 360–3.

Rodman LE, Farnell DR, Coyne JM, Allan PW, Hill DL, Duncan KLK, Tomaszewski JE, Smith AC, Page JG (1997) Toxicity of cordycepin in combination with the adenosine deaminase inhibitor 2-deoxycoformycin in beagle dogs. *Toxicol Appl Pharmacol* **147**: 39–45.

Schlesinger DP (1995) Methemoglobinemia and anemia in a dog with acetaminophen toxicity *Can Vet J* **36**: 515.

Scott MA, Stockham SL (2000) Basophils and mast cells. In *Veterinary Hematology*, Feldman BF, Zinkl JG, Jain NC (eds). Lippincott Williams and Wilkins, USA, pp. 308–17.

Shebuski RJ (1993) Interruption of thrombosis and hemostasis by anti-platelet agents. *Toxicol Pathol* **21**: 180–9.

Sicinska P, Bukowska B, Michalowicz J, Duda W (2006) Damage of cell membrane and antioxidative system in human erythrocytes incubated with microcystin -LR *in vitro*. *Toxicon* **47**: 387–97.

Smith GS (2000) Neutrophils. In *Veterinary Hematology*, Feldman BF, Zinkl JG, Jain NC (eds). Lippincott Williams and Wilkins, USA, pp. 281–97.

Thrall MA, Wieser G, Jain N (2004) Laboratory evaluation of bone marrow. In *Veterinary Hematology and Clinical Chemistry,* Thrall MA (ed.). Lippincott Williams and Wilkins, USA, p. 160.

Turton JA, Andrews CM, Havard AC, Robinson S, York M, Williams TC, GibsonFM (2002a) Hematotoxicity of thiamphenicol in the BALB/c mouse and Wistar Hanover rat. *Food Chem Toxicol* **40**: 1849–61.

Turton JA, Andrews CM, Havard AC, Williams TC (2002b) Studies on the hematotoxicity of chloramphenicol succinate in the Dunkin Hartley guinea pig. *Int J Exp Path* **83**: 225–38.

Turton JA, Sones WR, Andrews CM, Pilling AM, Williams TC, Molyneux G, Rizzo S, Gordon-Smith EC, Gibson FM (2006) Further development of a mouse model of chronic bone marrow aplasia in the busulphan-treated mouse. *Int J Exp Pathol* **87**: 49–63.

Weiss DJ (2000) Platelet production defects. In *Veterinary Hematology,* Feldman BF, Zinkl JG, Jain NC (eds). Lippincott Williams and Wilkins, USA, pp. 469–71.

Young KM (2000) Eosinophils. In *Veterinary Hematology* Feldman, BF, Zinkl JG, Jain NC (eds). Lippincott Williams and Wilkins, USA, pp. 297–307.

C H A P T E R

# 18

# Immunotoxicity

*Raghubir P. Sharma*

## INTRODUCTION

### Definition

Immunotoxicology is defined as the study of toxic effects of chemicals (or in some cases physical agents such as radiation) on the immune system. The concern about the undesirable effects of chemicals on the immune system or the immune system as a mediator of systemic toxic responses is relatively new; much of the advances have followed our understanding of the molecular events involved in immune mechanisms and discoveries in molecular biology. Immunotoxicity in veterinary practice is almost non-described even though interest in this area has emerged (Kende *et al*., 1984). In public health the interest in immunotoxicity increased after awareness of the unwanted effects of environmental chemicals, including air and water pollutants, food additives or contaminants. Even at present there is limited information on the harmful effects of chemicals on the immune system of domestic animals; much of the information is derived from experimental studies in laboratory models or the information is restricted to poultry or fish.

### Historical

Although the modern discipline of immunotoxicology is relatively new and immunology itself is nearly a century old, concern with infections and its possible prevention dates back to nearly one thousand years. It was realized long ago that survivors of smallpox or plagues were relatively resistant to the disease they have acquired earlier and people were inhaling dried smallpox scabs to prevent infection in China as early as in 11th century. It was not until late 19th century that boosting immunity with vaccination for specific infections was possible. The cellular basis of immune responses was recognized in the middle of 20th century. The same period also saw the realization that inadvertent exposure to chemicals and even the therapeutic use of drugs may influence the optimal mounting of immune responses against invading organisms. There were occasional published reports of immunotoxic effects against environmental chemicals, especially pesticides, prior to 1970; a systematic study of immunotoxicity of various chemicals followed that period.

The search for drugs that modulate immune functions predates the experimental approach in immunotoxicity and has also provided valuable information about immune mechanisms. It was also known sometime ago that environmental factors are important for development of cancer or certain autoimmune diseases; this information has led to screening of various chemicals for their untoward effects on the immune system. Yet, the information regarding the immunotoxicity of chemicals is often incomplete, controversial, and has little relevance to doses or concentrations which are encountered in normal environmental exposures.

### Importance of studying immunotoxicity

1 *Infectious diseases.* The immune response is an important defense mechanism for the host. In many cases a chemical or physical stress leads to immunosuppression that compromises the host defense mechanisms against invading organisms. Higher mortality or prolonged and serious illness may result as a consequence of such immune deficiency. If the immune responses are delayed or decreased even a mild infection may provide discomfort or serious disease. In some cases it leads to decreased growth or productivity in livestock.

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2 *Interference with effectiveness of vaccination.* Since the start of vaccinating animals against prevalent infectious diseases the general health of livestock has notably improved. Routine prophylactic immunization has prevented a number of epizootic problems and has nearly wiped out a variety of lethal diseases, resulting in reduced mortality and improved life expectancy. As a result of chemically elicited immunosuppression the effectiveness of immunization is likely to be compromised. The measure of the efficiency of immunization, either an antibody titer or lethal response to a controlled infection, is usually evaluated as a measure of immunotoxicity of chemicals; it is not known if this effect has relevance at low levels of exposure to which animals generally come in contact with chemicals.

3 *Cancer surveillance.* The concept of immune surveillance in tumor survival and growth is now widely accepted. A higher incidence of tumors has been observed in transplant patients receiving immunosuppressive therapy. Dietary supplements or therapeutic agents that enhance the immune effectiveness are generally used in conjunction with chemotherapy. It is also believed that stress-induced interference with immune mechanisms may lead to a number of health problems including neoplasia. Yet nude mice that are inherently immunodeficient do not show an increased frequency of spontaneous tumors. Such conflicting evidence leads to arguments whether the immune system has an important role in the development of incipient tumors. It is unlikely that immune responses are involved in the initiation of neoplasms but may ultimately influence their growth and propagation. Exposure to chemicals that are potent immunosuppressants may lead to higher incidence of tumors and resulting mortality.

4 *Allergy and autoimmunity*. The immune response against undesirable invaders is highly beneficial to general health; however, more of this is not necessarily good. There are a number of disorders that involve enhanced immune responses. Increased immunity may lead to allergic responses. Many chemicals being small molecules are not immunogenic in themselves but after entering the body they can combine with natural proteins and mount immune responses against either the normal proteins or the chemical moiety itself. Some chemicals are allergenic either because of their reactivity to biological molecules or may be metabolized to reactive products in the body. An activated immune system will increase the probability of such reactions. Production of immune response against tissue components is a step in the development of autoimmunity. There are a number of autoimmune disorders that occur in people or animals; the frequency of these diseases is genetically determined in most cases, however, exposure to environmental chemicals has been implicated as a trigger in some repressed conditions. It is not coincidental that allergic or autoimmune disorders are effectively treated with immunosuppressant therapy.

## THE IMMUNE SYSTEM

A brief description of the immune system is desirable here to review basic concepts of immunology. The following is not meant to be a complete description of the highly complex system that is activated after immunologic challenge and produces responses that are highly specific, cooperative, redundant, and interactive with each other or with other bodily functions, and in addition have properties such as immunologic memory and self-tolerance. For a comprehensive discussion of the immune responses it is desirable to consult textbooks on this topic (Mak and Saunders, 2006). The complexity and multiple stages involved in the immune responses provide a large number of target sites for toxicants to act upon and influence the immune system.

### Immune system is diffuse throughout the body

One of the characteristics of the immune system is that it is not confined to a body compartment but is present throughout the body. The immune system consists of many interdependent cell types that protect the body from various infections (bacterial, viral, fungal, parasitic) and from the development of tumor cells. The principal cellular components of the immune system, the lymphocytes, are found in nearly all tissues; sometimes they are modified to specialized cells. In addition to lymphocytes, the macrophages are an integral component of this system. Other leukocytes (particularly granular leukocytes) in circulation or in tissues help a variety of immune responses, particularly during inflammation. The immune cells are concentrated in some organs (indicated below) but may travel throughout the body via lymphatic and blood circulation. Many of these cell types have specialized functions, however, they work in concert by helping each other.

### Innate versus acquired immunity

Different parts of the body contain natural or innate immunity that is relatively non-specific. Components of innate immunity are principally localized at the sites of entry of invading organisms, such as skin and mucous membranes of digestive and respiratory systems. Lymph nodes underlying the mucous membranes of the digestive and respiratory tracts are integral part of this natural immunity; these can also partake in the adaptive immune

responses. These reactions are not specific to an antigen type and are produced without a prior challenge of the invading organism. Circulating and resident macrophages assist in phagocytosis and remove foreign material in addition to their important role in other aspects of immune responses.

Acquired immunity on the other hand is mounted in response to an antigenic challenge and is specific to the antigen type. It can be either cell mediated (that involves principally T lymphocytes) or humoral (also known as antibody mediated, involving specific antibodies produced by B lymphocytes). Antigens are initially engulfed by macrophages that process them and display parts of them on their surface together with some of their own proteins. This sensitizes T cells to recognize these antigens. All body cells are coated with various protein complexes called CD (cluster of differentiation); more than 160 clusters have been defined, each of which is a different chemical molecule that coats the surface.

## Functional units of the immune system

1 *Primary and secondary organs.* The primary organs of the immune system are bone marrow and thymus. In avian species the bursa is also a primary organ. Different cells of the immune system are derived initially from the bone marrow. During hemopoiesis, stem cells in bone marrow differentiate into either cells of the immune system or into precursors of cells that leave the bone marrow to continue their differentiation elsewhere.

   The other primary immune organ, thymus, differentiates lymphocyte precursors into T cells. Immature precursor lymphocytes leave bone marrow and migrate to the thymus. During the maturation in this organ T cells that are beneficial to the immune system are spared while those cells that might provoke autoimmune response are eliminated. The mature T cells are then released into the bloodstream. In birds the lymphocytes that migrate to bursa are differentiated into B cells. In most mammalian organisms differentiation of precursor cells to B lymphocytes is believed to occur either in the bone marrow or fetal liver.

   The secondary immune organs are spleen and lymph nodes. The spleen is the organ that eliminates both red and white unneeded blood cells. The cell types in spleen are T cells, B cells, natural killer (NK) cells, macrophages, dendritic cells, and red blood cells. Migratory macrophages and dendritic cells bring antigens to the spleen via the bloodstream where foreign antigens are captured from the blood that passes through the spleen. The lymph nodes, which function as a filter for lymph, are found throughout the body and drain interstitial fluid from most of tissues. The lymph nodes consist of T cells, B cells, dendritic cells, and macrophages. Lymph nodes are the organs where B cells are activated to produce large amounts of antibody.

2 *Cellular components.* Immune system comprises a variety of cells that communicate with each other or even other organs in the body to mount effective immune response against foreign invaders. Different types of cellular components of the immune system along with their characteristic CD markers are listed in Table 18.1. One of the major types of the immune cells consists of lymphocytes that are further classified in several subpopulations. Two major classes of lymphocytes are T cells (matured in the thymus) and B cells (termed after the bursa of Fabricius in birds, where these were first discovered). T lymphocytes are usually divided into two major subsets that are functionally and phenotypically different. The T and B cells each have about 100,000 molecules on their surfaces. T cells have CD2, CD3, CD4, CD28, CD45R, and other non-CD molecules on their surfaces. The T helper cell, also called the CD4+, is a pertinent coordinator of immune regulation. The main function of the T helper cell is to augment immune responses by the secretion of specialized factors or cytokines that activate other white blood cells to fight infection. Two further subsets of T helper cells, Th1 and Th2, have been identified based on the type of cytokines produced by them.

   Another important type of T cell is called the T killer/suppressor subset or CD8+ T cell. These cells are important in directly killing certain tumor cells, viral-infected cells, and sometimes parasites. The CD8+ T cells are important in down-regulation of immune responses. Both types of T cells can be found throughout the body, the distribution often depends on the secondary lymphoid organs where activation occurs, but they are also found in liver, lung, blood, and intestinal and reproductive tracts.

   Natural killer cells, often referred to as NK cells, are similar to the killer T cell subset (CD8+ T cells). These cells directly kill certain tumors such as melanomas, lymphomas, and viral-infected cells, including herpes and cytomegalovirus-infected cells. NK cells, unlike the CD8+ (killer) T cells, kill their targets without being primed in lymphoid organs. However, NK cells activated by secretions from CD4+ T cells kill their tumor or viral-infected targets more effectively.

   The major function of B cells is the production of antibodies in response to foreign proteins of bacteria, viruses, and tumor cells. Antibodies are specialized proteins that specifically recognize and bind to one particular protein. Antibody production and binding to a foreign substance or antigen, is critical as a means of signaling other cells to engulf, kill, or remove that substance from the body. B cells are coated with CD21, CD35, CD40, and CD45 in addition to other non-CD molecules.

TABLE 18.1 Cellular components of the immune system and their functions in mammals

| Cell types | Specific surface markers | Functions |
|---|---|---|
| Stem/progenitor cells | CD34, CD90, CD110, CD111, CD117, CD133, CD202, CD243 | Differentiate into hematopoietic cells, including erythrocytes and leukocytes |
| T lymphocytes | CD3, CD4, CD5, CD7, CD8, CD25, CD28, CD45R, CD52L, CD69, CD134, CD152, CD154 | Matured in thymus T cells are differentiated into subsets; cytokine production |
| T helper lymphocytes (Th1 and Th2) | CD2, CD3, CD4, CD28, CD45R (plus T cell markers) | Help maturation of T and B cells |
| Cytotoxic T lymphocytes (CTL) | Most lymphocytic markers + CD8 | Cause lysis of antigen-bearing cells; regulate Th cell action |
| NK T lymphocytes | CD11b, CD16a, CD56, CD69, CD94, CD152, CD158a, CD161 | Cytotoxicity of invading organisms and tumor cells |
| Null T cells | SWC6 (swine workshop cluster 6) cell surface antigen; devoid of other T cell markers | Not defined; may be involved in immunologic memory |
| B cells | Lymphocyte markers + CD21, CD35, CD40, CD45 | Differentiated into specific antibody producing cells |
| Plasma cells | CD38 | Production of antibodies |
| Monocytes, macrophages | CD11b, CD13, CD14, CD64, CD80, CD86, CD115, MHC class II | Monocytes are circulating cells whereas macrophages are both circulating and tissue residents; cytokine production; antigen presentation; phagocytosis |
| Dendritic cells | CD1a, CD11c, CD80, CD83, CD86, CD123, CD205, CD207, CD209 | Both circulating and tissue-specific; attract antigens and present to T cells |
| Mast cells | CD33, CD117, CD203c | Release of vasoactive products |
| Granulocytes (leukocytes, e.g. neutrophils, etc.) | CD15, CD16b, CD31, CD88, CD156a | Phagocytosis; help in inflammatory process |

Phenotypes indicated refer to human cells; only human and mouse cells are extensively analyzed. Only differentiating markers are indicated. Information on most domestic animals is limited. *Source*: eBioscience, San Diego, CA.

Macrophages are very important in the regulation of immune responses. They are often referred to as scavengers or antigen-presenting cells (APC) because they ingest foreign materials, process and present these antigens to other cells of the immune system such as T cells and B cells. This is the first step in the initiation of an immune response. Stimulated macrophages produce cytokines and exhibit increased levels of phagocytosis.

Another cell type in the immune system, only recently characterized, is the dendritic cell. Dendritic cells, originate in the bone marrow, and function as APC; these cells are more efficient APCs than macrophages. The dendritic cells are primarily found in the structural compartment of the lymphoid organs such as the thymus, lymph nodes, and spleen; they are also present in the bloodstream and other tissues. The dendritic cells are hard to isolate, which is often a prerequisite for the study of the functional qualities of specific cell types.

Other leukocytes or white blood cells also participate in the immune system, particularly via their contribution in inflammatory responses. These cells are granulocytes or polymorphonuclear leukocytes (PMNs), composed of three cell types, that is neutrophils, eosinophils, and basophils, so named for their staining characteristics with certain dyes. Eosinophils that are also found in certain tissues like lungs or beneath the skin are termed as mast cells. All granulocytes are especially important in the removal of bacteria and parasites from the body as they engulf these foreign bodies and degrade them using their enzymes.

3 *Cell–cell interactions*. A characteristic of the complex and diverse immune functions is the ability of cells to communicate with each other and with cells of other systems. These communications are made possible either by direct contact involving the cell surface receptors or by the soluble mediators termed as lymphokines or cytokines. The cytokines (see Table 18.2 for major cytokines and their functions) are usually small proteins that act on receptors either in the same cell or on other cells and carry out a variety of functions, including maintaining their homeostasis. Various cytokines possess properties that include redundancy and cascade effects, and have synergistic or antagonistic activity with other cytokines. Major cytokines stimulate cell proliferation and differentiation, or activate cells to mount an effective immune response.

## Cellular versus humoral immunity

The two major types of immune responses are mediated by T and B lymphocytes, respectively. These types of immune responses are distinct but not totally independent of each other. For example, T cells regulate the T dependent antibody formation whereas the antibody-dependent cell-mediated cytotoxicity (ADCC) has also

**TABLE 18.2 Major cytokines involved in immune responses***

| Cytokine | Source (s) | Function |
|---|---|---|
| TNFα | Macrophages, mast cells, NK cells | Regulation of cytokine expression and cell adhesion molecules; tumor cell death |
| TNFβ | Th1 and cytotoxic T cells | Phagocytosis, cell death; NO production |
| IFNγ | Macrophages, Th2 cells, activated B cells | Elimination of pathogens; switching of Ig classes; inhibits viral replication and cell proliferation |
| TGFβ | Monocytes, T cells | Chemotaxis of macrophages; IL-1 activation; IgA synthesis |
| MIF | Macrophages, lymphocytes | Chemotaxis of monocytes and T cells |
| IL-1α and β | Monocytes, macrophages, dendritic cells, B cells | Activation of Th and NK cells; B cell amplification; inflammation |
| IL-1Ra | Macrophages, monocytes | Inhibits IL-1 and IL-2 action |
| IL-2 | Th1 cells | Activation of T, B and NK cells |
| IL-3 | Th cells, NK cells, monocytes | Growth and differentiation of stem cells and mast cells; degranulation of mast cells |
| IL-4 | Th2 cells | T cell and B cell proliferation and differentiation; MHC II expression on macrophages |
| IL-5 | Th2 cells | Proliferation and differentiation of activated B cells; IgA synthesis; eosinophil growth |
| IL-6 | Macrophages, monocytes, Th2 cells, stromal cells | Maturation of B cells into plasma cells; antibody secretion; differentiation of stem cells; acute phase response; inflammation |
| IL-7 | Bone marrow and thymus stromal cells | Differentiates stem cells into progenitor T and B cells |
| IL-8 | Macrophages, endothelial cells | Attracts neutrophils |
| IL-10 | Th2 cells | Cytokine production by macrophages; activates B cells |
| IL-12 (p70 and p40) | B cells, monocytes, dendritic cells | Differentiation and activation of cytotoxic T cells and NK cells; induction of IFNγ |

*Abbreviations are defined in text. Only selected cytokines are listed; nearly three dozen interleukins (IL) and other cytokines have been described. Many cytokines are species-specific; others show cross reactivity.

been described. Maturation and differentiation of T cells may require cellular interactions or soluble messengers. B cells can be primed by antigen-presenting T cells or macrophages to produce a vast repertoire of antibodies specific to each antigen. The diversity of antibody production by B cells is accomplished by antigen recognition (depending on variation in antigen binding sequence, a characteristic of the variable region of antibody protein) and by antibody effector function (antibody isotype variability based on the constant region of antibodies). By using combinations from a multiple source of gene segments in the germ line DNA and hypermutations based on activation-induced deamination (Petersen-Mahrt *et al.*, 2002) an extremely large variety of specific antibodies can be created. Various isotypes of antibodies produced by B cells have been named as IgA, IgD, IgE, IgG, or IgM, with specific functions.

## Cooperation and interactions between immune and other systems

1 *Immune mechanisms subsequent to pathologic damage*. The purpose of the immune functions is to ward off undesirable organisms or materials from the body, its relationship with inflammatory processes and pathologic damage is implied. Many of the cellular components of the immune system are also primary components in the inflammation. Therefore it is natural that various components of this system are activated after damage to other tissue or organs of the body. The immune system responds to altered proteins or to other biological molecules formed by binding with reactive intermediates, which may be produced in the body subsequent to metabolic activation of a number of exogenous chemicals or due to tissue damage.
2 *Control of the immune system by nervous system*. The immune system interacts with other systems and is profoundly influenced by the central nervous system, both directly via innervations of lymphatic organs and indirectly via neuroendocrine mechanisms. The autonomic nervous system directly innervates thymus, spleen, lymph node, bone marrow, and other lymphoid tissues. Pharmacological manipulation of post-ganglionic noradrenergic nerve fibers affects immune functions. Increased levels of cyclic nucleotides, which respond to neurotransmitters, also alter the immune response. The cells of the immune system produce factors that influence the nervous system.
3 *Endocrine mechanisms.* Hormones, such as somatotrophin (growth hormone) and thymosin (thymic maturation factor), stimulate the immune responses, whereas steroids,

including sex hormones, generally suppress the immune responses. Role of stress on immune functions has been shown; it is presumed that exposure to exogenous chemicals may induce stress. Stress-like effects after stimulation of hypothalamic–pituitary–adrenal axis lead to cortisol production; the latter is a well-known immunomodulatory agent.

4 *The complement system*. A complex system that consists of a cascade of serum proteins, mostly of β-class globulins, is present in most vertebrates. The function of this system is to enhance the action of antibodies by causing cell membrane alterations, often cytolysis. The complement combines with the Fc receptors of an antibody molecule and is enzymatically activated to a cytotoxic membrane attack complex. Immunotoxic chemicals may modify the complement function and may alter the inflammatory processes.

## POSSIBLE MECHANISMS FOR IMMUNOTOXICITY

Exposure of animals to toxicants may have effects on their immune system by a variety of mechanisms. Not only is the immune system profoundly influenced by other organs or systems of the body, but the complexity of immune responses also provides multiple targets for effects of chemicals. A generalized scheme of how chemicals may influence the immune system is illustrated in Figure 18.1. Both immunosuppression and immunostimulation are undesirable for the general health of animals. Chemicals may affect protein synthesis required for cell proliferation as well as antibodies. Chemicals that have potential for alkylating nucleic acids or proteins are immunotoxic because they interfere with the function of various proteins in the immune responses. Some mycotoxins or their metabolites are highly reactive in mammals and may bind to or destroy tissues. The acquired immunity depends largely on cellular proliferation and any substance that interferes with cell production will cause immunosuppression.

Chemicals may interfere with the function of enzymes directly that are required in the immune processes. Activated esterases may degranulate mast cells, releasing histamine, and may be involved in the process of chemotaxis by polymorphonuclear cells. Protease inhibitors will interrupt the post-translational processing of various cytokines and can interrupt their release. Chemicals likely to modify cell membrane functions will have a similar effect. By altering cytokine signaling chemicals may profoundly alter differentiation of immune cells, such as lymphocyte subtypes, and since various cellular components have synergistic or antagonistic function, the homeostasis of the immune system is likely to be disrupted. Some chemicals, particularly biologically reactive immunogens, can selectively eliminate specific cells of this system. For example chemicals that can destroy macrophages will interrupt antigen processing and presentation to other cells for mounting an effective immune response.

Immune responses may cause tissue damage or can be mediators for causing health problems in animals. Allergy or hypersensitivity is a good example where the well being of the animal can be compromised. The allergic reactions

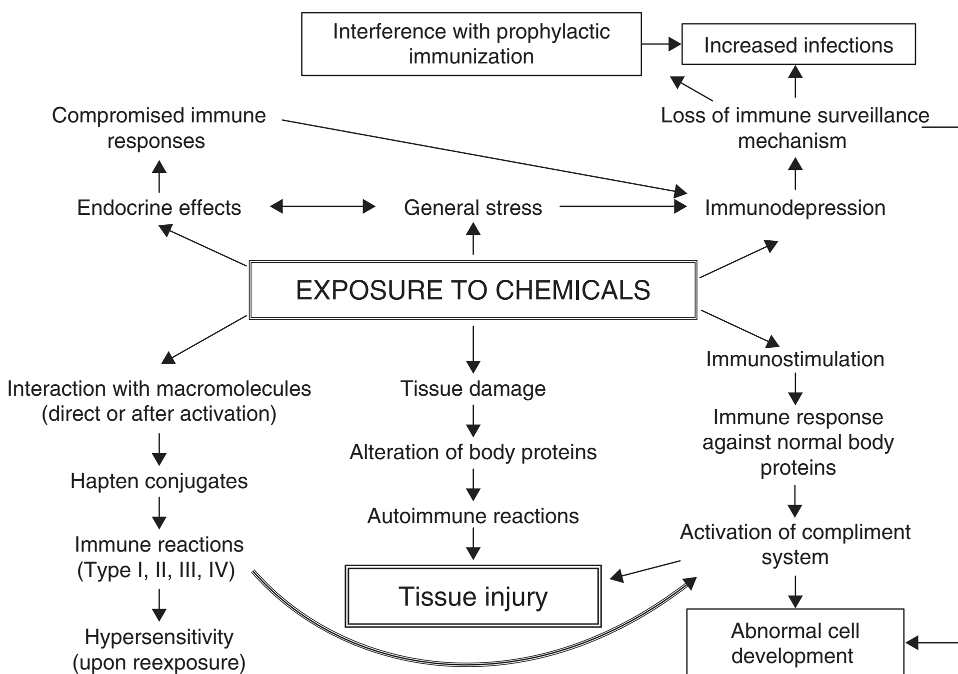


**FIGURE 18.1** A hypothetical scheme depicting various pathways indicating steps by which a chemical may produce immunotoxic effect and ultimately a disease or immune-mediated tissue damage (adapted from Sharma, RP (1984) Chemical interactions and compromised immune system. *Fundam Appl Toxicol* **4**: 345–51. Copyright 1984, with permission from Elsevier.

require initial sensitization and later a challenge exposure to a chemical to mount the response. Four distinct types of allergic mechanisms have been described (Gell and Coombs, 1975), all of which lead to inflammatory processes. Type I hypersensitivity is IgE mediated where specific IgE link on mast cells, a subsequent challenge to either the same or an antigenically similar chemical causes degranulation releasing bioactive substances. This type of reaction is exemplified by asthma, hay fever, food allergy, or anaphylactic reactions. Type II allergy is also antibody mediated; in this case the antigen may be cell bound and reacts to specific IgG or IgM type antibodies. The cell may be destroyed by either complement activation or phagocytosis. Drug-induced hemolytic anemia is a good example of such a reaction. Type III hypersensitivity requires a complex formation of the antigen with IgG or IgM antibodies, the immune complex may trigger complement activation leading to inflammatory changes, such as in Arthus reaction (localized edema or infiltration resulting in abscess or gangrene). The type IV hypersensitivity is cell mediated, where sensitized T helper lymphocytes react with the antigen, resulting in activation of macrophages and release of inflammatory mediators.

## IMMUNOTOXIC AGENTS

There is limited information on immunotoxic potential of different chemicals directly in animals of economic importance. Much of the available data relates to either laboratory animals or obtained from epidemiological studies in people. However, since there are common pathways of immune mechanisms in various higher mammals and also in birds, the conclusions of laboratory studies are applicable to domestic animals. In a few studies the target animal species have been employed but it is not always definite if the alterations of immune functions were primary or subsequent to damage to other organs or systems.

### Mycotoxins

Mycotoxins are of particular importance in animal management as these are common contaminants in animal feeds (Sharma, 1985, 1993). Feeds are contaminated in field with various molds or these can develop during storage. Some mycotoxins can indirectly influence the immunologic functions. Others can be neurotoxic or cause other organ pathology, and these compounds may activate the endocrine mechanisms. The stress-induced release of corticosteroids inhibits immune functions. Mycotoxins such as aflatoxin $B_1$ and fusarium T-2 toxin inhibit protein synthesis and cell proliferation, some may have selective effects on various subpopulations of lymphocytes. Several mycotoxins are cytotoxic to lymphocytes *in vitro*, perhaps because of their effects on membranes (including lymphocytic receptors) or interference with macromolecular synthesis and function. For example, cytochalasins (mycotoxins isolated from moldy rice) are highly cytotoxic and act on cytokinesis by binding to the filamentous actin; their immunotoxic potential has not been ascertained. Selected effects of immunotoxic mycotoxins that have been studied in domestic animals are listed in Table 18.3.

Immunomodulation by aflatoxin $B_1$ has been investigated in detail (Bondy and Pestka, 2000). In most species, resistance to infection is reduced by simultaneous exposure to aflatoxin $B_1$. Effects of aflatoxin $B_1$ are primarily on the cell-mediated immune functions; however, T cell-dependent humoral responses are also adversely affected. Often the humoral responses that are independent of T cells are not affected by low doses of aflatoxins. The immunosuppressive effects of aflatoxin $B_1$ can be explained on the basis of DNA binding of resulting epoxide derivative of aflatoxin $B_1$, thereby interfering with cell proliferation and protein synthesis. Limited studies suggest that aflatoxin $B_1$ alters cytokine production. The expression of macrophage derived cytokines, namely interleukin (IL)-1$\alpha$, IL-6, and tumor necrosis factor $\alpha$ (TNF$\alpha$), was suppressed in mitogen-stimulated macrophages derived from aflatoxin $B_1$ exposed mice; the effect of aflatoxin $B_1$ was greater on macrophages than on other types of immune cells (Dugyala and Sharma, 1996). Macrophages engulf large amounts of aflatoxin-bound macromolecules and thereby may be selectively sensitive to the toxin.

There have been a number of investigations involving fumonisin $B_1$ and immune responses (Bondy and Pestka, 2000). Fumonisin $B_1$ generally inhibited lymphocyte blastogenesis in cells obtained from exposed mammals; the poultry was relatively resistant. Inconsistent effects of fumonisin on immune functions have been reported. Decreased antibody formation to injected antigens has been reported in turkeys, pigs, calves, or even rodents; however, the levels of fumonisins used in these studies have been fairly large. Fumonisin-induced immunomodulation has been suggested in some studies; however, the results depend on the protocol of testing and sequence of exposure to toxin and the antigens. Fumonisin $B_1$ causes accumulation of free sphingoid bases, sphinganine, and sphingosine, by interfering with their conversion to ceramide and interrupts cell cycle (Johnson *et al*., 2003). The free sphingoid bases and their phosphates are important signaling agents in cells, the bases and their phosphates having opposite outcomes for cell survival. Cellular signaling is critical in mounting immune responses; fumonisin $B_1$ has been used as a tool to define the role of ceramide in signaling in lymphocytes. The exposure to fumonisin $B_1$ caused localized activation of the cytokine network, implying that innate immune responses are important in the hepatotoxic outcome (Bhandari and Sharma, 2002).

**TABLE 18.3 Selected examples of immunotoxic effects of mycotoxins in domestic or food animals**

| Mycotoxin | Species | Observed effects | Reference |
|---|---|---|---|
| Aflatoxin $B_1$ | Bovine | Decreased lymphocyte proliferation *in vitro* | Paul *et al*. (1977) |
| | Swine | Decreased lymphocyte proliferation, macrophage migration, DTH and titers to SRBC | Hoerr and D'Andrea (1983) |
| | | Increased sensitivity to *Salmonella* | Miller *et al*. (1978) |
| | Chicken | Decreased CMI and titers | Verma *et al*. (2004) |
| | | Decreased antibody titer | Thaxton *et al*. (1974) |
| | | Increased mortality against *Salmonella* | Boonchuvit and Hamilton (1975) |
| | | Reduced phagocytosis by reticuloendothelial cells | Chang and Hamilton (1979) |
| | Turkey | Thymic atrophy | Pier *et al*. (1972) |
| | | Reduced resistance to *Pasteurella* | Pier and Heddleston (1970) |
| Deoxynivalenol | Swine | Activation of immune responses at low doses | Zielonka *et al*. (2003) |
| | | Increase in serum IgA concentration | Drochner *et al*. (2004) |
| Fumonisin $B_1$ | Chicken | Depression in phagocytosis of peritoneal macrophages after *in vitro* exposure | Qureshi and Hagler (1992) |
| Ochratoxin | Dog | Necrotic mesenteric lymph nodes | Szczech *et al*. (1973b) |
| | Swine | Necrosis of gut-associated lymph nodes | Szczech *et al*. (1973a) |
| | Chicken | Lymphopenia and decreased phagocytosis by heterophils | Chang and Hamilton (1980) |
| | Turkey | Thymic atrophy, leukocytopenia, and heterocyclopenia | Chang *et al*. (1981) |
| Patulin | Rabbit | Reduced lymphocyte proliferation and serum immunoglobulins | Escoula *et al*. (1988a, b) |
| T-2 toxin | Cattle | Reduced lymphocyte proliferation and neutrophil function | Mann *et al*. (1984) |
| Zearalenone | Chicken | Decreased serum titers to Newcastle disease virus | Danicke *et al*. (2002) |

Ochratoxin A, a nephrotoxicant and widely encountered food and feed contaminant, has been investigated for its immunologic effects. Ochratoxin poisoning interferes with macromolecular synthesis, increases lipid peroxidation, and diminishes mitochondrial respiration. This mycotoxin is fairly cytotoxic and causes atrophy of gastrointestinal lymph nodes after oral ingestion (Bondy and Pestka, 2000). Systemic investigations suggest that exposure to ochratoxin A results in the inhibition of cellular, humoral, or innate immune responses; however, many of these effects are produced at exposure levels that are also nephrotoxic. Its immunotoxic effects were demonstrated in poultry and pigs.

Patulin has been investigated with regard to the immune system only to a limited extent. This toxin is less toxic; however, effects such as altered number of splenic T lymphocytes, diminished serum immunoglobulin concentrations, decreased delayed hypersensitivity responses, and increased neutrophil numbers have been reported. The observed changes in cellular phenotype distribution of immune cells may or may not indicate ultimate effects on immune functions.

T-2 toxin and deoxynivalenol cause severe depletion of T cells. In routine immunotoxicologic testing in rodents, both increase and decrease of immunologic functions have been reported depending on the protocol employed. T-2 toxin was immunosuppressive in murine *in vivo* and *in vitro* models. Effects of deoxynivalenol and T-2 toxin on cytokine production were not consistent. T-2 toxin caused increased expression and production of IL-2, IL-3 and interferon $\gamma$ in splenocytes from T-2 treated mice, but a decrease in IL-1$\alpha$, TNF$\alpha$, and IL-6 was noticed (Dugyala and Sharma, 1997). Deoxynivalenol, however, caused an increase in TNF$\alpha$ production by macrophages (Yang and Pestka, 2002).

Other mycotoxins occur in certain circumstances as food or feed contaminant. Many of these are toxic and influence immunologic functions. Rubratoxins that cause liver and kidney toxicity and diffuse hemorrhage, macrocyclic trichothecenes that include a large family of mycotoxins with varied toxicity, cytochalasins that bind to actin and also inhibit hexose transport in cells, ergotoxins with potent vasoconstrictor action, penicillic acid, etc. all produce inconsistent effects on immune functions.

## Metals

Different heavy metals, both in their inorganic and organic forms, elicit toxic responses on the immune system. Toxic metals such as lead, cadmium, and mercury possess immunotoxic properties at exposure levels that may not produce effects on other systems. The heavy metal lead is of particular concern in veterinary practice as exposure and poisoning of domestic animals with this metal has been encountered. Selected few studies with metals involving domestic animals are listed in Table 18.4. Lead, mercury, and nickel were reported to be immunosuppressive in different reports, whereas low doses of selenium, zinc, and manganese are immunostimulant. Organic tin compounds

**TABLE 18.4 Selected metals evaluated in domestic or food animals for immunotoxic potential**

| Metal | Reported or possible effects | Reference |
|---|---|---|
| Lead | Immunosuppression in cattle | Black *et al*. (1992) |
| | Decreased spleen plaque-forming cells in mallards | Rocke and Samuel (1991) |
| | Decreased peripheral lymphocyte counts and depressed interferon $\gamma$-like cytokine production by splenocytes of chicken | Lee *et al*. (2002) |
| | In Japanese quail, primary and total antibody response to immunization suppressed; secondary total antibody and IgG responses also reduced | Grasman and Scanlon (1995) |
| Mercury | Suppressed immunocompetence in fish | Sweet and Zelikoff (2001) |
| Molybdenum | Decreased antibodies to swine erythrocytes in calves | Gengelbach and Spears (1998) |
| Copper | Higher titers to ovalbumin in heifers | Salyer *et al*. (2004) |
| Zinc | Decreased antibody response at day 7 but increased phagocytosis by peritoneal exudates cells in pigs | van Heugten *et al*. (2003) |
| | Potentiation of immunity against *Salmonella* in chicken | Barbour *et al*. (2001) |
| | Higher antibody titers to ovalbumin in heifers | Salyer *et al*. (2004) |
| Chromium | Enhanced antibody response to ovalbumin in calves | Chang *et al*. (1996) |
| | Decreased lymphocyte proliferation and phagocytosis in carps | Steinhagen *et al*. (2004) |
| | Lower antibody titers and splenic and kidney plaque-forming cells in catfish | Khangarot *et al*. (1999) |
| Selenium | Enhanced cell mediated immune response in eiders | Wayland *et al*. (2002) |
| Vanadium | Increased IL-6 activity in macrophage supernatants | Qureshi *et al*. (1999) |

are highly thymotoxic. Cadmium has mixed effects, showing suppressive effects in certain test situations but either no effect or stimulation of the immune system in others. Large animals may consume selenium containing plants in locations with high soil contents for this metal; however, laboratory studies suggest that while inorganic selenium is immunotoxic the organic forms of this metal present in plants are not (Johnson *et al*., 2000).

The immunotoxic effects of metals are believed to be mediated by a variety of mechanisms. Metals form complexes with biological macromolecules, forming chelates and may replace essential metals. Protein synthesis, membrane integrity, and/or nucleic acid replication are often affected by metals. The *in vitro* and *in vivo* effects of the same metal are sometimes opposite. For example, lead produces immunosuppression when administered internally, whereas it acts as a mitogen in lymphocyte cultures. *In vitro* studies have promise for discerning cellular and molecular mechanisms involved; however, they offer little value in predicting the *in vivo* immunotoxic potential of a chemical.

## Pesticides

In spite of a vast amount of information dealing with the immunotoxic effects of organophosphorus and carbamate insecticides, there is little consensus that anticholinesterase pesticides are immunotoxic (Sharma, 2006). Rabbits fed low doses of methylparathion were reported to have atrophy of the thymus cortex and fewer antibody-forming cells in the lymph node; splenic morphology was considerably altered. Delayed hypersensitivity response was not altered in a consistent manner. There was no significant influence on circulating antibody levels against sheep erythrocytes. Diets containing carbaryl or carbofuran fed to rabbits reduced the numbers of activated lymphocytes in lymph nodes, decreased the number of splenic germinal centers, and produced atrophy of the thymic cortex.

Humoral immune response in rabbits exposed to malathion or dichlorvos was decreased against *Salmonella typhimurium.* A single high dose of malathion, parathion, or dichlorvos reduced primary IgM response to sheep erythrocytes at levels producing cholinergic symptoms. Dichlorvos inhibited the lysis of antibody-sensitized sheep erythrocytes in a dose-dependent manner. The organophosphates had no effect when given in multiple lower doses. A cholinomimetic agent arecoline reduced IgM antibody response only when given in a form that would sustain prolonged cholinergic symptoms.

Dimethoate caused a decrease in delayed type hypersensitivity reaction measured as foot-pad swelling in mice. Mitogen response of female mice splenic cells to phytohemagglutinin and lipopolysaccharide was reduced by this insecticide. Diazinon caused pathologic changes in spleen, thymus, and lymph nodes; however, these effects were dependent on dietary protein or lipid content. Organophosphates triphenylphosphate and triphenylphosphine oxide were reported to be immunomodulatory primarily to the innate immune functions.

Several impurities often present in organophosphates are potent cholinesterase inhibitors and may potentiate organophosphate toxicity. For example, of many impurities in malathion, *O*,*O*,*S*-trimethyl phosphorothioate (OOS-TMP) and *O*,*S*,*S*-trimethyl phosphorothioate (OSS-TMP) are potent cholinesterase inhibitors but they inhibit

immune responses at doses that produce no cholinergic symptoms.

Various chlorinated hydrocarbon insecticides such as dieldrin, *p′p′*-dichlorodiphenyl-trichloroethane (DDT), lindane, etc. have been tested for immunotoxic effects (Street, 1981). In most cases a reduced production of antibody to test antigen was noted; however, effects varied with the dose, species, and the protocol employed (Sharma and Zeeman, 1980).

## Industrial and environmental chemicals

Domestic animals may be accidentally exposed to industrial or environmental chemicals. Some of the investigations in domestic animals are summarized in Table 18.5. Contamination of cattle feed by a fire-retardant Firemaster, a polybrominated biphenyl (PBB) mixture, in Michigan in 1973 prompted several investigations on the immunotoxic potential of this compound. In cattle, small amounts of PBB did not induce alteration of immune functions (Kateley *et al*., 1982). In swine a decreased responsiveness of peripheral lymphocytes to mitogenic stimulation was observed when animals were given relatively large doses (200 ppm) of PBB. Chlorinated phenols, found in a variety of foods, were evaluated for their immunotoxic potential. Prompted by an episode of suspected poisoning in dairy cattle by pentachlorophenol, Forsell *et al*. (1981) investigated the immunologic profile of cattle given a commercial grade of this chemical and indicated no effect in treated animals; it appears that the immunotoxic effects are caused by tetrachlorodibenzo-*p*-dioxin (TCDD) impurity in the technical grade. The sensitive immune responses are likely T-cell mechanisms and are species-dependent; the effects are known to be reversible. TCDD and its analog 2,3,7,8-tetrachlorodibenzofuran (TCDF) produce immunotoxic effects discernible only at levels that induce other pathologic lesions.

A number of other industrial chemicals, primarily those used in the manufacture of plastics, have been studied for their effects on parameters of immune system. Toluene diisocyanate stimulated lymphocytes *in vitro*; vinyl chloride and styrene produced a similar effect. Dioxin depressed T-cell while stimulating B-cell responses. Benzene, vinylidene chloride, ethylenimine, and epichlorohydrin do not produce consistent *in vitro* effects on the immunocompetent cells, although many of these chemicals were cytotoxic in high concentrations.

## Miscellaneous chemicals and drugs

A number of drugs have the capability of altering the function of the immune system and for that reason are used therapeutically. Several drugs on the other hand have side effects involving the immune system or its functions. Diethylstilbestrol (DES) is a synthetic estrogen used in therapy. The immunosuppressive potential of this drug is of concern since the chemical has been used as a growth promoter in animals. DES is a suppressor of both cell mediated and humoral immunity, and a potent stimulant of macrophage function. DES increased tumor susceptibility and increased mortality against parasitic infection.

Cyclophosphamide, a potent immunosuppressive and an inhibitor of protein synthesis, impairs both T-cell and humoral functions. The effect of cyclophosphamide is selective for B-cell responses. In mammals, cyclophosphamide suppressed systemic and local graft versus host

**TABLE 18.5 Selected examples of contaminants in feed that may influence immune responses in domestic or food animals**

| Chemical | Species and effects | Reference |
|---|---|---|
| Organochlorine insecticide mixture | In swine elevated lymphocyte proliferation and CD4+/CD8+ ratio and leukocyte phagocytic activity after gestational and lactational exposure | Bilrha *et al*. (2004) |
| Organochlorine insecticides | Suppression of NK cell activity and T cell responses in harbor seals | de Swart *et al*. (1996) |
| Polyaromatic hydrocarbon mixture | Decreased lymphocyte response to T cell mitogens but increase to B cell mitogens in fish, aberration in NK cell activity in fish | Faisal *et al*. (1991a, b) |
| Polychlorinated biphenyls | Decreased T cell mediated immunity related to amount of pollutants in eggs of herring gull and Caspian tern | Grasman *et al*. (1996) |
| Polychlorinated biphenyls | Decreased antibody titers and delayed type hypersensitivity in fish-eating birds and reduced T cell proliferation and NK cell activity in seals | Luebke *et al*. (1997) |
| Polychlorinated biphenyls and other contaminants | Decreased NK cell activity, T cell mitogen response, antigen specific proliferation, mixed lymphocyte response and delayed hypersensitivity in harbor seals | van Loveren *et al*. (2000) |
| Tributyltin and polychlorinated biphenyls mixture | Suppressed antibody response and phagocyte oxidative burst activity in catfish | Regala *et al*. (2001) |
| Bleached kraft pulp mill effluent | Impairment of delayed type hypersensitivity but no effect on antibody response in minks | Smits *et al*. (1996) |

reaction, caused depletion of various leukocyte elements, and interfered with the mitogen-induced lymphocyte transformation, in addition to having a marked inhibitory effect on antibody synthesis.

Other drugs that have also been reported to have immunosuppressive properties include dexamethasone, azathiopurine, chlorpromazine, and chloral hydrate. Several other drugs may indeed have similar properties, but it does not appear that therapeutic doses of these in most instances will cause alteration of immunologic functions to any appreciable extent.

## TESTING FOR IMMUNOTOXIC POTENTIAL OF CHEMICALS

There is no single or simple test that can be used for the evaluation of immunotoxic effects of chemicals. A listing of selected tests usually employed for this purpose is provided in Table 18.6. The National Toxicology Program recommended a 2-tier approach for immunotoxicity testing; the first suggestive of immunotoxic potential, the second involving comprehensive testing if immunotoxic potential is apparent in preliminary testing (Luster *et al.*, 1988). Specific tests include testing of humoral and cell-mediated immunity and also interacting cells in various immune functions (i.e. macrophages). *In vitro* tests using heterogeneous mixtures of cells or their isolated subpopulations are useful. In many cases the reactivity of specific cells to surface-acting agents can be readily evaluated *in vitro* by using the mitogens. A number of chemicals decrease the response of lymphocytes to blastogenic agents by virtue of being cytotoxic. Direct effects of chemicals can be evaluated by their addition to cultures but should be viewed with caution. Some chemicals may have non-specific mitogenic properties themselves. Interaction of chemicals with mitogens in the medium may modify their function.

Lymphocyte cultures *in vitro* are useful when carried out after administering the test chemicals to the animals and then evaluating the mitogenic responsiveness of cells harvested from major lymphatic organs. In this case the test chemical can be transformed in the body if its action is dependent on the formation of reactive metabolite. Addition of metabolizing systems can be of value but their presence may modify lymphocyte transformation *in vitro*. Chemicals that modify the immunologic mechanisms indirectly (i.e. through alteration of other systems like steroid levels) can be suitably evaluated after *in vivo* administration.

## FUTURE TRENDS

Available information on the role of immune mechanisms in the manifestation of toxicity by various chemicals is inadequate. Immune suppression is sufficiently important in toxicology and more studies relating dose and effect need to be conducted. Immunosuppressive potentials of chemicals are of little importance if the effects occur only with exposure levels that equal or exceed those that directly affect other systems. Defining the mechanisms of action

**TABLE 18.6 Clinical or toxicological tests indicative of compromised immunologic responses**

| Tests | General principle |
|---|---|
| Hematology and lymphatic organ weights and pathology | Initial estimate of immunotoxic potential may be derived on general toxicity studies, both short and long term, by total and differential leukocyte counts, and when thymus, spleen and lymph nodes are examined. |
| Resistance to various pathogens | Bacterial, viral, or parasitic infections and tumor cell inoculations that have known effects on immune parameters can be used. In early stages mortality to sublethal infections can be used. |
| Lymphocyte typing | Phenotyping of cells bearing different markers can be employed in routine epidemiologic studies. |
| Lymphocyte proliferation | Responses to B and T cell mitogens, either on cells derived from exposed animals or cells treated *in vitro*, have been employed. |
| Antibody production | In domestic animals antibody titers to commonly used vaccinations are useful. In laboratory animals or small species (chicken, fish, etc.) antibodies against T dependent responses (sheep erythrocytes) or T independent responses (heterologous albumin) can be employed. Both antibody titers and enumeration of peripheral plaque-forming cells have been used. |
| Delayed hypersensitivity responses | These T cell dependent effects are common in smaller species; however, they can be used in large animals as well. |
| Cytokine levels | Measuring levels of various cytokines in serum or in tissues is useful after toxicant exposure. Production of cytokine after stimulation of target immunocytes *in vitro*, either from toxicant exposed animals or after *in vitro* treatment of cells with the toxicant, is useful. |
| Phagocytosis and/or cell migration | Leukocytes and macrophages can be evaluated for phagocytosis and/or chemotaxis; production of signaling agents can be measured. |

and the structure–activity relationships are needed for evaluating immunotoxic potential of chemicals.

Immunotoxicity in domestic animals should be considered as a part of their management. Housing and nutrition are of importance as they contribute substantially to the well being of animals. In particular, feed contaminants should be avoided and health surveillance to detect immunotoxic potential should be monitored. More studies involving target species are necessary to fully understand the toxicity potential; chicken are ideal and have been used when effects of chemicals on avian species are suspected. Veterinary diagnostic laboratories should realize the importance of potential immunotoxicity as it may effect productivity even when no other clinical symptoms are observed. Many of the analytical tests involving immune principals (such as for detection of infectious agents) are already available and can be modified to analyze immune functions; lymphocyte proliferation tests can easily be added to the available tools (Sharma *et al*., 1984).

## REFERENCES


Barbour EK, Hamadeh SK, Bejjani NE, Faroon OM, Eid A, Sakr W, Bouljihad M, Spasojevic R, Safieh-Garabedian B (2001) Immunopotentiation of a developed *Salmonella enterica* serotype enteritidis vaccine by thymulin and zinc in meat chicken breeders. *Vet Res Commun* **25**: 437–47.

Bhandari N, Sharma RP (2002) Fumonisin $B_1$-induced alterations in cytokine expression and apoptosis signaling genes in mouse liver and kidney after an acute exposure. *Toxicology* **172**: 81–92.

Bilrha H, Roy R, Wagner E, Belles-Isles M, Bailey JL, Ayotte P (2004) Effects of gestational and lactational exposure to organochlorine compounds on cellular, humoral, and innate immunity in swine. *Toxicol Sci* **77**: 41–50.

Black RD, McVey DS, Oehme FW (1992) Immunotoxicity in the bovine animal: a review. *Vet Hum Toxicol* **34**: 438–42.

Bondy GS, Pestka JJ (2000) Immunomodulation by fungal toxins. *J Toxicol Environ Health B Crit Rev* **3**: 109–43.

Boonchuvit B, Hamilton PB (1975) Interaction of aflatoxin and paratyphoid infections in broiler chickens. *Poult Sci* **54**: 1567–73.

Chang CF, Hamilton PB (1979) Impaired phagocytosis by heterophils from chickens during aflatoxicosis. *Toxicol Appl Pharmacol* **48**: 459–66.

Chang CF, Hamilton PB (1980) Impairment of phagocytosis by heterophils from chickens during ochratoxicosis. *Appl Environ Microbiol* **39**: 572–5.

Chang CF, Doerr JA, Hamilton PB (1981) Experimental ochratoxicosis in turkey poults. *Poult Sci* **60**: 114–19.

Chang GX, Mallard BA, Mowat DN, Gallo GF (1996) Effect of supplemental chromium on antibody responses of newly arrived feeder calves to vaccines and ovalbumin1. *Can J Vet Res* **60**: 140–4.

Danicke S, Ueberschar KH, Halle I, Matthes S, Valenta H, Flachowsky G (2002) Effect of addition of a detoxifying agent to laying hen diets containing uncontaminated or Fusarium toxin-contaminated maize on performance of hens and on carryover of zearalenone. *Poult Sci* **81**: 1671–80.

de Swart RL, Ross PS, Vos JG, Osterhaus AD (1996) Impaired immunity in harbour seals (*Phoca vitulina*) exposed to bioaccumulated environmental contaminants: review of a long-term feeding study. *Environ Health Perspect* **104**(Suppl. 4): 823–8.

Drochner W, Schollenberger M, Piepho HP, Gotz S, Lauber U, Tafaj M, Klobasa F, Weiler U, Claus R, Steffl M (2004) Serum IgA-promoting effects induced by feed loads containing isolated deoxynivalenol (DON) in growing piglets. *J Toxicol Environ Health A* **67**: 1051–67.

Dugyala RR, Sharma RP (1996) The effect of aflatoxin B1 on cytokine mRNA and corresponding protein levels in peritoneal macrophages and splenic lymphocytes. *Int J Immunopharmacol* **18**: 599–608.

Dugyala RR, Sharma RP (1997) Alteration of major cytokines produced by mitogen-activated peritoneal macrophages and splenocytes in T-2-toxin treated male CD-1 mice. *Environ Toxicol Pharmacol* **3**: 73–81.

Escoula L, Bourdiol D, Linas MD, Recco P, Seguela JP (1988a) Enhancing resistance and modulation of humoral immune response to experimental *Candida albicans* infection by patulin. *Mycopathologia* **103**: 153–6.

Escoula L, Thomsen M, Bourdiol D, Pipy B, Peuriere S, Roubinet F (1988b) Patulin immunotoxicology: effect on phagocyte activation and the cellular and humoral immune system of mice and rabbits. *Int J Immunopharmacol* **10**: 983–9.

Faisal M, Marzouk MS, Smith CL, Huggett RJ (1991a) Mitogen induced proliferative responses of lymphocytes from spot (*Leiostomus xanthurus*) exposed to polycyclic aromatic hydrocarbon contaminated environments. *Immunopharmacol Immunotoxicol* **13**: 311–27.

Faisal M, Weeks BA, Vogelbein WK, Huggett RJ (1991b) Evidence of aberration of the natural cytotoxic cell activity in *Fundulus heteroclitus* (Pisces: Cyprinodontidae) from the Elizabeth River, Virginia. *Vet Immunol Immunopathol* **29**: 339–51.

Forsell JH, Shull LR, Kateley JR (1981) Subchronic administration of technical pentachlorophenol to lactating dairy cattle: immunotoxicologic evaluation. *J Toxicol Environ Health* **8**: 543–58.

Gell PGH, Coombs RRA. (1975) *Clinical Aspects of Immunology*, 3rd edn. Blackwell Scientific, Oxford, 1754 pp.

Gengelbach GP, Spears JW (1998) Effects of dietary copper and molybdenum on copper status, cytokine production, and humoral immune response of calves. *J Dairy Sci* **81**: 3286–92.

Grasman KA, Scanlon PF (1995) Effects of acute lead ingestion and diet on antibody and T-cell-mediated immunity in Japanese quail. *Arch Environ Contam Toxicol* **28**: 161–7.

Grasman KA, Fox GA, Scanlon PF, Ludwig JP (1996) Organochlorine-associated immunosuppression in prefledgling Caspian terns and herring gulls from the Great Lakes: an ecoepidemiological study. *Environ Health Perspect* **104**(Suppl. 4): 829–42.

Hoerr FJ, D'Andrea GH (1983) Biological effects of aflatoxin in swine. In *Aflatoxin and Aspergillus flavus in Corn*, Diener UL, Asquith L, Dickens JW (eds). Craftmaster Printer Inc., Opelika, AL, 112 pp.

Johnson VJ, Tsunoda M, Sharma RP (2000) Increased production of proinflammatory cytokines by murine macrophages following oral exposure to sodium selenite but not to seleno-L-methionine. *Arch Environ Contam Toxicol* **39**: 243–50.

Johnson VJ, He Q, Kim SH, Kanti A, Sharma RP (2003) Increased susceptibility of renal epithelial cells to TNF$\alpha$-induced apoptosis following treatment with fumonisin $B_1$. *Chem Biol Interact* **145**: 297–309.

Kateley JR, Insalaco R, Codere S, Willett LB, Schanbacher FL (1982) Host defense systems in cattle exposed to polybrominated biphenyl. *Am J Vet Res* **43**: 1288–95.

Kende M, Gainer J, Chirigos M (eds) (1984) *Chemical Regulation of Immunity in Veterinary Medicine, Progress in Clinical and Biological research*, vol. 161. Alan R. Liss, New York, 599 pp.

Khangarot BS, Rathore RS, Tripathi DM (1999) Effects of chromium on humoral and cell-mediated immune responses and host

resistance to disease in a freshwater catfish, *Saccobranchus fossilis* (Bloch). *Ecotoxicol Environ Saf* **43**: 11–20.

Lee JE, Naqi SA, Kao E, Dietert RR (2002) Embryonic exposure to lead: comparison of immune and cellular responses in unchallenged and virally stressed chickens. *Arch Toxicol* **75**: 717–24.

Luebke RW, Hodson PV, Faisal M, Ross PS, Grasman KA, Zelikoff J (1997) Aquatic pollution-induced immunotoxicity in wildlife species. *Fundam Appl Toxicol* **37**: 1–15.

Luster MI, Munson AE, Thomas PT, Holsapple MP, Fenters JD, White Jr KL, Lauer LD, Germolec DR, Rosenthal GJ, Dean JH (1988) Development of a testing battery to assess chemical-induced immunotoxicity: National Toxicology Program's guidelines for immunotoxicity evaluation in mice. *Fund Appl Toxicol* **10**: 2–19.

Mak T, Saunders ME (2006) *The Immune Response*. Academic Press, New York, 1194 pp.

Mann DD, Buening GM, Osweiler GD, Hook BS (1984) Effect of subclinical levels of T-2 toxin on the bovine cellular immune system. *Can J Comp Med* **48**: 308–12.

Miller DM, Stuart BP, Crowell WA, Cole RJ, Goven AJ, Brown J (1978) Aflatoxicosis in swine – its effects on immunity and relationship to *Salmonellosis. Proc Am Assoc Vet Lab Diagn* **21**: 135–46.

Paul PS, Johnson DW, Mirocha CJ, Soper FF, Thoen CO, Muscoplat CC, Weber AF (1977) *In vitro* stimulation of bovine peripheral blood lymphocytes: suppression of phytomitogen and specific antigen lymphocyte responses by aflatoxin. *Am J Vet Res* **38**: 2033–5.

Petersen-Mahrt SK, Harris RS, Neuberger MS (2002) AID mutates *E. coli* suggesting a DNA deamination mechanism for antibody diversification. *Nature* **418**: 99–103.

Pier AC, Heddleston KL (1970) The effect of aflatoxin on immunity in turkeys. I. Impairment of actively acquired resistance to bacterial challenge. *Avian Dis* **14**: 797–809.

Pier AC, Heddleston KL, Cysewski SJ, Patterson JM (1972) Effect of aflatoxin on immunity in turkeys. II. Reversal of impaired resistance to bacterial infection by passive transfer of plasma. *Avian Dis* **16**: 381–7.

Qureshi MA, Hagler Jr WM (1992) Effect of fumonisin-B1 exposure on chicken macrophage functions *in vitro*. *Poult Sci* **71**: 104–12.

Qureshi MA, Hill CH, Heggen CL (1999) Vanadium stimulates immunological responses of chicks. *Vet Immunol Immunopathol* **68**: 61–71.

Regala RP, Rice CD, Schwedler TE, Dorociak IR (2001) The effects of tributyltin (TBT) and 3,3′,4,4′,5-pentachlorobiphenyl (PCB-126) mixtures on antibody responses and phagocyte oxidative burst activity in channel catfish, *Ictalurus punctatus*. *Arch Environ Contam Toxicol* **40**: 386–91.

Rocke TE, Samuel MD (1991) Effects of lead shot ingestion on selected cells of the mallard immune system. *J Wildlife Dis* **27**: 1–9.

Salyer GB, Galyean ML, Defoor PJ, Nunnery GA, Parsons CH, Rivera JD (2004) Effects of copper and zinc source on performance and humoral immune response of newly received, lightweight beef heifers. *J Anim Sci* **82**: 2467–73.

Sharma RP (1985) Immunotoxicology of food constituents. *Food Technol* **39**: 94–7.

Sharma RP (1993) Immunotoxicity of mycotoxins. *J Dairy Sci* **76**: 892–7.

Sharma RP (2006) Organophosphates, carbamates and the immune system. In *Toxicology of Organophosphate and Carbamates Compounds*, Gupta RC (ed.), pp. 495–507. Elsevier, San Diego, CA, 763 pp.

Sharma RP, Zeeman MG (1980) Immunologic alterations by environmental chemicals: relevance of studying mechanisms versus effects. *J Immunopharmacol* **2**: 285–307.

Sharma RP, James LF, Molyneux RJ (1984) Effect of repeated locoweed feeding on peripheral lymphocytic function and plasma proteins in sheep. *Amer J Vet Res* **45**: 2090–3.

Smits JE, Blakley BR, Wobeser GA (1996) Immunotoxicity studies in mink (*Mustela vison*) chronically exposed to dietary bleached kraft pulp mill effluent. *J Wildlife Dis* **32**: 199–208.

Steinhagen D, Helmus T, Maurer S, Michael RD, Leibold W, Scharsack JP, Skouras A, Schuberth HJ (2004) Effect of hexavalent carcinogenic chromium on carp *Cyprinus carpio* immune cells. *Dis Aquat Organ* **62**: 155–61.

Street JC (1981) Pesticides and the immune system. In *Immunologic Considerations in Toxicology*, Vol. I, pp. 45–66, Sharma RP (ed.), CRC Press, Boca Raton, FL, 174 pp.

Sweet LI, Zelikoff JT (2001) Toxicology and immunotoxicology of mercury: a comparative review in fish and humans. *J Toxicol Environ Health B Crit Rev* **4**: 161–205.

Szczech GM, Carlton WW, Tuite J (1973a) Ochratoxicosis in Beagle dogs. I. Clinical and clinicopathological features. *Vet Pathol* **10**: 135–54.

Szczech GM, Carlton WW, Tuite J, Caldwell R (1973b) Ochratoxin A toxicosis in swine. *Vet Pathol* **10**: 347–64.

Thaxton JP, Tung HT, Hamilton PB (1974) Immunosuppression in chickens by aflatoxin. *Poult Sci* **53**: 721–5.

van Heugten E, Spears JW, Kegley EB, Ward JD, Qureshi MA (2003) Effects of organic forms of zinc on growth performance, tissue zinc distribution, and immune response of weanling pigs. *J Anim Sci* **81**: 2063–71.

van Loveren H, Ross PS, Osterhaus AD, Vos JG (2000) Contaminant-induced immunosuppression and mass mortalities among harbor seals. *Toxicol Lett* **112/113**: 319–24.

Verma J, Johri TS, Swain BK, Ameena S (2004) Effect of graded levels of aflatoxin, ochratoxin and their combinations on the performance and immune response of broilers. *Br Poult Sci* **45**: 512–18.

Wayland M, Gilchrist HG, Marchant T, Keating J, Smits JE (2002) Immune function, stress response, and body condition in arctic-breeding common eiders in relation to cadmium, mercury, and selenium concentrations. *Environ Res* **90**: 47–60.

Yang GH, Pestka JJ (2002) Vomitoxin (deoxynivalenol)-mediated inhibition of nuclear protein binding to NRE-A, an IL-2 promoter negative regulatory element, in EL-4 cells. *Toxicology* **172**: 169–79.

Zielonka L, Gajecki M, Obremski K, Zwierzchowski W (2003) Influence of low doses of deoxynivalenol applied per os on chosen indexes of immune response in swine. *Pol J Vet Sci* **6**: 74–7.

# Part 3

## Nanoparticles, Radiation, and Carcinogenesis

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C H A P T E R

# 19

# Biomedical responses and toxicity of nanoparticles

*John A. Pickrell*

## INTRODUCTION

By some estimates, growth of nanotechnologies and nanomaterials (NMs) far exceed the impact of the Industrial Revolution; they are predicted to become a $1-trillion market by 2015 (Nel *et al.*, 2006). Investigators continue to discover unique properties of conventional materials at the submicrometer scale (Hoet *et al.*, 2004). Nanoparticles (NPs) are a diverse class of small-scale substances with one or more dimensions <100 nanometers (nm). They may be formed in nature, or by carbon combustion or by being released from power plants or from automobile exhausts. Alternatively, NP can be engineered from NM by molecular-level engineering to achieve unique mechanical, optical, electrical, and magnetic properties (Hoet *et al.*, 2004; Tsuji *et al.*, 2006).

NMs are expected to improve virtually all types of products; commercialization of products that exploit these unique properties is increasing. However, these same properties present new challenges to understanding, predicting, and managing potential adverse health effects following exposure. NMs have already found commercial application as sporting goods, tires, sunscreens, sanitary ware coatings, stain resistant textiles and clothing, food products and electronics (Hoet *et al.*, 2004; Nel *et al.*, 2006; Tsuji *et al.*, 2006).

Widespread application of NM confers potential for environmental release and subsequent human exposure. While the benefits of nanotechnology are widely publicized, the discussion of risks conferred by using consumer and industrial products are only beginning to emerge. Technological development and applications are outpacing research for safe use and a documentation of health and environmental risk (Hoet *et al.*, 2004; Tsuji *et al.*, 2006).

The future of nanotechnology will depend on public acceptance of the risks of inhaled NP relative to their benefits. Assuming public acceptance, toxicologist's experience with past "miracle" materials (e.g. asbestos) advises us that caution in using novel substances without fully evaluating potential health risks may be ill advised (Hoet *et al.*, 2004; Tsuji *et al.*, 2006). The purpose of this chapter is to describe the risks conferred on animals, especially companion animals from the use of NM.

As with conventional chemicals, risk assessment will be the basis of assessing and regulating releases from NMs to protect health and the environment. Many applications will likely have limited or manageable exposures and thus potential for effects; however, nanoscale compounds are not the same as their larger counterparts, and risks are expected to be product specific (Hoet *et al.*, 2004; Tsuji *et al.*, 2006). Some studies suggest that NMs are not inherently benign and that they may travel to organs throughout the body. In fact, preliminary studies suggest that pulmonary exposure to NMs induces inflammation, oxidative stress, and distant organ involvement. These factors are most likely related to small size of the particles and their ability to generate oxidative stress. They raise the question, "Do NM properties necessitate a new toxicological science?" (Nel *et al.*, 2006).

## BASIC NP CONCEPTS

### Particle size and agglomeration

The unusual physicochemical properties of NM are attributable to their small size (more than 1 dimension is

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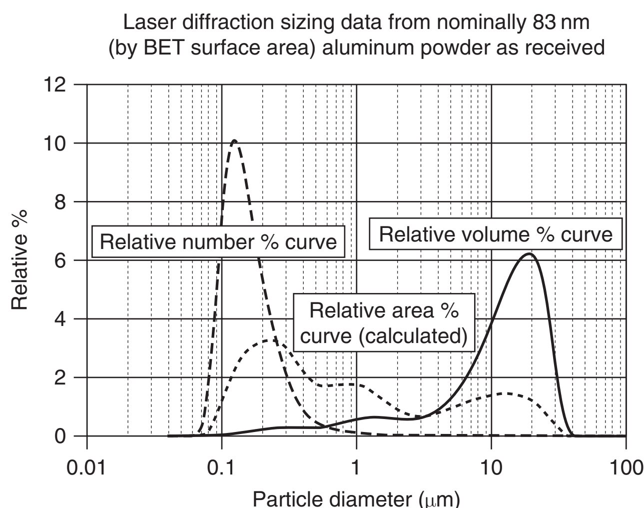


**FIGURE 19.1** Laser diffraction size data for "nanoscale" aluminum powder used for *in vitro* toxicity experiments. Note the apparent difference in size when depicted as a number distribution versus an area or volume distribution. This occurs because volume scales as the cube of the particle diameter and calculated area scales as the square. Each curve, if presented alone, would give an incomplete picture of the particle size distribution/state of agglomeration of the sample. The three curves will overlay only for an ideal spherical, monodisperse, and unagglomerated system. By comparing changes in particle size distribution to that of an "ideal" dispersion, a qualitative assessment of the degree of agglomeration can be made (reproduced with permission of Powers *et al.*, 2006).

less than 100 nm); *surface structure* – surface reactivity, groups, and coatings; *chemical composition* – purity, crystallinity, and electronic properties; *solubility* – surface morphology; and the effect of adsorbed chemicals; shape and aggregation (Nel *et al.*, 2006). The relationships between these factors are only beginning to be worked out.

Particles or particle agglomerates are usually not perfect spheres and are almost never singly dispersed. However, particle size is most often defined as the diameter of a sphere that is equivalent in the selected property to the particle measured; this makes it possible to conveniently plot size distributions of irregularly shaped particles or particle agglomerates using a single value (diameter) along a single axis. The property most often described is volume or mass; i.e. the diameter of a sphere of equal volume to the particle(s) in question (volume or mass diameter). In Figure 19.1, particle diameter is expressed as that which was the median (120 nm), surface area (250 nm), or volume (20 μm (20,000 nm)). Peak distributions in this figure contain some agglomerates of smaller particles.

## Cluster size

The average agglomeration number (AAN) is derived from the ratio of the volume-based median particle size to the average equivalent spherical volume derived from Brunauer Emmett Teller (BET) gas adsorption. An approximate AAN would be ~15,000/160$^3$ ~825,000 equivalent volumes.

## Solubility

Dissolution is affected by surface morphology and agglomeration. Larger more densely aggregated particles will dissolve more slowly. Adsorbed organic chemicals will slow solvation (Powers *et al.*, 2006).

Both particle size and solubility are important determinants of biological response after lung exposure. Particles must be small enough to be inhaled into deep lung, usually taken to be ≤4μm (4000 nm). Particles between 4000 and 500 nm will be phagocytized and cleared rapidly. Particles <500 nm aggregate size will be inefficiently phagocytized or ignored by phagocytes and translocate to the pulmonary interstitium and remain there as a potential toxin. If cleared rapidly they will not be injurious; if not they will remain as a chronic irritating source (Pickrell *et al.*, 2004, 2006).

Solubility of nano-sized particles (NSPs) can be considered to be a lack of biopersistence. Solubility and chemical reactivity are thought to be a function of environment; they should be measured in an environment as near to that of the biological environment as possible. This can sometimes be accomplished with *in vitro* conditions closely simulating biological solutions (Powers *et al.*, 2006). Solubility is thought to be a function of chemical activity, specific surface area, radius and curvature, agglomeration, and specific chemicals that are adsorbed to the NSP (Borm *et al.*, 2006). It is important to consider not just solubility in water, but in fluids that bear significant resemblance to that of the epithelial lining fluid (ELF). ELF is the fluid than lines the epithelial cells in the respiratory tract. A minimal volume is predicted –40–100 ml for mature humans. The pH varies from 6.9 at the end of inspiration to 7.5 at the end of expiration. Bicarbonate is a major buffer; the change is caused by removing $CO_2$ of expiration (Langmuir, 1965). Bicarbonate concentration in lung stimulant fluid increases the solubility of MgO (Pickrell *et al.*, 2006). Surprisingly, macrocrystalline (bulk) MgO is dissolved as extensively as microcrystalline NSP MgO in lung stimulant fluids in about 10–20 min (Pickrell *et al.*, 2006). This paradoxical result suggests that bicarbonate chemical activity may have a direct relation to the rapid dissolution of MgO in lung stimulant fluid related to its modest solubility in distilled water. Stoichiometry of the likely chemical species suggests a conversion of MgO to $Mg(OH)_2$ in aqueous media and a subsequent conversion to the hydrated carbonate (nesquehonite; Langmuir 1965; Pickrell *et al.*, 2006).

# ANIMAL OR TISSUE EXPOSURES

## *In vitro* exposures to NMs

Early indicators for NP-derived adverse health effects were needed for an adequate assessment cytotoxicity of different types of well-characterized NP; cytotoxicity was used to infer relative risk (Brunner *et al.*, 2006). For this purpose, the authors chose a mesothelioma and a fibroblast cell line. Two assays were performed. The first was mean culture activity, indicated by mitochondrial activity in converting a formazan type of dye from its leukoform to an active dye. In addition, DNA content, indicating cell number was measured (Brunner *et al.*, 2006).

The particles were ultrafine (UF) or NPs (Brunner *et al.*, 2006). Other than the positive asbestos control, none had >20% of the particles larger than 200 nm. Specific surface area was also higher than the asbestos positive control (90–190 $m^2/g$ versus the 8.5 $m^2/g$ of the asbestos). Mass median sizes were 20–50 nm and surface area median sizes were 6–21 nm, although they differed in terms of the shape and degree of agglomeration (Brunner *et al.*, 2006). Soluble amorphous silicate, a negative control caused minimal response in either cell line, consistent with its anticipated lack of toxicity (Brunner *et al.*, 2006). Crocidolite asbestos had a significant reduction in both cell activity and cell number (DNA) in both cell lines. The mesothelioma cell line was more severely affected, perhaps reflecting its metabolic activity. This toxicity was consistent with expectations from a positive control (Brunner *et al.*, 2006). Calcium phosphate had no significant cytotoxicity at 3 days; these data are consistent with it having no toxicity like the negative control particle – amorphous silica (Brunner *et al.*, 2006).

Insoluble metal oxides ($TiO_2$, $ZrO_2$, and $CeO_2$) had modest toxicity at 3 days of exposure that was most prominent in mesothelioma cells. By 6 days of exposure, these cells had returned to normal (Brunner *et al.*, 2006). Morphologically, the high dose of zirconium oxide caused the appearance of decreased cell number and function, even after 6 days incubation. These data are consistent with only a modest health effect relative to the positive control (Brunner *et al.*, 2006).

Zinc and ferric oxide were slightly soluble and quite toxic to both cell lines after 3 days incubation (Brunner *et al.*, 2006). The authors advance no explanation for zinc, beyond chemical toxicity. For iron, the toxicity resulted from free-radical production from the Haber–Weiss reaction. Because this reaction occurred at 40-fold less than the chemical toxicity of iron, it is said to be specific for the NP (Brunner *et al.*, 2006).

*In vitro* analysis of particulate matter (PM) effects on alveolar macrophages, epithelial cells, and neutrophils, demonstrates PM- and oxidant-dependent responses. These effects were observed after *in vitro* exposure to concentrated PM(2.5) ((concentrated ambient particles), CAPs) collected in real time. Oxidative stress mediated by reactive oxygen species (ROS) is an important mechanism of PM-induced lung inflammation (Tao *et al.*, 2003).

## Intratracheal instillation and pharyngeal aspiration

### *UF particles*

Exposure to ambient air pollution particles (PM) caused increased cardiopulmonary morbidity and mortality, particularly with pre-existing disease (Tao *et al.*, 2003). Exacerbation of pulmonary inflammation in susceptible people (e.g. asthmatics, chronic obstructive pulmonary disease (COPD) patients) is a central mechanism by which PM cause lung disease.

After intratracheal instillation, UF colloidal silica (UFCS) particles had greater ability to induce tissue damage and inflammation that did fine colloidal silica (FCS) particles. Electron microscopy demonstrated UFCS and FCS particles on both bronchiolar and alveolar wall surfaces; type I epithelial cell necrosis and basement membrane damage was greater in UFCS than FCS particles (Kaewamatawong *et al.*, 2005). A similar trend with $TiO_2$ required repeated exposure (Oberdorster *et al.*, 1995).

### *Carbon nanotubes*

Pharyngeal aspiration of single-walled carbon nanotubes (SWCNT) elicited an acute pulmonary inflammation in C57BL/6 mice that progressed to fibrosis and granulomas (Shvedova *et al.*, 2005). SWCNT caused a dose-dependent release of protein, lactate dehydrogenase (LDH), and gamma-glutamyl transferase activities into bronchoalveolar lung washings (lavage). In addition, accumulation of 4-hydroxynonenal (oxidative biomarker), depletion of glutathione in lungs, leukocyte accumulations, and cytokines were present in bronchoalveolar lavage fluid (BALF) at days 1–7. This was followed by fibrogenic transforming growth factor (TGF)-beta 1 that peaked on day 7. The progressive fibrosis found in mice exhibited two distinct morphologies – SWCNT-induced granulomas and diffuse interstitial fibrosis and alveolar wall thickening. Functional respiratory deficiencies and decreased bacterial clearance (*Listeria monocytogenes*) were found in mice treated with SWCNT (Shvedova *et al.*, 2005). *In vitro* exposures of multiwalled carbon nanotubes to fibroblasts at high doses induces a similar inflammation (Ding *et al.*, 2005).

Equal doses of UF carbon black particles or fine crystalline silica ($SiO_2$) did not cause either granulomas or alveolar wall thickening. Instead they caused weaker pulmonary inflammation and damage (Shvedova *et al.*, 2005).

## Intranasal exposure

### *Carbon black*

High doses of carbon black, given intranasally, showed adjuvant activity as indicated by enlargement of peribronchial lymph nodes and ovalbumin specific production of thymocyte (Th) 2 specific interleukin (IL) 4, IL5, and IL10; local cytokine production after carbon black exposure is predictive of allergic airway inflammation. This particle may predict adjuvant potential (de Haar *et al.*, 2005).

## Inhalation exposure

### *Polytetrafluoroethylene*

Inhalation of highly insoluble UF (nano) particles (diameter 20 nm) of low intrinsic toxicity ($TiO_2$), resulted in pulmonary inflammatory responses. However, these effects were not acute and occurred only after prolonged inhalation exposure of the aggregated UF particles at high levels $>1\,mg/m^3$ (Oberdorster *et al.*, 1995).

By way of comparison, recent studies with thermodegradation products of polytetrafluoroethylene (PTFE) we found that freshly generated PTFE fumes contained singlet UF particles (median diameter 26 nm); the fumes were toxic to rats at inhaled concentrations of $0.7–1.0 \times 10^6$ particles/$cm^3$. Exposure caused an acute hemorrhagic pulmonary inflammation and death after 10–30 min of exposure. Rat performance on a treadmill wheel was reduced by PTFE fume exposure (Oberdorster *et al.*, 1995).

These results confirm reports from other laboratories that the toxic nature of PTFE fumes cannot be attributed to gas-phase components of these fumes, HF, or to reactive compounds. Mass concentrations of the inhaled UF PTFE particles were low, $<60\,\mu g/m^3$. Aging of the fumes with concomitant aggregation of the UF particles significantly decreases their toxicity (Oberdorster *et al.*, 1995). Inhalation studies with UF particles ($\leqslant$100 nm; NP) in rats suggest that particles $\leqslant$50 nm in diameter, may contribute to increased mortality and morbidity.

# ENVIRONMENTAL EPIDEMIOLOGY

Recent epidemiological studies show an association between particulate air pollution and acute mortality and morbidity down to ambient particle concentrations below $100\,\mu g/m^3$. Whether this association also indicates causality between acute health effects and particle exposure at these low levels is unclear at this time; no mechanism is known that would explain such dramatic effects of low ambient particle concentrations.

## Sulfur dioxide (oxides of sulfur particles)

Sulfur dioxide (oxide of sulfur particles) comes mainly from burning sulfur containing fuels (Sullivan *et al.*, 2006), causing both indoor and outdoor pollution. For example, frequent or prolonged use of kerosene space heaters, especially poorly ventilated ones is a source of indoor sulfur dioxide. Sulfur dioxide particles are hygroscopic and tend to grow with increasing hydration of the atmosphere (Sullivan *et al.*, 2006).

Nasal breathing filters out much of the inhaled sulfur dioxide; it is very soluble and is often absorbed in the upper portion of the respiratory tract (Sullivan *et al.*, 2006). Sulfur dioxide is so irritating to the eyes, nose, and airways that its odor is detected as low as 0.5 ppm. At >6 ppm, companion animals often show acute clinical signs – tearing, runny nose, cough, bronchospasm, and shortness of breath. Additional chronic signs that come from prolonged exposure at lower exposure concentrations include cough, mucus hypersecretion, and clearing of the throat; these reflect airway inflammation and chronic bronchitis. Massive exposure is capable of inducing severe permanent pulmonary damage (Sullivan *et al.*, 2006). Low level prolonged human exposures correlate well with bronchial asthma. Future work will need to confirm this correlation in companion animals.

## Swine barn dust exposure

Healthy human volunteers weighing pigs for 3 h developed a neutrophilic pneumonitis; they had an increased bronchial responsiveness to methacholine. Wearing a mask reduced but did not abolish the inflammatory response. Gases and/or UF particles in this environment could be important factors in the development of increased bronchial responsiveness both in animals and their human caretakers (Larsson *et al.*, 2002). *In vitro* data suggest that airway epithelial cell swine dust extract exposure enhances subsequent lymphocyte adhesion to epithelial cells by a modulation of expression of intracellular adhesion molecule 1 and protein kinase C alpha (Mathisen *et al.*, 2004). Exposure caused inflammation characterized by increased numbers of neutrophils, macrophages, and to a lesser degree, lymphocytes in animals and swine barn caretakers. Interestingly, this inflammation is most pronounced in caretakers with no prior exposure to this environment. These findings suggested a tolerance to endotoxin or other substances in this environment was induced by repeated exposures. Interventions have been devised to reduce the risk of symptomatic respiratory disease (Von Essen and Romberger, 2003).

## NP exposure in the home

Use of engineered NM in sunscreens – zinc oxide or titanium dioxide or as bio-imaging probes, as cosmetics, or as

imaging probes of supra-magnetic iron oxides – has not led to reports of toxicities in humans (Nel *et al.*, 2006) or animals; however, ferric oxide NP are cytotoxic to mesothelioma cells and fibroblasts *in vitro* (Brunner *et al.*, 2006). As a point of comparison zinc oxide fumes at high doses (500 μg/m$^3$), in an occupational setting, caused fume fever (fatigue, chills, fever, myalgia, cough, and leukocytosis) (Nel *et al.*, 2006). Several factors may differ between the two situations. In occupational settings, exposures tend to be more persistent, have higher exposure levels and have more complex composition. Thus, they may confer more risks than in single inhalation exposure situations. None of these factors were evaluated in these situations.

Environmental tobacco smoke (ETS) from the burning of tobacco indoors can cause a health hazard for companion animals; because its aerosol can contain more than 4000 toxicants into the airway (Sullivan *et al.*, 2006). Some of them are carcinogens; the Environmental Protection Agency (EPA) has classified ETS a group A carcinogen, meaning that there is sufficient evidence to indicate that it will produce cancer in humans (Sullivan *et al.*, 2006).

Inhaled cigarette smoke often did not cause significant increases in numbers of malignant respiratory tumors in rats, mice, hamsters, dogs, or non-human primates even when exposed for long periods of time to very high concentrations of mainstream cigarette smoke. The results are different from the epidemiological evidence in human smokers. It is difficult to reconcile this major difference between observational studies in humans and controlled laboratory studies. Mice exposed to ETS experimentally and at high doses developed increased incidences of cancer (Hecht, 2005). Most investigators continue to compare the experimental dose pattern with that in humans, animals appear to be less sensitive than man (Coggins, 2002).

Beta-carotene supplementation attenuated cardiac remodeling induced by 1-month tobacco-smoke exposure in rats (Zornoff *et al.*, 2006). After 1-month exposure to ETS cardiac remodeling was present as indicated by disorganization or loss of myofilaments, infolding of plasma membrane, dilation of the sarcoplasmic reticulum, and polymorphic mitochondria with swollen or decreases cristae. Rats given beta-carotene during cigarette-smoke exposure had minimal or no significant cardiac remodeling. These data suggest beta-carotene attenuates, reduces or minimizes cardiac remodeling induced by cigarette smoke (Zornoff *et al.*, 2006). These data are probably most relevant to obese cardiac-compromised companion animals.

While smoke inhalation may have most of its clinical signs and health effects caused by toxic gases (e.g. carbon monoxide), airborne releases include gases, fumes, aerosols, and vapors. Irritant particles such as acrolein are capable of producing pulmonary damage and debris (Fitzgerald and Vera, 2006). Signs indicating increased risk from inhalation injury of smoke particles include confinement in a close space, alteration in breathing pattern, respiratory compromise, stridor, and singed hair. For example, wheezing, chronic cough, chronic hyper-reactive airways, and chronic bronchitis are frequently noted. Water-soluble toxins are absorbed and injure mostly the upper respiratory tract, while less soluble materials, organic materials, go deeper into the lungs and injure pulmonary parenchyma (Fitzgerald and Vera, 2006).

Differential diagnoses or conditions which must be adequately distinguished from smoke inhalation include asthma, heart disease, allergic pulmonary disease, inhalation of toxic solvents or sprays, pneumonia, neoplasia, trauma, COPD, and pneumothorax (Fitzgerald and Vera, 2006).

Overheated frying pans release only small amounts of Teflon™ (PTFE) which causes a fatal hemorrhagic pneumonitis in cockatiels directly adjacent in both time and space to the overheating (Anonymous, 2006; Blandford *et al.*, 1975). Within a short period of time, and in an adjacent room, there is only a minimal reaction. Current understanding implies that the small particles are capable of causing pulmonary injury directly after the overheating, but rapidly condense into larger and less biologically potent particles. As a point of comparison, the owner developed fume fever from which he recovered within 24 h (Blandford *et al.*, 1975; Lee and Seidel, 1991).

Aged particles collected as agglomerates were not toxic when given intratracheally to the rats. The particles became toxic when rats were given inhalation exposure to fumes evolved from the reheated agglomerate. The fumes contained numerous toxic submicron particles evolved from thermal decomposition of agglomerates by reheating. Rats died with pulmonary edema and hemorrhage reflecting type I pneumocyte damage. The edematous lungs revealed some agglomerated particles. However, small PTFE pyrolysis particles were difficult to differentiate from contaminating dust or cellular debris (Lee and Seidel, 1991).

Birds appear to be more sensitive than rats and humans to direct inhalation of Teflon™ fumes from overheated skillets; this sensitivity probably reflects their physiologically more efficient respiratory system. Oxygen is taken up more efficiently than in mammals. In addition, many birds are sufficiently small to have high metabolic rates.

## Stray dog exposures to automobile exhaust aerosols

The UF PM are seen in alveolar type I and II cells. Bronchoalveolar lavage show significant numbers of activated alveolar macrophages. Exposure to complex pollutant mixtures is causing lung structural changes induced by the sustained inflammatory process and resulting in airway and vascular remodeling and altered repair. Cytokines released from both, circulating inflammatory and resident lung cells in response to endothelial and

epithelial injury may be central to the pathology (Calderon-Garciduenas *et al.*, 2001a, b). Myocardial findings, lung epithelial and endothelial pathology, and chronic inflammatory lung changes were closely related (Calderon-Garciduenas *et al.*, 2001a, b).

UF particle exposures delivered at environmental levels, over long times relative to animal lifetimes to stray dogs in Mexico (City), UF were reported by Calderon-Garciduenas (2001a, b). These exposures are complicated by a heavy load of oxidant gases. The investigators report only a modest pulmonary fibrosis, approximately the amount which we see from our 1 ppm $O_3$ ozone exposure (Pickrell *et al.*, 1987; Calderon-Garciduenas *et al.*, 2001a, b). Although difficult to quantitate, the fibrosis is no more than we expected from the oxidant exposure alone in Mexico (City), DF. Thus, we must consider the possibility that UF particles cause so small a lesion, and disrupt so little architecture that healing is favored. Thus, the induction of procollagen and deposition of increased tissue collagen would be beneficial. Similar changes were seen in humans exposed to the same atmosphere (Churg *et al.*, 2003).

## RISK FROM PRODUCTION OF MANUFACTURED NPs

Risks from manufactured NP come from their preparation and subsequent use. For example, to assess risk of preparation, five representative NMs were selected for an analysis (Robichaud *et al.*, 2005). Their selection was based on their current or near-term potential for large-scale production and commercialization. The NMs were SWCNT, bucky balls ($C_{60}$), one variety of quantum dots (Q-dots), alumoxane NPs, and nano-titanium dioxide NPs. The assessment focused on the activities surrounding the fabrication of NM, exclusive of any impacts or risks with the NM themselves. A representative synthesis method was selected for each NM based on its potential for commercial production (Robichaud *et al.*, 2005).

A list of input materials, output materials, and waste streams for each step of fabrication was developed and entered into a database that included key process characteristics such as temperature and pressure (Robichaud *et al.*, 2005). The physical–chemical properties and quantities of the inventoried materials were used to assess relative risk based on factors such as volatility, carcinogenicity, flammability, toxicity, and persistence. These factors were first used to qualitatively rank risk. Then they were combined using an actuarial protocol developed by the insurance industry for the purpose of calculating insurance premiums for chemical manufacturers. This protocol ranks three categories of risk relative to incident risk, normal operations risk, and latent contamination risk (Robichaud *et al.*, 2005).

Results from this analysis determined that relative environmental risk from manufacturing each of these five materials was comparatively low in relation to other common industrial manufacturing processes (Robichaud *et al.*, 2005). Of these Q-dots, metal oxides, and SWCNT were especially low, in the range of silicon wafers, aspirin, and wine production. Only carbon black carried as high a risk as high density plastics and automotive lead batteries. All of these were lower than petroleum refining (Robichaud *et al.*, 2005).

A second study that examined health and safety funding for NM, showed that funding is expected to increase so that $85,000,000 is projected for environmental and health safety research globally (Dunphy-Guzman *et al.*, 2006). Nanotechnology exposure, environmental fate, and transport are considered fundamental to air pollution and possibilities for animal and human exposure. Cerium oxide particles have recently been shown to decarboxylate and polymerize small organic molecules. These molecules are considered to have potential as a gasoline additive. Thus, they have potential for exposure and impact on the ecosphere. Transportation studies to date have been limited (Dunphy-Guzman *et al.*, 2006).

Some smaller NPs show increased animal experimental toxicity due to their increased surface area. Certain carbon-based materials are of concern, because they may partition into cells. In addition, fullerenes can induce photodamage to lipids proteins and cells. In addition, NPs of metal oxides have been shown to induce DNA damage. Finally, hydroxyapatite, a material much like bone matrix has been shown to induce cell death (Oberdorster *et al.*, 2000; Dunphy-Guzman *et al.*, 2006).

Small particles have been shown to have atmospheric impact that is they band together in clouds (Dunphy-Guzman *et al.*, 2006). Titanium dioxide NPs have been shown to disinfect and degrade pollutants. There is a distinct possibility that they may have other activities that are photodegrative or induce biochemical transformations. In addition, NPs are key players in biochemical and geological processes. Global-scale impact should be considered; only life-cycle analyses will provide the information needed to directly predict the impact of NPs on the future of animals and the people who care for them (Dunphy-Guzman *et al.*, 2006).

## CONCLUSIONS

Swine barn dust inhaled in confined animal feeding operations is small enough to deposit in the deep lung; it is mostly allergenic and elicits low-grade allergenic lung disease that probably affects only their feed efficiency (feed required/pound of gain) and rate of growth (average

daily gain). Stray dogs exposed to the Mexico City polluted atmosphere sustain mild-to-moderate lung inflammation and scarring which probably does not affect them greatly. Whatever effect they sustain relates to both the toxic gases and the UF atmospheric particle co-exposure.

Much higher exposure levels of manufactured NP are required to cause health effects than are usually found. Although welder atmospheres may appear similar, they can only be compared to a limited extent, because they are occupational with persistently high levels and often complex containing several airborne toxins. ETS causes less lung cancer in animals than in man, although increased cancer rates compared to smoke free-environmental controls have been observed. Dogs with short- or medium-length noses are more likely to get lung cancer than longer nosed dogs that filter the toxic aerosols out in the nose and get nasal cancer.

Toxicity from overheated non-stick Teflon cookware is most likely to be seen in companion birds with more efficient respiratory systems. Only the particles that are the most recently after thermal decomposition are the smallest and have the most active surface elicit an acute adverse reaction.

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## REFERENCES

Anonymous (2006) Invisible Dangers to Protect Your Bird From. http://www.cockatielcottage.net/hazards.html (April 2006). http://www.ewg.org/reports/pfcworld/images/photo_nonstick_PTG368886.jpg (April 2006).

Borm P, Klaessig FC, Landry TD, Moudgil BM, Pauluhn J, Thomas K, Trottier R, Wood S (2006) Research strategies for safety evaluation of nanomaterials. Part VI. Characterization of nanoscale particles for toxicological evaluation. *Toxicol Sci* **90**: 23–32.

Blandford TB, Seamon PJ, Hughes R, Pattison M, Wilderspin MP (1975) A case of polytetrafluoroethylene poisoning in cockatiels accompanied by polymer fume fever in the owner. *Vet Rec* **96**(8): 175–8.

Brunner TI, Wick P, Manser P, Spohn P, Grass RN, Limback LK, Bruinink A, Stark WJ (2006) *In vitro* cytotoxicity of oxide nanoparticles: comparison to asbestos, silica and the effect of particle solubility. *Environ Sci Technol* **40**(14): 4374–81.

Calderon-Garciduenas L, Gambling TM, Acuna H, Garcia R, Gosnaya N, Monroy S, Villareal-Calderone A, Carson J, Koren HS, Devlin RB (2001a) Canines as a sentinel species for assessing chronic exposures to air pollutants. Part 2. Cardiac pathology. *Toxicol Sci* **61**: 356–67.

Calderon-Garciduenas L, Mora Tiscareno A, Fordham LA, Chung CJ, Garcia R, Osnaya N, Hernandez J, Acuna H, Gambling TM, Villareal-Calderone A, Carson J, Koren HS, Devlin RB (2001b) Canines as sentinel species for assessing chronic exposures to air pollutants. Part 1. Respiratory pathology. *Toxicol Sci* **61**: 342–55.

Churg A, Brauer M, del Carmen Avila-Casado M, Fortoul TI, Wright JL (2003) Chronic exposure to high levels of particulate air pollution and small airway remodeling. *Environ Health Perspect* **111**(5): 714–18.

Coggins CR (2002) A minireview of chronic animal inhalation studies with mainstream cigarette smoke. Inhal Toxicol **14**(10): 991–1002.

de Haar C, Hassing I, Bol M, Bleumink R, Pieters R (2005) Ultrafine carbon black particles cause early airway inflammation and have adjuvant activity in a mouse allergic airway disease model. *Toxicol Sci* **87**(2): 409–18.

Ding L, Stilwell J, Zhang T, Elboudwarej O, Jiang H, Selegue JP, Cooke PA, Gray JW, Chen FF (2005) Molecular characterization of the cytotoxic mechanism of multiwall carbon nanotubes and nano-onions on human skin fibroblast. *Nano Lett* **5**(12): 2448–64.

Dunphy-Guzman KA, Taylor MR, Banfiels LF (2006) Environmental risks of nanotechnology: National nanotechnology initiative funding, 2000–2004. *Environ Sci Technol* **40**: 1401–7.

Fitzgerald KT, Vera R (2006) Smoke inhalation. In *Small Animal Toxicology*, Peterson ME, Talcott PA (eds). Elsevier Saunders, St. Louis, MO, pp. 439–58.

Hecht SS (2005) Carcinogenicity studies of inhaled cigarette smoke in laboratory animals: old and new. *Carcinogenesis* **26**(9): 1488–92.

Hoet PHM, Bruski-Hohlfeld I, Salata OV (2004) Nanoparticles – known and unknown health risks. *J Nanobiotechnol* **2**: 1–15.

Kaewamatawong T, Kawamura N, Okajima M, Sawada M, Morita T, Shimada A (2005) Acute pulmonary toxicity caused by exposure to colloidal silica: particle size dependent pathological changes in mice. *Toxicol Pathol* **33**: 745–51.

Langmuir D (1965) Stability of carbonates in the system $MgO–CO_2–H_2O$. *J Geol* **73**: 730–54.

Larsson BM, Larsson K, Malmberg P, Palmberg L (2002) Airways inflammation after exposure in a swine confinement building during cleaning procedure. *Am J Ind Med* **41**(4): 250–8.

Lee KP, Seidel WC (1991) Pulmonary response to perfluoropolymer fume and particles generated under various exposure conditions. *Fundam Appl Toxicol* **17**(2): 254–69.

Mathisen T, Von Essen SG, Wyatt TA, Romberger DJ (2004) Hog barn dust extract augments lymphocyte adhesion to human airway epithelial cells. *J Appl Physiol* **96**(5): 1738–44.

Nel A, Xia T, Madler L, Ning L (2006) Toxic potentials of materials at the nanolevel – review. *Science* **311**: 622–7.

Oberdorster G, Gelein RM, Ferin J, Weiss B (1995) Association of particulate air pollution and acute mortality: involvement of ultrafine particles? *Inhal Toxicol* **7**(1): 111–24.

Oberdorster G, Finkelstein JN, Johnston C, Gelein R, Cox C, Baggs R, Elder ACP (2000) Acute pulmonary effects of ultrafine particles in rats or mice. *Health Effects Inst Res Rep* **96**: 1–95.

Pickrell JA, Hahn FF, Rebar AH, Horoda RA, Henderson RF (1987) Changes in collagen metabolism and proteinolysis after repeated inhalation exposure to ozone. *Exp Mol Pathol* **46**(2): 159–67.

Pickrell JA, Gakhar G, Mulukutla RS, Maghirang R, Klabunde JS, Malchesky PS, Green R, Oehme FW, Erickson L (2004) Safety of glycol, diesel fuel or combustion smokes in the presence of magnesium of titanium dioxide clearing agents. *Program of the 39th Midwest Regional Meeting (MWRM) of the American Chemical Society*, October, p. 183.

Pickrell JA, Castro SD, Gakhar G, Klabunde KJ, Hayden E, Hazarika S, Oehme FW, Erickson L (2006) Comparative solubility of nanoparticles and bulk oxides of magnesium in water and lung stimulant fluids. *Toxicol Sci* **90**: 451.

Powers KW, Brown SC, Krishna VB, Wasdo SC, Moudgil BM, Roberts SM (2006) Research strategies for safety evaluation of nanomaterials. Part VI. Characterization of nanoscale particles for toxicological evaluation. *Toxicol Sci* **90**: 296–303.

Robichaud CO, Tanzil D, Weilenmann U, Wiesner MR (2005) Relative risk analysis of several manufactured nanomaterials: an insurance industry context. *Environ Sci Technol* **39**(22): 8985–94.

Shvedova AA, Kisin ER, Mercer R, Murray AR, Johnson VJ, Potapovich AI, Tyurina YY, Gorelik O, Arepalli S, Schwegler-Berry D, Hubbs AF, Antonini J, Evans DE, Ku BK, Ramsey D, Maynard A, Kagan VE, Castranova V, Baron P (2005) Unusual inflammatory and fibrogenic pulmonary responses to single-walled carbon nanotubes in mice. *Am J Physiol Lung Cell Mol Physiol* **289**(5): L698–708.

Sullivan JB, Van Ert MD, Krieger GR, Peterson ME (2006) Indoor environmental quality and health. In *Small Animal Toxicology*, Peterson ME, Talcott PA (eds). Elsevier Saunders, St. Louis, MO, pp. 244–72.

Tao F, Gonzalez-Flecha B, Kobzik L (2003) Reactive oxygen species in pulmonary inflammation by ambient particulates. *Free Rad Biol Med* **35**(4): 327–40.

Tsuji JS, Maynard AD, Howard PC,‡ James JT, § Lam C-W, Warheit DB, Annette B, Santamaria AB (2006) Forum series: research strategies for safety evaluation of nanomaterials. Part IV. Risk assessment of nanoparticles. *Toxicol Sci* **89**: 42–50.

Von Essen S, Romberger D (2003) The respiratory inflammatory response to the swine confinement building environment: the adaptation to respiratory exposures in the chronically exposed worker. *J Agric Saf Health* **9**(3): 185–96.

Zornoff LAM, Matsubara LS, Matsubara BB, Okoshi MP, Okoshi K, Pai-Silva MD, Carvalho RF, Cicogna AC, Padovani CR, Novelli EL, Norvo R, Campana AO, Pieva AR (2006) Beta-carotene supplementation attenuates cardiac remodeling induced by 1-month tobacco-smoke exposure in rats. *Toxicol Sci* **90**: 259–66.

CHAPTER

# 20

# Oxidative stress and neurodegeneration

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## INTRODUCTION

Oxidative stress is characterized by the presence of an excess of oxidants caused by an imbalance between the formation and neutralization of reactive oxygen species (ROS) (Behl, 1999). Oxidative stress can lead to the formation of chain reaction by-products, which create a potentially unstable cellular environment by causing massive cellular injury and extensive damage to the lipids, proteins, and biological macromolecules. Oxidative stress has also been linked to tissue damage, advancing age associated dysfunctions, and a broad spectrum of degenerative diseases including neurodegeneration (Barone *et al.*, 2000).

Free radicals or oxidants are highly reactive, unstable molecules that have an unpaired electron in their outermost electron orbit which react with or oxidize various cellular components and biological macromolecules including proteins, DNA, RNA, lipids, fatty acids, and advanced glycation end products, i.e. carbonyls (Stohs and Bagchi, 1995). These reactions between cellular components and free radicals lead to DNA-single-strand break (DNA-SSB) and DNA-double-strand break, DNA fragmentation, lipid peroxidation, protein oxidation products, mitochondrial malfunction, cell membrane damage, lactate dehydrogenase (LDH) leakage, and eventually programmed cell death (apoptosis) and unprogrammed cell death (necrosis). Examples of free radicals or oxidizing molecules are hydrogen peroxide, singlet oxygen, superoxide anion, hydroxyl radical, peroxyl radical, reactive nitrogen oxides, and peroxynitrite. Superoxide is produced deleteriously by a one-electron transfer in the mitochondrial-electron transfer chain. Hydrogen peroxide is also produced by a wide variety of enzymes including monoxygenases and oxidases. Peroxynitrite is a potent free radical which is 1000 times more potent as an oxidizing compound than hydrogen peroxide (Stohs, 1995; Stohs and Bagchi, 1995). Specific markers of free-radical peroxynitrite formation, such as nitrotyrosines or isoprostanes, have been linked to chronic heart disease, liver disease, areas of inflammation, and as well as neurodegenerative diseases including Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis, and Huntington's disease (Stohs, 1995; Stohs and Bagchi, 1995).

The steady-state formation of free radicals is normally balanced by a similar rate of consumption of antioxidants. Various pathological processes disrupt this balance by increasing the formation of free radicals in proportion to the available antioxidants and antioxidant enzymes. Enhanced formation of free radicals induces a compromised effect on the body's immune system leading to inflammation, ischemia–reperfusion injury of different organs, apoptosis, necrosis, and altered gene expression. Enhanced formation of free radicals and oxidative stress, and inadequate antioxidant activity are associated with a broad spectrum of disease states such as cardiovascular dysfunctions, chronic fatigue syndrome, hepatitis, cancer, autoimmune disorders, HIV and AIDS, cancer, and diverse neurodegenerative diseases (Stohs, 1995; Stohs and Bagchi, 1995).

## OXIDATIVE STRESS AND NEURONAL INJURY

Numerous studies confirmed the damaging effects of oxidative stress on the nervous system. In a study conducted by Urano *et al.* (1997) rats were exposed to 100% oxygen in a chamber, in which many morphological changes,

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e.g. swollen astrocytes around vessels, deformed nuclei in nerve cells, pigmentation, swollen mitochondria, and abnormal accumulation of synaptic vesicles in swollen nerve terminals, were observed by electron microscopy. When synaptosomes isolated from oxygen-exposed rats were stimulated by KCl, acetylcholine release from the terminal decreased more significantly than in synaptosomes from control rats ($p < 0.01$). Synaptic plasma membrane fluidity decreased in response to oxygen exposure, while plasma membrane permeability to sucrose increased significantly ($p < 0.05$). Cholesterol: phospholipids ratio of the plasma membranes was increased by oxidative stress and the content of unsaturated fatty acids, especially arachidonic acid and docosahexaenoic acid, decreased. The levels of lipid peroxidation (as determined by the amount of thiobarbituric-acid-reactive substances (TBARS)) in the plasma membranes of oxygen-exposed rats were significantly higher than in control rats ($p < 0.01$). Results suggested that free radicals derived from oxygen may attack nerve terminals and peroxidize the plasma membrane (Davies, 1990; Urano *et al.*, 1997).

## OXIDATIVE STRESSORS: HEAVY METALS, ENVIRONMENTAL TOXICANTS, AND TOBACCO

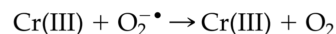
Numerous evidence suggests that exposure to structurally diverse environmental toxicants, including heavy metals, polyhalogenated or polycyclic aromatic hydrocarbons, and tobacco and its carcinogenic counterparts, may involve a common cascade of events which includes the production of ROS and oxidative stress leading to lipid peroxidation (Bagchi *et al.*, 1998a, 2002), DNA-SSB and fragmentation (Stohs *et al.*, 1997), membrane damage with decreased membrane fluidity (Bagchi *et al.*, 1992), apoptosis (Stohs, 1995), glutathione depletion (Bagchi *et al.*, 1996a), altered calcium homeostasis (Bagchi *et al.*, 1997, 1998a, b), enhanced release of tumor necrosis factor alpha (TNF-$\alpha$), induction of stress/heat shock protein (HSP) 90 (Von Burg and Lui, 1993), stimulation of oncogene expression, and inhibition of tumor suppressor genes (Schwarz *et al.*, 1995).

### Chromium, lead, cadmium, and other heavy metals

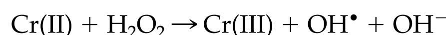
#### *Chromium*

Chromium exists predominantly in two valence states: Cr(III) and Cr(VI). Cr(III) is an essential micronutrient, while Cr(VI) is carcinogenic. Cr(VI) is a highly reactive transition metal widely known to cause allergic dermatitis as well as toxic and carcinogenic effects (Von Burg and Lui, 1993; Goyer, 1996). Chromate, $(CrO_4)^{2-}$, is the most dominant form of Cr(VI) in neutral aqueous solutions and can readily cross cellular membranes via non-specific anion carriers (Danielsson *et al.*, 1982).

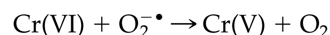
The ability of Cr(VI) to induce an oxidative stress is well known. Von Burg and Lui (1993) and Barceloux (1999) summarized the acute and chronic toxicity, neurotoxicity, dermotoxicity, genotoxicity, carcinogenicity, immunotoxicity, and general environmental toxicity of chromium. Chromium (VI)-induced internucleosomal DNA fragmentation, inhibition of macromolecular synthesis, and apoptosis have been demonstrated in cultured Chinese hamster ovary cells, and low-dose exposure to chromium has been shown to potentiate cell proliferation and apoptosis (Von Burg and Lui, 1993; Manning *et al.*, 1994; Barceloux, 1999). Vitamin E, glutathione, and oxygen scavengers can decrease chromate-induced cytotoxicity, lipid peroxidation, and DNA damage, with vitamin E exhibiting the greatest protective effect (Sugiyama, 1991). Both forms of chromium undergo redox cycling (Stohs and Bagchi, 1995). Various investigators have examined the mechanism of DNA cleavage by Cr(VI). The results indicate that Cr(VI) complexes are produced in the reduction of Cr(VI) by cellular reductants such as NADPH that react with hydrogen peroxide to generate hydroxyl radicals (Shi and Dalal, 1989). The hydroxyl radicals are believed to be the initiators of the primary events in Cr(VI) cytotoxicity and are responsible for causing DNA-strand breaks. The reactions involved in the redox cycling of Cr(III) and Cr(VI) are:



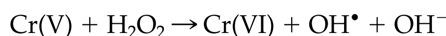
$$Cr(III) + O_2^{-\bullet} \rightarrow Cr(III) + O_2$$



$$Cr(II) + H_2O_2 \rightarrow Cr(III) + OH^{\bullet} + OH^-$$



$$Cr(VI) + O_2^{-\bullet} \rightarrow Cr(V) + O_2$$



$$Cr(V) + H_2O_2 \rightarrow Cr(VI) + OH^{\bullet} + OH^-$$

Although Cr(III) is much less non-toxic compared with Cr(VI), Cr(III) can be reduced to Cr(II) by biological reductants such as NADH and L-cytenine (Ozawa *et al.*, 1993). The newly formed Cr(II) reacts with hydrogen peroxide to produce hydroxyl radical, which is presumably responsible for tissue-damaging effects including the production of DNA damage. In summary, both Cr(VI) and Cr(III) are biologically active oxidation states of chromium, although Cr(VI) is much more toxic and produces greater oxidative stress and carcinogenesis. Both oxidation states of chromium are involved in redox cycling with the production of ROS.

Investigations have shown that sodium dichromate [Cr(VI)] more effectively induces the formation of ROS and causes oxidative tissue and DNA damage compared with Cr(III) (Manning *et al.*, 1994; Bagchi *et al.*, 1995a). The effect of an oral low dose (0.05 median lethal dose ($LD_{50}$))

of Cr(VI) (2.5 mg/kg/day) on brain mitochondrial and microsomal lipid peroxidation, excretion of urinary lipid metabolites, and brain nuclear DNA-SSB in Sprague–Dawley rats has shown that maximum increases in these parameters occur between 60 and 75 days of treatment. The results of these experiments clearly indicate that low-dose subchronic administration of Cr(VI) induces oxidative stress, resulting in tissue-damaging effects that may contribute to the toxicity and carcinogenicity of Cr(VI) (Bagchi *et al*., 2002).

The *p53* tumor suppressor gene plays a major role in the regulation of cellular stress response including oxidative stress, in part, through the transcriptional activation of genes involved in cell cycle control, DNA repair, and apoptosis (Amundon *et al*., 1998). Many factors have been shown to contribute to control the activation of *p53*, and the downstream response to *p53* activation may vary depending on the cellular environment or other modifying factors in the cell, including exposure to diverse xenobiotics. Because *p53* is activated in response to DNA damage and different signaling mechanisms, the role of the *p53* gene was assessed to unveil the mechanism of structurally diverse xenobiotics-induced oxidative stress and toxicity in the brain tissues (Bagchi *et al*., 2000). Measured biomarkers included superoxide anion production (cytochrome *c* reduction), DNA fragmentation, and lipid peroxidation (TBARS). The levels of these parameters were not significantly different in the brains of untreated C57BL/6NTac- and C57BL/6TSG p53-deficient mice. A comparative study was conducted using different types of environmental stressors including 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), endrin, naphthalene, and Cr(VI) in p53-deficient mice. Treatment of C57BL/6NTac mice with a 0.01 $LD_{50}$ dose of TCDD, endrin, naphthalene, and Cr(VI) increased cytochrome *c* reduction by 1.5- to 1.6-fold in brain tissues as compared to the control animals, although under the same conditions, approximately 1.7- to 2.4-fold increases in cytochrome *c* reduction were observed in brain tissues of *p53*-deficient mice (Table 20.1). Similar results were obtained in brain lipid peroxidation (Table 20.2) and DNA fragmentation (Table 20.3) (Bagchi *et al*., 2000).

Approximately 2.5-, 3.0-, 3.4-, and 2.5-fold increases in cytochrome *c* reduction were observed in brain tissues of C57BL/6NTac mice after treatment with 0.10 $LD_{50}$ doses of TCDD, endrin, naphthalene, and Cr(VI) compared to corresponding values in untreated control animals. Under the same conditions, approximately 4.0-, 6.9-, 5.1-, and 3.7-fold increases in cytochrome *c* reduction were observed in brain tissues of TSG-p53 mice compared to control animals. In comparison, at a 0.10 $LD_{50}$ dose, TCDD, endrin, naphthalene, and Cr(VI) induced 1.6-, 2.3-, 1.5-, and 1.5-fold greater increases in cytochrome *c* reduction in brain tissues of p53-deficient mice than in C57BL/6NTac animals (Table 20.1). Similar results were obtained in brain

**TABLE 20.1 Cytochrome c reduction in brain tissue (nmol reduced/15 min/mg of protein)**

| | 0.50 $LD_{50}$ | | 0.10 $LD_{50}$ | | 0.01 $LD_{50}$ | |
|---|---|---|---|---|---|---|
| **Treatment** | **C57BL/6NTac** | **p53-deficient** | **C57BL/6NTac** | **p53-deficient** | **C57BL/6NTac** | **p53-deficient** |
| Control | $5.78 \pm 0.41^{a}$ | $7.34 \pm 0.65^{a}$ | $5.78 \pm 0.41^{a}$ | $7.34 \pm 0.65^{a}$ | $5.78 \pm 0.41^{a}$ | $7.34 \pm 0.65^{a}$ |
| TCDD | $33.07 \pm 5.22^{b}$ | $59.60 \pm 11.71^{a}$ | $14.21 \pm 1.80^{b}$ | $29.38 \pm 4.74^{b}$ | $8.66 \pm 1.25^{b}$ | $12.31 \pm 1.68^{b}$ |
| Endrin | $31.28 \pm 6.64^{b}$ | $74.12 \pm 16.27^{b}$ | $17.55 \pm 2.19^{b,c}$ | $50.32 \pm 9.07^{c}$ | $9.29 \pm 1.05^{b}$ | $16.67 \pm 1.79^{c}$ |
| Naphthalene | $30.61 \pm 6.45^{b}$ | $70.33 \pm 8.29^{b}$ | $19.81 \pm 2.40^{c}$ | $37.58 \pm 6.44^{b}$ | $9.05 \pm 1.35^{b}$ | $17.30 \pm 3.41^{c}$ |
| Cr(VI) | $23.83 \pm 5.56^{b}$ | $56.80 \pm 8.94^{b}$ | $14.57 \pm 2.08^{b}$ | $26.94 \pm 4.58^{b}$ | $9.39 \pm 1.22^{b}$ | $12.85 \pm 1.76^{b}$ |

Cytochrome *c* reduction as an index of superoxide anion production was determined in brain tissue homogenates of C57Bl/6NTac and *p53*-deficient mice 24 h after treatment with a 0, 0.01, 0.10, or 0.50 $LD_{50}$ dose of the xenobiotics. Each value is the mean ±SD of four experiments

[a-c]Values within a column with non-identical superscripts are significantly different ($p < 0.05$) from each other

**TABLE 20.2 Lipid peroxidation in brain tissue (nmol MDA/mg of protein)**

| | 0.50 $LD_{50}$ | | 0.10 $LD_{50}$ | | 0.01 $LD_{50}$ | |
|---|---|---|---|---|---|---|
| **Treatment** | **C57BL/6NTac** | **p53-deficient** | **C57BL/6NTac** | **p53-deficient** | **C57BL/6NTac** | **p53-deficient** |
| Control | $1.69 \pm 0.19^{a}$ | $2.02 \pm 0.24^{a}$ | $1.69 \pm 0.19^{a}$ | $2.02 \pm 0.24^{a}$ | $1.69 \pm 0.19^{a}$ | $2.02 \pm 0.24^{a}$ |
| TCDD | $5.36 \pm 0.50^{b}$ | $11.53 \pm 1.82^{b}$ | $3.32 \pm 0.29^{b}$ | $7.56 \pm 0.80^{b}$ | $1.74 \pm 0.21^{a}$ | $2.53 \pm 0.19^{b}$ |
| Endrin | $6.68 \pm 0.74^{c}$ | $19.16 \pm 2.32^{c}$ | $2.90 \pm 0.41$ | $5.52 \pm 0.79^{c}$ | $2.07 \pm 0.31^{a}$ | $2.44 \pm 0.36^{a}$ |
| Naphthalene | $6.52 \pm 0.75^{c}$ | $23.43 \pm 4.19^{c}$ | $1.99 \pm 0.30^{a}$ | $4.25 \pm 0.50^{d}$ | $1.83 \pm 0.47^{a}$ | $2.20 \pm 0.17^{a}$ |
| Cr(VI) | $5.88 \pm 0.81^{b,c}$ | $14.71 \pm 1.69^{d}$ | $2.84 \pm 0.42^{b}$ | $4.79 \pm 0.33^{c,d}$ | $1.87 \pm 0.32^{a}$ | $2.92 \pm 0.36^{b}$ |

Lipid peroxidation was based on the formulation of TBARS. C57B1/6NTac and p53-deficient mice were killed 24 h after treatment with a 0, 0.01, 0.10, or 0.50 $LD_{50}$ dose of xenobiotics. Each value is the mean ±SD of four experiments. MDA was used as the standard.

[a–d]Values within a column with non-identical superscripts are significantly different ($p < 0.05$) from each other.

TABLE 20.3 DNA fragmentation in brain tissue (exposed as a percentage of total DNA in supernatant fraction)

| Treatment | 0.50 $LD_{50}$ | | 0.10 $LD_{50}$ | | 0.01 $LD_{50}$ | |
|---|---|---|---|---|---|---|
| | C57BL/6NTac | p53-deficient | C57BL/6NTac | p53-deficient | C57BL/6NTac | p53-deficient |
| Control | $2.96 \pm 0.40^{a}$ | $2.76 \pm 0.24^{a}$ | $2.96 \pm 0.40^{a}$ | $2.76 \pm 0.24^{a}$ | $2.96 \pm 0.40^{a}$ | $2.76 \pm 0.24^{a}$ |
| TCDD | $7.12 \pm 0.63^{b}$ | $11.57 \pm 1.50^{b,c}$ | $4.01 \pm 0.29^{b}$ | $6.48 \pm 0.75^{b}$ | $3.43 \pm 0.38^{a,b}$ | $4.45 \pm 0.37^{b}$ |
| Endrin | $7.84 \pm 0.94^{b}$ | $12.23 \pm 1.09^{b}$ | $3.94 \pm 0.26^{b}$ | $6.85 \pm 0.55^{b}$ | $3.68 \pm 0.45^{a,b}$ | $5.29 \pm 0.60^{b}$ |
| Naphthalene | $5.34 \pm 0.71^{c}$ | $10.46 \pm 0.88^{c}$ | $3.00 \pm 0.22^{a}$ | $8.26 \pm 1.04^{c}$ | $3.03 \pm 0.47^{a}$ | $4.63 \pm 0.48^{b}$ |
| Cr(VI) | $6.20 \pm 0.51^{b,c}$ | $12.55 \pm 1.22^{b}$ | $3.90 \pm 0.41^{b}$ | $5.81 \pm 0.66^{b}$ | $3.74 \pm 0.31^{b}$ | $4.30 \pm 0.61^{b}$ |

DNA fragmentation was determined in brain tissues of C57Bl/6NTac and p53-deficient mice 24 h after treatment with a 0, 0.01, 0.10, or 0.50 $LD_{50}$ dose of the four xenobiotics. Each value is the mean ±SD of four experiments.

[a–c] Values within a column with non-identical superscripts are significantly different ($p < 0.05$) from each other.

lipid peroxidation and DNA fragmentation (Tables 20.2 and 20.3) (Bagchi *et al.*, 2000).

Treatment of C57BL/6NTac mice with 0.50 $LD_{50}$ dose of TCDD, endrin, naphthalene, and Cr(VI) increased cytochrome *c* reduction by 5.7-, 5.4-, 5.3-, and 4.1-fold in brain tissues compared to corresponding values in untreated control animals, although under the same conditions, approximately 8.1-, 10.1-, 9.6-, and 7.7-fold increases in cytochrome *c* reduction were observed in brain tissues of p53-deficient mice. In comparison, at a 0.50 $LD_{50}$ dose, TCDD, endrin, naphthalene, and Cr(VI) induced 1.4-, 1.9-, 1.8-, and 1.9-fold higher increases in cytochrome *c* reduction in brain tissue of p53-deficient animals than in C57BL/6NTac mice (Table 20.1) (Bagchi *et al.*, 2000). These results demonstrate the massive production of ROS and oxidative DNA damage by Cr(VI) and other xenobiotics. Similar results were obtained in brain lipid peroxidation and DNA fragmentation (Tables 20.2 and 20.3) (Bagchi *et al.*, 2000).

### *Lead*

Lead is another major environmental toxicant that causes neurological, hematological, and gastrointestinal dysfunction. Prolonged exposure to lead may also cause reproductive impairment, hypertension, and nephropathy. Furthermore, lead slows nerve conduction, alters calcium homeostasis, inhibits enzymes, and stimulates synthesis of binding proteins (Stohs and Bagchi, 1995; Goyer, 1996). Although lead is not considered a transition metal, the catalysis of peroxidative reactions by lead may be a major contributor to the toxic effects of this metal. Various studies have shown that administration of lead to experimental animals results in the production of lipid peroxidation in the brain tissues (Stohs and Bagchi, 1995). Administration of vitamin E prevents enhanced lipid peroxidation and toxicity of lead (Ramstoeck *et al.*, 1980), providing additional evidence for the role of oxidative stress in lead toxicity. As observed with cadmium and nickel, lead produces a compensatory increase in tissue glutathione levels. Results involving the role of ROS and oxidative stress in the toxicity of lead are consistent with the results for other heavy metals (Stohs and Bagchi, 1995).

### *Iron, copper, manganese, and zinc*

The brain, particularly the gray matter, is rich in metal ions such as iron (Fe), copper (Cu), manganese (Mn), and zinc (Zn) which may consist of significant concentrations within the range 0.1–0.5 mM (Hamai *et al.*, 2001). Experimental data has shown these metal ions are key neurochemical factors in both the generation and defense against ROS and neurological diseases. In neurological diseases, an abnormal reaction occurs between a protein and a redox-active metal ion promoting the formation of ROS, which can be detrimental to the nervous system (Jenner, 1996). ROS and oxidative stress significantly contribute to the pathophysiology of Alzheimer's disease, and the amyloid precursor protein (APP) has been shown to alter expression in response to selected metal supplementation and chelation (Cheng and Trombetta, 2004). Other studies have shown that rabbits fed a high-cholesterol diet in the presence of copper ions developed amyloid brain lesions and cognitive deficiencies (Bush *et al.*, 2003; Sparks and Schreurs, 2003). Currently, several independent research groups are conducting investigation to unveil the mechanism of action.

## Oxidative stress by organophosphate and carbamate pesticides

Residential and industrial use of organophosphate and carbamate pesticides is widespread in the United States. According to the 1997 findings of the US Environmental Protection Agency, over 40 organophosphate pesticides and 22 carbamate pesticides are the among 900 pesticides registered for use in the United States which pose the highest risks to human health (Bagchi *et al.*, 2006). organophosphates have been used extensively to control wide range of

(sucking and chewing) pests of field crops, fruits, and vegetables. The widespread use of well-known organophosphate pesticide chlorpyrifos has raised major concerns about its potential to cause fetal or neonatal neurobehavioral damage, even at doses that do not evoke acute toxicity. Chlorpyrifos has been shown to inhibit replication of brain cells, to elicit alterations in neurotrophic signaling, governing cell differentiation, and apoptosis, as well as to evoke oxidative stress (Bagchi *et al.*, 2006). Both organophosphate and carbamate pesticides primarily target of action in the nervous system of insects. Exhibiting many structural similarities with naturally occurring compounds, organophosphate and carbamate interfere with the conduction of signals and cholinergic reactions in the nervous system of insects via inhibiting the release of the enzyme acetylcholinesterase at the synaptic junction. Eserine, parathion, and malathion are further examples of cholinesterase inhibitors responsible for the hydrolysis of body choline esters, including acetylcholine at cholinergic synapses (Bagchi *et al.*, 2006).

Experimental exposure of animals to these pesticides elicits potential of neurotoxicity and DNA damage as well as development of birth defects, abnormal spermocytes and oocytes, and even fetal death (Kaplan *et al.*, 1993). Present case reports of residential exposure to chlorpyrifos resulting in such symptoms as cognitive slowing, cognitive problems, and sensory neuropathy may arise weeks to months after application.

Recent studies indicate that toxic manifestations induce by these pesticides are associated with the enhanced production of ROS which provides an explanation for the multiple types of toxic responses as well as a characteristic wasting syndrome. Most of these pesticides may also serve as common mediators in the activation of protein kinase C (PKC), oncogene expression, apoptosis, and tumor formation. The ability to produce ROS *in vivo* with successive tissue damage was examined by brain lipid peroxidation and DNA-SSB. Chemiluminescence, LDH leakage, and DNA-SSB were assessed to determine the *in vitro* production of ROS. Organophosphates have also shown the mechanism involved in the induction of oxidative-tissue-damaging effects, including lipid peroxidation and nuclear DNA-SSB in *in vivo* models.

A comparative *in vitro* and *in vivo* effect of organophosphates such as chlorpyrifos and fenthion were assessed in cultured neuroactive PC-12 cells and in treated animals (Bagchi *et al.*, 1995b). Dissimilar polyhalogenated cyclic hydrocarbons, such as endrin and chlordane, and chlorinated acetamide herbicides, such as alachlor were also compared to these organophosphates insecticides.

The ability to produce ROS *in vivo* with successive tissue damage was examined by hepatic and brain lipid peroxidation and DNA-SSB. Chemiluminescence, LDH leakage, and DNA-SSB were assessed to determine the *in vitro* production of ROS. These results clearly demonstrated that administration of organophosphates result in the *in vitro* and *in vivo* induction of brain lipid peroxidation, chemiluminescence response, LDH leakage, and DNA-SSB suggesting that the ROS and/or free radicals may be involved in the toxic manifestations of organophosphates and other structurally dissimilar pesticides. Furthermore, results indicate the tissue specificity of organophosphates with respect to the responses.

#### In vitro *and* in vivo *generation of ROS and lipid peroxidation in the brain tissue by structurally dissimilar pesticides*

Organophosphate- and other xenobiotics-induced *in vivo* production of ROS and membrane damage in the brain tissue were assessed by brain lipid peroxidation and DNA damage. Endrin, chlordane, alachlor, chlorpyrifos, and fenthion were administered orally to female Sprague–Dawley rats in two 0.25 $LD_{50}$ doses at 0 and 21 h and killed at 24 h. Lipid peroxidation assays were determined on the brain whole homogenates from control and treated animals according to the method of Buege and Aust (1972), based on the formation of TBARS. Following treatment of the rats with chlorpyrifos, fenthion, endrin, chlordane, and alachlor approximately 4.6- to 5.3-fold increases in brain lipid peroxidation were observed in brain homogenates. Similar results were obtained in cultured PC-12 cells using these pesticides. The effects of these pesticides on lipid peroxidation and DNA damage in brain homogenates are summarized in Table 20.4 (Bagchi *et al.*, 1995a, b).

The effect of dichlorvos exposure on lipid peroxidation and antioxidant defense system in different regions of the rat central nervous system was investigated. An inhibition of acetylcholinesterase activity was used as an index of dichlorvos neurotoxicity. Dichlorvos exposure resulted in a significant decrease in glutathione peroxidase (GSHPx) activity. The decreased levels of both reduced and oxidized glutathione as observed following dichlorvos exposure affected the GSH/GSSG ratio. The results also supported that the enzymes superoxide dismutase (SOD) and catalase may enhance the disposal of potentially toxic free radicals. Furthermore, the decrease in GSH levels may be a mechanism for the detoxification of dichlorvos in the brain (Julka *et al.*, 1992).

#### *Chemiluminescence response by chlorpyrifos, fenthion, and other structurally diverse pesticides for generation of ROS*

The chemiluminescence assay is a non-specific test for the identification of ROS. The sustained chemiluminescence produced by brain tissues following treatment with chlorpyrifos, fenthion, and other pesticides including alachlor, chlordane, and endrin is presumably due to the continued production of ROS. The chemiluminescence responses produced following *in vitro* administration of these

**TABLE 20.4 Effects of pesticides on nuclear DNA-SSB in brain tissue and cultured PC-12 cells and lipid peroxidation (TBARS content) in Sprague–Dawley rats**

| | DNA-SSB (elution constant $\times 10^{-3}$) | | |
|---|---|---|---|
| Treatment | Brain tissue | Cultured PC-12 cells | TBARS (nmol/mg protein) |
| Control | 7.9 ± 2.1[a] | 3.4 ± 0.4[a] | 5.4 ± 0.6[a] |
| Endrin | 15.0 ± 2.3[b] | 8.5 ± 0.9[b] | 12.7 ± 0.9[b] |
| Chlordane | 13.0 ± 1.0[b,c] | 7.6 ± 0.7[b] | 11.4 ± 1.1[b] |
| Alachlor | 17.0 ± 1.7[b] | 7.2 ± 0.6[b] | 19.6 ± 1.3[c] |
| Fenthion | 11.0 ± 0.8[c] | 8.6 ± 0.3[b] | 28.6 ± 2.0[d] |
| Chlorpyrifos | 11.0 ± 1.6[c] | 8.3 ± 0.5[b] | 25.0 ± 2.1[d] |

Female Sprague–Dawley rats received two 0.25 $LD_{50}$ doses of the pesticides in corn oil at 0 and 21 h and killed at 24 h. Control animals received the vehicle. Effects of 100 nM concentrations of the pesticides on DNA-SSB of cultured PC-12 cells at 24-h post-treatment were determined. DNA-SSB were determined by the alkaline elution method and are expressed as DNA elution constants. Lipid peroxidation was determined as the content of TBARS, using MDA as the standard. Each value is the mean ±SD of at least four to six animals in each group. Values with non-identical superscripts are significantly different ($p < 0.05$).

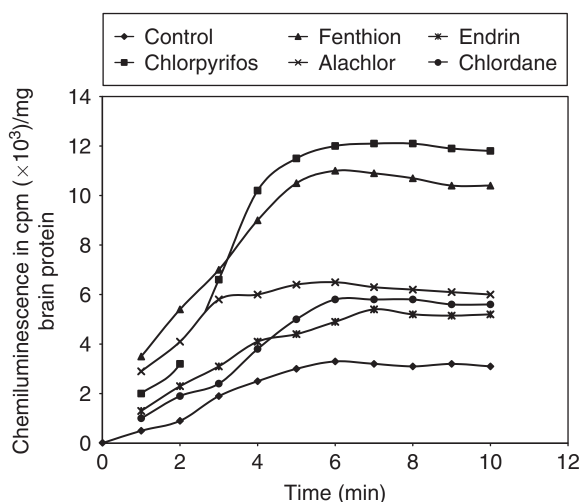



**FIGURE 20.1** Chemiluminescence production by brain homogenates *in vitro* following exposure to structure diverse pesticides.

pesticides in brain tissues are shown in Figure 20.1. The chemiluminescence response produced by brain homogenates from pesticide(s)-treated rats rapidly increases, reaching a maximum between 6 and 8 min of incubation, while brain homogenates from control animals reach a peak chemiluminescence at 6 min. The chemiluminescence persisted for over 10 min. Increases of 2.9- and 2.4-fold were observed in the chemiluminescence responses in the brain homogenates of the animals treated with chlorpyrifos and fenthion. The greatest chemiluminescence responses in brain tissues were induced by chlorpyrifos (Bagchi *et al.*, 1995b, 2002).

### *LDH leakage by chlorpyrifos, fenthion, and other pesticides*

Organophosphates produce toxicological problems due to their high environmental persistence and ability to accumulate in the adipose tissue. Organophosphates exhibit similar abilities as inducers of hepatic drug metabolizing enzymes (Viviani *et al.*, 1978), and act as potent competitive and stereospecific inhibitors of ligand binding to specific types of brain receptors (Botham, 1990).

As an index of membrane and cellular damage, the release of LDH from cultured PC-12 cells was measured as a function of concentration of the pesticides. Increased release of LDH into the media of cultured cells is an indicative of cellular and membrane damage. Cultured PC-12 cells were incubated in the presence of 0, 50, 100, or 200 nM of chlorpyrifos, fenthion, and other pesticides, and the release of LDH by the cells was measured after 24 h of incubation as an index of cytotoxicity. The amount of LDH released by these pesticides was concentration dependent. However, the differences in the release of LDH into the media were not significantly different at concentrations of 100 and 200 nM. Maximal release of LDH from cultured PC-12 cells was observed at 100 nM concentrations of the pesticides. Increases of 2.3-, 2.5-, 2.8-, 3.1-, and 3.4-fold were observed in LDH leakage following incubation of the PC-12 cells with endrin, chlordane, alachlor, chlorpyrifos, and fenthion, respectively (Table 20.5) (Bagchi *et al.*, 1995b, 2002).

A sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis study demonstrated the overexpression of 60 and 90 kDa protein in hepatic, brain, and lung tissues following treatment with structurally dissimilar pesticides (Figure 20.2). Enhanced expression of 90 kDa protein was also observed in cultured PC-12 cells following treatment with these pesticides (Figure 20.2) (Bagchi *et al.*, 1996b, 2002). Table 20.6 depicts the radioactivity ($P^{32}$ counts/min) associated with Hsp89$\alpha$ and Hsp89$\beta$ mRNA expression in control and pesticide-treated cultured PC-12 cells. The cultured PC-12 cells were incubated with 50, 100, or 200 nM concentrations of the pesticides and the concentration-dependent effects of the pesticides were determined. At various concentrations of alachlor, endrin, chlorpyrifos, and fenthion, the expression of Hsp89$\alpha$ and Hsp89$\beta$ significantly increased as compared to the control values.

**TABLE 20.5 Concentration-dependent effects of pesticides on the release of LDH from cultured PC-12 cells**

| Treatment | Concentration of LDH in the media (U LDH/l) | | |
|---|---|---|---|
| | 50 nM | 100 nM | 200 nM |
| Control | 77.4 ± 9.2[a] | 76.9 ± 10.3[a] | 79.2 ± 8.6[a] |
| Endrin | 123.8 ± 16.5[b,c] | 178.1 ± 11.6[b] | 183.6 ± 20.2[b] |
| Chlordane | 100.6 ± 9.8[b] | 193.5 ± 20.4[b,c] | 212.4 ± 19.5[b,c] |
| Alachlor | 131.7 ± 11.4[c] | 216.7 ± 19.3[c] | 230.3 ± 22.6[c] |
| Chlorpyrifos | 147.1 ± 15.3[c,d] | 239.9 ± 20.8[c,d] | 241.0 ± 25.7[c,d] |
| Fenthion | 162.5 ± 13.7[d] | 263.2 ± 24.6[d] | 266.5 ± 20.4[d] |

PC-12 cells ($25 \times 10^4$ cells/35-mm Petri-dish) in 2 ml of RPMI 1640 were incubated for at least 3 h to allow cell adherence and 50, 100, or 200 nM concentrations of pesticides were added to the cultures in two equally divided portions at 0 and 24 h. The incubation was continued at 37 °C in an atmosphere of 5% $CO_2$ for 24 h. Media were collected from the cultures and assayed for LDH activity. Data are expressed as the mean value of six experiments ±SD. Values with non-identical superscripts are significantly different ($p < 0.05$).

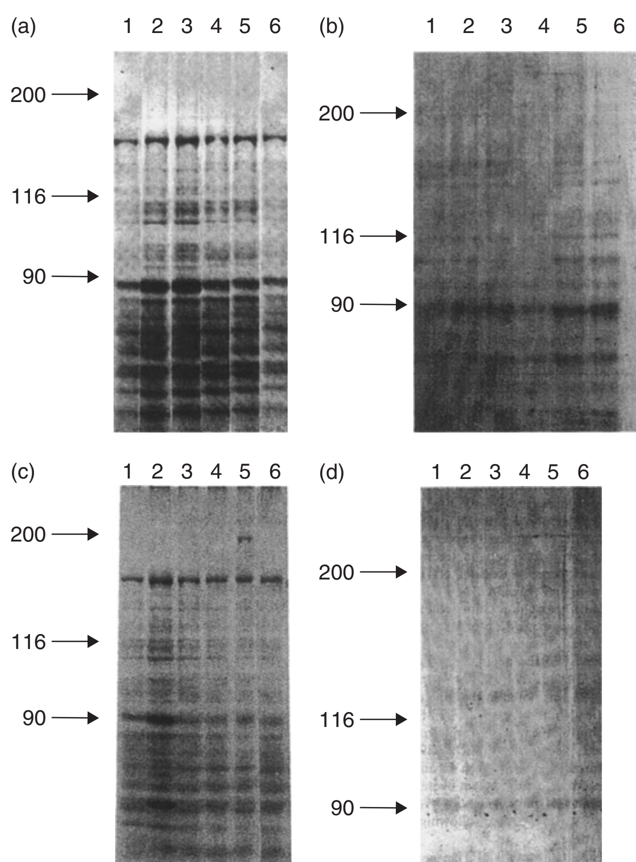



**FIGURE 20.2** SDS polyacrylamide gel electrophoresis of the hepatic (a), brain (b), and lung (c) tissues from control and pesticide-treated rats, and cell lysates (d) from control and pesticide-treated cultured PC-12 cells. Lanes as follows: 1, control sample; 2, alachlor-treated sample; 3, endrin-treated sample; 4, chlordane-treated sample; 5, chlorpyrifos-treated sample; and 6, fenthion-treated sample.

### *Genotoxicity of organophosphate*

The detection of genotoxicity caused by organophosphate pesticides has also been determined using the single-cell gel electrophoresis assay or comet assay. Chlorpyrifos and acephate were tested for their ability to induce *in vivo* genotoxic effect in leukocytes of Swiss albino mice (Rahman *et al.*, 2002). The mice were administered orally with doses ranging from 0.28 to 8.96 mg/kg body weight of chlorpyrifos, or 12.25 to 392.00 mg/kg body weight of acephate. A comet assay was performed on whole blood at 24, 48, 72, and 96 h. A significant increase in mean comet tail length indicating DNA damage was observed at 24-h post-treatment ($p < 0.05$) with both pesticides in comparison to cyclophosphamide (positive control) demonstrating the DNA damage was dose related. The mean comet tail length revealed a clear dose-dependent increase. A gradual decrease in mean tail length was noted from 48-h post-treatment. By 96 h of post-treatment the mean comet tail length reached control levels indicating repair of the damaged DNA.

Organophosphate-induced oxidative stress at the tubular level has been hypothesized as playing a role in the pathogenesis of acute tubular necrosis. Bidrin®, an organophosphate insecticide formulation with dicrotophos as the active ingredient, has been associated with renal tubular epithelial cell (LLC-PK1) toxicity. A study conducted by Poovala *et al.* (1999) assessed LDH release, $H_2O_2$ levels (μmol/mg protein per h), and malondialdehyde (MDA) formation (nmol/mg protein). Results showed LDH which significantly increased with concentration and time, after exposure of the cells to 1000, 1250, 1500, 1750, and 2000 ppm of Bidrin® for 6, 12, 24, and 48 h. Antioxidants 2-methylaminochroman (2-MAC) and desferrioxamine reduced cell damage induced by 1250 ppm of Bidrin® over a 24-h incubation. The greatest reductions in the percentage of LDH were produced by desferrioxamine 2 mM and 2-MAC 2.5 μM, both significantly lower than Bidrin® alone. $H_2O_2$ levels were significantly elevated after exposure to 1250 ppm of Bidrin®. Significantly increased MDA formation compared with control was also found in Bidrin®-exposed cells indicating enhanced lipid peroxidation. MDA generation was significantly

**TABLE 20.6 Radioactivity ($^{32}P$ counts/min) associated with Hsp89$\alpha$ and Hsp89$\beta$ mRNA expression in control and pesticide-tre`ated, cultured PC-12 cells**

| | Hsp89$\alpha$ | | | Hsp89$\beta$ | | |
|---|---|---|---|---|---|---|
| **Treatment** | **50 nM** | **100 nM** | **200 nM** | **50 nM** | **100 nM** | **200 nM** |
| Control (DMSO) | 637 ± 52[a] | 716 ± 73[a] | 754 ± 66[a] | 596 ± 34[a] | 634 ± 39[a] | 787 ± 55[a] |
| Alachlor | 2427 ± 211[b] | 3219 ± 255[b] | 3957 ± 265[b] | 2392 ± 181[b] | 3611 ± 263[b] | 4456 ± 328[b] |
| Endrin | 2042 ± 187[c] | 3315 ± 308[b] | 2821 ± 257[c] | 1808 ± 178[b] | 3544 ± 295[b] | 4030 ± 413[b] |
| Chlordane | 787 ± 64[d] | 893 ± 62[c] | 987 ± 71[d] | 682 ± 71[c] | 756 ± 84[c] | 941 ± 90[c] |
| Chlorpyrifos | 2287 ± 218[b,c] | 3626 ± 275[b] | 4573 ± 422[b] | 2938 ± 232[b,d] | 3885 ± 367[b,d] | 4744 ± 411[b] |
| Fenthion | 2642 ± 223[b] | 3711 ± 307[b] | 4388 ± 414[b] | 3182 ± 274[d] | 4312 ± 323[d] | 4418 ± 409[b] |

Cultured PC-12 ($1 \times 10^7$) cells were incubated individually with either 50, 100, or 200 nM of alachlor, chlordane, chlorpyrifos, or fenthion, and total RNA was isolated after 24 h using the guanidium isothiocyanate method. The RNAs were run on 1% agarose gels, transferred to nylon membranes, and Northern blot analyses were performed by using $^{32}P$-labeled HSP89$\alpha$ and HSP89$\beta$ probes. Values are expressed as mean counts/min ±SD of four to six animals. Values within each column with non-identical superscripts are significantly different ($p < 0.05$).

suppressed by 2-MAC and desferrioxamine. These results demonstrated that Bidrin® can cause direct tubular cytotoxicity, and implicate, at least in part, a role for ROS and accompanying lipid peroxidation in cytotoxicity.

### In vitro *and* in vivo *PKC activation by structurally diverse pesticides and chromium (VI) and cadmium (II)*

Various pesticides and transition metals induce oxidative deterioration of biological macromolecules in which PKC may play a major regulatory role. PKC is a family of isoenzymes with distinct roles in normal and pathogenic activities within cells. PKC is involved in signaling pathways mediating the regulation of many cell processes including cell differentiation, cell survival, gene expression, secretion, cytoskeletal function, and cell–cell interactions (Lord and Pongracz, 1995). Thus, PKC is involved in a cascade of events associated with cell regulation which is subject to both internal and external factors. A growing body of evidence indicates that free radicals and ROS may be involved in mediating signal transduction through interaction with PKC (Brawn *et al.*, 1995; Lander *et al.*, 1995). Recent studies have demonstrated that PKC is rapidly activated in cells following oxidative exposure (Brawn *et al.*, 1995). For example, the modulation of PKC activity by oxidant tumor promoters and the phorbol esters is well known (Prasad and Jones, 1992; Lord and Pongracz, 1995). The modulation of PKC by carbon tetrachloride has been shown to depend on the degree of oxidative unbalance provoked by various concentrations of this haloalkane.

The comparative *in vitro* and *in vivo* abilities of structurally diverse pesticides including TCDD, endrin, chlordane, lindane, dichlorodiphenyltrichloroethane (DDT), alachlor, chlorpyrifos, and fenthion, and chromium (VI) and cadmium (II) to modulate PKC activity were assessed in both cultured neuroactive PC-12 cells *in vitro* and in the brain tissues of these xenobiotics-treated animals (Bagchi *et al.*, 1996a, b, 1997).

**TABLE 20.7 Comparative PKC activity in female Sprague–Dawley rats following treatment with pesticides, cadmium chloride (Cd(II)), and sodium dichromate (Cr(VI)) in brain**

| Treatment | PKC activity/μg of protein/min |
|---|---|
| Control | 10.7 ± 1.1[a] |
| TCDD | 25.2 ± 2.9[b] |
| Endrin | 24.4 ± 3.7[b,c] |
| Chlordane | 20.8 ± 1.7[c] |
| Lindane | 22.0 ± 2.5[c] |
| DDT | 21.1 ± 1.6[c] |
| Alachlor | 19.1 ± 1.4[c,e] |
| Chlorpyrifos | 37.4 ± 4.2[d] |
| Fenthion | 35.4 ± 2.9[d] |
| Chromium (VI) | 16.7 ± 1.9[e] |
| Cadmium (II) | 26.4 ± 3.6[b] |

Female Sprague–Dawley rats were treated orally with two 0.25 $LD_{50}$ doses at 0 and 21 h with pesticides, cadmium chloride (Cd(II)), or sodium dichromate (Cr(VI)), and killed at the 24-h time point. PKC activity was monitored using a kit from Upstate Biotechnology, NY. Values with non-identical superscripts are significantly different ($p < 0.05$).

Approximately 2.0- to 3.5-fold increases in PKC activities in the brain tissues were observed as compared to the control tissues (Table 20.7). Concentration-dependent effect of these xenobiotics was observed on enhanced PKC activities (2.9- to 4.3-fold) in neuroactive pheochromocytoma PC-12 cells (Table 20.8). In these *in vitro* experiments, maximum activation of PKC was also observed primarily with 100 nM concentrations of these xenobiotics. No further increases in PKC activity were observed following treatment with 200 nM concentrations of these xenobiotics. Thus, organophosphates pesticides as well as these xenobiotics can modulate a vital component in the cell signaling pathway (Tables 20.7 and 20.8) (Bagchi *et al.*, 1997).

### *Developmental neurotoxicity of chlorpyrifos on cultured PC-12 and gliotypic C6 cells*

Studies have demonstrated that glial-type cells have been targeted by chlorpyrifos through the same multiple

**TABLE 20.8 Comparative PKC activity in cultured neuroactive PC-12 cells following treatment with pesticides, cadmium chloride (Cd(II)), and sodium dichromate (Cr(VI))**

| Treatment | PKC activity/μg of protein/min | | |
|---|---|---|---|
| | 50 nM | 100 nM | 200 nM |
| Control (DMSO) | 0.78 ± 0.08[a] | 0.77 ± 0.10[a] | 0.95 ± 0.13[a] |
| TCDD | 2.28 ± 0.16[b] | 2.31 ± 0.26[b] | 2.02 ± 0.15[b] |
| Endrin | 2.10 ± 0.12[b,c] | 2.45 ± 0.32[b] | 2.28 ± 0.32[b] |
| Chlordane | 1.87 ± 0.20[c] | 2.41 ± 0.16[b] | 2.12 ± 0.14[b] |
| Lindane | 1.37 ± 0.09[d] | 2.09 ± 0.24[b] | 2.21 ± 0.18[b] |
| DDT | 1.59 ± 0.22[d] | 2.44 ± 0.27[b] | 2.45 ± 0.21[b] |
| Chlorpyrifos | 2.26 ± 0.20[b] | 3.31 ± 0.38[c] | 3.22 ± 0.40[c] |
| Fenthion | 1.88 ± 0.14[c] | 3.26 ± 0.43[c] | 3.12 ± 0.35[c] |
| Alachlor | 1.86 ± 0.24[c] | 2.37 ± 0.28[b] | 2.33 ± 0.17[b] |
| | 0.2 μM | 0.4 μM | 0.6 μM |
| Chromium (VI) | 1.95 ± 0.22[c] | 3.17 ± 0.37[c] | 3.21 ± 0.32[c] |
| Cadmium (II) | 1.21 ± 0.14[d] | 2.95 ± 0.32[c] | 2.88 ± 0.30[c] |

Cultured cells were treated individually with 50, 100, or 200 nM concentrations of pesticides, or 0.2, 0.4, or 0.6 μM concentration of cadmium chloride (Cd(II)) or sodium dichromate (Cr(VI)) in two equally divided concentrations at 0 and 21 h and incubated at 37°C. PKC activity was monitored using a kit from Upstate Biotechnology, NY at the 24-h time point. Values with non-identical superscripts are significantly different ($p < 0.05$).

mechanisms that have been demonstrated for the effects of chlorpyrifos on brain development *in vivo* (Garcia *et al.*, 2001). Post-neurogenesis, glial development continues and given that chlorpyrifos targets events in both glial cell replication and the later stages of differentiation, the vulnerable period for developmental neurotoxicity of chlorpyrifos is likely to extend well into childhood or even early adolescence. Early *in vitro* studies have also demonstrated the methods causing neurotoxicity by chlorpyrifos, by means of assessment of cultures of immature brain tissue (Roy *et al.*, 1998; Monnet-Tschudi *et al.*, 2000) or transformed neural cell lines, such as neuronotypic PC-12 cell lines. PC-12 cell lines are less receptive to neurotoxins and have shown to effectively establish cell replication as a major target because they maintain a fixed pattern of mitosis until differentiation is triggered by addition of trophic factors and deletion of serum (Crumpton *et al.*, 2000). To demonstrate the antimitotic effects of chlorpyrifos, a study in two *in vitro* models, PC-12 cells and gliotypic C6 cells were compared (Qiao *et al.*, 2001). In the first set of experiments, PC-12 and gliotypic C6 cells were exposed to chlorpyrifos or chlorpyrifos metabolites for 1 h in the absence of serum, to obviate any potential protective effect of serum proteins (Garcia *et al.*, 2001) selecting a chlorpyrifos concentration (30 μM) which was previously found to cause robust but submaximal inhibition of DNA synthesis *in vitro*. Equimolar concentrations of chlorpyrifos oxon also produced significant inhibition of DNA synthesis, again with gliotypic C6 cells showing a greater effect than PC-12 cells; however, chlorpyrifos oxon was also significantly less effective than was chlorpyrifos itself (Monnet-Tschudi *et al.*, 2000).

The effects of chlorpyrifos were also compared to those of other cholinesterase inhibitors, using equivalent concentrations (30 μM) of each compound demonstrated that both diazinon, an organophosphate, and physostigmine, a competitive cholinesterase inhibitor, caused significant inhibition of DNA synthesis in gliotypic C6 cells. For PC-12 cells, diazinon caused a significant decrement, though smaller than the effect of chlorpyrifos, and physostigmine was ineffective. The current results are consistent with previous concepts where it was demonstrated that chlorpyrifos exerts antimitotic actions on developing neural cells independently of cholinesterase inhibition (Pope, 1999; Slotkin, 1999). It was also observed that chlorpyrifos was more effective than chlorpyrifos oxon, despite the fact that the latter is a far more potent cholinesterase inhibitor.

The effects of chlorpyrifos and its major metabolites in two *in vitro* models, PC-12 cells and gliotypic C6 cells showed chlorpyrifos inhibited DNA synthesis in both cell lines but had a greater effect on gliotypic cells. Chlorpyrifos oxon, the active metabolite that inhibits cholinesterase, also decreased DNA synthesis in PC-12 and gliotypic C6 cells with a preferential effect on the latter. However, diazinon, another organophosphate pesticide, also inhibited DNA synthesis with predilection toward gliotypic C6 cells, was less effective than was chlorpyrifos. It was also found that the addition of sera protected the cells from the adverse effects of chlorpyrifos and that the effect could be reproduced by addition of albumin. These results indicate that chlorpyrifos and other organophosphates such as diazinon have immediate, direct effects on neural cell replication, preferentially for gliotypic cells. In light of the protective effect of serum proteins, the fact that the fetus and newborn possess lower concentrations of these proteins suggests that greater neurotoxic effects on them and may occur at blood levels of chlorpyrifos that are non-toxic to adults (Aschner, 1999; Monnet-Tschudi *et al.*, 2000; Garcia *et al.*, 2001).

Adverse effects of chlorpyrifos on glial cell replication are of critical importance in defining the sensitive period for effects on central nervous system development. Glia cells provide nutritional, structural, and homeostatic support essential to architectural modeling of the brain (Morita *et al.*, 1999; Barone *et al.*, 2000) and because glial development continues well into the postnatal period, glial targeting implies a prolonged vulnerability, extending into childhood. Chlorpyrifos administration *in vivo* inhibits DNA synthesis and causes loss of brain cells during gliogenesis (Dam *et al.*, 1998) with maximal effects on neural functions appearing during peaks of glial development (Monnet-Tschudi *et al.*, 2000). In aggregating brain-cell cultures, chlorpyrifos affects glial markers, again unrelated to cholinesterase inhibition (Monnet-Tschudi *et al.*, 2000). The above results thus confirm conclusively that chlorpyrifos, rather than its active metabolite, chlorpyrifos oxon, is the primary agent in these effects (Bagchi *et al.*, 2006).

Taking together, TCDD, endrin, chlordane, lindane, alachlor, naphthalene, Cr(VI), and Cd(II) are environmental as well as industrial pollutants, and extensive research has provided evidence that ROS and oxidative stress are involved in the toxicity of these xenobiotics in the brain tissues.

## Naphthalene: a bicycylic aromatic hydrocarbon

Naphthalene is widely used in various commercial and industrial applications including lavatory scent disks, soil fumigants, and moth balls. Exposure to naphthalene is associated with the development of hemolytic anemia in humans and laboratory animals (Germansky and Jamall, 1988). The toxic manifestations induced by naphthalene appear to involve the conversion of naphthalene to the naphthoquinones as 1,2-naphthoquinone and 1,4-naphthoquinone (Lubek *et al.*, 1989) as well as hydroxylated products including 1-naphthol, 2-naphthol, and 1,2-dihydro-1,2-dihydroxynaphthalene (Seaton and Tjeerdema, 1995). In general, quinones are believed to be toxic by a mechanism involving redox cycling and oxidative stress. The role of ROS in the cytotoxicity of various quinine derivatives has been reviewed extensively. Menadione (2-methyl-1,4-naphthoquinone) has been the most extensively studied model compound with respect to the toxicity of quinones and naphthoquinones (Stohs, 1995).

### *Naphthalene-induced membrane microviscosity*

In a study by Vuchetich *et al.* (1996), membrane fluidity studies were performed on mitochondrial and microsomal membranes of brain tissues by steady-state fluorescence spectroscopy. The membranes were treated with 0.5 mM diphenyl-hexatriene (DPH) in tetrahydrofuran as the fluorescent probe and incubated for 2 h at 37°C. The membranes were kept at 4°C for 3–5 h for complete incorporation of DPH. Fluorescence polarization, a measure of membrane fluidity, was determined at 25°C with a Perkin-Elmer spectrofluorometer equipped with perpendicular and parallel polarizers, using an excitation wavelength of 365 nm and an emission wavelength of 430°nm. Fluorescence polarization and the apparent microviscosity were calculated as described by Shintzky and Barrenholz (1978) and Bagchi *et al.* (1992) (Table 20.9).

**TABLE 20.9 NAP-induced changes of membrane microviscosity in the brain mitochondrial and microsomal membranes, and the protective ability of VES**

| | Membrane microviscosity (in Poise) | | | |
|---|---|---|---|---|
| **Treatment** | **Mitochondria** | **% Control** | **Microsomes** | **% Control** |
| *12 h* | | | | |
| Control | 0.161 ± 0.028[a,d] | – | 0.183 ± 0.014[a] | |
| VES | 0.193 ± 0.023[a,c] | 120 | 0.119 ± 0.032[b] | 65 |
| NAP | 0.336 ± 0.060[b] | 209 | 0.320 ± 0.030[c] | 175 |
| NAP + VES | 0.231 ± 0.037[c,d] | 143 | 0.245 ± 0.036[d] | 134 |
| *24 h* | | | | |
| Control | 0.182 ± 0.004[a] | – | 0.179 ± 0.009[a] | |
| VES | 0.159 ± 0.007[d] | 87 | 0.117 ± 0.010[b] | 65 |
| NAP | 0.335 ± 0.013[b] | 184 | 0.295 ± 0.012[c] | 165 |
| NAP + VES | 0.208 ± 0.009[c] | 114 | 0.220 ± 0.005[d] | 123 |
| *48 h* | | | | |
| Control | 0.182 ± 0.005[a] | – | 0.160 ± 0.011[a] | |
| VES | 0.073 ± 0.290[a] | 95 | 0.121 ± 0.019[b] | 76 |
| NAP | 0.259 ± 0.022[d] | 142 | 0.210 ± 0.012[c] | 131 |
| NAP + VES | 0.211 ± 0.065[c,d] | 116 | 0.170 ± 0.035[d] | 106 |

Female Sprague–Dawley rats were individually treated with vitamin E succinate (VES) 100 mg/kg for 3 consecutive days and 40 mg/kg on days 4, 5, 6, and 7. Naphthalene (NAP) (1100 mg/kg) in corn oil was administered on day 4, 2 h after VES, and the animals were killed 12, 24, 48, and 72 h after NAP administration. Control animals received the corresponding vehicle. Each value represents the mean ±SD of four to six animals. Values within each column with non-identical superscripts are significantly different ($p < 0.05$).

The ability of naphthalene to induce an oxidative stress was determined by measuring lipid peroxidation (TBARS), DNA-SSB, glutathione depletion, and membrane microviscosities of the brain tissues. The effects of administering naphthalene (1100 mg/kg) and/or vitamin E succinate to rats were assessed on lipid peroxidation in brain mitochondria and microsomes. Following naphthalene administration, maximum increases in lipid peroxidation in brain subcellular fractions were observed at the 12-h time point. Increases in lipid peroxidation of 2.4- and 2.0-fold occurred at 12-h post-treatment were observed in the brain mitochondria and microsomes, respectively, as compared to control values (Bagchi *et al.*, 1998a).

The results indicate that naphthalene induces the production of ROS. The ROS produced may lead to enhanced lipid peroxidation, as well as other cell-damaging effects, including membrane and DNA damage and glutathione depletion, contributing to the toxic manifestations of naphthalene. The administration of vitamin E succinate significantly attenuates these effects following an acute dose of naphthalene in rats (Table 20.10).

In another study, low-dose (0.05 $LD_{50}$) chronic effects of naphthalene (110 mg/kg/day p.o. in corn oil) were investigated for 120 consecutive days on increased lipid peroxidation and DNA fragmentation in brain tissues of female Sprague–Dawley rats. The results of this study demonstrated the low dose, chronic administration of naphthalene may induce an oxidative stress leading to brain lipid peroxidation and DNA fragmentation (Bagchi *et al.*, 1998a, b).

## Tobacco and neurotoxicity

The use of tobacco products constitutes the most significant cause of morbidity and mortality in the world causing respiratory disease, cancer, and cardiovascular diseases related to atherosclerosis and stroke leading to neuronal injury (Euler *et al.*, 1996). Tobacco smoke is a complex mixture of more than 3800 known components including a variety of trace elements, many of which are implicated in free-radical-producing reactions, and subsequently lead to the formation of highly reactive secondary radicals and toxic organic products (Deliconstantinos *et al.*, 1994a). Nitric oxide may be the most important free radical in the gas phase of tobacco smoke, and may participate in the formation of other free radicals (Deliconstantinos *et al.*, 1994b). Other free radicals present in tobacco smoke include carbon-centered radicals as isoprene radicals, alkoxyl radicals, peroxyl radicals,

**TABLE 20.10 NAP-induced DNA-SSB and glutathione depletion in the brain nuclei and the protective ability of VES**

| Groups | DNA-SSB in brain (elution constant × $10^{-3}$) | % Control | Glutathione depletion | % Control |
|---|---|---|---|---|
| *12 h* | | | | |
| Control | 7.9 ± 0.6[a] | – | 3.61 ± 0.48[a] | – |
| VES | 15.0 ± 3.0[b] | 190 | 3.92 ± 0.47[a] | 109 |
| NAP | 19.0 ± 0.9[c] | 241 | 2.33 ± 0.41[b] | 65 |
| NAP + VES | 15.0 ± 3.0[b] | 190 | 2.72 ± 0.36[b,d] | 75 |
| *24 h* | | | | |
| Control | 7.0 ± 0.5[a] | – | 3.82 ± 0.51[a] | – |
| VES | 13.0 ± 5.0[b] | 186 | 3.54 ± 0.29[a] | 93 |
| NAP | 17.0 ± 8.0[b,c] | 243 | 1.96 ± 0.30[c] | 51 |
| NAP + VES | 15.8 ± 7.5[b,c] | 226 | 3.03 ± 0.38[d] | 79 |
| *48 h* | | | | |
| Control | 7.3 ± 0.6[a] | – | 3.54 ± 0.37[a] | – |
| VES | 16.3 ± 0.4[b] | 223 | 3.6 ± 0.42[a] | 102 |
| NAP | 16.0 ± 6.0[b,c] | 219 | 2.15 ± 0.36[b,c] | 61 |
| NAP + VES | 13.8 ± 0.7[b] | 189 | 3.26 ± 0.45[a,b] | 92 |
| *72 sh* | | | | |
| Control | 7.5 ± 0.5[a] | – | 3.88 ± 0.50[a] | – |
| VES | 16.5 ± 9.0[b] | 220 | 3.72 ± 0.44[a] | 96 |
| NAP | 17.0 ± 9.0[b,c] | 227 | 2.42 ± 0.38[b] | 62 |
| NAP + VES | 16.0 ± 0.3[b] | 213 | 3.47 ± 0.42[a] | 89 |

*Note*: See footnote of Table 20.9.

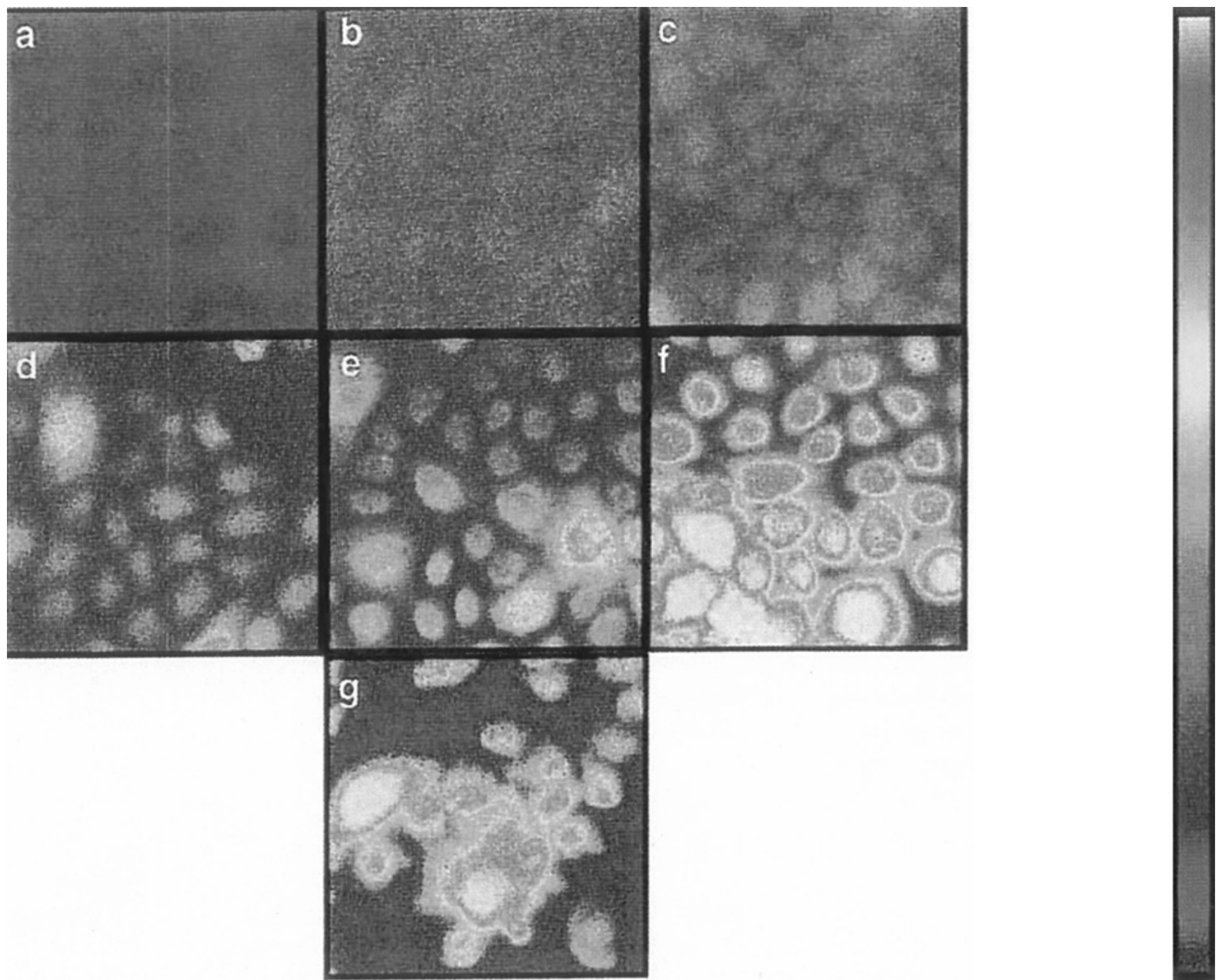



**FIGURE 20.3** Changes in intracellular redox states of human cells after treatment with a tobacco extract. Cells (70% confluent) were treated with the tobacco extract for 24 h. After 24 h, medium was replaced with fresh medium containing 5 μm 2,7-dichlorofluorescein diacetate and fluorescence intensity was measured 5 m later at 513 nm with a confocal laser scanning microscope. a, Control; b, 100 μg/ml; c, 150 μg/ml; d, 200 μg/ml; e, 250 μg/ml; f, 300 μg/ml; and g, 3 mM hydrogen peroxide (positive control). This figure is reproduced in color in the color plate section.

superoxide anion, hydrogen peroxide, nitrogen dioxide, and peroxynitrite radical (Pryor and Stone, 1993). The peroxynitrite radical is formed from the condensation of superoxide anion and nitric oxide.

The ability of tobacco to induce the production of ROS and an oxidative stress is demonstrated in Figure 20.3 (Bagchi *et al.*, 1999). Human oral keratinocytes were incubated with an aqueous extract of tobacco for 24 h, and the cells were treated with 2,7-dichlorofluoroscein diacetate as the fluorescent probe. The cells were then examined by scanning laser confocal microscopy. The cells had been incubated with the following concentrations of the tobacco extract: a, control; b, 100 μg/ml; c, 150 μg/ml; d, 200 μg/ml; e, 250 μg/ml; f, 300 μg/ml; and g, 3 mM hydrogen peroxide (positive control). The hydrogen peroxide was used as a positive control. A concentration-dependent increase in fluorescence intensity occurred was demonstrated, indicating an increase in the intracellular oxidized states of the cells (data not shown). The relative intensities of the fluorescence were quantitated, and a concentration-dependent increase in oxidative states was observed in the cells. Results indicated that tobacco products can initiate the formation of ROS and produce an increase in the intracellular oxidized state of exposed cells. ROS may also be produced in smokers by phagocytic and neuronal cells that are activated in response to enhanced ROS production as a result of exposure to various constituents in tobacco smoke (Anderson, 1991; Becker *et al.*, 1996; Bagchi *et al.*, 1999).

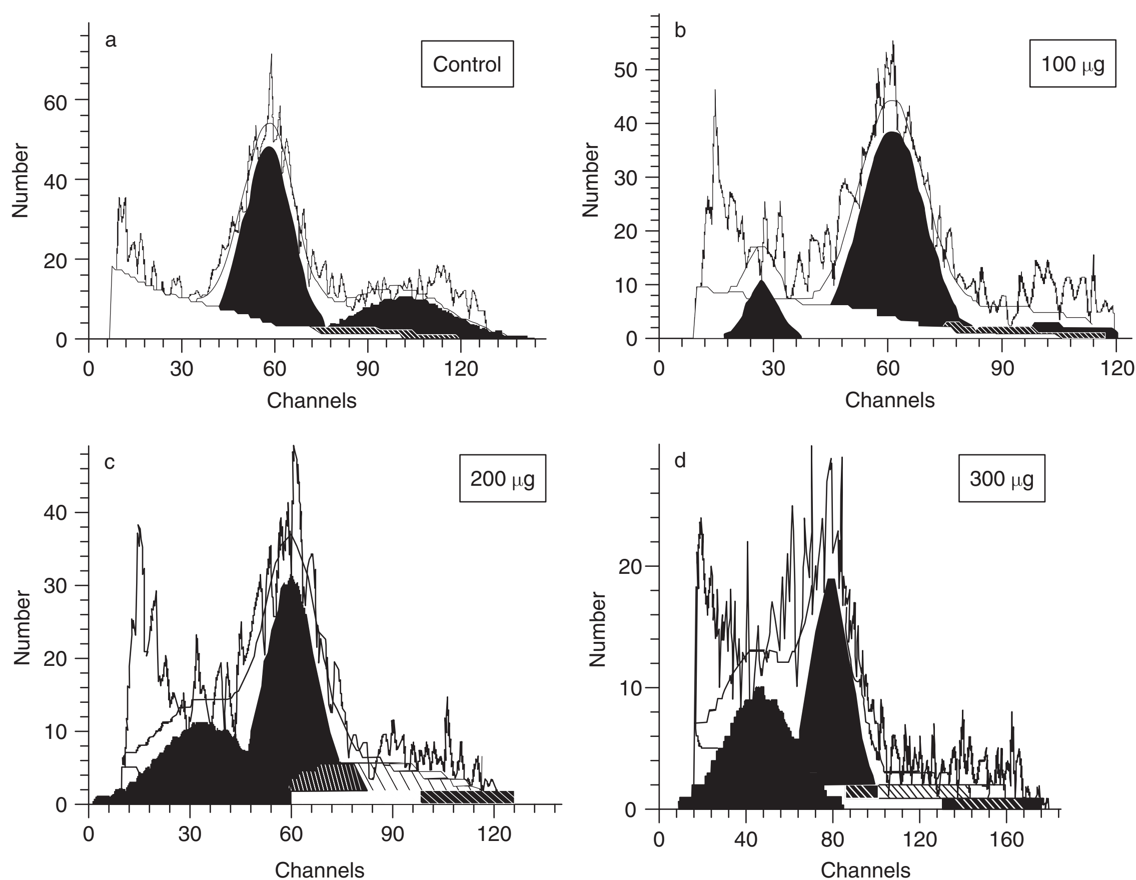



**FIGURE 20.4** Flow cytometric analysis of cell cycle distribution and apoptosis in human cells in response to increasing concentrations of tobacco extract. Primary keratinocytes were grown approximately 70% confluence and then treated with various concentrations of tobacco extract for 24 h. Following incubation, the cells were removed from culture surfaces by trypsinization and DNA content and apoptosis were determined using the method of Bagchi *et al.* (1999). a, control; b, tobacco extract (100 μg/ml); c, tobacco extract (200 μg/ml); and d, tobacco (300 μg/ml).

### *DNA cell cycle modulation and apoptotic cell death*

Concentration-dependent effects of tobacco extract on apoptotic cell death of human cells were determined using flow cytometry (Figure 20.4). The cells were incubated with tobacco extract for 24 h prior to labeling with propidium iodide. The percentages of the apoptopic peak (cell death) increased with increasing concentrations tobacco extract. The percentages of the cells exhibiting apoptotic cell death as analyzed by ModFit cell cycle analysis were approximately 0%, 9%, 29%, and 35% when cells were treated with 0, 100, 200, and 300 μg/ml of tobacco extract, respectively. A distinct loss of cells in the G2-M phase coincides with the appearance of the apoptotic cell population. The results indicate that tobacco treatment to human cells enhanced the production of free radicals which may initiate destructive pathways via indirect mechanism (Bagchi *et al.*, 1999).

## ROLES OF OXIDATIVE STRESS, GLUTATHIONE DEPLETION, HSP AND MITOCHONDRIAL DYSFUNCTION IN NEURODEGENERATIVE DISEASES

Oxidative stress has been extensively studied in neurological diseases including Alzheimer's disease, Parkinson's disease, multiple sclerosis, amyotrophic lateral sclerosis, and Huntington's disease. Compared with other organs, the brain is believed to be particularly susceptible to the damaging affects of ROS due to its high metabolic rate and relatively reduced capacity for cellular regeneration. Neuronal cells are especially susceptible to oxidative stress and subsequent damage including cell death. In a study conducted by Urano *et al.* (1997) active oxygen species have been shown to induce oxidative damage to the nervous system. Results suggested that free radicals derived from oxygen may attack nerve terminals and peroxidize the

plasma membrane. However, in response to the oxidative stress, the status of the defense system in synapse, i.e. the concentration of vitamin E, activities of SOD, and GSHPx changed, and that many of the changes observed were reduced remarkably by the intraperitoneal administration of vitamin E prior to stress. Therefore, results also supports the idea that vitamin E contributes to the protection against nerve dysfunction caused by oxidative stress (Urano *et al*., 1997).

In cases of Parkinson's disease, Alzheimer's disease, and amyotrophic lateral sclerosis, various indices of ROS damage have been reported within the specific brain region that undergoes selective neurodegeneration. For example, markers for lipid peroxidation, such as 4-hydroxynonenal (4-HNE) and MDA, have been identified in the cortex and hippocampus of patients with Alzheimer's disease, the substantia nigra of patients with Parkinson's disease and in spinal fluid from patients with amyotrophic lateral sclerosis (Butterfield *et al*., 2002). Protein nitration, a marker of protein oxidation, has also been demonstrated to be elevated in the hippocampus and neocortex of individuals with Alzheimer's disease, in lewy bodies in cases of Parkinson's disease and within motor neurons in amyotrophic lateral sclerosis (Smith *et al*., 1997).

Alternative explanations for the pathogenesis of neurodegenerative diseases include subsequent depletion or reduced function of necessary antioxidant enzymes. Antioxidant enzymes such as SOD, catalase, GSHPx, and glutathione reductase (GSHRd) display reduced activities in affected brain regions in Alzheimer's disease (Behl, 1999). Glutathione and oxidized glutathione depletion is the earliest known biochemical indicator of substantia nigral degeneration (Behl, 1999).

## Depletion of glutathione

Oxidative stress may be initiated by a decline in the antioxidative defense system or oxidative stress caused by other factors may decrease the concentrations of antioxidants. Alterations of antioxidant defenses support the hypothesis that oxidative stress may play an important role in the pathophysiology of Parkinson's disease. The most robust and significant alteration in the antioxidant defense is a decrease in GSH concentration. Initially, a complete absence of GSH in the presence of high GSSG concentrations was reported (Perry and Yong, 1986; Perry *et al*., 1988).

## Glutathione transferases

Glutathione transferase is a group of detoxification enzymes involved in the metabolism of pesticides and other toxins. Glutathione transferases have direct antioxidant activity and are involved in the metabolism of dopamine. The activity of glutathione transferase has been reported to be normal in the brains of Parkinson's disease patients. Although there is no association between idiopathic Parkinson's disease and glutathione transferase polymorphism, the distribution of the glutathione transferase 1 genotype differed significantly between patients and controls that had been exposed to pesticides (Menegon *et al*., 1998). Therefore, glutathione transferase 1, which is expressed in the blood–brain barrier, may influence response to neurotoxins and may explain the susceptibility of some people to the parkinsonism-inducing effects of pesticides.

## Roles of ROS, HSP, and neurodegenerative diseases

The concept that oxidative stress occurs in Parkinson's disease derives primarily from the fact that the metabolism of dopamine, by chemical or enzymatic means, can generate free radicals and other ROS. Auto-oxidation of dopamine leads to the formation of neuromelanin and can generate quinone and semiquinone species and other ROS. Perhaps more importantly, enzymatic oxidation of dopamine catalyzed by monoamine oxidase leads to the formation of hydrogen peroxide ($H_2O_2$) as well as its deaminated metabolites 3,4-dihydroxybenzoic acid (DOPAC) and homovanillic acid (HVA). Normally, $H_2O_2$ is inactivated by catalase or by GSHPx in a reaction in which GSH is used as a cosubstrate. Because catalase is compartmentalized into peroxisomes the detoxification of cytosolic and mitochondrial peroxides depends predominantly on GSHPx. $H_2O_2$ can react with $Fe^{2+}$ and forms the highly reactive and cytotoxic hydroxyl radical ($^{\bullet}OH$) via the Fenton reaction. The situation is likely to be self-perpetuating because dopamine depletion caused by a decrease in dopamine neurons leads to a compensatory increase in dopamine turnover, with increased formation of $H_2O_2$ and increased demands on the glutathione system in the remaining neurons. This hypothesis is supported by experimental studies demonstrating that enhanced dopamine turnover is associated with increased formation of oxidized glutathione (GSSG) which, in turn, can be prevented by inhibitors of dopamine metabolism (Spina and Cohen, 1989). A variety of critical biomolecules, including lipids, proteins, and DNA, can be damaged by ROS, thereby potentially leading to neurodegeneration.

Increased oxidative alterations to proteins such as $\alpha$-synuclein in Parkinson's disease, $\beta$-amyloid (A$\beta$) in Alzheimer's disease, and SOD1 in amyotrophic lateral sclerosis might result in increased protein misfolding and impaired degradation. This, in turn, might cause the toxic accumulation of soluble protofibrils or insoluble aggregates within the diseased brain that can contribute to neurodegeneration. Oxidative stress increases with age in the brain, and neurons might be particularly affected because they are postmitotic. The ability of cells to respond to

oxidative protein damage also seems to decline with age and might contribute to protein buildup. A decline in induction of HSPs, for instance, can result in an increase in oxidatively damaged proteins that might be resistant to ubiquitinylation and degradation by the 26S proteasome. Depletion of soluble HSPs has been proposed to be an important influence in the cellular neurodegeneration that is associated with amyotrophic lateral sclerosis. Indeed, pharmacological induction of HSPs by the agent arimoclomol has recently been demonstrated to slow disease progression in amyotrophic lateral sclerosis – SOD1 transgenic mouse models (Kieran *et al*., 2004). Increased expression of HSP70 has been suggested to protect against the toxic effects associated with human Parkinson's disease mutations in both the $\alpha$-synuclein and parkin genes, including oxidative damage that results in protein misfolding (Takeuchi *et al*., 2002; Winklhofer *et al*., 2003).

### Roles of oxidative stress and mitochondrial dysfunction in neurodegenerative diseases

Epidemiological studies have identified certain environmental agents, including pesticides and herbicides, as being risk factors for Parkinson's disease. Animals that are treated with the insecticide rotenone display selective neuropathology that is extremely reminiscent of the disease state (Betarbet *et al*., 2006). Both 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and rotenone are selective complex I inhibitors. Complex I inhibition by MPTP can directly result in increased oxidative stress, particularly through the production of $O_2^{-\bullet}$. It also results in reduced mitochondrial function, including decreased ATP generation. ATP depletion can result in increased cytoplasmic dopamine levels, which might in turn result in indirect production of oxidative stress through the generation of dopamine oxidation by-products (German *et al*., 2000).

## PATHOPHYSIOLOGY OF NEURODEGENERATIVE DISEASES

Substantial evidence that oxidative stress is either a causative or an ancillary factor in the pathogenesis of major neurodegenerative diseases, including Parkinson's disease (Ebadi *et al*., 1996), Alzheimer's disease (Behl, 1999), and amyotrophic lateral sclerosis (Hall *et al*., 1998), as well as in cases of stroke, trauma, and seizures (Facchinetti *et al*., 1998). Important new data showing the role of free-radical damage in transmissible spongiform encephalopathies has also come to light. In Parkinson's diseased patients, decreased levels of antioxidant enzyme activities have been found (Fahn and Cohen, 1992) and evidence of oxidative stress in the form of increased lipid peroxidation and oxidation of DNA bases is seen in the substantia nigra, the area of the brain affected by Parkinson's disease (Jenner, 1996). Similar increased lipid peroxidation and oxidation of DNA and proteins are seen in Alzheimer's disease (Retz *et al*., 1998).

### Alzheimer's disease

Alzheimer's disease is the most common neurodegenerative disorder worldwide. In the United States alone, approximately 4.5 million people suffer from this devastating condition and affect almost half of all patients with dementia. The 2–3% of persons aged 65 years show signs of the disease; while 25–50% of persons aged 85 years have symptoms. Alzheimer's disease is characterized clinically by progressive cognitive deterioration together with declining activities of daily living and neuropsychiatric symptoms or behavioral changes. Early symptom is amnesia that becomes steadily more pronounced with illness progression, with relative preservation of older memories. As the disorder progresses, extensive cognitive impairment includes aphasia, apraxia, agnosia, and frontal lobe functions such as decision-making and planning.

Alzheimer's disease consists principally of neuronal loss or atrophy of the hippocampus and cerebral cortex together with the deposition of an abnormal protein or A$\beta$ in the form of extracellular amyloid (or senile) plaques and intracellular neurofibrillary tangles of abnormally hyperphosphorylated tau protein (Selkoe, 2003). Amyloid plaques contain small, toxic cleavage products, denoted as A$\beta$40 and A$\beta$42 of the APP. The apoE4 (apolipoprotein E4) genotype is a powerful risk factor for developing Alzheimer's disease, and it may possibly affect A$\beta$ deposition and neurofibrillary tangle formation (Roses, 1996).

#### *Glutathione in Alzheimer's disease*

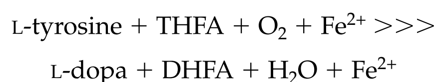
Strong evidence that oxidative stress is involved in the pathogenesis of Alzheimer's disease comes from a clinical study showing that oral vitamin E intake delayed progression in patients with moderately severe impairment from Alzheimer's disease (Sano *et al*., 1997). A role of oxidative stress in Alzheimer's disease is further supported by increased levels of TBARS, a measure of lipid peroxidation (Lovell *et al*., 1995; Marcus *et al*., 1998). However, studies reporting a disturbance of glutathione homeostasis are less clear. The total brain levels of glutathione appeared to be unaffected in Alzheimer's disease (Perry *et al*., 1987), whereas GSHPx and GSSG reductase were found to be elevated in different brain regions (Lovell *et al*., 1995) or unchanged (Marcus *et al*., 1998). Transcription of GSH peroxidase and GSSG reductase was elevated in hippocampus and inferior parietal lobule, but not in cerebellum of

Alzheimer's disease patients, which may reflect the protective gene response to the increased peroxidation in the brain regions showing severe Alzheimer's disease pathology (Aksenov *et al*., 1998). 4-HNE, one marker of lipid peroxidation, is neurotoxic in neuronal culture and *in vivo* and is elevated in Alzheimer's disease brain and cerebrospinal fluid (CSF). The levels of glutathione transferase, a protective enzyme against aldehydes and especially 4-HNE were decreased in the brain and ventricular CSF of autopsied Alzheimer's disease and normal control subjects (Lovell *et al*., 1998). Together these data imply that oxidative stress plays an important role in the pathogenic process but that alterations in the glutathione system are secondary to other events leading to neurodegeneration.

## Parkinson's disease

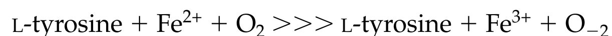
Parkinson's disease also known as paralysis agitans is the most common neurodegenerative movement disorder. According to the CDC's National Center for Health Statistics (NCHS), approximately 1% of the United States population older than 65 years suffers from this slowly progressive neurodegenerative disease. Although uncommon, cases of Parkinson's disease have been reported at all ages, even as low as 11. It occurs in all parts of the world, but appears to be more common in people of European ancestry than in those of African ancestry. Those of East Asian ancestry have an intermediate risk. It is more common in rural than urban areas and men are affected slightly more often than women. About 2% of the population develops the disease some time during life.

Parkinson's disease is primarily due to the insufficient formation and action of dopamine, a substance produced in the dopaminergic neurons in the brain. The disease was first formally recognized and documented in 1817 in *An Essay on the Shaking Palsy* by the British physician Dr. James Parkinson. The biochemical changes in the brain of Parkinson's disease patients were identified in the 1960s. Parkinson's disease or "shaking palsy" is a brain disorder that causes muscle tremor, stiffness, and weakness. Early symptoms of Parkinson's disease include muscular stiffness, fatigue, and trembling that usually begins with a slight tremor in the hand, arm, or leg. As the disease progresses, muscle dysfunctions occur such as stiffness, weakness, trembling, and rigidity. In the later stages of Parkinson's disease the affected person loses the ability to control their movements, making everyday activities hard to manage, and the intellect begins to be affected by the disease. About one-third of Parkinson's disease sufferers eventually show signs of dementia. The disease runs an average 10-year course and ultimately results in death usually by an infection or aspiration pneumonia. Selective and progressive degeneration of pigmented dopaminergic neurons in the substantia nigra pars compacta is the principal pathology that underlies Parkinson's disease. Pathological changes to the dopamine neurons of the substantia nigra pars compacta are also thought to be involved in schizophrenia and psychomotor retardation sometimes seen in clinical depression. Several reasons for the death of dopamine neurons include age-related liver deficiency, genetic influences, viral infections such as encelphalitis, or environmental toxins. Some studies have suggested free-radical damage may also play a part in Parkinson's disease by allowing dopamine to be lost via oxidation and may lead to further neuronal damage (Xu *et al*., 2002). Cellular damage occurs due to the lack of L-dopa formation, the metabolic precursor of dopamine. Normal L-dopa formation occurs as a result of the following reaction from L-tyrosine and tetrahydrofolate (THFA) (product of vitamin folic acid and nicotinamide; DHFA, dihydrofolate):



$$\text{L-tyrosine} + \text{THFA} + O_2 + Fe^{2+} >>> \text{L-dopa} + \text{DHFA} + H_2O + Fe^{2+}$$

However, lack of L-tyrosine or THFA will result in the unsuccessful formation of L-dopa. In a THFA-deficient reaction, superoxide anion formation occurs in the following reaction:



$$\text{L-tyrosine} + Fe^{2+} + O_2 >>> \text{L-tyrosine} + Fe^{3+} + O_{-2}$$

### *Glutathione and oxidative phosphorylation in Parkinson's disease*

The mutual effects of glutathione homeostasis and inhibition of oxidative phosphorylation in the pathogenesis of Parkinson's disease were also studied. However, whether a decrease in glutathione precedes the defect of oxidative phosphorylation or conversely remains controversial. Past research in Parkinson's disease patients demonstrated a defect in oxidative phosphorylation via decrease in complex I activity of the electron transport chain in the substantia nigra (Schulz and Beal, 1994). However, in a contrasting study, depletion of glutathione by buthionine sulphoximine (BSO) produces enlargement and degeneration of mitochondria in neonatal rats (Jain *et al*., 1991) and a decrease in the activity of complexes I and IV in weaning rats (Heales *et al*., 1995); therefore, disputing that pathogenesis of Parkinson's disease results from the primary dysfunction of glutathione homeostasis and secondary inhibition of oxidative phosphorylation.

A recent study in the intersecting pathways to neurodegeneration in Parkinson's disease was studied in the relationship of mitochondrial impairment at complex I, oxidative stress, as well as the aggregation of Parkinson's-disease-related gene $\alpha$-synuclein, and dysfunctional protein degradation. Betarbet *et al*. (2006) evaluated the pathogenesis Parkinson's disease in *in vivo* and *in vitro* models of chronic low-grade complex I inhibition with the pesticide

rotenone. Chronic rotenone exposure *in vivo* caused oxidative modification of DJ-1, a gene linked to Parkinson's disease, accumulation of $\alpha$-synuclein, and proteasomal impairment. The effects became more regionally restricted such that systemic complex I inhibition eventually resulted in highly selective degeneration of the nigrostriatal pathway, one of the major dopamine pathways in the brain. DJ-1 modifications, $\alpha$-synuclein accumulation, and proteasomal dysfunction were also seen *in vitro* and these effects could be prevented with $\alpha$-tocopherol. Thus, chronic exposure to a pesticide and mitochondrial toxin brings into play three systems, DJ-1, $\alpha$-synuclein, and the ubiquitin–proteasome system, and thus implies that mitochondrial dysfunction and oxidative stress link environmental and genetic forms of the disease (Betarbet *et al*., 2006).

## Huntington's disease

Huntington's disease or Huntington's chorea is an autosomal-dominant inherited neurodegenerative disorder characterized by abnormal body movements and a reduction of various mental abilities. Huntington's disease was discovered by the Ohio physician George Huntington who first described it in 1872. Afflicted individuals lose their ability to walk, talk, think, and reason and may become depressed, and lose their short-term memory capacity. Huntington's disease affects males and females equally and crosses all ethnic and racial boundaries. It typically begins in mid-life, between the ages of 30 and 45 years, though onset may occur as early as the age of 2 years. Children who develop the juvenile form of the disease rarely live to adulthood. In the United States alone, about 30,000 people have Huntington's disease and at least 150,000 others have a 50 percent risk of developing the disease (National Institute of Neurological Disorders and Stroke, 2006).

Clinical cases of Huntington's disease illustrate selective neuronal dysfunction and subsequent loss of neurons in the striatum, cerebral cortex, and other parts of the brain. Several mechanisms of neuronal cell death have been proposed for Huntington's disease, including excitotoxicity, oxidative stress, impaired energy metabolism, and apoptosis. Excitotoxicity refers to the neurotoxic effect of excitatory amino acids in the presence of excessive activation of postsynaptic receptors. Oxidative stress in Huntington's disease is caused by the presence of large amounts of free radicals or highly reactive oxygen derivatives and may occur as a consequence of mitochondrial malfunction or excitotoxic cell death. Striatal damage induced by quinolinic acid can be ameliorated by the administration of spin-trap agents, which reduce oxidative stress, providing indirect evidence for the involvement of free radicals in excitotoxic apoptosis.

Candidate treatments to slow the progression of the disease are under study, yet have been slow to reach Huntington's disease patients. According to recent research, eicosapenaenoic acid (EPA) an omega-III fatty acid has shown to slow and possibly reverse the progression of the disease. In the United States, it is available over the counter in lower concentrations in omega-III and fish oil supplements. Other agents and measures such as creatine, CoQ10, the antibiotic minocycline, trehalose, exercise, antioxidant-containing foods and nutrients, and select dopamine antagonists, such as tetrabenazine might also slow the progress of Huntington's disease.

## Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis also known as Lou Gehrig's disease or Maladie de Charcot, is a progressive, and perpetually fatal motor neuron disease. Amyotrophic lateral sclerosis involves degeneration of both the upper and lower motor neurons resulting in progressive muscle, weakness, fasciculation, and atrophy. Eventually, all muscles under voluntary control are affected resulting in paralysis and respiratory failure. Most people with amyotrophic lateral sclerosis die from respiratory failure within 3–5 years from the onset of symptoms. Amyotrophic lateral sclerosis is one of the most common neuromuscular diseases worldwide which affects people of all races and ethnic backgrounds. As many as 30,000 Americans have amyotrophic lateral sclerosis, and an estimated 5000 people in the United States are diagnosed with the disease each year.

Amyotrophic lateral sclerosis is associated with mutations in the gene that produces the SOD1 enzyme. SOD1 is a powerful antioxidant that protects the body from damage caused by free radicals. Although it is not yet clear how the SOD1 gene mutation leads to motor neuron degeneration, researchers have theorized that an accumulation of free radicals may result from the faulty functioning of this gene. Animal studies have shown that motor neuron degeneration and deficits in motor function accompany the presence of the SOD1 mutation. However, studies involving transgenic mice have yielded a different theory about the role of SOD1 in amyotrophic lateral sclerosis. Mice lacking the SOD1 gene entirely do not develop amyotrophic lateral sclerosis, or exhibit any adverse reactions to the missing gene. This and the presence of SOD1 mutant proteins in amyloid-like neurofilaments indicate that SOD1 may act as an amyloid protein. Amyloid proteins exhibit a mutant conformation that can associate with other amyloids to form higher-order structures which may block cellular transport and other machinery necessary to the cell's proper function. Recent structural evidence suggests that some copper and zinc SOD1 mutations result in destabilization of normal dimers of the enzyme and promote aggregation, forming amyloid or pores (Koo *et al*., 1999; Hough *et al*., 2004). Therefore, stabilization of dimers has been proposed as a therapeutic intervention (Ray and Lansbury, 2004).

Autoimmune responses, which occur when the body's immune system attacks normal cells, have been suggested as one possible cause for motor neuron degeneration in amyotrophic lateral sclerosis. Some scientists theorize that antibodies may directly or indirectly impair the function of motor neurons, interfering with the transmission of signals between the brain and muscles. In searching for the cause of amyotrophic lateral sclerosis, researchers have also studied environmental factors such as exposure to toxic or infectious agents. Studies have also focused on the role of glutamate in motor neuron degeneration. Scientists have found that, compared to healthy people, amyotrophic lateral sclerosis patients have higher levels of glutamate in the serum and spinal fluid. Studies have demonstrated that neurons begin to die off when they are exposed over long periods to excessive amounts of glutamate. Scientists are trying to understand what mechanisms lead to a buildup of unneeded glutamate in the spinal fluid and how this imbalance could contribute to the development of amyotrophic lateral sclerosis. Some scientists also theorize that antibodies may directly or indirectly impair the function of motor neurons, interfering with the transmission of signals between the brain and muscles. Researchers have also studied environmental factors such as exposure to toxic or infectious agents.

### *SOD in amyotrophic lateral sclerosis*

Numerous studies on autosomal-dominant amyotrophic lateral sclerosis have provided evidence that oxidative stress may play an important role in the pathogenesis of amyotrophic lateral sclerosis. Transgenic expression of mutated CuZn SOD gene may lead to an amyotrophic lateral sclerosis phenotype despite normal or increased SOD activity. Although mutations in the SOD gene may not lead directly to the generation of ROS by a failure to detoxify superoxide, the gain of function mutations are likely to cause oxidative stress, e.g. by the formation of peroxynitrite. 3-Nitrotyrosine, a marker of peroxynitrite formation, is increased in cortex, spinal cord, and CSF of patients with autosomal-dominant or sporadic amyotrophic lateral sclerosis, as well as in transgenic animals (Ferrante *et al*., 1997; Tohgi *et al*., 1999). In addition, other markers of oxidative damage such as protein carbonyl and nuclear DNA 8OH20dG levels are increased in the motor cortex of sporadic amyotrophic lateral sclerosis patients (Ferrante *et al*., 1997).

Although the evidence for oxidative stress is strong in amyotrophic lateral sclerosis, the role of altered glutathione metabolism is less clear. Although changes in glutathione metabolism suggest enhanced generation of ROS and implicate increased concentrations of glutamate, insufficient research demonstrates glutathione concentrations in the cortex or spinal cord of amyotrophic lateral sclerosis patients. An increase of glutathione-binding sites in the spinal cord of amyotrophic lateral sclerosis patients may be construed as an upregulation caused by a deficiency of glutathione. (Lanius *et al*., 1993; Bains and Shaw, 1997).

## PROTECTION AGAINST NEURODEGENERATIVE DISEASES: TOCOPHEROL, ASCORBIC ACID, AND OTHER ANTIOXIDANTS

Nutrients have been used in clinical studies and are widely used by people with neurodegenerative diseases in order to partially treat the disease or slow down its deterioration. It has been suggested that following diets high in antioxidants may reduce the chances of developing neurodegenerative diseases (Grant, 1997). A number of *in vitro* studies have shown that antioxidants, both endogenous and dietary, can protect the nervous tissue from damage by oxidative stress (Urano *et al*., 1997). In an animal study by Tagami *et al*. (1998), tocopherol was shown to prevent cell death in rat neurons subjected to hypoxia followed by oxygen reperfusion and prevent neuronal damage from reactive nitrogen species. Tocopherol and $\beta$-carotene was also shown to protect rat neurons from oxidative stress caused by exposure to ethanol (Mitchell *et al*., 1999).

Most *in vivo* and clinical studies of the effects of lipid-soluble antioxidant supplementation on neurological diseases have focused on tocopherol or vitamin E and $\beta$-carotene. Vitamin C and tocopherol in large doses are commonly used by patients in order to lessen the cell damage that occurs in Parkinson's disease. The enzymes SOD and catalase require these vitamins in order to nullify the superoxide anion, a toxin commonly produced in damaged cells. Coenzyme Q10 has more recently been used for similar reasons.A report in 1991 demonstrated that ascorbic acid and synthetic tocopherol slowed the rate at which Parkinson's disease progressed in patient by 2.5 years (Fahn, 1991). In a Dutch study, it was found that the risk of Parkinson's disease was lower in subjects who had higher dietary intakes of antioxidants, particularly tocopherol (de Rijk *et al*., 1997). In another study, it was found that patients suffering from Parkinson's disease had consumed less of the small-molecule antioxidants $\beta$-carotene and ascorbic acid than did non-sufferers of the disease, implying that dietary antioxidants do play a protective role in this disease (Hellenbrand *et al*., 1996).

The L-dopa precursor L-tyrosine was also shown to relieve an average of 70% of neurological disease symptoms (Jankovic, 2001). Ferrous iron, the essential cofactor for L-dopa biosynthesis was shown to relieve between 10% and 60% of symptoms in 110 out of 110 patients (Birkmayer and Birkmayer, 1986).

## CONCLUSIONS

Neurodegenerative disorders constitute one of the major challenges of modern medicine and health professionals. Although these diseases are relatively common and often highly debilitating, the mechanisms responsible for their pathologies are poorly understood, and there are currently no effective preventative or therapeutic strategies. As demonstrated earlier, common themes occur in several neurodegenerative disorders. Slowly progressive neurodegenerative diseases are probably not the result of a single event, but rather a multi-cascade event/process involving environmental, epigenetic and genetic events. ROS may serve as common mediators in programmed cell death (apoptosis) or unprogrammed cell death (necrosis) in response to many different toxicants and pathological conditions. Recent studies demonstrated that oxidative stress and oxidative DNA damage may be involved in the cytotoxicity of chlorpyrifos, fenthion, and other pesticides, as well as heavy metals. Neurotransmitter systems play a key role in behavioral function and disturbances, and structurally dissimilar pesticides and heavy metal significant disrupt the system. Figure 20.5 exhibits a schematic diagram of the mechanistic pathways involved in the disease pathophysiology. Finally, much experimental evidence demonstrates that structurally diverse and dissimilar environmental pollutants including pesticides, tobacco, and heavy metals have adverse effects on dopamine, neurotransmitters, and brain functions. These cascade of events lead to altered gene expression and downregulation of antioxidant defense system and cause neurodegeneration.

Thus, the next generation of drug treatment will focus on combined therapies selective for several decisive events that characterize these disorders. Lowering the burden of protein aggregation, oxidative and nitrosative stress, mitochondrial injury, inflammatory response, and heavy metal accumulation in the brain so as to re-establish neurotransmission and block excitotoxicity may prove beneficial in the treatment of several neurodegenerative diseases.

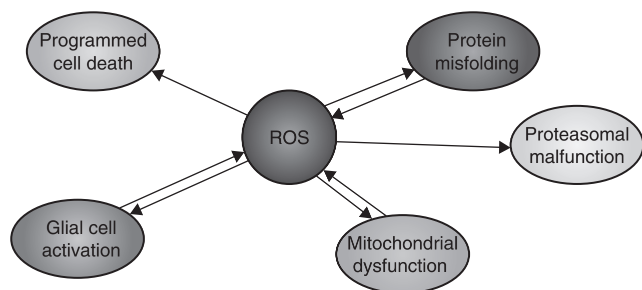



**FIGURE 20.5** ROS production as a major player in the cycle of events leading to neurodegeneration.

## REFERENCES


Aksenov MY, Tucker HM, Nair P, Aksenova MV, Butterfield DA, Estus S, Markesbery WR (1998) The expression of key oxidative stress-handling genes in different brain regions in Alzheimer's disease. *J Mol Neurosci* **11**: 151–64.

Amundon SA, Myers TG, Fornance Jr AJ (1998) Roles of p53 in growth arrest and apoptosis: putting on the brakes after genotoxic stress. *Oncogene* **17**: 3287–99.

Anderson R (1991) Assessment of the roles of vitamin C, vitamin E, and beta-carotene in the modulation of oxidant stress mediated by cigarette smoke-activated phagocytes. *Am J Clin Nutr* **53**: 358S–61S.

Aschner M, Allen JW, Kimelberg HK, LoPachin RM, Streit WJ (1999) Glial cells in neurotoxicity development. *Annu Rev Pharmacol Toxicol* **39**: 151–73.

Bagchi D, Hassoun EA, Bagchi M, Stohs SJ (1995a) Chromium-induced excretion of urinary lipid metabolites, DNA damage, nitric oxide production, and generation of reactive oxygen species in Sprague–Dawley rats. *Comp Biochem Physiol C Pharmacol Toxicol Endocrinol* **110**: 177–87.

Bagchi D, Bagchi M, Hassoun EA, Stohs SJ (1995b). *In vitro* and *in vivo* generation of reactive oxygen species, DNA damage and lactate dehydrogenase leakage by selected pesticides. *Toxicology* **104**: 129–40.

Bagchi D, Bagchi M, Hassoun EA, Stohs SJ (1996a) Cadmium-induced excretion of urinary lipid metabolites, DNA damage, glutathione depletion, and hepatic lipid peroxidation in Sprague–Dawley rats. *Biol Trace Elem Res* **53**: 143–54.

Bagchi D, Bhattacharya G, Stohs SJ (1996b) *In vitro* and *in vivo* induction of heat shock (stress) protein (Hsp) gene expression by selected pesticides. *Toxicology* **112**: 57–68.

Bagchi D, Bagchi M, Tang L, Stohs SJ (1997) Comparative *in vitro* and *in vivo* protein kinase C activation by selected pesticides and transition metal salts. *Toxicol Lett* **91**: 31–9.

Bagchi D, Bagchi M, Balmoori J, Vuchetich PJ, Stohs SJ (1998a) Induction of oxidative stress and DNA damage by chronic administration of naphthalene to rats. *Res Commun Mol Pathol Pharmacol* **101**: 249–57.

Bagchi D, Tran MX, Newton S, Bagchi M, Ray SD, Kuszynski CA, Stohs SJ (1998b) Chromium and cadmium induced oxidative stress and apoptosis in cultured J774A.1 macrophage cells. *In Vitro Mol Toxicol* **11**: 171–81.

Bagchi D, Balmoori J, Bagchi M, Ye X, Williams CB, Stohs SJ (2000) Role of p53 tumor suppressor gene in the toxicity of TCDD, endrin, naphthalene and chromium (VI) in liver and brain tissues of mice. *Free Rad Biol Med* **28**: 895–903.

Bagchi D, Stohs SJ, Downs BW, Bagchi M, Preuss HG (2002) Cytotoxicity and oxidative mechanisms of different forms of chromium. *Toxicology* **180**: 5–22.

Bagchi M, Hassoun EA, Bagchi D, Stohs SJ (1992) Endrin-induced increases in hepatic lipid peroxidation, membrane microviscosity, and DNA damage in rats. *Arch Environ Contam Toxicol* **23**: 1–5.

Bagchi M, Balmoori J, Bagchi D, Ray SD, Kuszynski C, Stohs SJ (1999) Smokeless tobacco, oxidative stress, apoptosis and antioxidants in human oral keratinocytes. *Free Rad Biol Med* **26**: 992–1000.

Bagchi M, Zafra S, Bagchi D (2006) DNA damage, gene expression, and carcinogenesis by organophosphates and carbamates. In *Toxicology of Organophosphate and Carbamate Compounds*, Gupta RC (ed.). Elsevier Academic Press, Amsterdam/New York, pp. 533–48.

Bains JS, Shaw CA (1997) Neurodegenerative disorders in humans: the role of glutathione in oxidative stress-mediated neuronal death. *Brain Res Rev* **25**: 335–58.

Barceloux D (1999) Chromium. *Clin Toxicol* **37**: 173–94.

Barone S, Das KP, Lassiter TL, White LD (2000) Vulnerable processes of nervous system development: a review of markers and methods. *Neurotoxicology* **21**: 15–36.

Becker S, Soukup JM, Gilmour M I, Devlin RB (1996) Stimulation of human and rat alveolar macrophages by urban air particulate: effects on oxidant radical generation and cytokine production. *Toxicol Appl Pharmacol* **141**: 637–48.

Behl C (1999) Alzheimer's disease and oxidative stress: implications for novel therapeutic approaches. *Prog Neurobiol* **57**: 301–23.

Betarbet R, Canet-Aviles RM, Sherer TB, Mastroberardino PG, McLendon C, Kim JH, Lund S, Na HM, Taylor G, Bence NF, Kopito R, Seo BB, Yagi T, Yagi A, Klinefelter G, Cookson MR, Greenamyre JT (2006) Intersecting pathways to neurodegeneration in Parkinson's disease: effects of the pesticide rotenone on DJ-1, alpha-synuclein, and the ubiquitin–proteasome system. *Neurobiol Dis* **22**: 404–420.

Birkmayer W, Birkmayer JGD (1986) Iron, a new aid in the treatment of Parkinson patients. *J Neural Transm* **67**: 287–92.

Botham PA (1990) Are pesticides immunotoxic. Adverse drug react. *Acute Poison Rev* **9**: 91–101.

Brawn MK, Chiou WJ, Leach KL (1995) Oxidant-induced activation of protein kinase C in UC11MG cells. *Free Rad Res* **22**: 23–37.

Buege JA, Aust SD (1972) Microsomal lipid peroxidation. *Method Enzymol* **52**: 302–10.

Bush AI, Masters CL, Tanzi RE (2003) Copper, $\beta$-amyloid, and Alzheimer's disease: tapping a sensitive connection. *Proc Natl Acad Sci USA* **100**: 11193–4.

Butterfield DA, Castegna A, Lauderback CM, Drake J (2002) Evidence that amyloid-peptide-induced lipid peroxidation and its sequelae in Alzheimer's disease brain contribute to neuronal death. *Neurobiol Aging* **23**: 655–64.

Cheng SY, Trombetta LD (2004) The induction of amyloid precursor protein and $\alpha$-synuclein in rat hippocampal astrocytes by diethyldithiocarbamate and copper with or without glutathione. *Toxicol Lett* **146**: 139–49.

Crumpton TL, Seidler FJ, Slotkin TA (2000) Developmental neurotoxicity of chlorpyrifos *in vivo* and *in vitro*: effects on nuclear transcription factor involved in cell replication and differentiation. *Brain Res* **857**: 87–98.

Dam K, Seidler FJ, Slotkin TA (1998) Developmental neurotoxicity of chlorpyrifos: delayed targeting of DNA synthesis after repeated administration. *Dev Brain Res* **108**: 39–45.

Danielsson BRG, Hassoun E, Dencker L (1982) Embryo toxicity of chromium: distribution in pregnant mice and effects on embryonic cells *in vitro*. *Arch Toxicol* **51**: 233–45.

Davies JE (1990) Neurotoxic concerns of human pesticides exposure. *Am J Ind Med* **18**: 327–31.

Deliconstantinos G, Villiotou V, Stavrides JC (1994a) Scavenging effects of hemoglobin and related heme containing compounds on nitric oxide, reactive oxidants and carcinogenic volatile nitrosocompounds of cigarette smoke. A new method for protection against the dangerous cigarette constituents. *Anticancer Res* **14**: 2717–26.

Deliconstantinos G, Villiotou V, Stavrides JC (1994b) Pathophysiology of nitric oxide in cancer. *Cancer Mol Biol* **1**: 77–86.

Ebadi M, Srinivasan SK, Baxi MD (1996) Oxidative stress and antioxidant therapy in Parkinson's disease. *Prog Neurobiol* **48**: 1–19.

Euler DE, David SJ, Guo H (1996) Effect of cigarette smoking on pentane excretion in alveolar breath. *Clin Chem* **42**: 303–8.

Facchinetti F, Dawson VL, Dawson TM (1998) Free radicals as mediators of neuronal injury. *Cell Mol Neurobiol* **18**: 667–82.

Fahn S (1991) An open trial of high-dosage antioxidants in early Parkinson's disease. *Am J Clin Nutr* **53**: 380S–2S.

Fahn S, Cohen G (1992) The oxidant stress hypothesis in Parkinson's disease: evidence supporting it. *Ann Neurol* **32**: 804–12.

Ferrante RJ, Browne SE, Shinobu LA, Bowling AC, Baik MJ, MacGarvey U, Kowall NW, Brown Jr RH, Beal MF (1997) Evidence of increased oxidative damage in both sporadic and familial amyotrophic lateral sclerosis. *J Neurochem* **69**: 2064–74.

Garcia SJ, Seidler FJ, Crumpton TL, Slotkin TA (2001) Does the developmental neurotoxicity of chlorpyrifos involve glial targets? Macromolecule synthesis, adenylyl cyclase signaling, nuclear transcription factors, and formation of reactive oxygen in C6 glioma cells. *Brain Res* **891**: 54–68.

German DC, Liang CL, Manaye KF, Lane K, Sonsalla PK (2000) Pharmacological inactivation of the vesicular monoamine transporter can enhance 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced neurodegeneration of midbrain dopaminergic neurons, but not locus coeruleus noradrenergic neurons. *Neuroscience* **101**: 1063–9.

Germansky M, Jamall S (1988) Organ-specific effects of naphthalene on tissue peroxidation, glutathione peroxidases, and superoxide dismutase in the rat. *Arch Toxicol* **61**: 480–3.

Goyer RA (1996) Toxic effects of metals. In *Casarett and Doull's Toxicology: The Basic Science of Poisons*, 5th edn, Klaassen CD (ed.). McGraw-Hill, New York, pp. 691–736.

Grant WB (1997) Dietary links to Alzheimer's disease. *Alzheimer's Dis Rev* **2**: 42–55.

Hall ED, Andrus PK, Oostveen JA, Fleck TJ, Gurney ME (1998) Relationship of oxygen radical-induced lipid peroxidative damage to disease onset and progression in a transgenic model of familial amyotrophic lateral sclerosis. *J Neurosci Res* **53**: 66–77.

Hamai D, Bondy SC, Becaria A, Campbell A (2001) The chemistry of transition metals in relation to their potential role in neurodegenerative processes. *Curr Top Med Chem* **1**: 541–51.

Heales SJ, Davies SE, Bates TE, Clark JB (1995) Depletion of brain glutathione is accompanied by impaired mitochondrial function and decreased *N*-acetyl aspartate concentration. *Neurochem Res* **20**: 31–8.

Hellenbrand W, Boeing H, Robra BP, Seidler A, Vieregge P, Nischan P, Joerg J, Oertel WH, Schneider E, Ulm G (1996) Diet and Parkinson's disease. II. A possible role for the past intake of specific nutrients. Results from a self-administered food frequency questionnaire in a case–control study. *Neurology* **47**: 644–50.

Hough MA, Grossmann JG, Antonyuk SV, Strange RW, Doucette PA, Rodriguez JA, Whitson LJ, Hart PJ, Hayward LJ, Valentine JS, Hasnain SS (2004) Dimer destabilization in superoxide dismutase may result in disease-causing properties: structures of motor neuron disease mutants. *Proc Natl Acad Sci USA* **101**: 5976–81.

Jain A, Martensson J, Stole E, Auld PA, Meister A (1991) Glutathione deficiency leads to mitochondrial damage in brain. *Proc Natl Acad Sci USA* **88**: 1913–17.

Jankovic J (2001) Parkinson's disease. A half century of progress. *Neurology* **57**: 1–3.

Jenner P (1996) Oxidative stress in Parkinson's disease and other neurodegenerative disorders. *Pathol Biol (Paris)* **44**: 57–64.

Julka D, Pal R, Gill KD (1992) Neurotoxicity of dichlorvos: effect of antioxidant defense system in rat central nervous system. *Exp Mol Pathol* **56**: 144–52.

Kaplan JG, Kessler J, Rosenberg N, Pack D, Schaumburg HH (1993) Sensory neuropathy associated with Dursban (chlorpyrifos) exposure. *Neurology* **43**: 2193–6.

Kieran D, Kalmar B, Dick JR, Riddoch-Contreras J, Burnstock G, Greensmith L (2004) Treatment with arimoclomol, a coinducer of heat shock proteins, delays disease progression in amyotrophic lateral sclerosis mice. *Nat Med* **10**: 402–5.

Koo EH, Lansbury Jr PT, Kelly JW (1999) Amyloid diseases: abnormal protein aggregation in neurodegeneration. *Proc Natl Acad Sci USA* **96**: 9989–90.

Lander HM, Ogiste JS, Teng KK, Novogrodsky A (1995) p21ras as a common signaling target of reactive free radicals and cellular redox stress. *J Biol Chem* **270**: 21195–8.

Lanius RA, Krieger C, Wagey R, Shaw CA (1993) Increased [35S] glutathione binding sites in spinal cords from patients with sporadic amyotrophic lateral sclerosis. *Neurosci Lett* **163**: 89–92.

Lord JM, Pongracz J (1995) Protein kinase C: a family of isoenzymes with distinct roles in pathogenesis. *Clin Mol Pathol* **48**: M57–64.

Lovell MA, Ehmann WD, Butler SM, Markesbery WR (1995) Elevated thiobarbituric acid-reactive substances and antioxidant enzyme activity in the brain in Alzheimer's disease. *Neurology* **45**: 1594–601.

Lovell MA, Xie C, Markesbery WR (1998) Decreased glutathione transferase activity in brain and ventricular fluid in Alzheimer's disease. *Neurology* **51**: 1562–6.

Lubek BM, Kabow S, Basu PK, Wells PG (1989) Cataractogenicity and bioactivation of naphthalene derivatives in lens culture and *in vivo*. *Lens Eye Toxicol Res* **6**: 203–9.

Manning FC, Blankenship LJ, Wise JP, Xu J, Bridgewater LC, Patierno SR (1994) Induction of internucleosomal DNA fragmentation by carcinogenic chromate, relationship to DNA damage, genotoxicity and inhibition of macromolecular synthesis. *Environ Health Perspect* **102**: 159–67.

Marcus DL, Thomas C, Rodriguez C, Simberkoff K, Tsai JS, Strafaci JA, Freedman ML (1998) Increased peroxidation and reduced antioxidant enzyme activity in Alzheimer's disease. *Exp Neurol* **150**: 40–4.

Menegon A, Board PG, Blackburn AC, Mellick GD, Le Couteur DG (1998) Parkinson's disease, pesticides, and glutathione transferase polymorphisms. *Lancet* **352**: 1344–6.

Mitchell JJ, Paiva M, Heaton MB (1999) Vitamin E and beta-carotene protect against ethanol combined with ischemia in an embryonic rat hippocampal culture model of fetal alcohol syndrome. *Neurosci Lett* **263**: 189–92.

Monnet-Tschudi F, Zurich MG, Schilter B, Costa LG, Honegger P (2000) Maturation-dependent effects of chlorpyrifos and parathion and their oxygen analogs on acetylcholinesterase and neuronal and glial markers in aggregating brain cell cultures. *Toxicol Appl Pharmacol* **165**: 175–83.

Morita K, Ishimura K, Tsuruo Y, Wong DL (1999) Dexamethasone enhances serum deprivation-induced necrotic death of rat C6 glioma cells through activation of glucocorticoid receptors. *Brain Res* **816**: 309–16.

National Institute of Neurological Disorders and Stroke, www.ninds.nih.gov (accessed December 8, 2006).

Ozawa T, Ueda J, Shimazu Y (1993) DNA single strand breakage by copper (II) complexes and hydrogen peroxide at physiological conditions. *Biochem Mol Biol Int* **31**: 455–61.

Perry TL, Yong VW (1986) Idiopathic Parkinson's disease, progressive supranuclear palsy and glutathione metabolism in the substantia nigra of patients. *Neurosci Lett* **67**: 269–74.

Perry TL, Yong VW, Bergeron C, Hansen S, Jones K (1987) Amino acids, glutathione, and glutathione transferase activity in the brains of patients with Alzheimer's disease. *Ann Neurol* **21**: 331–6.

Perry TL, Hansen S, Jones K (1988) Brain amino acids and glutathione in progressive supranuclear palsy. *Neurology* **38**: 943–6.

Poovala VS, Huang H, Salahudeen AK (1999) Role of reactive oxygen metabolites in organophosphate–bidrin-induced renal tubular cytotoxicity. *J Am Soc Nephrol* **10**: 1746–52.

Pope CN (1999) Organophosphorus pesticides: do they all have the same mechanism of toxicity? *J Toxicol Environ Health* **2**: 161–81.

Prasad MR, Jones RM (1992) Enhanced membrane protein kinase C activity in myocardial ischemia. *Basic Res Cardiol* **87**: 19–26.

Pryor WA, Stone K (1993) Oxidants in cigarette smoke. Radicals, hydrogen peroxide, peroxynitrate, peroxynitrite. *Ann NY Acad Sci* **686**: 12–28.

Qiao D, Seidler FJ, Slotkin TA (2001) Developmental neurotoxicity of chlorpyrifos modeled *in vitro*: comparative effects of metabolites and other cholinesterase inhibitors on DNA synthesis in PC12 and C6 cells. *Environ Health Perspect* **109**: 909–13.

Rahman MF, Mahboob M, Danadevi K, Saleha Banu B, Grover P (2002) Assessment of genotoxic effects of chloropyriphos and acephate by the comet assay in mice leucocytes. *Mutat Res* **516**: 139–47.

Ramstoeck ER, Hoekstra WG, Ganther HE (1980) Triakyllead metabolism and lipid peroxidation *in vivo* vitamin E- and selenium-deficient rats as measured by ethane production. *Toxicol Appl Pharmacol* **54**: 251–7.

Ray SS, Lansbury Jr PT (2004) A possible therapeutic target for Lou Gehrig's disease. *Proc Natl Acad Sci USA* **101**: 5701–2.

Retz W, Gsell W, Münch G, Rösler M, Riederer P (1998) Free radicals in Alzheimer's disease. *J Neural Transm* **54**, 221–36.

de Rijk MC, Breteler MM, den Breeijen JH, Launer LJ, Grobbee DE, van der Mechè FG, Hofman A (1997) Dietary antioxidants and Parkinson disease: the Rotterdam study. *Arch Neurol* **54**: 762–5.

Roses AD (1996) A lipoprotein E in neurology. *Curr Opin Neurol* **4**: 265–70.

Roy TS, Andrews J, Seidler FJ, Slotkin TA (1998) Chlorpyrifos elicits mitotic abnormalities and apoptosis in neuroepithelium of cultured rat embryos. *Teratology* **58**: 62–8.

Sano M, Ernesto C, Thomas RG, Klauber MR, Schafer K, Grundman M, Woodbury P, Growdon J, Cotman CW, Pfeiffer E, Schneider LS, Thal LJ (1997) A controlled trial of selegiline, alpha-tocopherol, or both as treatment for Alzheimer's disease. The Alzheimer's Disease Cooperative Study. *N Engl J Med* **336**: 1216–22.

Schulz JB, Beal MF (1994) Mitochondrial dysfunction in movement disorders. *Curr Opin Neurol* **7**: 333–9.

Schwarz M, Buchmann A, Stinchcombe S, Luebeck G, Moolgavkar S, Bock KW (1995) Role of receptors in human and rodent carcinogenesis. *Mutat Res* **333**: 69–79.

Seaton CL, Tjeerdema RS (1995) Comparative disposition and biotransformation of naphthalene in fresh- and seawater-acclimated striped bass (*Morone saxatilas*). *Xenobiotica* **25**: 553–62.

Selkoe DJ (2003) Folding proteins in fatal ways. *Nature* **426**: 900–4.

Shi X, Dalal NS (1989) Chromium (V) and hydroxyl radical formation during the glutathione reductase-catalyzed reduction of chromium (VI). *Biochem Biophys Res Commun* **163**: 627–34.

Shintzky M, Barrenholz Y (1978) Dynamics of the hydrocarbon layer in liposomes of lecithin and sphingomyelin containing diacetal phosphate. *J Biol Chem* **249**: 2652–7.

Slotkin TA (1999) Developmental cholinotoxicants: nicotine and chlorpyrifos. *Environ Health Perspect* **107**: 71–80.

Smith MA, Richey Harris PL, Sayre LM, Beckman JS, Perry G (1997) Widespread peroxynitrite-mediated damage in Alzheimer's disease. *J Neurosci* **17**: 2653–7.

Sparks DL, Schreurs BG (2003) Trace amounts of copper in water induce $\beta$-amyloid plaques and learning deficits in a rabbit model of Alzheimer's disease. *Proc Natl Acad Sci USA* **100**: 11065–9.

Spina MB, Cohen G (1989) Dopamine turnover and glutathione oxidation: implications for Parkinson disease. *Proc Natl Acad Sci USA* **86**: 1398–1400.

Stohs SJ (1995) Synthetic pro-oxidants: drugs, pesticides and other environmental pollutants. In *Oxidative Stress and Antioxidant Defenses in Biology*, Ahmad S (ed.). Chapman and Hall, New York, pp. 117–80.

Stohs SJ, Bagchi D (1995) Oxidative mechanisms in the toxicity of metal ions. *Free Rad Biol Med* **18**: 321–36.

Stohs SJ, Bagchi D, Bagchi M, Hassoun EA (1997) Generation of reactive oxygen species, DNA damage and lipid peroxidation in liver by structurally dissimilar pesticides. In *Liver and Environmental Xenobiotics*, Rana SVS, Taketa K (eds). Narosa-Springer Verlag Publishing House, New Delhi, pp. 102–13.

Sugiyama M (1991) Effects of vitamins on chromium(VI)-induced damage. *Environ Health Perspect* **92**: 63–70.

Tagami M, Yamagata K, Ikeda K, Nara Y, Fujino H, Kubota A, Numano F, Yamori Y (1998) Vitamin E prevents apoptosis in cortical neurons during hypoxia and oxygen reperfusion. *Lab Invest* **78**: 1415–29.

Takeuchi H, Kobayashi Y, Yoshihara T, Niwa J, Doyu M, Ohtsuka K, Sobue G (2002) Hsp70 and Hsp40 improve neurite outgrowth and suppress intracytoplasmic aggregate formation in cultured neuronal cells expressing mutant SOD1. *Brain Res* **949**: 11–22.

Tohgi H, Abe T, Yamazaki K, Murata T, Ishizaki E, Isobe C (1999) Remarkable increase in cerebrospinal fluid 3-nitrotyrosine in patients with sporadic amyotrophic lateral sclerosis. *Ann Neurol* **46**: 129–31.

Urano S, Asai Y, Makabe S, Matsuo M, Ohtsubo K, Izumiyama N, Endo T (1997) Oxidative injury of synapse and alteration of antioxidative defense systems in rats, and its prevention by vitamin E. *Eur J Biochem* **245**: 64–70.

Viviani A, Lutz WK, Schlatter C (1978) Time course of the induction of aryl hydrocarbon hydroxylase in rat liver nuclei and microsomes by phenobarbital, 3-methyl cholanthrene, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, dieldrin and other inducers. *Biochem Pharmacol* **27**: 2103–8.

Von Burg R, Lui D (1993) Chromium and hexavalent chromium. *J Appl Toxicol* **13**: 225–30.

Vuchetich PJ, Bagchi D, Bagchi M, Hassoun EA, Tang L, Stohs SJ (1996) Naphthalene-induced oxidative stress in rats and the protective effects of vitamin E succinate. *Free Rad Biol Med* **21**: 577–90.

Winklhofer KF, Henn IH, Kay-Jackson PC, Heller U, Tatzelt J (2003) Inactivation of parkin by oxidative stress and C-terminal truncations: a protective role of molecular chaperones. *J Biol Chem* **278**: 47199–208.

Xu J, Kao SY, Lee FJ, Song W, Jin LW, Yankner BA (2002) Dopamine-dependent neurotoxicity of $\alpha$-synuclein: a mechanism for selective neurodegeneration in Parkinson disease. *Nat Med* **8**: 600–6.

C H A P T E R

# 21

# Radiation and radioactive materials

*John A. Pickrell*

## INTRODUCTION

Although radiation cannot be seen, its interaction with matter is understood at a theoretical and partially at an experimental level. Its effects in humans have been extensively explored in large studies (Harley, 2001). Studies include the radium dial painters, survivors of the atomic bomb, patients with ankylosing spondylitis, children treated for ringworm (*Tinea captis*) and uranium miners exposed to radon soil gas, and to the short-lived radon daughter isotopes (Harley, 2001).

Models of radiation damage have been developed. For many years, inadvertent releases of ionizing radiation have been universally feared. Alternatively it has been used in controlled, defined amounts as radiation therapy for certain tumors in both companion animals and man. All studies provide the same picture of radiation toxicity. There are sufficient details of radiation effects to make credible estimates of risk from radiation exposure (Harley, 2001).

Since acute radiation toxicity becomes apparent shortly after exposure, history is an important criterion in determining whether the radiation is related to the cause of a particular complication or adverse effect. In contrast, late radiation toxicity in organs such as the kidneys, liver or central nervous system (CNS) will not be seen until months or perhaps even years after radiation exposure (Center for Drug Evaluation, 2005).

## BASIC RADIATION CONCEPTS

The four main types of irradiation are X-rays, gamma rays, electrons (negatively charged beta particles or positively charged positrons) and alpha particles. An atom can decay by the loss of mass (helium – loss of two protons and neutrons), the loss of a positively or negatively charged electron (beta particle or positron). Gamma irradiation occurs when excess energy is released from the nucleus (alpha, beta or positron transition) (Harley, 2001).

### Energy

$$\text{Energy} = \frac{1}{2}(\text{particle mass}) \times (\text{particle velocity})^2$$

Both high amounts of mass and high velocity can be used experimentally (artificially) to contrive releases of large amounts of energy (neutron activation (bombarding) of an atom).

Alpha particles have sufficiently low velocity relative to the speed of light that no correction is required. Thus, alpha particle energy = 0.511 (1 − velocity of particle$^2$/velocity of light$^2$) + 0.511. Gamma rays are pure electromagnetic radiation with energy = ($6.626 \times 10^{-34}$ J/s − Planck's constant) × (frequency of the radiation). Conventional units of radiation to be considered are one electron volt (1 eV) = $1.6 \times 10^{-19}$ J and one million electron volts (1 MeV) (Harley, 2001).

### Alpha particles

Alpha particles are a helium nucleus (two protons and two neutrons) ejected from the nucleus of a radioactive element $^{226}$Radium$_{86}$ decomposes to $^{222}$Radon$_{84}$ and an alpha particle ($^{2}He^{2++}$) + energy (5.2 MeV, range 4–8 MeV). There are several, discrete monoenergetic alphas emitted from an alpha emitter, not a continuous spectrum of emissions (Harley, 2001).

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### Beta particles, positron and electron capture

Beta particles are emitted when a neutron is converted to a proton plus an electron and the electron is lost. Positrons are emitted when a proton becomes a neutron and decays by beta emission or an electron is captured. These are competing processes, and both occur with about the same frequency (Harley, 2001).

### Gamma ray (photon) emission

Gamma ray emission is mostly secondary to an alpha, beta or positron emission or electron capture. When all of the energy is not used by the emission, the nucleus is in an excited state with energy available for emission. This excitation energy is emitted as a photon when the particle is emitted. In many cases the photon will not be emitted, but bind to an electron which is ejected as a monoenergetic particle equal to the photon energy minus the binding energy (Harley, 2001).

## INTERACTION OF RADIATION WITH MATTER

Ionizing radiation loses energy by producing ion pairs (an electron and a positively charged atom). About 33.85 eV is needed to produce an ion pair. This is ~2 times the energy needed for ionization, which is lost in the ion pair formation. Alpha and beta particles and gamma rays lose energy in somewhat different ways (Harley, 2001).

### Alpha particles

An alpha particle is heavily charged with a mass equal to 7300 times that of an electron. Yet energy can be transferred to particles going only twice as fast as the alpha particle. Since it requires 33.85 eV to produce an electron pair, a 5 MeV can produce ~7400 pairs within 1 μm of the decay. One micrometer (1/1,000,000 m; 1/1000 mm; 15% of the diameter of a human red blood cell) is much thinner than a sheet of paper. This linear energy transfer (LET) is said to be much greater than is needed to have deoxyribonucleic acid (DNA) strand breakage. Thus, it is said to have a high LET.

### Beta particles

Energy loss in matter cannot be simplified as it can in alpha particles because:

1 Even low energy beta emitters are traveling near the speed of light and must be corrected for mass increase.
2 Electrons are interacting with particles of the same mass; large energy losses are possible.
3 Radioactive (Bremstrahlung) energy loss from slowing down is appreciable (Harley, 2001) (with alpha particles they were not).

### Gamma rays

$$E\ (\mathrm{MeV\,m^2/kg}) = \text{mass energy absorption coefficients } (\mathrm{m^2/g^2})\ (\text{initial photon energy})$$

Mass energy absorption coefficients in air and in muscle are somewhat similar between 0.01 and 3.0 MeV. Energy loss/unit length is obtained by multiplying absorption coefficients by density ($kg/m^3$). Density is usually between 1 and 2.6 for most tissues. Thus, relative biological effectiveness (RBE) is not too different from 1. Rad (dose in air) is ~ roentgen equivalent in man (rem). This LET particle can penetrate much farther into tissue. Thus, radiation dose is distributed over much greater areas, tending toward diffuse as opposed to focal (Harley, 2001).

If lead shielding were used, density would be much greater, and penetration would be much less. If energy emitted is sufficiently low penetration can be taken to be negligible. A lead shield is often used when obtaining diagnostic X-rays as a safety feature. Some fraction of energetic gamma emitters (e.g. $^{60}$Cobalt) irradiation can penetrate even lead and added earth shielding. This radiation leakage may be as high as 1–10% of the source strength depending on the amount of shielding used if therapeutic gamma instead of X-irradiation is used.

## ABSORBED DOSE

### Total dose and dose rate

Total dose is the total energy absorbed per unit of mass; this measurement is independent of the time taken to deliver it (the dose rate) (Harley, 2001). If the dose is delivered quickly in a single exposure (high dose rate), there is almost no time to repair radiation damage and damage/unit of dose absorbed is high. Alternatively if the total dose is delivered quite slowly, say over the lifespan of the animal, there is more time to repair radiation damage and damage/unit of dose is much lower.

Very little time is required to initiate repair. For example, if multiple fractionated doses are delivered per hour, the damage/unit of total dose decreases significantly. Fractionated radiotherapy leads to the emergence of a more homogeneous population from cell repopulation

and by the high degree of radiosensitivity of the G2 cells that is a decrease in the percentage of G2 cells. Variations in the chromatin pattern of the cells may be explained by DNA repair processes (El-Khattabi *et al.*, 1997; Murata *et al.*, 1998; Harley, 2001). While these data may be relevant to higher radiation doses required for therapy where present tolerable doses are merely palliative, protraction does lengthen the time and therefore the cost required to deliver that dose.

Absorbed dose = mean energy deposited in mass divided by the mass of the target organ (Harley, 2001). Sometimes absorbed dose is called kerma (kinetic energy released in matter). Dose is delivered to target organs. Because exposure and dose are often used interchangeably, dose is often confused with exposure level. For comparison, exposure level is radiation in air = total radiation ions (one sign) per unit mass divided by mass.

## Equivalent dose and cancer risk

For alpha particles, with large mass produce intense ionization tracks per unit distance relative to beta particles. Beta particles produce more intense ionization than gamma rays. LET measures this transfer of energy per unit distance. If dose is considered to be proportional to response, that dose must be normalized for LET. For a particular endpoint (e.g. cell death in mouse fibroblasts), frequently one calculates relative biological effectiveness (RBE). RBE = dose of radiation under study/dose of gamma rays to achieve the same effect.

We calculate equivalent dose as equivalent dose = (dose) (weighting factor (RBE)).

Weighting factors recommended are ~1 for X-rays, gamma rays, beta particles and electrons and 20 for conventional neutrons (>>>0.1–2 MeV), protons, alpha particles and charged particles of unknown energy.

For example, the weighting factor and RBE of plutonium-238 ($^{238}Pu$) is about 10–30. Thus, the rem is 10–30 roentgen dose in air (rad s). This radiation dose pattern following alpha emission is confined to an immensely small area and is said to be focal as opposed to being diffuse if LET were operating. It is not uncommon to focal dose corresponding to focal response in alpha particle exposure (Harley, 2001). Thus, one might see millions of *burned out zones* or indicators of cell death following alpha emission if microscopic resolution was sufficiently good.

The term effective dose allows comparison of cancer and genetic risks from different partial body and whole body doses, to be integrated for the purposes of estimating a whole body response. Some tissues (gonads weighting factor = 0.2) are more sensitive than others (red bone marrow, colon, stomach and lung weighting factors = 0.12; bladder, breast, liver, esophagus and thyroid weighting factors = 0.05). Skin and bone surface are the least sensitive (weighting factors = 0.01). Other tissues not specified are taken to have a weighting factor of 0.05 as a conservative estimate (cited in Harley, 2001).

It is useful to calculate doses using the tissue weighting factor in protracted exposures (exposures over significant periods of time). Most non-experimental companion or domestic animals do not receive protracted exposures. A major exception to this is the animal receiving repeated fractionated doses as radiation therapy, or the relatively rare animals in which radiation has been implanted so that it acts as an internal emitter of radioactivity (Harley, 2001).

## Committed equivalent dose

Once radioactivity is placed internally it becomes an internal emitter. Internal emitters are used infrequently either therapeutically or with accidental exposure via ingestion or inhalation; internal emitters may be removed only with varying degrees of difficulty (Harley, 2001). Usually they cannot be removed simply or easily and irreversible committed equivalent dose is said to exist. For radionuclides with half-lives 0–3 months the equivalent dose is equal to the annual dose of the year of intake (Harley, 2001). For a radionuclide with a half-life of 3 months, a years exposure would allow the passage of four half-lives so that fraction of dose delivered was $(1-\frac{1}{2}^{4})$ ~94% of the total dose for all time. Those with shorter half-lives would approach 100% more closely (half-life of 1 month $(1-\frac{1}{2}^{12})$ ~99.98% of the total dose for all time).

## Negligible individual risk level (negligible dose)

Radiation is feared because its delivery cannot be seen, and is often not measured, because it is only in retrospect that one realizes that radiation exposure has occurred. We accept the concept of linear, non-threshold cancer induction from ionizing radiation (Harley, 2001); this allows calculation of cancer risk regardless of how small (or large) the dose may be. Calculating very low risks leads us to calculating a risk that one would consider acceptably low, that is below the ability to detect a response (Harley, 2001).

The National Council for Radiation Protection (NCRP) has identified a negligible individual risk level (NIRL) as a level of annual excess risk fatal risks of health effects attributable to irradiation below which further effort to reduce radiation to the individual is unwarranted. The NCRP emphasized that this level should not be confused with an acceptable risk level, a level of significance or a

standard. The NCRP recommended a level ~½ the natural background radiation level; the final recommended NIRL level is 1 mrem; this level is now called negligible individual dose (NID) level (Harley, 2001).

Effective dose was a parameter used to assess biological risk related to radiation exposure, from dual-energy X-ray absorptiometry (DXA). Children are a worst-case estimate because they absorb higher doses than adults. With the exception of the hip scans in 1- and 5-year-old children, the effective doses were below the NID limit of 1 mrem/year (Thomas *et al.*, 2005).

By way of comparison, NCRP has set the allowable human exposure to avoid cancer at 2000 mrem/year (2 rem/year). Total lifetime exposure was taken to be 1000 mrem/year (1 rem/year) × the person's age. This level is more than 1000 times the level defined as NIRL (NID) (Harley, 2001).

### Radiation safety for diagnostic imaging

Diagnostic imaging with radiation must be performed with proper protection (Kahn and Line, 2005). Although exposure factors are substantially lower than with past diagnostic imaging, considerable radiation dose may be delivered over time if proper protection is not worn. Lead gloves will lower external radiation dose ~1000-fold from scatter radiation, not in the direct radiation beam, but only 10-fold from radiation when directly in the beam of irradiation. Thyroid and eye shields are recommended. For example, upper limb and skull studies are especially likely to result in substantial exposure to anyone holding the film or horse (Kahn and Line, 2005).

## MECHANISMS OF DNA DAMAGE AND MUTAGENESIS

### Energy deposition in the cell nucleus

Radiation is released and slows down by forming ion pairs (Harley, 2001). Different ion densities result in formation of alpha particles, beta particles or gamma rays. Tract structure is roughly characterized into sparsely populated (low LET) and densely populated (high LET) tracts. Each tract from X-irradiation of gamma rays results in a 70 events across the width of the nucleus for ~0.5 rad s. Alternatively, an alpha track may produce many 30,000 of events across the same nucleus for ~300 rad s.

Within a cell indirect effects occur within nanometers of the direct effects. Bigger tracts are caused by radiation that is high LET. Low doses of high LET result in a few cells being hit by a single track. Low LET radiation tends to be spread out over the cells more evenly. One gray of radiation will produce 1000 single strands and less double strand breaks (~40); intermediate levels of damage were seen in base damage (500 breaks) and DNA protein crosslinks (150 breaks). About 30% of the double strand breaks are complex because of additional breaks (Harley, 2001).

### *In vitro* irradiation studies

Small lymphocytes from horses were transformed into larger radioresistant lymphoblast-like cells following stimulation by phytohemagglutinin, *in vitro*; 100 rad s X-irradiation killed all the small lymphocytes, but only ~1/3 of the lymphoblast-like cells (Dewey and Brannon, 1976). Horse lymphocytes were more radiosensitive than human lymphocytes. The ratios between doses inducing the same effect are 1.3, 1.7 and 9.4 for the number of binucleated cells with micronuclei, micronucleus frequency in binucleated cells and DNA synthesis inhibition, respectively (Catena *et al.*, 1997).

In cattle, at any LET value, repair is much slower after heavy ion exposure than after X-irradiation. For ions with an LET of <10,000 keV/μm more than 90% of the strand breaks induced are repaired within 24 h. At the highest LET value (16,300 keV/μm) no significant repair is observed (Baumstark-Khan *et al.*, 2003). Thus, a steep dose–response effect is seen at higher doses.

The base-line frequency of sister chromatic exchange (SCEs) was similar in the three species and no significant variation in man, cattle or muntjac (deer) cells was not different even after administration of 400 rad of X-rays (Das and Sharma, 1983). Pre-irradiation of artificial vessel walls substantially decreased the wall's capabilities to resist fibrosarcoma-induced lysis and abilities of blood vessels to limit extravasation and perhaps invasion (Heisel *et al.*, 1984).

X-rays induce DNA damage including strand breaks that lead to formation of micronuclei and chromosomal aberrations; X-irradiation was associated with an increased number of apoptotic cells (Konopacka and Rzeszowska-Wolny, 2006). This phenomenon was termed "bystander effect". A number of studies suggest that bystander effect appears to be associated with up-regulation of oxidative metabolism. The factors causing micronucleation by X-irradiation, oxidative DNA damage and incomplete repair may be regulated by apoptosis-independent pathways (Konopacka and Rzeszowska-Wolny, 2006).

Immature B lymphocytes in the chicken bursa of fabricius have previously been reported to undergo apoptosis by low doses of ionizing radiation. Increase of the pyknotic cells in number was observed at a dose of as little as 1 Gy. The chicken bursal cells are hypersensitive to X-irradiation with regard to induction of apoptosis, and that the apoptotic bursal cells exhibit most of the ultrastructural features known to be typical of apoptosis (Arai *et al.*, 1996).

# ANIMAL EXPOSURES AND RADIATION TOXICITY

## Introduction

Animals may show acute irradiation-induced changes from high single doses of radiation either from external or internal emitters. Alternatively, irradiation regimens may be devised which have high radiation dose rates. Irradiation of cells likely to be most sensitive to high doses of irradiation may be used to model the changes expected to be seen from these radiation doses. High external doses are most commonly accumulated from either an X-irradiation source or a $^{60}$Cobalt gamma ray source. Usually diagnostic X-irradiation will not be sufficiently high to produce acute symptoms. However, if therapeutic irradiation is given as a single dose, high doses of external irradiation may be sustained. Most often very high doses of external irradiation given for therapeutic purposes are fractionated over several to many doses, allowing more total radiation dose to be sustained without causing clinical signs of radiation toxicity to appear, because of the lower radiation dose rate. Mammary tumors are sometimes treated with external irradiation. The radiation field commonly includes the areas occupied by the intestines and lungs. Less commonly, fugitive releases (reactor accidents) may release radioactive iodine which localizes to irradiate the thyroid glands.

The highest levels of this type of radiation produce CNS changes. It is unlikely that anything but a gross miscalculation of radiation dose and dose rate would lead to this high level of radiation. At ultra high external irradiation doses, people and animals die quickly with associated CNS nervous signs and death at <1 week. Disruption of the blood–brain barrier (BBB) occurs after radiation injury; it is detectable by magnetic resonance imaging or horseradish peroxidase studies. For example, rats, irradiated at 60 Gy, were serially sacrificed at 2–24 weeks showed a detectable disruption of the BBB at 2 weeks post-irradiation; disruption of BBB preceded white matter necrosis (Rubin *et al*., 1994). In humans, one pilot study administered 55–60 Gy which as a single exposure would have been fatal; however, they hyperfractionated this dose in 110 cGy fractions. The hyperfractionation allowed completion of the course of radiotherapy. Tumor regression was noted, with minimal reoccurrence of tumors and no overt radiation toxicity (Fontanesi *et al*., 1995). Thus, hyperfractionation allows delivery of high doses that were therapeutic to tumors.

At high levels of this type of irradiation, but below the levels capable of inducing CNS signs, the short-lived intestinal epithelial cells die in massive amounts and gastrointestinal (GI) mucosal changes are said to occur. Death usually occurs in 1–2 weeks. Again, only the worst radiation accident or miscalculation of dose would lead to exposures of this level. For example, Chernobyl had fatalities in 30 of ~3000 staff members, but no record of GI changes being the predominant cause of death (Harley, 2001).

At lower levels there can still be appreciable clinical contributions from GI mucosal changes; great effort is made to minimize the importance of these to people or animal survival. One such way of improving chances of survival from radiation therapy is directing the radiation toward the field of the tumor; we call this involved field radiation therapy (IFRT) (Albuquerque *et al*., 2005). This is relatively simple to effect in large animals such as humans; it is more difficult to sufficiently focus the beam sufficiently for the same advantages in smaller dogs. Twenty ovarian cancer patients treated with 50.4 Gy of directed radiation were 66% 5-year recurrence free and 33% disease free. Thus, directed beam radiation therapy that would have been fatal if not directed minimized the consequences of human ovarian cancer (Albuquerque *et al*., 2005). After radiation treatment for gynecological cancer, patients gained more body fat than expected in Chilean women around menopause; the loss of fat-free mass observed during radiation treatment was probably associated with infrequent physical activity (Pia de la Maza *et al*., 2004). The extent to which this effect occurs older dogs has not been determined.

At still lower doses failure renewal of blood cells from bone marrow aplasia or hypoplasia may occur. These occur at low enough doses that radiotherapy may induce them clinically. Hyperfractionation of radiation dose helps minimize the consequences of these changes, by lowering the overall dose rate. When human external beam irradiation 50–70 Gy was combined with cisplatin chemotherapy to treat head and neck cancer 6% of the patients had groin hematomas requiring no treatment. In addition, 6% of the patients had mucosal events; hematologic changes were less frequent at 2% (Gemmette, 2003). Intensive induction chemotherapy combined with external beam irradiation therapy of children with brain stem gliomas produced objective improvements in 50% of the cases; this treatment course also produced severe but manageable hematologic toxicities. The data suggest that future radiation therapy regimens may require hematologic toxicities to be managed (Benesch *et al*., 2001). IL-17A is a T-cell-derived proinflammatory cytokine required for microbial host defense (Tan *et al*., 2006). *In vivo* expression profoundly stimulates granulopoiesis. Knockout mice establish IL-17A as an inducible mechanism that is required for recovery of granulopoiesis after radiation injury (Tan *et al*., 2006).

Depending on dose, external irradiation may lead to different types of reproductive toxicity in the male companion animal (Scialli *et al*., 1995 and De Celis *et al*., 1996 cited in Ellington and Wilker, 2006). At very high doses, it may lead to permanent aspermia. At intermediate doses it may lead to reduction in sperm numbers. Finally, at lower doses

external irradiation may lead to DNA alterations in sperm cells (Scialli *et al.*, 1995; and De Celis *et al.*, 1996 cited in Ellington and Wilker, 2006).

Finally, at lower levels, but with continuing irradiation, degenerative changes, for example cancer, are of the greatest concern. Usually, these changes are caused by fugitive releases of radiation (reactor accidents). The Chernobyl Reactor Accident released massive quantities of $^{131}$Iodine causing increased thyroid cancer in Belarus (>100 thyroid cancers/year). Less than 1/3 of this increase was seen in the Ukraine and the Russian Federation (Harley, 2001). No such tumors are reported for companion dogs, but they were similarly exposed and some tumors may have occurred. Widespread radioactivity was released across much of Europe and has been recorded >10,000 miles around the world at Fiji Atoll. Increased $^{90}$Strontium was observed in European cow's milk near the time of Chernobyl.

Thirteen dogs with invasive thyroid carcinoma were treated by four once-weekly fractions of 9 Gy of 4 MeV X-rays of external beam irradiation (Brearley *et al.*, 1999). Four of the dogs died from primary thyroid carcinoma and four from metastatic spread. Of the remaining five dogs, three died of unrelated problems, and two were still alive at the time of the census. Median survival time from first dose to death from either primary or metastatic disease was 96 weeks; with a range was 6–247 weeks. Radiation therapy should be considered an important modality for the control of invasive canine thyroid carcinoma (Brearley *et al.*, 1999).

Animals receiving fractionated external radiation therapy for mammary tumors may develop chronic pulmonary degenerative disease because the thorax is irradiated. To achieve the desired therapeutic effect, the dose may be too high to heal by primary intent; thus, they will heal by secondary intent – fibrosis. Type II cell proliferation is a common early event in radiation toxicity to the lungs. Following 10–15 Gy of X-irradiation, first wave of type II cell proliferation correlated with an increase in surfactant in alveolar fluids (Coggle, 1987). The second wave follows an additional delay allowing the alveolar epithelial continuity to be sufficiently compromised by the low rates of type I alveolar pneumocyte loss; type I cell loss triggered a compensatory wave of type II cell divisions that contrasted with the dramatic and immediate hyperplastic responses which many toxic irritants produce in type II epithelial cells (Coggle, 1987). At later times, fractionated external thoracic irradiation 1–2 times weekly to achieve 4000–6000 rad s modestly increased total lung collagen (Pickrell *et al.*, 1975; Pickrell *et al.*, 1983). Collagen production was initially increased, leading to the increase in total lung collagen; collagen metabolism returned to a more normal level at later times (Pickrell *et al.*, 1978; Pickrell *et al.*, 1983).

The relation of static compliance of excised lungs to collagen accumulation and histologic fibrosis was examined in Syrian hamsters inhaling sufficient high LET $^{238}PuO_2$ particles to achieve initial lung burdens of 50 or 100 nCi (Pickrell *et al.*, 1983). Hamsters exposed to 50 nCi $^{238}PuO_2$ showed normal collagen content and static lung compliance with minimal histologic fibrosis 288 days after exposure. In contrast, hamsters exposed to 100 nCi had significant pulmonary fibrosis at that time and the highest incidence of dense scars at any time period. Such findings are consistent with a stiffening of lung parenchyma. Hamsters had increased total lung collagen, reduced lung compliance and histologic evidence of diffuse interstitial fibrosis. The diffuse interstitial fibrosis developed by this injury resolves spontaneously as indicated by total lung collagen, compliance and histology; dense fibrous scars, however, do not resolve (Pickrell *et al.*, 1983; Pickrell and Abdel-Mageed, 1995).

Total body irradiation-induced changes in expression of CD25 and CD71 activation markers on the surface of lymphocytes, suggesting that radiation may alter tumor surveillance. Taken together, the relative percentages and activation status of immune cell compartments support the conclusion that these total body irradiation-induced changes function to slow tumor progression.

## Consequences of radiation therapy

When X-ray therapy (XRT) has been given, radiation injury is often limited to organs within the radiation beams. The risk of radiation injury to an organ is determined by the organ's radiosensitivity and by the concentration time–activity curve of the agents in that organ or at a specific anatomical target. For example, late radiation effects can occur if the kidneys receive a significant radiation absorbed dose. The kidneys are known to have a relatively low radiation tolerance dose (23 Gy for conventionally fractionated XRT); therefore, late radiation nephritis may be a dose-limiting toxicity. Radiation fibrosis is a common sequelae to XRT for lung cancer (Pickrell *et al.*, 1975; Pickrell *et al.*, 1978; Pickrell and Pickrell-Mageed, 1995; Center for Drug Evaluation, 2005).

It is possible to predict the effect of treatment in cancer in a non-invasive manner by apoptosis imaging *in vivo* after radiotherapy, by using $^{125}$I-annexin V. Brain tumors were implanted in susceptible (nude) mice. By 6 h after receiving levels of X-irradiation as low as 2 Gy both autoradiography and immunohistochemical staining showed more apoptosis in the tumors of irradiated groups than in the control group (Watanabe *et al.*, 2006).

Therapeutic brain irradiation can cause progressive decline in cognitive function, particularly in children, but the reason for this effect is unclear. Neocortical neurons of very young mice are more susceptible to radiation-induced apoptosis than are older mice. However, this sensitivity decreases rapidly after birth. By 14 days after parturition, acute cell loss due to radiation occurs primarily in non-neuronal populations (Nakaya *et al.*, 2005).

Poly (ADP-ribose) polymerase-1 (PARP-1) facilitates the repair of DNA strand breaks (Calabrese *et al*., 2004). Inhibiting PARP-1 increases the cytotoxicity of DNA-damaging chemotherapy and radiation therapy *in vitro*. But classical PARP-1 inhibitors have limited clinical utility. AG14361 is, to our knowledge, the first high-potency PARP-1 inhibitor with the specificity and *in vivo* activity to enhance chemotherapy and radiation therapy of human cancer (Calabrese *et al*., 2004). GR205171 has the most potent anti-emetic activity of any tachykinin NK1 receptor antagonist described to date. The compound is orally active in the ferret and dog, long-lasting, and warrants further investigation as a potential broad-spectrum anti-emetic agent (Gardner *et al*., 1996).

Three-dimensional dose-rate/time/response surfaces for chronic exposure to carcinogens and ionizing radiation clarify the interactive roles of competing risks (Raabe, 1987). The three dimensions are average dose rate, exposure time and risk. The improved conceptualization afforded by them contributes to the planning and evaluation of epidemiological analyses and experimental studies involving chronic exposure to radiation toxicants (Raabe, 1987).

## Conclusions

Radiation is of concern because it cannot be seen, exposure is frequently not painful, but it can cause significant biological effects. Alpha and neutron particles are the largest radiation particles, they penetrate the least and deliver the highest radiation energy per unit distance. Their dose patterns resemble multiple tiny foci of radiation damage. When irradiation interacts with matter, alpha and neutrons have 20 times the injurious cancer causing potential than beta irradiation and gamma rays have.

Cancer causing or apoptotic potentials are related to the interaction of ionizing radiation (neutron, alpha, beta or gamma) with the critical target cell nucleus. In veterinary medicine, the greatest potential for acute radiation damage lies in accidents releasing the contents of nuclear reactors such as Chernobyl in Belarus, radiation cancer therapy, most commonly for dog mammary tumors, or a gross miscalculation of irradiation dose needed for diagnostic imaging. Radiation fatalities at the highest dose can affect the CNS, perhaps by damaging the BBB. At a lower dose they can damage intestinal epithelium with rapid turnover, causing the GI syndrome, at a still lower level that can damage blood cells causing the hematologic syndrome.

Fractionating the radiation dose dramatically reduces the probability of getting an acute reaction from radiation therapy. If the dose is protracted over long time periods (months instead of hours to minutes), there is danger of chronic degenerative disease. For example, children who receive relatively low radiation doses appear to lost measurable cognitive function. The degree to which this is a lower dose manifestation of the CNS syndrome is not understood. When radiation is in the lung area, pulmonary fibrosis may develop; post-radiation nephritis is not uncommon. Dr. Raabe's 1987 model for three-dimensional dose-rate/time/response surfaces after chronic exposure to ionizing radiation by dose fractionation has helped us to understand the interactive roles of competing risks from therapeutic irradiation.

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## REFERENCES

Albuquerque KV, Singla R, Potkul RK, Smith DM, Creech S, Lo S, Emami B (2005) Impact of tumor volume-directed involved field radiation therapy integrated in the management of recurrent ovarian cancer. *Gynecol Oncol* **96**(3): 701–4.

Arai S, Kowada T, Takehana K, Miyoshi K, Nakanishi YH, Hayashi M (1996) Apoptosis in the chicken bursa of fabricius induced by X-irradiation. *J Vet Med Sci* **58**(10): 1001–6.

Baumstark-Khan C, Heilmann J, Rink H (2003) Induction and repair of DNA strand breaks in bovine lens epithelial cells after high LET irradiation. *Adv Space Res* **31**(6): 1583–91.

Benesch M, Lackner H, Moser A, Kerbl R, Schwinger W, Oberbauer R, Eder HG, Mayer R, Wiegele K, Urban C (2001) Outcome and long-term side effects after synchronous radiochemotherapy for childhood brain stem gliomas. *Pediatr Neurosurg* **35**(4): 173–80.

Brearley MJ, Hayes AM, Murphy S (1999) Hypofractionated radiation therapy for invasive thyroid carcinoma in dogs: a retrospective analysis of survival. *J Small Anim Pract* **40**(5): 206–10.

Calabrese CR, Almassy R, Barton S, Batey MA, Calvert AH, Canan-Koch S, Durkacz BW, Hostomsky Z, Kumpf RA, Kyle S, Li J, Maegley K, Newell DR, Notarianni E, Stratford IJ, Skalitzky D, Thomas HD, Wang LZ, Webber SE, Williams KJ, Curtin NJ (2004) Anticancer chemosensitization and radiosensitization by the novel poly(ADP-ribose) polymerase-1 inhibitor AG14361. *J Natl Cancer Inst* **96**(1): 56–67.

Catena C, Asprea L, Carta S, Tortora G, Conti D, Parasacchi P, Righi E (1997) Dose–response of X-irradiated human and equine lymphocytes. *Mutat Res* **373**(1): 9–16.

Center for Drug Evaluation and Research (2005) Pharmacology and Toxicology, United States Department of Health and Human Services, Rockville, MD.

Coggle JE (1987) Proliferation of type II pneumonocytes after X-irradiation. *Int J Radiat Biol Relat Stud Phys Chem Med* **51**(3): 393–9.

Das BC, Sharma T (1983) Blood lymphocyte culture system: quantitative analysis of X-ray-induced chromosome aberrations in man, muntjac and cattle. *Mutat Res* **110**(1): 111–39.

De Celis R, Pedron-Nuevo N, Feria-Velasco A (1996) Toxicology of male reproduction in animals and humans. *Arch Androl* **37**: 201–18.

Dewey WC, Brannon RB (1976) X-irradiation of equine peripheral blood lymphocytes stimulated with phytohaemagglutinin *in vitro*. *Int J Radiat Biol Relat Stud Phys Chem Med* **30**(3): 229–46.

El-Khattabi O, Pauwels O, Simon S, Gasperin P, Fruhling J, Kiss R, Van Houtte P (1997) *In vivo* characterization by means of digital cell image analysis of early-induced fractionated radiotherapy effects on the MXT mouse mammary tumor. *Int J Radiat Oncol Biol Phys* **37**(3): 673–8.

Ellington JE, Wilker CE (2006) Reproductive toxicology of the male companion animal. In *Small Animal Toxicology*, Peterson ME, Talcott PA (eds). Saunders-Elsevier, St Louis, MO, pp. 500–18.

Fontanesi J, Heideman RL, Muhlbauer M, Mulhern R, Sanford RA, Douglass EC, Kovnar E, Ochs JJ, Kuttesch JF, Tai D, *et al*. (1995) High-activity $^{125}$I interstitial irradiation in the treatment of pediatric central nervous system tumors: a pilot study. *Pediatr Neurosurg* **22**(6): 289–97.

Gardner CJ, Armour DR, Beattie DT, Gale JD, Hawcock AB, Kilpatrick GJ, Twissell DJ, Ward P (1996) GR205171: a novel antagonist with high affinity for the tachykinin NK1 receptor, and potent broad-spectrum anti-emetic activity. *Regul Pept* **65**(1): 45–53.

Gemmette JJ (2003) Complications associated with selective high-dose intraarterial cisplatin and concomitant radiation therapy for advanced head and neck cancer. *J Vasc Interv Radiol* **14**(6): 743–8.

Harley NH (2001) Toxic effects of radiation and radioactive materials. In *Casarett and Doull's Toxicology*: *The Basic Science of Poisons*, 6th edn, Klaassen CD (ed.). McGraw Hill Inc., New York, pp. 917–42.

Heisel MA, Laug WE, Stowe SM, Jones PA (1984) Effects of X-irradiation on artificial blood vessel wall degradation by invasive tumor cells. *Cancer Res* **44**(6): 2441–5.

Kahn CM, Line S ( 2005) Diagnostic imaging. In *The Merck Veterinary Manual*. Merck and Co., Whitehorse Station, NJ, pp. 1368–79.

Konopacka M, Rzeszowska-Wolny J (2006) The bystander effect-induced formation of micronucleated cells is inhibited by antioxidants, but the parallel induction of apoptosis and loss of viability are not affected. *Mutat Res* **593**(1–2): 32–8.

Miller GM, Kim DW, Andres ML, Green LM, Gridley DS (2003) Changes in the activation and reconstitution of lymphocytes resulting from total-body irradiation correlate with slowed tumor growth. *Oncology* **65**(3): 229–41.

Murata O, Sakurai H, Mitsuhashi N, Hasegawa M, Yamakawa M, Kurosaki H, Hayakawa K, Niibe H (1998) 31P NMR spectroscopy can predict the optimum interval between fractionated irradiation doses. *Anticancer Res* **18**(6A): 4297–301.

Nakaya K, Hasegawa T, Flickinger JC, Kondziolka DS, Fellows-Mayle W, Gobbel GT (2005) Sensitivity to radiation-induced apoptosis and neuron loss declines rapidly in the postnatal mouse neocortex. *Int J Radiat Biol* **81**(7): 545–54.

Pia de la Maza M, Agudelo GM, Yudin T, Gattas V, Barrera G, Bunout D, Hirsch S (2004) Long-term nutritional and digestive consequences of pelvic radiation. *J Am Coll Nutr* **23**(2): 102–7.

Pickrell JA, Schnizlein CT, Hahn FF, Snipes MB, Jones RK (1978) Radiation-induced pulmonary fibrosis: study of changes in collagen constituents in different lung regions of Beagle dogs after inhalation of beta-emitting radionuclides. *Radiat Res* **74**: 363–77.

Pickrell JA, Harris DL, Hahn FF, Belasich JJ, Jones RK (1975) Biological alterations resulting from chronic lung irradiation. III. Effect of partial $^{60}$Co thoracic irradiation upon pulmonary collagen metabolism and fractionation in Syrian hamsters. *Radiat Res* **62**: 133–44.

Pickrell JA, Diel JH, Slauson DO, Halliwell WH, Mauderly JL (1983) Radiation-induced pulmonary fibrosis resolves spontaneously if dense scars are not formed. *Exp Mol Pathol* **38**(1): 22–32.

Pickrell JA, Abdel-Mageed AB (1995) Radiation-induced pulmonary fibrosis. In *Pulmonary Fibrosis*, *Volume 80 Lung Biology in Health and Disease*, Phan SH, Thrall RS (eds), Marcel Dekker, New York, NY, pp. 363–81.

Raabe OG (1987) Three-dimensional dose–response models of competing risks and natural life span. *Fundam Appl Toxicol* **8**(4): 465–73.

Rubin P, Gash DM, Hansen JT, Nelson DF, Williams JP (1994) Disruption of the blood–brain barrier as the primary effect of CNS irradiation. *Radiother Oncol* **31**(1): 51–60.

Scialli AR, Lione A, Boyle PGK (1995) *Reproductive Effects of Chemical, Physical and Biologic Agents*, *REPROTOX*. The Johns Hopkins Press, Baltimore, MD.

Tan W, Huang W, Zhong Q, Schwarzenberger P (2006) IL-17 receptor knockout mice have enhanced myelotoxicity and impaired hemopoietic recovery following gamma irradiation. *J Immunol* **176**(10): 6186–93.

Thomas SR, Kalkwarf HJ, Buckley DD, Heubi JE (2005) Effective dose of dual-energy X-ray absorptiometry scans in children as a function of age. *J Clin Densitom* **8**(4): 415–22.

Watanabe H, Murata Y, Miura M, Hasegawa M, Kawamoto T, Shibuya H (2006) *In-vivo* visualization of radiation-induced apoptosis using $^{125}$I-annexin V. *Nucl Med Commun* **27**(1): 81–9.

C H A P T E R

# 22

# Carcinogenesis: mechanisms and models*

*Supratim Choudhuri, Kirk Arvidson, and Ronald Chanderbhan*

## INTRODUCTION

Animals, like humans, have always been exposed to thousands of chemical substances in their daily lives. This exposure may come from the food they eat, the water they drink, the air they breathe, etc. A high level of exposure to many of these chemicals may cause cancer in humans and animals. Cancer has become an increasingly prominent disease in recent times but incidence of cancer had been documented through writings and drawings thousands of years ago. The earliest known descriptions of cancer were found from the writings in Egyptian papyri, discovered and deciphered in the late 19th century. Two of these papyri, which were written around 1600 BC and called "Edwin Smith" and "George Ebers" papyri, contain descriptions of various cancers and Egyptian medical practice of treating cancer. Hippocrates is credited with naming cancer as "karkinoma" (carcinoma) because a tumor looked like a "crab" (karkinoma is Greek for crab). Hippocrates believed that the body contained four types of "humors" (body fluids): blood, phlegm, yellow bile, and black bile. An excess of black bile collecting in various body sites was thought to cause cancer. The black-bile theory of cancer was supported by the influential Greek physician Galen and it dominated the scientific thought for over 1300 years. The black-bile theory of cancer was eventually replaced by the lymph theory of cancer in the 17th century. The lymph theory was developed from the discovery of the lymphatic system by the Italian surgeon Gaspare Aselli, and abnormalities of lymph was viewed as the primary cause of cancer. The lymph theory gained rapid support. The Scottish surgeon John Hunter (1723–1792) lent support to the lymph theory by hypothesizing that tumors grew from lymph that is constantly thrown out by the blood. The cellular origin of cancer was propounded by Rudolph Virchow in the late 19th century when he recognized that cells, even cancerous cells, were derived from other cells.

In keeping with the theories on the possible physiological causes of cancer, an increasing number of reports from Europe and England documented the association between occupation/chemical exposure and the development of cancer. For example, Paracelsus was probably the first to identify a chemical substance, arsenic disulfide, as the causative agent of lung cancer in the miners of Schneeberg and Joachimsthal, Germany. The Italian physician Bernadrino Ramazzini reported in 1713, the absence of cervical cancer and higher incidence of breast cancer in nuns and thought this was related to their celibacy. The English physician Percival Pott observed that young men in their twenties who had been chimney sweeps, had a high rate of cancer of the scrotum. He suggested that the causative agent might be chimney soot (tar) and recommended frequent washing and changing of clothes in order to reduce exposure to the soot. In 1761, John Hill in UK implicated the use of tobacco as snuff in the development of nasal polyps. In 1795, Sammuel Thomas von Soemmering in Germany reported the occurrence of lip cancer among pipe smokers. In 1895, Ludwig Wilhelm Carl Rehn reported the occurrence of bladder

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cancer among workers in German dye industry. Various epidemiological studies have since identified major environmental causes of cancer, which include both naturally occurring chemicals as well as man-made chemicals.

Experimental cancer research began in the early 20th century and has been continuing since. Recent advances in molecular genetics have provided researchers with additional tools to study the mechanisms and the molecular biology of cancer. The knowledge gained from such studies form the foundation of our understanding of the process of carcinogenesis. Today, chemical carcinogenesis is regarded as a multistep process with a long latency period. For a relatively detailed historical perspective of cancer, the reader is referred to Marquardt (1999), Pitot (2002), and the American Cancer Society website.

# TERMINOLOGY

*Cancer* is a term that is commonly used to indicate a group of diseases characterized by uncontrolled cell proliferation and usually the spread of these abnormal cells. In common parlance, the word cancer is near-synonymous with other expressions, such as *malignant tumor*, *malignant neoplasm*, *malignancy*, and *neoplasia*. Tumors or neoplasms (new growth of tissue) can thus be benign or malignant (cancerous). In this chapter, the expression "cancer" and "malignant neoplasm" will be used interchangeably. Agents causing cancer are called *carcinogens* and the process of cancer development is called *carcinogenesis*. Most neoplasms arise from the clonal expansion of a single cell that has undergone neoplastic transformation (hence monoclonal). According to Pitot (2002), *neoplasia* or the constituent lesion *neoplasm*, can be defined as a heritably altered, relatively autonomous growth of tissue. The "heritably altered" aspect reflects the abnormal genetic expression, which is either inherent in the neoplastic cells or is induced in response to environmental stimuli. Another term relevant to cancer is hyperplasia. *Hyperplasia* means an increase in cell number. Hyperplasia can be found in neoplastic as well as non-neoplastic conditions. Examples of non-neoplastic hyperplasia include callus formation, hepatomegaly (liver enlargement), etc. Hepatomegaly in response to various xenobiotic treatments usually involves both hyperplasia and hypertrophy (enlargement of cells).

In the parlance of cancer biology, the suffix "oma" means tumor, benign, or malignant. For example, *fibroma* and *lipoma* are benign neoplasms, while *melanoma*, *hepatoma*, and *seminoma* are malignant neoplasms. Sometimes even non-neoplastic lesions end with the "oma" suffix, such as *hematoma* and *granuloma*. Cancer of the embryonic tissue is denoted with the suffix "blastoma", such as *neuroblastoma* and *retinoblastoma*. Likewise, cancer of connective tissue, such as bone, cartilage, fat, muscle, and blood vessels, is denoted with the suffix "sarcoma", such as *fibrosarcoma*, *liposarcoma*, and *rhabdomyosarcoma*.

# CANCER EPIDEMIOLOGY

Cancer incidence data are available in many countries from registries that monitor the occurrence of different types of cancers in various populations. Epidemiological data show that certain cancers tend to occur more frequently among people in certain geographic locations. For example, breast and prostate cancers have lower incidence rates in Asian countries but higher incidence rates in Europe and North America (Haas and Sakr, 1997; McPherson *et al.*, 2000). In contrast, gastric cancer has lower incidence rate in economically developed Western countries including the USA, but higher incidence in Japan and Korea (Alberts *et al.*, 2003). These differences in cancer incidence reflect the role of both genetic and environmental factors. Strong evidence for the importance of environmental factors comes from studies on migrant populations. When there is a significant difference in the incidence of a specific cancer in the native country and the new host country, the migrant population acquires the cancer incidence rate of the host country. For example, Japanese populations in western countries typically acquire the higher breast and colon cancer rates of the host country within a generation or two. Incidence of cancer may also be influenced by gender. For example, gastric cancer shows about two-fold higher incidence in males than females in every region of the world studied (Alberts *et al.*, 2003). While knowledge of the genetic basis of cancer helps understand the molecular mechanism of carcinogenesis, knowledge of cancer causing environmental factors is crucial to making important health and environmental policy decisions that may have far-reaching impact on human and animal health.

# AGENTS CAUSING CARCINOGENESIS

The three main classes of agents (carcinogens) causing cancers are chemicals, radiation, and viruses. In this chapter, chemical carcinogenesis will be emphasized, while viral and radiation carcinogenesis will be discussed briefly.

## Chemical carcinogenesis

### *Chemical carcinogens*

Chemical carcinogens originate from both industrial and natural processes. In 1915, Yamagawa and Ichikawa pioneered the field of experimental cancer research when they produced tumors by repeated application of coal tars

**TABLE 22.1 Some known/suspected chemical carcinogens and their target organs**

| Chemicals/suspected carcinogens | Organs affected |
|---|---|
| Aflatoxin | Liver |
| 4-Aminobiphenyl | Bladder |
| Arsenicals | Lung, skin |
| Diesel exhaust | Lung |
| Benzene | Leukemia |
| Cigarette smoking | Lung |
| Pipe smoking | Lip |
| Soot | Scrotum |
| Dyes (aromatic amines, such as 2-naphthylamine) | Urinary bladder |
| Nickel compounds | Lung |
| Vinyl chloride | Liver |
| Radium (radioactive watch colors) | Bone |
| Formaldehyde | Nose |
| Snuff | Nose |
| Diethylstilbestrol | Genital tract |

Adapted from Marquardt, H. 1999. Chemical Carcinogens. In *Toxicology* (Eds. H. Marquardt, S.G. Schäfer, R.O. McClellan and F. Welsch), pp. 151–178, Copyright (1999) with permission from Elsevier.

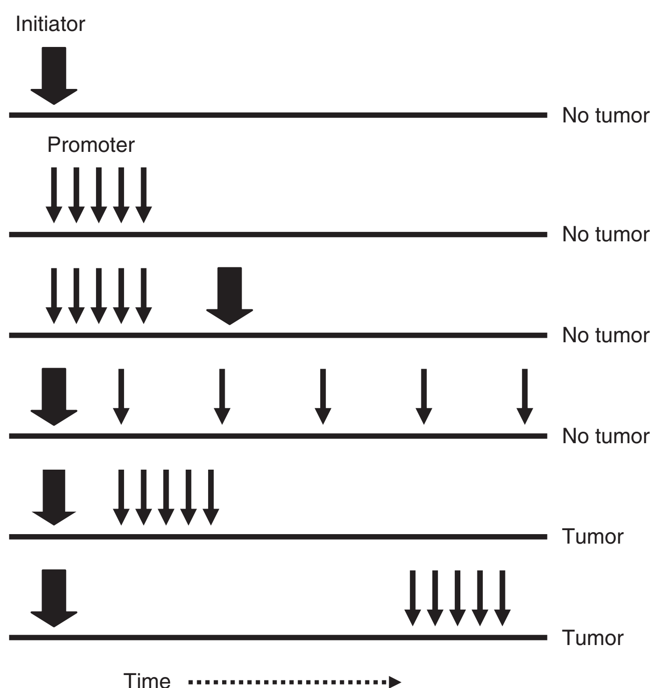


**FIGURE 22.1** The target tissue must be exposed to an initiator first followed by repeated exposure to promoters in order for the tumors to develop. If the time gap between the exposure to initiator and promoter varies from a week to a year, tumors will still develop. However, if the exposure to promoter is first and is followed by exposure to initiator, tumors will not develop. Adapted from Okey *et al*. 1998. In *The Basic Science of Oncology*, 3rd ed., edited by I.F. Tannock and R.P. Hill, with permissions from the McGraw-Hill Companies.

on the skin of rabbits. Many of the chemical carcinogens identified in early research were by-products of industrial processes. Subsequent studies have revealed the carcinogenic potential of many other industrial, naturally occurring chemicals, as well as man-made chemicals. Chemical carcinogens may be synthetic ("man made") or of natural origin, they are extremely diverse in structure and belong to very different chemical classes, such as inorganic, organic, fibers, plastic, hormones, etc.

Based on their biological activities, chemical carcinogens can be classified as *genotoxic carcinogens* (DNA-reactive) and *non-genotoxic carcinogens* (non-DNA-reactive, epigenetic). Genotoxic carcinogens can be further classified as *direct carcinogens* (active without metabolic activation) and *indirect carcinogens* or *procarcinogens* (active after metabolic activation).

Table 22.1 lists some of these chemical carcinogens and the organs affected by them.

### *Biological process of chemical carcinogenesis*

Experimental cancer research with carcinogenic chemicals led to the realization that chemical carcinogenesis is a multistep process. The pathogenesis of cancer involves many individual events, such as metabolic activation (biotransformation) of a procarcinogenic chemical to a DNA-reactive compound (in the case of indirect genotoxic carcinogens), covalent binding to DNA, induction of heritable mutations, expression of the mutant phenotype at the molecular level (e.g. altered gene expression) resulting in altered cellular function, cellular transformation and neoplastic growth, and spreading of the transformed cells to other parts of the body. These events in the pathogenesis of cancer have been distilled into three distinct steps to describe the multistep nature of carcinogenesis: the steps are *initiation*, *promotion*, and *progression* (Foulds, 1954). *Tumor initiation* involves introduction of heritable genetic change in a normal cell; *tumor promotion* involves clonal expansion of the initiated cell resulting in the formation of a benign tumor; and *tumor progression* involves the conversion of a benign tumor into a malignant one.

In order for the tumors to develop *the target tissue must be exposed to an initiator first, followed by repeated exposure to promoters*. If the time gap between the exposure to initiator and promoter varies from a week to a year, tumors will still develop. However, if the exposure to promoter is first and is followed by exposure to initiator, tumors will not develop. After the promotion event, the cancer cell acquires further heritable changes that lead to malignancy and metastasis (the progression step). Figure 22.1 shows how sequential exposure to tumor initiator and tumor promoter leads to the development of cancer.

#### *Initiation*

In the initiation phase, the chemical or its reactive metabolite causes a permanent change in the DNA of the target cell(s), such as a mutation, a distortion of the DNA structure with further consequences, elimination of a component of DNA (bases or sugars), or errors in DNA repair (Pitot, 2002). Thus, initiation is a genetic process. The chemical

causing initiation is called an *initiator*. Once a target cell is initiated, initiation is irreversible. An initiated cell by itself is not a cancer cell because it has not acquired the property of uncontrolled growth. Additionally, all initiated cells do not produce tumors because many undergo programmed cell death or *apoptosis*. In order for an initiated cell to transform into a cancer cell and eventually produce a detectable tumor, promotion is necessary.

Examples of tumor initiators are benz[a]pyrene, dimethylbenz[a]anthracene, 3-methylcholanthrene, 2-acetylamino-fluorene, dimethylnitrosamine (DMN), diethylnitrosamine, etc. Exposure to the tumor initiator *N*-methyl-*N*-nitrosourea (MNU) results in the modification of guanine into $O^6$-methylguanine in the DNA of the target tissue. $O^6$-methylguanine is a major tumor-initiating lesion because if it is not repaired, it leads to G:C→A:T transition mutation. The "suicide enzyme" $O^6$-methylguanine-DNA methyltransferase (MGMT) repairs this lesion. MGMT catalyzes the transfer of a methyl group from $O^6$-methylguanine as well as $O^4$-methylthymidine to an internal cysteine residue, thereby repairing the DNA lesion in a single step but at the same time inactivating itself. Transgenic mice overexpressing MGMT are less sensitive to tumor development in the tissue overexpressing the enzyme because of the increased repair of $O^6$-methylguanine (Becker *et al.*, 1996, 2003; Liu *et al.*, 1999; Qin *et al.*, 2000). Experiments with MGMT transgenic mice have also demonstrated that the $O^6$-methylguanine lesion is possibly also involved in tumor progression (Qin *et al.*, 2000; Becker *et al.*, 2003).

*Promotion*

Tumor promoters alter the expression of genetic information of the cell, as well as, in many cases, inhibit programmed cell death (Pitot, 2002). Tumor promoters usually influence the proliferation of initiated cells, which results in the proliferation of preneoplastic cells and formation of benign focal lesions, such as enzyme-altered foci in the liver, nodules in the mammary gland, polyps in the colon, and papillomas in the skin. Therefore, tumor promotion is an epigenetic process. In addition to causing cell proliferation, tumor promoters appear to block apoptosis, thus leading to accumulation of the initiated cells as dysfunctional, non-differentiated cells within a tissue. Some of the lesions that develop due to promotion regress, but others acquire additional mutations and progress to malignant neoplasm. Tumor promotion is a reversible process up to a certain stage if the promoter is withdrawn.

Tumor promoters tend to be organ specific. For example, phenobarbital, TPA (12-*O*-tetradecanoylphorbol-13-acetate, a phorbol ester isolated from croton oil) is skin specific. Chlordane, DDT (dichlorodiphenyltrichloroethane), TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin), β-naphthoflavone, peroxisome proliferators, and polybrominated biphenyls are hepatic tumor promoters; mirex (an organochlorine) is a promoter in both skin and liver; saccharin is a bladder tumor promoter.

*Progression*

Tumor progression involves autonomous growth and metastasis of the malignant neoplasm. At the cellular level, chromosomal breakage and deletion, duplication, or translocation of chromosomal fragments are hallmarks of tumor progression phase. Additional mutations in the oncogenes and tumor suppressor genes also accumulate during this phase. This is why tumor initiators can also cause tumor progression (Qin *et al.*, 2000; Becker *et al.*, 2003). Karyotypic instability and accumulation of additional mutations may reflect an ongoing selection of cells suitable for neoplastic growth and metastasis (Okey *et al.*, 1998).

Some putative tumor progression agents (with no initiator activity) that cause transition from promotion to progression include benzene, benzoyl peroxide, and 2,5,2′,5′ -tetrachlorobiphenyl (Okey *et al.*, 1998).

***Mode of action of chemical carcinogens***

Both direct and indirect carcinogens (procarcinogens) interact with DNA, forming DNA adducts. Indirect carcinogens acquire carcinogenic properties after metabolic activation, forming the *ultimate carcinogen*, which is the reactive metabolite that covalently modifies DNA. The biological activity of the carcinogens depends on a balance between their activation and detoxification in the target tissue.

The majority of these compounds interact with DNA through three different types of chemical reactions (Dipple, 1995; Okey *et al.*, 1998). The reactions involve the transfer of (1) an alkyl group, (2) an arylamine group, or (3) an aralkyl group to DNA. An arylamine contains an amine-substituted aromatic ring, while an aralkyl (arylalkyl) contains an alkyl-substituted aromatic ring. An example of a reactive arylamine group is arylnitrenium ion while an example of a reactive aralkyl group is benzyl radical.

The DNA-reactive groups are generated through specific reaction chemistries, such as oxidation at carbon—carbon double bonds yielding alkylating or aralkylating agents; oxidation or reduction at nitrogen producing arylaminating agents; conjugations of hydroxy compounds producing aralkylating or arylaminating agents; and conjugation between glutathione with dihaloalkanes producing alkylating agents. There are, however, some carcinogens that do not fit these categories, such as acylating agents, α,β-unsaturated aldehydes, chloroethylene oxide, etc. (Dipple, 1995).

*Alkylation and alkylating agents*

Alkylating agents add an electrophilic alkyl group ($R\text{-}CH_2^+$) to electron-rich (nucleophilic) sites in DNA. Carcinogens that transfer alkyl residues to DNA include

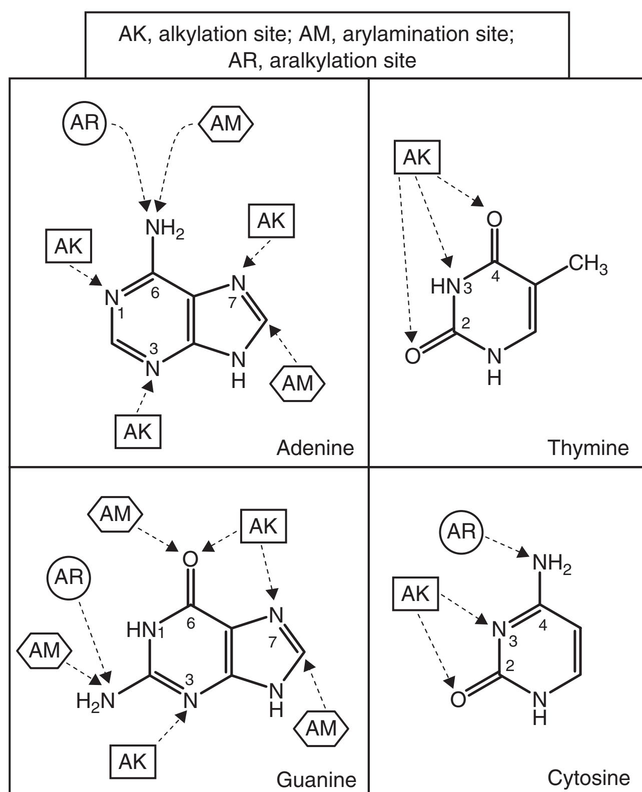


**FIGURE 22.2** DNA bases showing the sites where alkylation, arylamination, and aralkylation reactions can occur resulting in the formation of DNA adducts. N3 alkylation of guanine, although reported in the literature and shown in the figure, is less common than N7 alkylation. Adapted from Okey *et al.* 1998. In *The Basic Science of Oncology*, 3rd ed., edited by I.F. Tannock and R.P. Hill, with permissions from the McGraw-Hill Companies.

nitrosamines, aliphatic epoxides, aflatoxins, lactones, nitrosoureas, mustards, haloalkanes, aryl triazenes, and sultones (Dipple, 1995). The sites of substitution in DNA bases by alkylating agents are many and are indicated in Figure 22.2. Alkylating agents are produced by enzymatic reactions (e.g. P450 mediated). An example is cytochrome P450 2E1 (CYP2E1)-mediated metabolism of DMN (Figure 22.3). CYP2E1-mediated hydroxylation of a methyl group in DMN results in the formation of $\alpha$-hydroxynitrosamine, which undergoes spontaneous demethylation, releasing one molecule of formaldehyde and forming MMN (monomethyl nitrosamine). The MMN undergoes tautomerization to form diazonium hydroxide, which undergoes spontaneous decomposition to form the carbonium ion ($CH_3^+$). The carbonium ion is the alkylating agent. Larger *N*-nitroso compounds, such as tobacco-smoke-derived NNK ((4-methylnitrosamino)-1-(3-pyridyl)-1-butanone), are activated by cytochrome P450 1A2 (CYP1A2). Aflatoxin B1 is also bioactivated by P450. In humans, cytochromes P450 1A2 (CYP1A2) and P450 3A4 (CYP3A4) appear to be the most important in the metabolic activation of aflatoxin B1 (Van Vleet *et al.*,

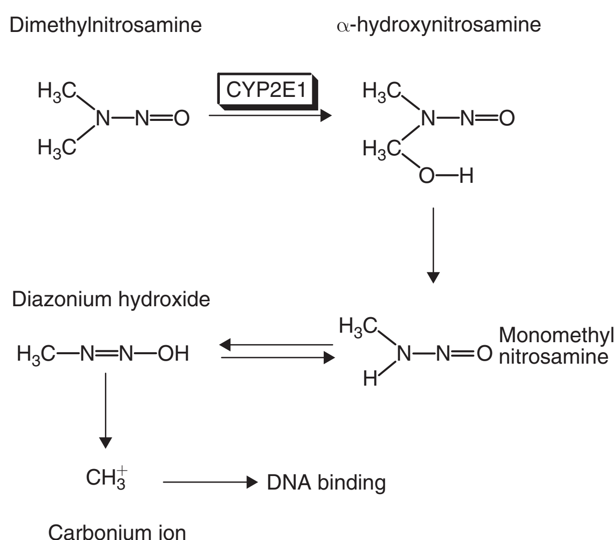


**FIGURE 22.3** Metabolic activation pathway for DMN. Adapted from Okey *et al.* 1998. In *The Basic Science of Oncology*, 3rd ed., edited by I.F. Tannock and R.P. Hill, with permissions from the McGraw-Hill Companies.

2002). Hydroxylation of the unsaturated C—C bond in aflatoxin B1 results in the formation of a reactive epoxide intermediate, the 8,9-epoxide (Figure 22.4). This aliphatic epoxide can intercalate into DNA by forming an alkyl adduct with guanine. This intercalation, resulting in a GC→TA transversion mutation at the third position of codon 249 of the p53 gene, is thought to be the major reason for aflatoxin-induced carcinogenesis (Smela *et al.*, 2001).

*Arylamination and arylaminating agents*

Aromatic amines (arylamines), amides, aminoazo dyes, heterocyclic amines, etc. all undergo metabolic activation forming highly reactive, electrophilic aryl nitrenium ions ($Ar—NH^+$). The major sites of substitution in DNA by arylaminating agents appear to be the C-8 position and the amino group of the purine nucleotides (Dipple, 1995). Potential arylamination sites in DNA bases by arylaminating agents are indicated in Figure 22.2. A prototypical example is the metabolic activation of 2-naphthylamine. *N*-oxidation by CYP1A2 forms the corresponding *N*-hydroxyarylamine (e.g. *N*-hydroxynaphthylamine), which can undergo a number of conjugation reactions forming sulfate, acetate, or glucuronide conjugates. These conjugates can be excreted in the urine. In the acidic pH of urine, the conjugate dissociates and the *N*-hydroxynaphthyl moiety is protonated to form the nitrenium ion (Figure 22.5). Formation of the nitrenium ion, which is the ultimate carcinogen, explains why 2-naphthylamine is carcinogenic in the urinary tract and urinary bladder. *N*-acetyltransferase (NAT), which catalyzes the transfer of acetyl groups from acetyl-CoA to arylamines forming acetyl

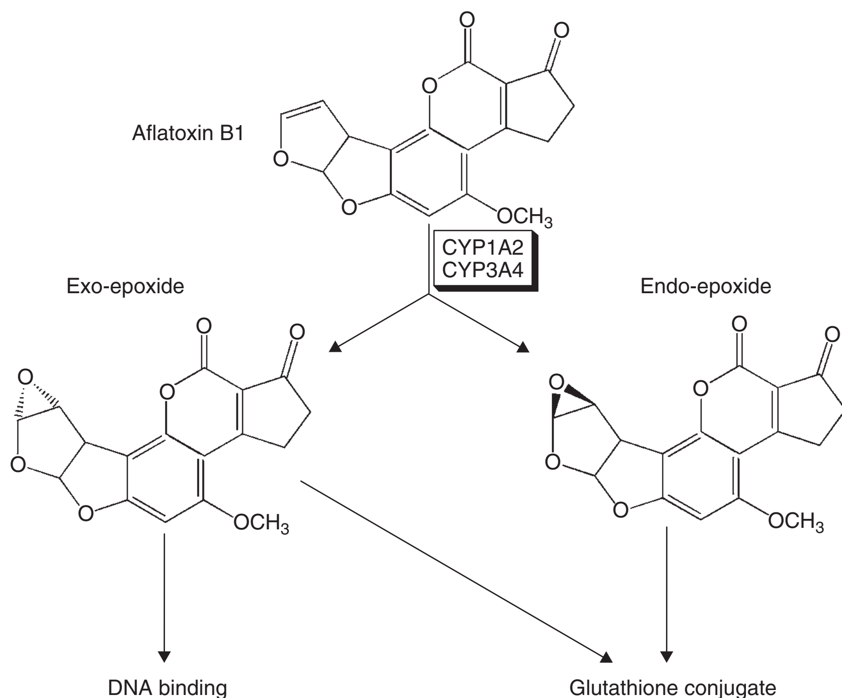


**FIGURE 22.4** Metabolic activation pathway for aflatoxin B1. Adapted from Okey *et al*. 1998. In *The Basic Science of Oncology*, 3rd ed., edited by I.F. Tannock and R.P. Hill, with permissions from the McGraw-Hill Companies.

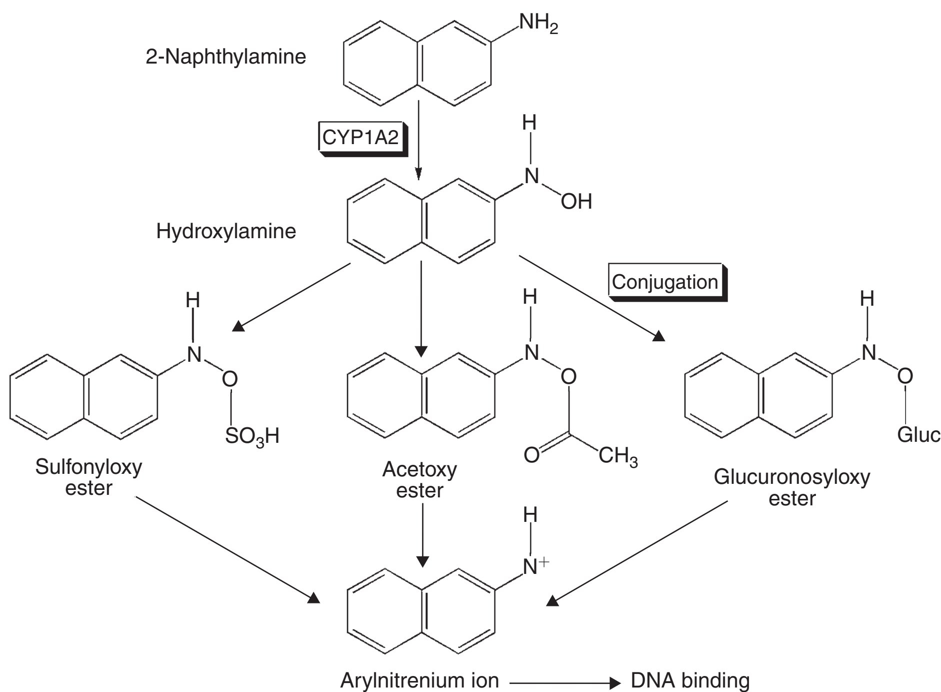


**FIGURE 22.5** Metabolic activation pathway for 2-naphthylamine. Adapted from Okey *et al*. 1998. In *The Basic Science of Oncology*, 3rd ed., edited by I.F. Tannock and R.P. Hill, with permissions from the McGraw-Hill Companies.

esters, shows a great deal of polymorphism across species. Among humans, there are slow and fast acetylators. In hamsters, acetylation is fast while in dogs and rats, acetylation is slow. These species differences in NAT activity as well as intraspecies polymorphism can greatly influence the toxicity outcome of the substances in question.

*Aralkylation and aralkylating agents*

Polycyclic aromatic hydrocarbons (PAHs) and related compounds are capable of transferring an aralkyl group to DNA. Carcinogens that transfer an aralkyl group to DNA include the PAHs, alkyl benzenes, pyrrolizidine alkaloids,

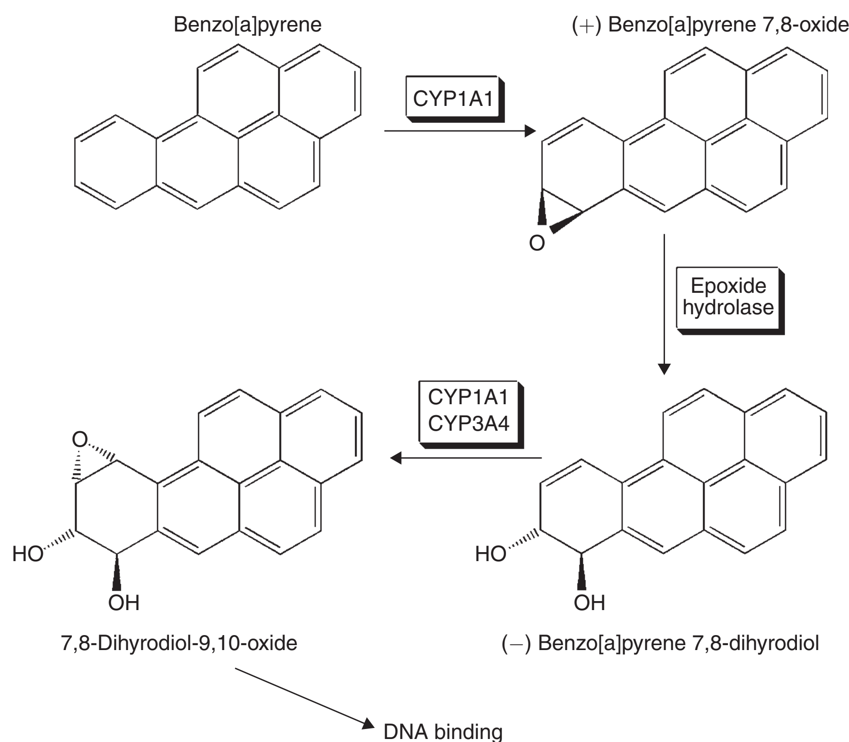


**FIGURE 22.6** Metabolic activation pathway for benzo[a]pyrene. Adapted from Okey *et al*. 1998. In *The Basic Science of Oncology*, 3rd ed., edited by I.F. Tannock and R.P. Hill, with permissions from the McGraw-Hill Companies.

and nitroaromatics that are activated through the formation of dihydrodiol epoxide (Dipple, 1995). A classic example of this class is benzo[a]pyrene, which is converted by cytochrome P450 1A1 (CYP1A1) to the 7,8-epoxide. This epoxide is hydrolyzed by epoxide hydrolase to the 7,8-dihydrodiol of benzo[a]pyrene, which again can be converted by CYP1A1 to the 7,8-dihydrodiol-9,10-epoxide of benzo[a]pyrene. This epoxide is stable, electrophilic and therefore it attacks the DNA bases (Figure 22.6). Potential aralkylation sites in DNA bases by aralkylating agents are indicated in Figure 22.2.

### *Cellular defense against DNA damage: DNA repair, and removal of DNA adducts*

DNA adducts (the chemical induced, covalently modified DNA bases) can alter the DNA structure and in turn, molecular processes, such as replication and transcription. If not repaired or repaired incorrectly, these modifications may ultimately lead to mutations and eventually cancer, particularly if the adduct is located in an oncogene or tumor suppressor gene. There are a number of mechanisms to repair DNA adducts: (1) direct repair, (2) base excision repair (BER), (3) nucleotide excision repair (NER), and (4) mismatch repair (MMR).

In *direct repair*, the bond between the nucleotide and the adduct is broken restoring the original conformation. For example, $O^6$-methylguanine ($O^6$-MeG) is repaired by MGMT (methylguanine-DNA methyltransferase) by direct demethylation of the methylguanine. If methylguanine is not repaired, it will pair with thymine instead of cytosine during replication. In the following replication cycle, this thymine will pair with adenine in the complementary strand. The net result is a G:C→A:T transition mutation in the DNA. Such G:C→A:T transition mutations are known to activate the *ras* oncogenes in nitrosourea-induced mouse lymphomas and rat mammary tumors, as well as in azoxymethane (AOM)-induced aberrant crypt foci (ACF) (Dumenco *et al.*, 1993, Zaidi *et al.*, 1995).

*BER* mechanism works mainly on non-bulky base modifications, such as those produced by methylation (in positions where it does not interfere with H-bonding, such as 3- or 7-methyladenine or 8-hydroxyguanine). These types of methylation patterns are produced by MNU. These inappropriate bases are recognized and removed by specific DNA glycosylases, which cleave the *N*-glycosidic bond between the base and the sugar. As a result, the base is removed and the sugar–phosphate backbone of the DNA remains intact. This creates a base gap in the DNA, also called an AP site (**ap**urinic or **ap**yrimidinic site). The AP site is then recognized by AP endonuclease, which introduces a nick 5′ to the AP site. A third enzyme, deoxyribophosphodiesterase (an excision endonuclease) then produces a second nick 3′ to the AP site and removes the baseless sugar–phosphate backbone. The resultant one nucleotide gap is filled in by DNA polymerase, and DNA ligase seals the nick.

*NER* is a versatile mechanism that can eliminate a wide range of structurally unrelated lesions. Bulky base modifications, such as benzo[a]pyrene–guanine adducts caused by smoking, cisplatin–guanine adducts and psoralen–thymine adducts resulting from chemotherapy,

UV-induced multiple thymine dimers, etc. are all repaired by NER. Humans do not have photolyase, so thymine dimers are corrected in humans by NER. The mechanism of NER involves generation of two nicks by an excision nuclease, one on each side of the lesion. As a result, a small portion of the affected DNA strand is removed. DNA polymerase then continues the repair synthesis and ligase seals the gap.

*MMR* mechanism repairs bases that violate Watson–Crick base pairing rules. The classic example is that of *Escherichia coli*. The sequence 5′ -GATC-3′in *E. coli* DNA is methylated at adenine, and the sequences 5′-CCAGG-3′and 5′ -CCTGG-3′are methylated at cytosine. When DNA replicates, the daughter-strand methylation is delayed. As a result, the newly synthesized daughter strand is always undermethylated compared to the parental strand. If there is a base misincorporation, the MMR machinery (MutS–MutH–MutL complex) identifies the misincorporated base by scanning the methylation status of both strands. The mismatched base is excised from the undermethylated daughter strand. The same principle applies to eukaryotic DNA MMR. Three eukaryotic protein heterodimers composed of Msh (**M**ut**S** **h**omologue) subunits recognize DNA mismatches with different but overlapping specificities: Msh2 · Msh3, Msh2 · Msh6, and (only in plants) Msh2 · Msh7. A second heterodimer, Mlh1 (**M**ut**L** **h**omologue1) · Pms2, couples mismatch recognition to excision of the error-containing nascent DNA. Deficiencies in Msh2 dramatically increase mutation rates (Hoffman *et al.*, 2004). For example, in HNPCC (hereditary non-polyposis colorectal cancer) and other human cancers, mutations in MMR genes, such as MSH2, MSH6, MLH1, PMS2, have been observed (Buermeyer *et al.*, 1999); by the same token, mice transgenic for Msh2 are tolerant to the carcinogenic effects of methylating agents (deWind *et al.*, 1995).

Apart from the above-mentioned mechanisms, two other mechanisms of DNA repair are recombination repair and strand break repair. In *recombination repair*, when DNA polymerase encounters an unrepaired lesion on the parental (template) strand during replication, it stops synthesis at that point and resumes synthesis at the next priming site. This leaves a gap in the daughter strand. This gap is filled by recombination with the other parental strand. This donor parental strand is then repaired by polymerase and ligase. *Strand break repair* may involve single- or double-strand breaks. *Single-strand breaks* do not disrupt the integrity of the DNA. The intact single strand is coated by PARP 1 (poly(ADP-ribose) polymerase-1) protein near the lesion site of the other strand. The single-strand break is then repaired as in excision repair. *Double-stranded breaks* are dangerous because they damage the integrity of the DNA. Double-stranded breaks can be repaired in two ways: either by crossing over or by synthesis-dependent strand-annealing repair. Various proteins (such as Ku) bind to the broken ends of the DNA to protect it and initiate the repair.

## Viral carcinogenesis

### *Tumor viruses, oncogenes, and tumor suppressor genes*

A number of cellular genes are now implicated in carcinogenesis. These genes are of two types: oncogenes and tumor suppressor genes. Broadly speaking, activation of oncogenes and inactivation of tumor suppressor genes may have similar consequences in terms of carcinogenesis. Oncogenes are found in cancer causing viruses which are of two types: DNA viruses with DNA genomes and RNA viruses with RNA genomes. DNA viruses with oncogenic potential are from six distinct viral families: hepatitis B viruses, simian virus 40 (SV40) and polyomavirus, papillomaviruses, adenoviruses, herpesviruses, and poxviruses. In contrast, members of only one family of RNA viruses, the retroviruses, are capable of inducing oncogenic potential (Cooper, 1995).

#### *Retroviral oncogenes and their evolution*

The discovery of retroviral oncogenes significantly advanced our understanding about the function of genes specifically responsible for the induction of abnormal cell proliferation. They replicate inside the cell through a DNA intermediate, called *provirus*, which is integrated into the chromosomal DNA of the infected cell. Transforming viruses (viruses capable of inducing malignant transformation of animal cells) do not need the oncogene for their replication. Viral oncogenes (*v-onc*) are derived from cellular proto-oncogenes (*c-onc*). Cellular proto-oncogenes contain introns while the corresponding viral oncogenes generally lack the introns. Thus, viral oncogenes originated in the host through recombination between the genome of a non-transforming retrovirus and a cellular oncogene. This has been demonstrated in studies on the *v-src* oncogene by Hanafusa and colleagues in the early 1980s.

Figure 22.7 shows how a cellular proto-oncogene (*c-onc*) could be acquired by the viral genome. Transforming viruses preferentially integrate at the fragile sites on the host genome. These fragile sites correspond to the hot spots of recombination. Integration of the provirus in the genome next to the *c-onc* produces a transcription unit under the control of the viral LTR (long terminal repeats). Transcription of this unit produces a viral/cellular hybrid RNA. Intact viral RNAs are also produced from other integrated proviruses. These intact viral RNAs and hybrid RNAs, through reverse transcription and recombination, captures the processed cellular proto-oncogene as part of the viral genome. The captured processed cellular proto-oncogene now becomes a retroviral oncogene (*v-onc*).

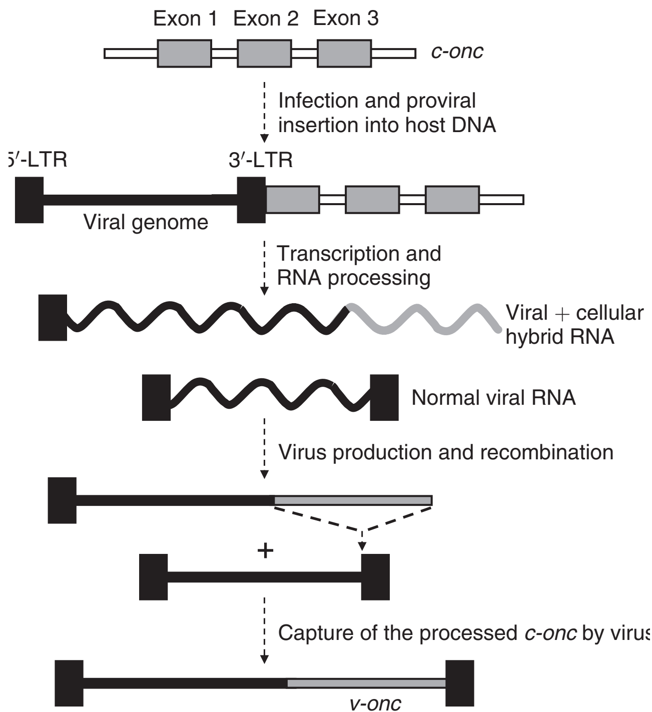


**FIGURE 22.7** Mechanism by which a cellular proto-oncogene (*c-onc*) is captured by retrovirus to give rise to a viral oncogene (*v-onc*).

*Activation of oncogenes*

Retroviral oncogenes (*v-onc*) are altered versions of normal cellular genes called proto-oncogenes. The function of the *v-onc* product is similar or identical to that of the proto-oncogene (*c-onc*) product, but its expression is generally unregulated. Proto-oncogenes may be activated by mutation, chromosomal re-arrangement, or gene amplification. Chromosomal re-arrangements that include translocations and inversions can activate proto-oncogenes by dysregulation of their transcription (e.g. transcriptional activation) or by gene fusion. For example, the product of the *ras* oncogene differs from that of the normal proto-oncogene by one base which is caused by a point mutation in the 12th codon (GGC→GTC) resulting in one amino acid change (Gly→Val). An example of oncogene activation through gene amplification is *myc*, which codes for a transcription factor that plays a role in cell division. One-third of all neuroblastomas shows *myc* oncogene amplification that inversely correlates with the prognosis. Such amplification of the *myc* oncogene results in the generation of a very high amount of its product. Generation of a high amount of *myc* oncogene product can also be due to high levels of transcription. This has been reported in Burkitt's lymphoma where translocation of the *myc* proto-oncogene from its normal location (chromosome 8) to chromosome 14 brings it close to the immunoglobulin heavy chain gene promoter. As a result, c-*myc* now finds itself in a region of vigorous gene transcription, with a consequent overproduction of its product. Another example of chromosomal translocation and dysregulation of proto-oncogene is found in chronic myelogenous leukemia (CML). In CML, a reciprocal translocation occurs between chromosomes 9 and 22 [t(9;22)]. A portion on the long arm of chromosome 9 (9q) containing the *abl* gene is translocated next to the *bcr* (breakpoint cluster region) gene on the long arm of chromosome 22 (22q). The altered chromosome 22 is called the Philadelphia chromosome (Ph′ ). The *bcr–abl* produces higher levels of a fusion protein Bcr–Abl that has protein kinase activity. Some other oncogenes associated with cancers of various organs are *hst* (stomach cancer), *met* (osteosarcoma), *bcl-1* (B-cell leukemia), etc.

*Inactivation of tumor suppressor genes*

Tumor suppressor genes, which also participate in the regulation of normal cell growth, are usually inactivated by point mutations or truncation of their protein sequence coupled with the loss of the normal allele. The first mutation may be inherited or somatic. The second mutation will often be a gross event leading to loss of heterozygosity and tumor suppressor function. This mechanism provides support to the two-hit hypothesis. In 1971, Alfred Knudson proposed the *two-hit hypothesis* in which he applied statistical analysis to compare patients with hereditary and non-hereditary forms of retinoblastoma. Knudson hypothesized that one germline copy of the damaged gene that is inherited in the hereditary form of retinoblastoma was not sufficient to trigger the development of this cancer. A second hit to or loss of the good copy in the gene pair was necessary to produce retinoblastoma. Frequent loss of heterozygosity in the tumor cells provides support to Knudson's hypothesis. Analysis of linkage markers in retinoblastoma cells and normal cells from the same individual shows that the markers that are heterozygous in normal cells are hemizygous in tumor cells, indicating the loss of the normal allele in the tumor cells (second hit).

*Functions of oncogene products (oncoproteins)*

Proto-oncogenes encode proteins that are mostly involved in the regulation of cell growth, division, and differentiation. Consequently, proto-oncogenes encode products that can act as growth factors, growth-factor-receptor-associated tyrosine kinases, membrane-associated non-receptor tyrosine kinases, G-protein-coupled receptors, membrane-associated G-proteins, serine–threonine kinases, transcription factors, and regulators of programmed cell death.

Growth factors play important roles in cell cycle control, and they signal a cell to either enter the G1 phase or bypass it. The right amount of growth factors should be produced at the right time, or else cell cycle control may be dysregulated. Examples of oncogene-encoded growth factors are *sis* oncogene-encoded platelet-derived growth factor (PDGF) B chain and *int*-2 oncogene-encoded

fibroblast growth factor (FGF)-related growth factor. Receptor-associated and non-receptor tyrosine kinases as well as G-proteins, G-protein-coupled receptors, and serine–threonine kinases all encode products that are important in regulating the transduction of mitogenic signals in the cell, thereby regulating normal cell division. In most cases, these oncogenes encode mutant forms of the proteins so that they are not subject to the on–off regulation in response to mitogenic signals. In other words, the mitogenic signal is perpetually "on", resulting in uncontrolled cell proliferation.

Oncogenes, such as *src* and *abl*, encode tyrosine kinases that are membrane associated, while *erb*B, *neu*, and *fms* encode transmembrane kinases. An example of an oncogene encoding a GTP-binding GTPase is *ras*. Normal GTP-binding proteins (G-proteins), including *ras*, are important signal transducers. The active form binds GTP and transduces the mitogenic signal; hydrolysis of GTP to GDP inactivates the protein and terminates the signal. Further activation involves exchange of GDP for GTP. Mutant forms of *ras* exist in an inactive GDP-bound form, resulting in a perpetual "on" mode of the mitogenic signal. Oncogenes encoding transcription factors also act by the same principle. The proto-oncogene products of *fos* (Fos) and *jun* (Jun) form a heterodimer to produce the transcription factor *activator protein-1* (AP-1). Mutant AP-1 may act in a constitutive manner without activation by agents, such as phorbol esters. Other oncogenes, such as *myc* and *myb*, also code for transcription factors that, when mutated, can cause aberrant transcriptional dysregulation.

The cell growth and division suppressor effects are also lost in mutant *p53* which is a tumor suppressor gene and whose product (p53 protein) is a transcription factor. The three dimensional structure of p53 protein with its target DNA molecule defined the core domain and the amino acids involved in DNA binding (Cho *et al.*, 1994). These amino acids that are involved in DNA binding show the highest mutation rate in various cancers. This demonstrates how mutations in tumor suppressor genes can abrogate their tumor suppressor function by disrupting the transcriptional regulation of their target genes. Another role of p53 is to regulate apoptosis or programmed cell death by upregulating the pro-apoptotic gene *Bax*. Mutant p53 cannot mediate apoptosis; thus cells with unrepaired DNA damage are prevented from undergoing apoptosis. Survival of these cells and their subsequent division may lead to the development of cancer cells.

Mutagenic chemicals that can disrupt the structure and/or expression of these proto-oncogenes can cause their oncogenic activation. For example, phorbol ester is a known activator of AP-1-mediated transcription. The procarcinogen 1,2-dimethylhydrazine has been reported to induce mutation in *ras* which was detectable in preneoplastic and neoplastic rat colonic mucosa (Jacoby *et al.*, 1991).

## Radiation carcinogenesis

### *Radiation dose and risk*

In the following discussion, radiation will refer to only ionizing radiation. A radiation dose to tissue is expressed as *absorbed energy per unit tissue mass*. *Gray* (Gy) is the unit of radiation dose and is quantified as 1 J/kg. The older unit *rad* is still used and 1 rad = 0.01 Gy. Carcinogenic potential depends on the absorbed dose (energy).

LET (linear energy transfer, $L$) is a measure of the rate at which energy ($E$) is deposited to the absorbing medium per unit distance ($l$) traversed by the radiation ($L = dE/dl$; if the distance traversed is measured in mm, then $L = \text{keV/mm}$). Consequently, high-LET radiations (e.g. $\alpha$-particles, neutrons, heavy ions, pions, also known pi mesons) will loose, i.e. deposit in the absorbing medium, greater amounts of energy than low-LET radiations (e.g. $\gamma$-rays, X-rays, electrons). In the case of tissue exposure, the energy deposited by the radiation causes ionizations and the generation of free radicals, which cause macromolecular damage. Thus, high-LET radiations are more destructive to biological materials than low-LET radiations because at the same dose, low-LET radiations induce the same number of radicals more sparsely within a cell, whereas high-LET radiations transfer most of their energy to a small region of the cell. The localized DNA damage caused by dense ionizations from high-LET radiations is more difficult to repair than the diffuse DNA damage caused by the sparse ionizations from low-LET radiations.

Experimental studies with animals as well as epidemiological studies and data indicate that higher or continual radiation exposure increases the incidence of specific cancers. Increased incidence of lung cancer has been observed among uranium miners, fluorspar miners, zinc and iron ore miners (Adams and Cox, 1997). Gottlieb and Husen (1982) studied the incidence of lung cancer among American Indians. According to the authors, lung cancer has been a rare disease among the Indians of the southwestern United States, but the advent of uranium mining in the area has been associated with an increased incidence of lung cancer among Navajo uranium miners. The study centered on Navajo men with lung cancer who were admitted to the hospital from February 1965 to May 1979. Of a total of 17 patients with lung cancer, 16 were uranium miners. The cause of the lung cancers are the $\alpha$-particle radiation from the inhaled radon gas emanating from the radium present in the ore. Another well-documented example of radiation-induced cancer is the occurrence of osteosarcoma among workers in luminous dial watch factories. Workers used to lick the paint brush to maintain sharp edges and in the process consumed radium-226 and radium-228. The ingested radium deposited in the bone and was the source of short-range $\alpha$-particles. A recent example of radiation-induced increases in thyroid cancer comes from the Chernobyl incident.

The nuclear reactor explosion at Chernobyl in 1986 resulted in substantial contamination of some parts of Belarus, Ukraine, and Bryasnk. Immediately after the accident, these regions were contaminated with cesium-137 as well as various isotopes of iodine, including iodine-131 (Stsjazhko *et al.*, 1995). Cesium-137 is a very dangerous radioisotope to the environment because of its long-term effects. Its half-life of about 30 years ensures that it stays in the environment for a very long time. The authors reported that those who continued to live in the contaminated region and to consume locally produced milk for the 3 months after the accident, most (about 85%) of the radiation dose to the thyroid was derived from iodine-131, while the remainder was from short-lived isotopes of iodine. The incidence of thyroid cancer among children under 15 years was 30.6 per million during 1991–1994 as compared to 0.3 during 1981–1985. Another source of human data on carcinogenesis by ionizing radiation is from the A-bomb survivors from Hiroshima and Nagasaki. Data showed that in the first 5–10 years after the exposure, the risk of leukemia increased rapidly but declined thereafter. The risk of solid tumors in many organs also increased significantly (Okey *et al.*, 1998).

#### *Mechanism of radiation-induced carcinogenesis*

An absorbed dose of 1 Gy generates about $2 \times 10^5$ ionizations within the mammalian cell. Approximately 1% of these ionizations occur in the DNA itself (Adams and Cox, 1997). Consequent DNA damage involves both single- and double-strand breaks, most of which are repaired within a few hours. Single-strand breaks, which are more frequently caused by low-LET radiations, are more readily repaired than double-strand breaks. UV radiation is known to distort the DNA strands by causing thymine dimers. In animals that have photolyase, thymine dimers are repaired by the direct repair mechanism that involves breaking of the bond causing the lesion. Humans do not have photolyase, so thymine dimers in humans are corrected by the NER mechanism.

Ionizing radiation and oxidative stress are closely associated. Irradiated cells produce damaging reactive oxygen species (ROS), which can cause severe damage to cellular macromolecules including nuclear DNA (Wu *et al.*, 1999; Spitz *et al.*, 2004). A cell's oxidative status plays an important role not only at the time of radiation exposure, but also long after exposure. Irradiation may produce ROS for several minutes or even hours after exposure (Spitz *et al.*, 2004).

At the cytological level, an extension of radiation-induced DNA damage is chromosome breakage. Radiation can induce aberrant intra-chromosomal crossing over that involves one or both chromatids. Radiation can also induce non-disjunction of homologous chromosomes resulting in trisomy in the $F_1$ offsprings, as well as other chromosomal aberrations, such as translocations and deletions (Adams and Cox, 1997). Chromosomal breaks have been shown to occur at a higher frequency in certain fragile sites. In other words, depending on the energy, radiation can cause increased genomic instability.

Unrepaired DNA-strand/chromosomal breaks can lead to deletion or scrambling of gene sequences. The hypoxanthine–guanine phosphoribosyltransferase (HPRT) locus on X-chromosome is known to undergo such aberrations following high radiation exposure (Thacker, 1986). If the target gene is an oncogene or a tumor suppressor gene, then radiation-induced mutations in oncogenes or inactivation of tumor suppressor genes may have serious consequences.

It should be emphasized that most of the molecular data on radiation-induced carcinogenesis have been obtained from laboratory studies, including *in vitro* studies. For humans, epidemiological studies from accidents and disasters involving ionizing radiation also demonstrate increased risk of cancer owing to high exposure to radiation. Nevertheless, little reliable information is currently available on the increased risk factors for high-LET radiations or about dose-rate effects of low-LET radiations (Okey *et al.*, 1998).

## CLASSIFICATION OF CARCINOGENS

The most widely used systems for classifying carcinogens comes from the International Agency for Research on Cancer (IARC). The US Environmental Protection Agency (EPA) has also developed a very similar classification scheme. In the past 30 years, the IARC has evaluated about 900 likely candidates for their cancer causing potential in humans. Most of the agents are of probable, possible, or unknown risk. Only about 90 are classified as carcinogenic to humans. The IARC classification scheme is described below:

- *Known human carcinogen*

*Group 1*: There is sufficient evidence of carcinogenicity in humans.

- *Probable human carcinogen*

*Group 2A*: There is limited evidence of carcinogenicity in humans, but sufficient evidence of carcinogenicity in experimental animals, and a strong evidence that the carcinogenesis in experimental animal is mediated by a mechanism that also operates in humans.

- *Possible human carcinogen*

*Group 2B*: There is limited evidence of carcinogenicity in humans, and less than sufficient evidence of carcinogenicity in experimental animals.

- *Not classifiable for human carcinogenicity*

*Group 3*: The evidence of carcinogenicity is inadequate or limited in experimental animals.

- *Not likely to be a human carcinogen*

*Group 4*: Not carcinogenic to humans.

## ASSAYS FOR CARCINOGENS

Tests most frequently used to determine the carcinogenic activity include (1) long-term bioassays and (2) short-term assays. Recently, building of databases of known/suspected carcinogenic compounds has helped in the development of computer programs that can predict the carcinogenic potential of new compounds, using quantitative structure–activity relationships (QSAR). These programs provide additional tools when there is ambiguous data or insufficient data to infer safety. Some of the common tests under each category are discussed below.

### Long-term bioassays

The National Toxicology Program (NTP) rodent cancer bioassay evolved out of the National Cancer Institute (NCI) cancer bioassay protocol in the 1970s. Groups of 50 or more rodents are assigned to control or treatment groups. Test substances are given by intubation, dietary or drinking water consumption, or dermal or inhalation exposure. Dosing starts at age 5–6 weeks and lasts for 2 years, at which point surviving animals receive a complete histopathological examination (Bucher, 2002). In a classic lifetime tumor bioassay, the maximum tolerated dose (MTD) for a particular chemical is determined in a dose-range finding study, then doses close to the MTD are administered to male and female rats and mice for 2 years. A statistically significant, chemical-associated increase in tumor count is taken as evidence of carcinogenicity (Parsons and McKinzie, 2001). These assays are expensive and time consuming. Additionally, the use of MTD is debatable since the high doses of chemical used to develop tumors often lead to cytotoxic effects in animal tissues. This cytotoxicity may lead to cell proliferation and other promoter-like events. Nevertheless, the rodent tumor bioassay has been used for carcinogenicity testing for many years, primarily for four reasons. First, the rodent tumor bioassay fulfills an obvious need for an experimental system. Second, *in vivo* exposure and tumor development are considered more relevant endpoints for assessing human cancer risk than *in vitro* tests with bacterial or mammalian cells or non-tumor endpoints. Third, the rodent tumor bioassay has been used for many years without disastrous human health consequences, and finally, at the present time there is no other "ideal" assay with which to replace the rodent tumor bioassay (Parsons and McKinzie, 2001).

### Short-term assays

These assays may be used to determine a chemical's ability to cause *mutational events*, *chromosomal aberrations* or *DNA damage*, *in vitro* or *in vivo*.

Two major assays used to detect *mutational events* are the *Ames test* for bacterial mutagenesis, and the *mammalian cell gene mutation assays*. The Ames test was developed in the early seventies by Bruce Ames at The University of California, Berkeley. The Ames method is based on inducing growth in genetically altered strains of *Salmonella typhimurium*. These strains need histidine in order to grow. If the chemical agent under investigation is a mutagen, it should cause some of the bacteria to undergo mutations so that some of these bacteria (revertants) can grow without histidine, just like the wild-type bacteria. To evaluate the effect of metabolism on the chemical, such tests are performed in the presence and absence of S9 fraction (post-mitochondrial supernatant fraction). The S9 fraction contains liver enzymes, generally from a rat treated with a broad-spectrum xenobiotic-metabolizing enzyme inducer, such as Arochlor 1254. There are several altered *Salmonella* strains used in the Ames test, such as TA97, TA98, TA100, TA1535, and TA1537; each varies in sensitivity to specific mutagens. The Ames test yields a number, such as the number of revertants (could be as high as several hundred thousands) per microgram of a pure chemical (mutagen) or per gram of food containing that mutagen. The mammalian cell gene mutation assays measure forward mutations at a specific locus. Two of the well-studied loci are the thymidine kinase (TK) locus in mouse lymphoma cells and the HPRT locus in Chinese hamster ovary (CHO) cells. To evaluate the effect of metabolism on the chemical, such tests are performed in the presence and absence of S9 fraction.

Cytogenetic analysis for *chromosomal aberrations* enables direct observation of various chromosomal aberrations. Cells are arrested in metaphase, examined microscopically, and the chromosomal aberrations are enumerated. The cells used could be CHO cells, human peripheral blood lymphocytes, or rat lymphocytes. An alternate cytogenetic approach is the observation of *micronuclei*. A micronucleus is formed during the metaphase/anaphase transition. It may arise from a lagging chromosome (*aneugenic* event resulting in chromosome loss) or an acentric chromosome fragment detaching from a chromosome after breakage (*clastogenic* event). Micronuclei are easily scored. Another assay for measuring chromosomal aberrations is the *sister chromatid exchange* (SCE) assay, which detects the ability of a chemical to enhance the exchange of DNA between two sister chromatids of a duplicating chromosome. SCE means a reciprocal interchange of the two chromatid arms within a single chromosome. This exchange is visualized during metaphase. Detection of SCEs requires some means of differentially labeling sister chromatids, such as by the incorporation of bromodeoxyuridine (BrdU) into chromosomal DNA for two cell cycles. The most commonly used assays employ mammalian bone marrow cells or peripheral blood lymphocytes,

often from rodent species. All these assays could be performed in the presence and absence of metabolic activation systems (S9).

Detection of *DNA damage* can be determined using the *comet assay*. It is also known as the *single-cell gel electrophoresis assay* and it is a very useful and sensitive technique for detecting DNA damage at the level of a single cell. The technique acquired its name from the comet-like shape of the DNA of the cells which can be seen under the microscope after the procedure. This technique was developed by Swedish researchers Östling and Johansson in 1984, and was later modified by Singh and coworkers in 1988 as the alkaline comet assay. The alkaline comet assay is much more sensitive than the original comet assay. Briefly, the single suspension of the cells of interest are suspended in low-melting agarose and layered onto slides pre-coated with agarose. Cells are lysed under high salt concentration to release the damaged DNA. The DNA is subjected to unwinding under alkaline/neutral conditions to allow DNA supercoils to relax. Electrophoresis of this DNA under neutral or highly alkaline (pH > 13) conditions allows the broken ends to migrate forming the "comets". After neutralization, staining is done using fluorescent DNA dyes (e.g. ethidium bromide, propidium iodide, Hoescht 33258, acridine orange, etc.) and the DNA is visualized under a fluorescent microscope. The slides are then scored. About 50–100 cells are counted per sample, by manual counting or using specific software. Both qualitative and quantitative assessment of DNA damage is carried out. The alkaline comet assay enables the detection of single-strand DNA breaks in individual cells. Therefore, the alkaline comet assay can measure low levels of DNA-strand breaks. The ability of a genotoxic agent to induce DNA lesions can also be investigated by measuring the *unscheduled DNA synthesis* (UDS). The assay measures DNA repair synthesis after excision and removal of a stretch of DNA containing the lesion induced by chemical and physical agents. The assay is based on the incorporation of tritium-labeled thymidine into the DNA of mammalian cells which are not in the S phase (DNA synthesis phase) of the cell cycle. The uptake of tritium-labeled thymidine can be determined by autoradiography or by liquid scintillation counting of DNA from the treated cells.

## Structure–activity relationships and carcinogenicity

Over 70,000 chemicals are used commercially and more than 100,000 naturally occurring chemicals have been identified (Cramer *et al.*, 1978). It would be virtually impossible, impractical, economically unfeasible, and not sensible to test all these compounds for potential carcinogenicity. This then begs the question – how do scientists (toxicologists, food technologists, drug developers, regulatory scientists, etc.) make decisions as to the safety of chemicals for addition to food, as drugs, for industrial use and/or for prioritizing those that do require additional, extensive toxicity testing?

One of the ways toxicologists have prioritized the study of chemicals is through the use of structure–activity relationship (SAR) analysis. SAR can be divided into two classes: (1) qualitative relationships and (2) quantitative relationships.

### *Qualitative structure–activity relationships*

A qualitative structure–activity relationship is an association between a molecular (sub)structure and the presence or absence of a biological activity, or the capacity to modulate a biological activity imparted by another substructure. A substructure associated with the presence of a biological activity is sometimes called a structural alert. Therefore, chemicals with similar chemical structures and physicochemical properties should exhibit similar biological activity whether it is a specific toxic effect or a pharmacological activity.

Qualitative SAR analyses rely upon a finite knowledge base of compounds that have been previously tested and the researcher's ability to identify key structural similarities between the compound of interest and compounds in the previously established knowledge bases. A variety of classification schemes, such as those detailed in Cramer *et al.* (1978), Cheeseman *et al.* (1999), and FDA's Red-book (Rulis *et al.*, 1984), have been developed that exploit these relationships, most of which are based on classifying either mutagens or carcinogens into broad categories. The Ashby and Tennant classification scheme (Ashby *et al.*, 1989) for structural alerts (Figure 22.8) is probably one of the simplest approaches to identifying potential carcinogenic activity of a compound that has unknown toxicity. This classification scheme correlates a structural alert (a fragment of a chemical's structure that may participate in an electrophilic attack on DNA) with a compound's carcinogenic potential. These alerts can be used in the evaluation of a chemical to give a qualitative sense of the compound's likelihood of being carcinogenic. A list of structural alerts proposed by Ashby *et al.* (1991) and functional groups cited by Munro *et al.* (1996) are summarized in Table 22.2.

In addition to using classification schemes to identify compounds for potential concerns, there are a number of structurally searchable database that allow one to identify close structural analogs of the compound in question. Publicly available examples are the National Library of Medicine's (NLM) ChemIDPlus (NLM, 2005) and EPA's Distributed Structure-Searchable Toxicity (DSSTox) Public Database Network (EPA, 2005). The data from these analogs may be used to extrapolate a numerical risk value for a potential toxic effect.

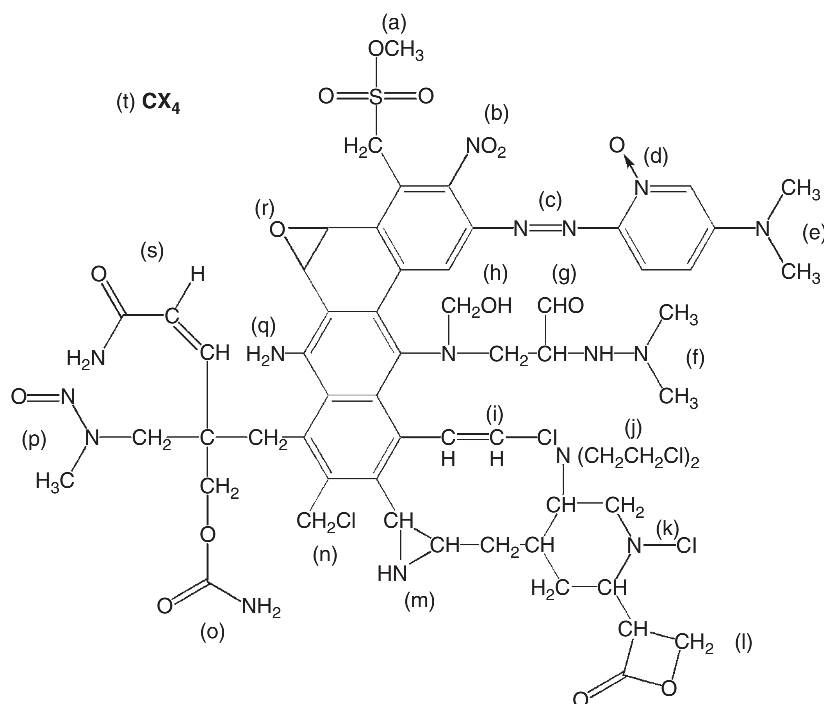


**FIGURE 22.8** Hypothetical chemical structure highlighting (in black) the Ashby and Tennant structural alerts for carcinogenicity. Adapted from Ashby *et al*. 1989. Classification according to chemical structure, mutagenicity to Salmonella and level of carcinogenicity of a further 42 chemicals tested for carcinogenicity by the U.S. National Toxicology Program. *Mut Res* **223**: 73–103, with permission from Elsevier. (a) alkyl ester of either phosphonic or sulfonic acids; (b) aromatic nitro groups; (c) aromatic azo groups, by virtue of their reduction to an aromatic amine; (d) aromatic ring *N*-oxides; (e) aromatic mono- and di-alkylamino groups; (f) alkyl hydrazines; (g) alkyl aldehydes; (h) *N*-methylol derivatives; (i) monohaloalkenes; (j) a large family of *N* and *S* mustards (*β -haloethyl*); (k) *N*-chloramines (substructure has not been associated with carcinogenicity, but potent genotoxic activity has been reported); (l) propiolactones and propiosultones; (m) aromatic and aliphatic aziridinyl derivatives; (n) both aromatic- and aliphatic-substituted primary alkyl halides; (o) derivatives of urethane (carbamates); (p) alkyl *N*-nitrosamines; (q) aromatic amines, their *N*-hydroxy derivatives and the derived esters; (r) aliphatic epoxides and aromatic oxides; (s) $\alpha,\beta$-unsaturated carbonyls; and (t) halogenated methanes (X = H, Cl, Br, I in any combination).

### *Quantitative structure–activity relationships*

QSAR is a quantitative relationship between a biological activity (e.g. toxicity) and one or more descriptors that are used to predict the activity. In other words, QSAR utilizes the existing scientific data to make decisions about the potential carcinogenicity of new and/or untested chemicals using predictive models based on some type of algorithm (e.g. relating structural topology, chemical bond types, bioavailability, solubility, etc. to some toxic endpoint – genotoxicity, carcinogenicity, etc.) powered by computer software.

There are several commercially available predictive toxicity programs currently available. These programs can be divided into two general categories: (1) human expert/ruled-based programs such as OncoLogic and DEREK and (2) human expert/statistical/correlative programs such as MDL QSAR, TOPKAT, and MCASE-MC4PC.

An example of a human expert/rule-based program is OncoLogic, which predicts carcinogenicity based on the chemical structure of a substance (Woo *et al.*, 1995). Analysis is done by applying a decision tree approach that incorporates the knowledge of mechanisms of action and human epidemiological studies. OncoLogic contains four carcinogenicity modules focused on fibers, metals or organometallics, polymers, and organics. The output consists of a justification of the potential carcinogenicity, or lack thereof, using a tiered level of concern (low, marginal, low moderate, moderate, high moderate, or high) with identification of the chemicals used in the decision tree analysis that led to the conclusions.

An example of a statistical/correlative program is MCASE-MC4PC (MultiCase, 2005), which has several predictive modules, including mutagenicity, carcinogenicity, and teratogenicity. MultiCASE uses an algorithm to identify all possible 2–10-atom fragments for each discrete chemical structure within a diverse training set of substances. The program then identifies fragments (structural alerts or biophores) that are primarily associated with the biological activity (e.g. carcinogenicity) of the training set and modulators of the activity, such as bioavailability and conformational comparisons. New substances with unknown toxicity are screened by entering the structure of the untested substance using a structural drawing program. The molecule in question is reduced to all possible 2–10-atom fragments and these fragments are compared to the list of structural alerts (biophores) and modulators

**TABLE 22.2 A list of Ashby and Tennant structural alerts and functional-group list compiled by Munro (Me = —$CH_3$, Et = —$CH_2CH_3$)**

| |
|---|
| *Aryl and heterocyclic ring-substituted amino- and nitro- derivatives (Ashby AA class)* |
| Bioactivation to nitrenium ions (Ar—$N^+$—H) by *N*-hydroxylation to arylhydroxylamines (Ar—N(OH)R) |
| 1°- and 2°-amines [Ar—NHR, where R may be —H, —Me, —Et, or activated Me or Et] (examples mostly 1°-amines and include hydrochloride salts) |
| 3°-amines [Ar—$NR^1R^2$, where $R^1$ and $R^2$ = —Me, —Et] (few examples mostly di-Me) |
| 2°-acetamides and -formamides [Ar — NHCOR, where R = —H, —Me, or activated Me] (examples mostly R═NHCOMe) |
| Nitroarenes [Ar— $NO_2$] (reduction to nitroarene; many examples) |
| Nitrosoarenes [Ar—N═O] (reduction to arylhydroxylamine) |
| Arylhydroxylamines [Ar—N(OH)R] |
| *Nitroso compounds (Ashby NO class)* |
| Bioactivation to carbonium ($R_2CH^+$) or diazonium ions ($RN^+$≡N) by oxygenation at α-C to diazohydroxides (R-N═N—OH); substrate must possess —H, —OH on α-C or α-C═O relative to nitrogen |
| *N*-nitroso-*N*-dialkylamines [$R^1R^2$N—N═O, most examples are from this class] |
| *N*-nitroso-*N*-alkylamides [$R^1R^2$N—N═O, where $R^2$ = $COR^3$, $R^3$ = alkyl or aryl] |
| *N*-nitroso-*N*-alkylureas [$R^1R^2$N—N═O, where $R^2$ = $CONR^3R^4$, $R^3$ = H, $R^4$ = alkyl or aryl] |
| *N*-nitroso-*N*-alkylcarbamates (a.k.a. urethanes) [$R^1R^2$N—N═O, where $R^2$ = $CO_2R^3$, $R^3$ =alkyl or aryl] |
| *N*-nitroso-*N*-alkylnitriles [$R^1R^2$N—N═O, $R^2$ = CN] |
| *N*-nitroso-*N*-hydroxylamines [R-N(OH)NO] |
| *Hydrazo derivatives (Ashby NZN class)* |
| Bioactivation to carbonium ($R_2CH^+$) or diazonium ions ($RN^+$≡N) by oxygenation at α-C; R groups may be alkyl or aryl, but one must possess —H, —OH on α-C or α-C═O relative to nitrogen |
| Hydrazines [$R^1R^2$N—$NR^3R^4$] (oxidation to azo then azoxy derivative; examples mostly terminal $R_2$N—$NH_2$) |
| Azoxy alkane [$R^1$-$N^+$($O^-$)═N-$R^2$, R is $C_4$ or less] |
| *Natural electrophiles (Ashby ALK class)* |
| Aliphatic halides [R-$CH_2$X, where R is ⩽$C_4$ and contains activating groups; not more than one —X per C] |
| Benzylic halides [Ar—$CH_2$X, where X = Cl, Br, I] |
| Oxiranes and aziridines [3-membered *O*- and *N*-containing rings] |
| Propiolactones [4-membered ring-containing —$CO_2^-$] |
| Alkyl esters of sulfonic [$RSO_2OR^1$] and sulfuric [$ROSO_2OR^1$] acids (where $R^1$ =Me or Et) |
| Alkyl esters of phosphonic [RP(═O)$(OR^1)_2$] and phosphoric [$(RO)_2$P(═O)$OR^1$] acids (where $R^1$ = Me or Et) |
| Mixed alkyl esters of phosphoric acid [$(RO)_2$P(═S)$OR^1$, where $R^1$ = Me or Et] |
| Haloethylamines [—$NCH_2CH_2X$] |
| Haloalkylethers [ethyl (—$OCH_2CH_2X$) and methyl (—$OCH_2X$)] |
| α-ualocarbonyl [R(C═O)$CH_2$X] or (α-halohydroxy [R(CHOH)$CH_2$X] |
| Haloamines [$R_2$N—X] |
| α,β-Hunsaturated carbonyls [$R_2$C═C—C(═O)R, where R is an aldehyde, ketone, ester, amide group] |
| Allylic halides and alkoxides [$R_2$C—C—$CH_2$X, where X = Cl, Br, I, or OR] |
| *Other alerting groups (Ashby SA+ class)* |
| Halogenated methanes [$CH_mX_n$, where $m + n = 4$] |
| Vinyl halides [$R^1R^2$C═CHX, where X = Cl, Br, or I] |
| PAHs |
| Isocyanate [R-N═C═O] |
| Isothiocyanate [R-N═C═S] |
| Azoarenes [$R^1$-N═N—$R^2$, $R^1$ and $R^2$ are aryl] (—$SO_3$H group on both rings non-alerting) |

Adapted from Bailey *et al.* 2005. The use of structure-activity relationship analysis in the food contact notification program. *Regul Toxicol Pharmacol* **42**: 225–235, with permission from Elsevier.

identified within the training set. The output consists of scalar units, referred to as CASE units, in increasing activity values ranging from 10 to 80. A CASE rating of 10–19 is considered non-toxic, 20–29 are marginal (equivocal, weak, or inconsistent findings), 30–49 are moderately potent toxins (*trans*-gender, single site tumors), and 50–80 are potent toxins (*trans*-species, *trans*-gender, multiple site tumors in rodents). The biophores identified by the program are subjected to a set of human expert rules that take into account specificity, positive predictivity, false positives, and coverage to arrive at a decision as to whether the test molecule is carcinogenic, level of confidence with the prediction, and whether the program-generated CASE-unit score is appropriate.

These types of predictive software continue to be refined and their concordance, specificity, and predictivity are approaching the 90+ percent range. As long-term testing becomes increasingly expensive and controversial (animal rights activism, etc.), look for these QSAR software packages to play an increasing and more prominent role in the safety assessment of new chemical substances.

The accumulated knowledge of the metabolism, chemical reactivity, exposure, SAR, and other relevant information on chemicals, when scientifically and systematically

woven together, can allow us to make expert judgments as to the potential risks due to exposure to chemicals in food, air, and water. These judgments can be used to identify those compounds that merit further toxicity testing and those not worth pursuing. All schemes used to make these decisions must be thoroughly tested, be transparent, and evoke public confidence that such decisions now require.

## CONCLUSION

After more than three decades since President Nixon declared the "War on Cancer" with the enactment of the National Cancer Act, the war still continues. Routine checkup has been effective in the early diagnosis and cure of certain forms of cancer, such as colon cancer and breast cancer. Inventions in modern medicine have further added to better prognosis. Nevertheless, cancer largely remains an incurable disease unless it is detected pretty early.

In spite of significant advances in our knowledge on the molecular mechanisms of carcinogenesis, scientists seem to have a long way to go before all these advances in knowledge could be translated into effective and curative therapy. In the meantime, more work is needed to understand certain aspects of carcinogenesis, such as the mechanisms of action of non-genotoxic carcinogens. However, the accumulated knowledge on various aspects of carcinogenesis has definitely helped scientists make appropriate risk assessment and health policy recommendations. Finally, recent progress in the science of genomics has provided scientists with renewed hope that further breakthroughs in cancer detection and therapy are not far away, allowing us to effectively control the disease.

## REFERENCES

Adams GE, Cox R (1997) Radiation carcinogenesis. In *Cellular and Molecular Biology of Cancer*, 3rd edn, Franks LM, Teich NM (eds). Oxford University Press, Oxford, pp. 130–50.

Alberts SR, Cervantes A, van de Velde, CJ (2003) Gastric cancer: epidemiology, pathology and treatment. *Ann Oncol* **42**(Suppl. 2): ii31–6.

Ashby J, Tennant RW (1991) Definitive relationships among chemical strucutre, carcinogencity and mutagenicity for 301 chemicals tested by the US NTP. *Mut Res* **257**: 229–306.

Ashby J, Tennant RW, Zeiger E, Stasiewicz S (1989) Classification according to chemical structure, mutagenicity to *Salmonella* and level of carcinogenicity of a further 42 chemicals tested for carcinogenicity by the US National Toxicology Program. *Mut Res* **223**: 73–103.

Bailey AB, Chanderbhan R, Collazo-Braier N, Cheeseman MA, Twaroski ML (2005) The use of structure-activity relationship analysis in the food contact notification program. *Regulat Toxicol* **42**: 225–35.

Becker K, Dosch J, Gregel CM, Martin BA, Kaina B (1996) Targeted expression of human $O^6$-methylguanine-DNA methyltransferase (MGMT) in transgenic mice protects against tumor initiation in two-stage skin carcinogenesis. *Cancer Res* **56**: 3244–9.

Becker K, Gregel C, Fricke C, Komitowski D, Dosch J, Kaina B (2003) DNA repair protein MGMT protects against *N*-methyl-*N*-nitrosourea-induced conversion of benign into malignant tumors. *Carcinogenesis* **24**: 541–6.

Bucher JR (2002) The National Toxicology Program rodent bioassay: designs, interpretations, and scientific contributions. *Ann NYAcad Sci* **982**: 198–207.

Buermeyer AB, Deschenes SM, Baker SM, Liskay RM (1999) Mammalian DNA mismatch repair. *Annu Rev Genet* **33**: 533–64.

Cheeseman MA, Machuga EJ, Bailey AB (1999) A tiered approach to threshold of regulation. *Food Chem Toxicol* **37**: 387–412.

Cho Y, Gorina S, Jeffrey PD, Pavletich NP (1994) Crystal structure of a p53 tumor suppressor-DNA complex: understanding tumorigenic mutations. *Science* **265**: 346–55.

Cooper GM (1995) *Oncogenes*, 2nd edn, Jones and Bartlett, Boston.

Cramer GM, Ford RA, Hall RL (1978) Estimation of toxic hazard – a decision tree approach. *Food Cosmet Toxicol* **16**: 255–76.

deWind N, Dekker M, Berns A, Radman M, te Riele H (1995) Inactivation of the mouse Msh2 gene results in mismatch repair deficiency, methylation tolerance, hyperrecombination, and predisposition to cancer. *Cell* **82**: 321–30.

Dipple A (1995) DNA adducts of chemical carcinogens. *Carcinogenesis* **16**: 437–41.

Dumenco LL, Allay E, Norton K, Gerson SL (1993) The prevention of thymic lymphomas in transgenic mice by human $O^6$-*alkylguanine*-DNA alkyltransferase. *Science* **259**: 219–22.

EPA (2005) DSSTox Public Database Network. Accessed online at http://www.epa.gov/nheerl/dsstox/.

Foulds L (1954) The experimental study of tumor progression: a review. *Cancer Res* **14**: 327–39.

Gottlieb LS, Husen LA (1982) Lung cancer among Navajo uranium miners. *Chest* **81**: 449–52.

Haas GP, Sakr WA (1997) Epidemiology of prostate cancer. *CA Cancer J Clin* **47**: 273–87.

Hoffman PD, Leonard JM, Lindberg GE, Bollmann SR, Hays JB (2004) Rapid accumulation of mutations during seed-to-seed propagation of mismatch-repair-defective *Arabidopsis*. *Genes Dev* **18**: 2676–85.

Jacoby RF, Llor X, Teng BB, Davidson NO, Brasitus TA (1991) Mutations in the K-ras oncogene induced by 1,2-dimethylhydrazine in preneoplastic and neoplastic rat colonic mucosa. *J Clin Invest* **87**: 624–30.

Knudson Jr AG (1971) Mutation and cancer: statistical study of retinoblastoma. *Proc Natl Acad Sci USA* **68**: 820–3.

Liu L, Qin X, Gerson SL (1999) Reduced lung tumorigenesis in human methylguanine DNA—methyltransferase transgenic mice achieved by expression of transgene within the target cell. *Carcinogenesis* **20**: 279–84.

Marquardt H (1999) Chemical carcinogens. In *Toxicology*, Marquardt H, Schäfer SG, McClellan RO, Welsch F (eds). Academic Press, San Diego, pp. 151–78.

McPherson K, Steel CM, Dixon JM (2000) ABC of breast diseases. Breast cancer-epidemiology, risk factors, and genetics. *Br Med J* **321**: 624–8.

MultiCase Incorporated (2005) Corporate website accessed at http://www.multicase.com/.

Munro IC, Ford RA, Kennepohl E, Sprenger JG (1996) Thresholds of toxicological concern based on structure-activity relationships. *Drug Metab Rev* **28**: 209–17.

NLM (2005) Specialized Information Services. ChemIDPlus. Accessed online at http://chem.sis.nlm.nih.gov/chemidplus/chemidlite.jsp and http://chem.sis.nlm.nih.gov/chemidplus/.

Okey AB, Harper PA, Grant DM, Hill RP (1998) Chemical and radiation carcinogenesis. In *The Basic Science of Oncology*, 3rd edn, Tannock IF, Hill RP (eds). McGraw-Hill, New York, pp. 166–96.

Parsons BL, McKinzie PB (2001) Developing methods of genetic analysis to improve cancer risk assessment. *Regul Res Perspec* **1**: 1–11.

Pitot HC (2002) *Fundamentals of Oncology*, 4th edn. Marcel and Dekker, Inc, New York.

Qin X, Zhang S, Matsukuma S, Zarkovic M, Shimizu S, Ishikawa T, Nakatsusru Y (2000) Protection against malignant progression of spontaneously developing liver tumors in transgenic mice expressing $O^6$-methylguanine-DNA methyltransferase. *Jpn J Cancer Res* **91**: 1085–9.

Rulis AM, Hattan DG, Morgenroth III VH (1984) FDA's priority-based assessment of food additives. I. Preliminary results. *Regulat Toxicol Pharmacol* **4**: 37–56.

Smela ME, Currier SS, Bailey EA, Essigmann JM (2001) The chemistry and biology of aflatoxin B(1): from mutational spectrometry to carcinogenesis. *Carcinogenesis* **22**: 535–45.

Spitz DR, Azzam EI, Li JJ, Gius D (2004) Metabolic oxidation/reduction reactions and cellular responses to ionizing radiation: A unifying concept in stress response biology. *Cancer Metas Rev* **23**: 311–22.

Stsjazhko VA, Tsyb AF, Tronko ND, Souchkevitch G, Baverstock KF (1995) Childhood thyroid cancer since accident at Chernobyl. *Br Med J* **310**: 801.

Thacker J (1986) The use of recombinant DNA techniques to study radiation-induced damage, repair and genetic change in mammalian cells. *Int J Rad Biol* **50**: 1–30.

Van Vleet TR, Klein PJ, Coulombe Jr RA (2002) Metabolism and cytotoxicity of aflatoxin b1 in cytochrome p-450-expressing human lung cells. *J Toxicol Environ Health* A **65**: 853–67.

Woo YT, Lai DY, Argus MF, Arcos JC (1995) Development of structure-activity relationships rules for predicting carcinogenic potential of chemicals. *Toxicol Lett* **79**: 219–28.

Wu LJ, Randers-Pehrson G, Xu A, Waldren CA, Geard CR, Yu Z, Hei TK (1999) Targeted cytoplasmic irradiation with alpha particles induces mutations in mammalian cells. *Proc Natl Acad Sci USA* **96**: 4959–64.

Zaidi NH, Pretlow TP, O'Riordan MA, Dumenco LL, Allay E, Gerson SL (1995) Transgenic expression of human MGMT protects against azoxymethane-induced aberrant crypt foci and G to A mutations in the K-ras oncogene of mouse colon. *Carcinogenesis* **16**: 451–6.

# Part 4

## Drugs of Use and Abuse

CHAPTER

# 23

# Toxicity of over-the-counter drugs

*Karyn Bischoff*

## INTRODUCTION

The topic of over-the-counter (OTC) drugs is complicated. It encompasses a large number of products. Many contain more than one active ingredient. Products are available for oral, topical, intraocular, intranasal, and intrarectal administration. Most veterinary exposures are through ingestion. These products are readily available in many homes. Toxicosis may result when animals are medicated by well-intentioned animal owners or even veterinarians, or exposure may be accidental, in which case the amount of medication ingested may not be known. Response to a given drug may be species specific or different between individuals of the same species. Idiosyncratic reactions to drugs must also be considered, though reports in domestic animals are rare (Papich, 1990; Brumbaugh, 2001)

Approximately one-quarter of the calls to human poison control centers in 1990 dealt with OTC drugs, and similar reports are common with the American Society for the Prevention of Cruelty to Animals' National Animal Poison Control Center (Murphy, 1994; Villar *et al.*, 1998). It has been estimated that there are over 300,000 OTC drug formulations available. Approximately 700 active ingredients in various combinations may be present in these formulations (Papich, 1990). Only a small fraction can be addressed in this chapter.

Important classes of drugs that will be addressed include analgesics, cold, flu, and allergy medications, and drugs used to treat gastrointestinal symptoms. Nutritional supplements, for the most part, will not be addressed here. A few herbal preparations, notably ma juang and guarana, are discussed in this chapter. Toxicologically important minerals such as iron, and important vitamins such as vitamin D, are addressed elsewhere in the text. Stimulants and diet pills may contain methylxanthines such as caffeine, addressed elsewhere in the text, as well as sympathomimetic amines, discussed below with decongestants.

Though this chapter deals mostly with drugs intended for per os (PO) dosing, a few topical preparations are discussed below.

### Suspected OTC drug reactions

Obtaining a thorough history is of great importance when dealing with suspect OTC drug-related problems (Talcott, 2006). Animal owners may not volunteer critical information. Well meaning pet owners may administer OTC drugs to treat perceived symptoms in their pets (Papich, 1990; Jones *et al.*, 1992; Villar *et al.*, 1998; Roder, 2005a; Sellon, 2006). Veterinarians may share the blame for inappropriate dosing (Papich, 1990). Chronic analgesic administration to treat orthopedic problems is a particular hazard affecting all classes of animals that may present to the veterinary practice (Roder, 2005a). Accidental ingestions may occur if drugs are improperly stored (Papich, 1990; Jones *et al.*, 1992; Villar *et al.*, 1998). Unexpected circumstances may arise, making it difficult to properly assess the history. One such example concerns severe clinical signs prompting euthanasia in a kitten. It was later discovered that the feline in question had been allowed to play with an empty acetaminophen container (Allen, 2003).

If an adverse reaction to an OTC drug is suspected, administration of this drug should be immediately discontinued. The owner should be instructed to bring the drug container to the veterinarian as a source of information on the active ingredients, indications for use, and manufacturer identification and telephone number or address. The manufacturer may have information on treatment

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and prognosis and, in the United States, will use the information you provide for adverse events reporting to the US Food and Drug Administration (FDA), and thus should be contacted. More information on adverse event reporting can be found at the FDA website: http://www.fda.gov/cvm/adetoc.htm. Any material remaining in the container may be analyzed to verify that the contents are as expected (Brumbaugh, 2001).

Early gastrointestinal decontamination may be helpful in the asymptomatic animal and may involve emetics, gastric lavage, and instillation of activated charcoal and cathartics. If drugs are used topically, removal with a mild detergent bath is usually beneficial (Brumbaugh, 2001). Careful monitoring and maintenance of body temperature is required after bathing, and bath towels may be warmed by tumbling in heated clothes drier to help prevent hypothermia.

# ANALGESICS

According to Jones *et al.* (1992), about 5% of dog- and cat-related calls to one poison control center were in response to analgesic ingestion, and nearly 80% involved dogs. Commonly used OTC analgesics include acetaminophen and nonsteroidal anti-inflammatory drugs (NSAIDs), such as aspirin, ibuprofen, naproxen, and ketaprofen. Aspirin shares many properties with other NSAIDs, but will be addressed separately due to some of its unique features.

## Acetaminophen

Acetaminophen does not have the anti-inflammatory properties of NSAIDs, though it is an analgesic and antipyretic (Hjelle and Grauer, 1986). Acetaminophen is found in a variety of OTC pain relievers and may be combined with other drugs in cold, flu, and allergy medications (Roder, 2005a). Common brand names in the United States include Tylenol® and Anacin-3®. Acetaminophen is sold under the name Paracetamol® in Australia and Great Britain, and is a metabolite of the analgesic phenacetin.

Acetaminophen toxicosis is most commonly reported in cats (Rumbeiha *et al.*, 1995). Clinical acetaminophen toxicosis is usually associated with a single exposure, though adverse effects as a result of multiple dosing have been reported (Hjelle and Grauer, 1986; Villar *et al.*, 1998). The most common clinical situation results from the owner medicating their animal without a veterinarian's advice or supervision (Hjelle and Grauer, 1986; Aronson and Drobatz, 1996). One report documents severe poisoning in a kitten that had played with an empty acetaminophen container (Allen, 2003).

### *Toxicity*

Individual differences in sensitivity to acetaminophen are reported within species (Webb *et al.*, 2003), but the use of acetaminophen is always contraindicated in cats due to their sensitivity to this drug (Jones *et al.*, 1992; Villar *et al.*, 1998; Wallace *et al.*, 2002; Roder, 2005a). Clinical signs of acetaminophen toxicosis in cats, including death, have been reported at doses of 10 mg/kg (Aronson and Drobatz, 1996). Most poisonings have been associated with doses of 50 mg/kg and greater (Murphy, 1994; Aronson and Drobatz, 1996; Allen, 2003; MacNaughton, 2003; Roder, 2005a; Sellon, 2006). Villar *et al.* (1998) reported 50% methemoglobinemia within 4 h in cats dosed with 120–140 mg/kg acetaminophen, and one of four cats dosed with 143 mg/kg died. One regular acetaminophen tablet contains 352 mg of the active ingredient, and an extra-strength tablet contains 500 mg.

The recommended dose for acetaminophen in dogs is 15 mg/kg PO every 8 h of 10 mg/kg PO every 12 h (Plumb, 2002). Toxicosis has been reported at a dose of 46 mg/kg (Sellon, 2006), though doses of 100 mg/kg or greater are more likely to be associated with clinical signs (Jones *et al.*, 1992; Boothe, 2001a; Roder, 2005a). Most dogs recover if dosed with less than 500 mg/kg (Villar *et al.*, 1998). Doses over 460 mg/kg have been associated with methemoglobinemia in dogs and deaths have occurred (Schlesinger, 1995; Villar *et al.*, 1998; Wallace *et al.*, 2002). A dose of 900 mg/kg caused "fulminant liver failure" (Boothe, 2001a). Doses greater than 1000 mg/kg (1g /kg) are reported to cause unconsciousness and cyanosis within hours and death within 12 h (Villar *et al.*, 1998).

### *Toxicokinetics*

After ingestion, acetaminophen is rapidly absorbed in the stomach and small intestine (Schlesinger, 1995; Wallace *et al.*, 2002). Peak plasma concentrations occur 4 h after ingestion in cats (Rumbeiha *et al.*, 1995).

Circulating acetaminophen is minimally bound to plasma protein and distributed widely (Wallace *et al.*, 2002). The therapeutic plasma concentration of acetaminophen in a dog is about 30 μg/ml, and toxicosis is associated with levels of 300 μg/ml and greater (Court and Greenblatt, 1997).

Metabolism of acetaminophen occurs primarily in the liver. There are three major pathways: direct glucuronide conjugation, direct sulfate conjugation, and oxidation mediated by cytochrome P450 enzymes (Hjelle and Grauer, 1986; Wallace *et al.*, 2002).

Acetaminophen has an available hydroxyl group. Immediate phase II conjugation is the primary route of metabolism in most species and involves glucuronide and sulfate (Dahm and Jones, 1996). Due to limitations of cat physiology, they have only about one-tenth the acetaminophen biotransformation ability of dogs (Hjelle and

Grauer, 1986; Sellon, 2006). Glucuronide conjugation is the fate of 50–60% of a dose of acetaminophen given to either a human or dog (Aronson and Drobatz, 1996). This pathway is deficient in cats due to decreased microsomal UDP-glucuronyltransferase 1,6 enzyme activity (Court and Greenblatt, 1997; Wallace *et al.*, 2002). The amount of acetaminophen that is conjugated to glucuronide in cats is dependent on dose, but is always a relatively small proportion. After an oral dose of 20 mg/kg acetaminophen, 1% undergoes glucuronidation. Five percent of a 60 mg/kg dose is conjugated to glucuronide, and 16% of a 120 mg/kg dose enters this pathway (Hjelle and Grauer, 1986).

Sulfate conjugation is less important than glucuronide conjugation in the disposition of acetaminophen in most species (Aronson and Drobatz, 1996; Boothe, 2001; Allen, 2003). Dogs metabolize about 10–20% of a given dose of acetaminophen via sulfation. However, this is the more important, though still limited, pathway in cats. Again, use of this pathway is dependent on dose of acetaminophen. After dosing with 20 mg/kg, 92% of the acetaminophen underwent sulfation, but after a dose of 60 mg/kg acetaminophen, 78% was sulfated, and if the dose was 120 mg/kg, only 57% was sulfated. The sulfation pathway may be saturated due to the limited availability of inorganic sulfates (Hjelle and Grauer, 1986).

Metabolism of acetaminophen by phase I processes is relatively minimal in most species, but is very important to the mechanism of action of this drug, as will be described below. Cytochrome P450 oxidation increases as phase II pathways become saturated (MacNaughton, 2003; Sellon, 2006). Approximately 5% of a dose of acetaminophen undergoes oxidation by cytochrome P450s in the dog. When cats are dosed with 20 mg/kg acetaminophen, 5% undergoes oxidation, but this number increased to 10% at doses of 60–120 mg/kg (Hjelle and Grauer, 1986). The product of the oxidation pathway is *N*-acetyl-*p*-benzoquinoneimine (NAPQI). NAPQI is conjugated to reduced glutathione (GSH), forming an inactive product (Savides and Oehme, 1985; Schlesinger, 1995; Jones *et al.*, 1992; Aronson and Drobatz, 1996; Sturgill and Lambert, 1997; Wallace *et al.*, 2002; Allen, 2003; MacNaughton, 2003; Roder, 2005a; Sellon, 2006).

The by-products of acetaminophen metabolism are excreted predominantly through the urine (Sturgill and Lambert, 1997; MacNaughton, 2003; Sellon, 2006). Less than 5% of a dose is excreted as the parent compound in humans (Wallace *et al.*, 2002). Some conjugates are eliminated in the bile (Sturgill and Lambert, 1997). Rate of elimination is dependent on the species and the dose. The elimination half-life of a 100–200 mg/kg dose of acetaminophen is 72 min in dogs and increases to 210 min when the dose is increased to 500 mg/kg. The elimination half-life in cats for a 20 mg/kg dose is 36 min, but the half-life for a 60 mg/kg dose is 144 min, and that for a 120 mg/kg dose is 288 min (Hjelle and Grauer, 1986). The half-life is prolonged in male cats compared to females (Rumbeiha *et al.*, 1995).

#### *Mechanism of action*

Unlike NSAIDs, the therapeutic effects of acetaminophen are independent of cyclooxygenase (COX) but based on its interference with endoperoxidase (Boothe, 2001a). Toxic effects of acetaminophen are due to the formation of the metabolite NAPQI. Large doses of acetaminophen overwhelm the sulfide and glucuronide conjugation pathways and lead to increased formation of the active metabolite (Hjelle and Grauer, 1986; Dahm and Jones, 1996; Sturgill and Lambert, 1997; MacNaughton, 2003; Roder 2005a). NAPQI is usually conjugated with GSH, as noted above. GSH stores become depleted 16–24 h after exposure to acetaminophen (Rumbeiha *et al.*, 1995). This is due to oxidation and decreased production of GSH (Hjelle and Grauer, 1986; Allen, 2003; Webb *et al.*, 2003; Sellon, 2006). GSH was 28% depleted in one dog 48 h after acetaminophen dosing (Wallace *et al.*, 2002).

Erythrocyte injury is the predominant problem associated with acetaminophen ingestion in cats. Oxidative injury to erythrocytes may take the form of methemoglobin production or Heinz body production (Rumbeiha *et al.*, 1995; Webb *et al.*, 2003). GSH becomes depleted in erythrocytes and hemoglobin is oxidized to methemoglobin, which cannot carry oxygen (Hjelle and Grauer, 1986; Aronson and Drobatz, 1996). Animals with 30% of their hemoglobin converted to methemoglobin show clinical signs and cyanotic mucous membranes (Rumbeiha *et al.*, 1995; Aronson and Drobatz, 1996). Methemoglobinemia is reversible (Schlesinger, 1995). Methemoglobin reductase is the enzyme responsible for converting methemoglobin to hemoglobin. Cats have less methemoglobin reductase activity than other domestic species (MacNaughton, 2003). Acetaminophen may produce methemoglobinemia in dogs, but this change is not seen in humans, rats, or hamsters (Hjelle and Grauer, 1986).

Heinz body formation is an irreversible change caused by the precipitation of hemoglobin (Schlesinger, 1995; Aronson and Drobatz, 1997). Heinz bodies increase red cell fragility and decrease survival time of erythrocytes, and thus may cause hemolysis and anemia (Schlesinger, 1995; Aronson and Drobatz, 1997; Allen, 2003). NAPQI binds to the sulfhydryl groups on hemoglobin (Allen, 2003). There are eight such groups on feline hemoglobin, but only four on the hemoglobin of other domestic species and two on the human molecule (Hjelle and Grauer, 1986; Rumbeiha *et al.*, 1995; Aronson and Drobatz, 1996; Allen, 2003; Sellon, 2006).

Hepatic effects predominate in dogs, mice, rats, and humans (Wallace *et al.*, 2002; Sellon, 2006). Oxidative damage to hepatocytes leads to zone 3 (centrilobular) hepatocyte degeneration and necrosis (Hjelle and Grauer, 1986;

Dahm and Jones, 1996; Treinen-Moslen, 2001; Wallace *et al.*, 2002). NAPQI acts as an electrophile. It causes tissue damage by forming covalent adducts with biological macromolecules (Savides and Oehme, 1985; Hjelle and Grauer, 1986; Jones *et al.*, 1992; Dahm and Jones, 1996; Sturgill and Lambert, 1997; Villar *et al.*, 1998; Zimmerman, 1999; Treinen-Moslen, 2001). Mitochondria may be a primary target for NAPQI, which may alter their functional integrity and bind to adenine nucleotides (Dahm and Jones, 1996; Treinen-Moslen, 2001). NAPQI may bind to membrane proteins that regulate calcium homeostasis, leading to increased intracellular calcium ions (Dahm and Jones, 1996; Sturgill and Lambert, 1997). Cytoskeletal damage or activation of endonucleases with DNA fragmentation has been proposed in the mechanism of cell death (Dahm and Jones, 1996). Production of superoxide anions and peroxidative injury occur during phase I metabolism of acetaminophen and cause oxidative stress to the cell, particularly after GSH depletion (Hjelle and Grauer, 1986; Dahm and Jones, 1996; Zimmerman, 1999; Sellon, 2006). Endothelial damage may cause some of the clinical signs reported, such as edema of the face and extremities and hemorrhage (Wallace *et al.*, 2002).

Factors that enhance the toxic effects of acetaminophen include GSH depletion due to fasting (Treinen-Moslen, 2001) and induction of P4502E1, as occurs with exposure to barbiturates (Sturgill and Lambert, 1997). Cimetidine inhibits cytochrome P450s and has been used therapeutically (Murphy, 1994; Schlesinger, 1995; Sellon, 1996; Aronson and Drobatz, 1997; Zimmerman, 1999; MacNaughton, 2003). Young animals may be less sensitive to acetaminophen toxicosis than mature animals. This is the case in children and neonatal mice, and is believed to be due to the immaturity of the mixed-function oxidase system and more rapid GSH synthesis in young animals (MacNaughton, 2003).

### *Clinical signs*

Clinical signs of acetaminophen toxicosis are attributable to its toxic effects on erythrocytes and hepatocytes. Clinical methemoglobinemia is the most common problem in cats and occurs in dogs. Centrilobular hepatic necrosis is more common in dogs and also occurs in other species, including cats, humans, rats, and hamsters (Hjelle and Grauer, 1986).

Clinical signs in cats usually occur within an hour or two of exposure. Anorexia is reported in 35% of cats presenting for acetaminophen exposure, as is vomiting. Hypersalivation is reported in 24% (Aronson and Drobatz, 1996), and commonly occurs within 2 h of exposure (Savides and Oehme, 1985). Diarrhea occurred in 18% of cats. Mental depression was reported in 76% (Aronson and Drobatz, 1996) and usually takes place within 3 h (Savides and Oehme, 1985).

Methemoglobinemia usually occurs within the first 4 h after acetaminophen ingestion and is dose dependent. Cats given a dose of 60 mg/kg acetaminophen had 21.7% of their hemoglobin converted to methemoglobin, and the methemoglobin concentration in cats dosed with 120 mg/kg acetaminophen was 45.5% (Hjelle and Grauer, 1986). Clinically evident cyanosis may occur at 30% methemoglobinemia (Rumbeiha, 1995; Aronson and Drobatz, 1996). Fifty-nine percent of the cats in Aronson and Drobatz (1996) study had pale or dark mucous membranes. An equal percent had evident respiratory distress, and blood was noted to be brown in 12%. Animals may appear weak and depressed (Hjelle and Grauer, 1986; Jones *et al.*, 1992; Allen, 2003) and 12% presented comatose (Aronson and Drobatz, 1997). Tachycardia was seen in 18%. Hemolysis, anemia, icterus, and pigmenturia have been described and may be seen for up to 48 h after acetaminophen ingestion (Savides and Oehme, 1985; Rubin and Papich, 1990; Rumbeiha, 1995; Allen, 2003; Villar *et al.*, 1998; Sellon, 2006).

Edema of the face and forelimbs or front paws is commonly described in affected cats (Savides and Oehme, 1985; Hjelle and Grauer, 1986; Papich, 1990; Murphy, 1994; Rumbeiha, 1995; Aronson and Drobatz, 1996; Villar *et al.*, 1998; Roder, 2005a). Aronson and Drobatz (1996) reported an incidence of 29%. Death is usually due to methemoglobinemia, but fatal liver failure (Rumbeiha *et al.*, 1995). Evidence of hepatic necrosis is unusual in cats, but has been reported with high-dose exposures, particularly in males (Roder, 2005a; Sellon, 2006).

Dogs may vomit soon after ingesting a high dose of acetaminophen (Schlesinger, 1995). More severe clinical signs in dogs are usually attributable to hepatic necrosis (Hjelle and Grauer, 1986; Schlesinger, 1995; Sellon, 2006). Signs may commence about 36 h after ingestion and include nausea and vomiting, anorexia, abdominal pain, and depression (Hjelle and Grauer, 1986; Murphy, 1994; Villar *et al.*, 1998; Boothe, 2001; Roder, 2005a). Tachycardia and tachypnea have also been noted (Roder, 2005a; Sellon, 2006). Mild cases may recover in another 2–3 days, but severe cases progress to icterus and death, usually within 4 days (Murphy, 1994; Sellon, 2006).

Methemoglobinemia also occurs in dogs, is often associated with high doses of acetaminophen, and may be more likely to cause death than liver failure (Schlesinger, 1995; Villar *et al.*, 1998; Wallace *et al.*, 2002; Roder, 2005a; Sellon, 2006). Methemoglobinemia usually occurs 4–12 h after ingestion but dogs may not present for 48 h (Wallace *et al.*, 2002; MacNaughton, 2003). Doses of 200 mg/kg acetaminophen PO converted 18.8% of hemoglobin to methemoglobin in dogs, and 500 mg/kg produced 51.5% methemoglobinemia (Hjelle and Grauer, 1986). Signs reported included cyanosis, brown color to blood, lethargy and recumbence (Schlesinger, 1995; Wallace *et al.*, 2002; MacNaughton, 2003). Hemolysis, anemia, icterus, and

shock have been described (Schlesinger, 1995). Several authors report pigmenturia. Occasionally, signs of methemoglobinemia occur in acetaminophen intoxicated dogs in the absence of clinically evident liver damage (Schlesinger, 1995; Wallace *et al.*, 2002).

Facial edema, edema of conjunctiva and nictitating membrane and edema of the forelimbs or paws have been reported in dogs (Hjelle and Grauer, 1986; Schlesinger, 1995; Villar *et al.*, 1998; MacNaughton, 2003; Sellon, 2006).

### *Clinical chemistry*

Methemoglobinemia and hemolysis may be noted on blood work. Whole blood exposed to air may have a brown tinge (Schlesinger, 1995; MacNaughton, 2003; Sellon, 2006). Heinz bodies in cats and dogs may be evident on a blood smear stained with new methylene blue (Murphy, 1994; Schlesinger, 1995). Heinz bodies are reported to occur 3 days after dosing (Webb *et al.*, 2003) and were evident in 12% of accidentally intoxicated cats (Aronson and Drobatz, 1997). Anemia occurred in 75% of cats (Aronson and Drobatz, 1997) and has been reported in dogs (Schlesinger, 1995; Wallace *et al.*, 2002; MacNaughton, 2003). Hyperbilirubinemia may occur within 48 h of acetaminophen ingestion in cats and has been reported in dogs with hemolysis (Schlesinger, 1995; Sellon, 2006). Regeneration was apparent in one dog 11 days after acetaminophen ingestion (Schlesinger, 1995).

Alanine transaminase (ALT) may be elevated in both cats and dogs. This change was reported by Aronson and Drobatz (1996) in 35% of cats exposed to acetaminophen. It has been reported to occur 3 days after dosing and may represent hepatic injury due to hypoxia or toxic insult in cats (Rumbeiha *et al.*, 1995; Webb *et al.*, 2003; Sellon, 2006). Increased ALT is usually believed to be due to toxic hepatic injury in dogs and occurs within 24 h of dosing (Roder, 2005a; Sellon, 2006). Hypoxic injury may contribute to increases in ALT in dogs (Schlesinger, 1995). Increases in aspartate transaminase (AST) and alkaline phosphatase (ALP) have been recorded (Schlesinger, 1995; Wallace *et al.*, 2002; Roder, 2005a). Prothrombin time (PT) and partial thromboplastin time (PTT) may be elevated if liver damage is severe (Sellon, 2006). Progressive decreases in serum cholesterol and serum albumin have been described (Sellon, 2006). Forty-one percent of cats presenting for acetaminophen toxicosis had hypocholesterolemia and 12% had hypoalbuminemia (Aronson and Drobatz, 1996).

Hemoglobinuria and hematuria may be noted in dogs and cats with acetaminophen toxicosis (Hjelle and Grauer, 1986; Murphy, 1994; Villar *et al.*, 1998; Wallace *et al.*, 2002; Sellon, 2006).

### *Diagnosis and management*

Diagnosis of acetaminophen poisoning is usually based on a history of clinical exposure and appropriate clinical signs. Plasma, serum, and urine are commonly tested for acetaminophen at human hospitals and some veterinary laboratories (Murphy, 1994; Allen, 2003; Sellon, 2006). Test results are not be available until hours or days after the sample is submitted, but acetaminophen toxicosis often presents as an emergency and treatment should be initiated immediately.

If the animal presents within 6 h of ingestion, decontamination measures should be instituted to prevent further absorption (Sellon, 2006). Emetics may be used in the alert animal (Wallace *et al.*, 2002; Roder, 2005a; Sellon, 2006). If large doses are ingested, gastric lavage of the anesthetized, intubated animal is appropriate (Roder, 2005a; Sellon, 2006). After gastric emptying, the patient is given activated charcoal and a cathartic such as sorbitol (Wallace *et al.*, 2002; Roder, 2005a; Sellon, 2006).

Antidotal therapy should be initiated as soon as possible in animals suspected of ingesting toxic doses of acetaminophen (Hjelle and Grauer, 1986; Villar *et al.*, 1998; Roder, 2005a). Antidotal therapy involves use of sulfate sources to bind NAPQI and enhance GSH production. Several compounds are available that act as sulfur donors, and include *N*-acetylcysteine, *S*-adenosylmethionine (SAMe), and sodium sulfate. *N*-acetylcysteine therapy is the most commonly recommended treatment for acetaminophen toxicosis. Additionally, antioxidants may be used to reduce methemoglobin.

*N*-acetylcysteine is a source of sulfhydryl groups that may be used for phase II sulfation or for production of GSH (Savides and Oehme, 1985; Hjelle and Grauer, 1986; Villar *et al.*, 1998). *N*-acetylcysteine is hydrolyzed to L-cysteine by deacetylation and may be oxidized in the liver to inorganic sulfate (Hjelle and Grauer, 1986). Use of *N*-acetylcysteine promotes sulfation of acetaminophen in cats. Rumbeiha *et al.* (1995) report a 50% decrease in the plasma half-life of acetaminophen in cats treated with *N*-acetylcysteine and Savides and Oehme (1985) reported an increase in the total fraction excreted as a sulfate conjugate. Cats treated with *N*-acetylcysteine have a more rapid recovery of blood GSH levels than untreated cats (Savides and Oehme, 1985). *N*-acetylcysteine decreased the half-life of methemoglobin in the blood of cats from more than 10–5 h (Rumbeiha *et al.*, 1995). *N*-acetylcysteine is reported to prevent anemia in acetaminophen intoxicated cats (Webb *et al.*, 2003).

*N*-acetylcysteine therapy should be initiated to any suspected case of acetaminophen toxicosis, even if exposure cannot be confirmed or time until treatment is delayed (Hjelle and Grauer, 1986; Villar *et al.*, 1998). Treatment is most effective if given within 8 h of exposure (Aronson and Drobatz, 1996; Roder, 2005a). Treatment within 24 h decreases mortality, but liver damage may occur (Roder, 2005a). *N*-acetylcysteine is sold in 10% and 20% solutions. Twenty percent solutions may be diluted 1:1 in normal saline or 5–10% dextrose solution (Plumb, 2002;

MacNaughton, 2003; Sellon, 2006). An initial dose of 140 mg/kg may be given intravenously (IV) slowly (Hjelle and Grauer, 1986; Murphy, 1994; Schlesinger, 1995; Aronson and Drobatz, 1996; MacNaughton, 2003; Roder, 2005a; Sellon, 2006). This dose may be given orally if no vomiting is evident and activated charcoal was not recently administered (Schlesinger, 1995; Sellon, 2006). Doses of 70 mg/kg should be given IV or PO every 6h thereafter for 42–48 h (Hjelle and Grauer, 1986; Schlesinger, 1995; Aronson and Drobatz, 1997; MacNaughton, 2003; Roder, 2005a).

SAMe is another possible sulfate source that has been used to treat acetaminophen toxicosis in dogs and cats. Decreased hemolysis and overall improvement was reported in treated dogs (Wallace *et al.*, 2002). SAMe may prevent methemoglobin production in cats, though this was not evident in one study. The number of Heinz bodies was lower and the packed cell volume (PCV) did remain higher in treated versus untreated cats (Webb *et al.*, 2003; Sellon, 2006). Dogs are given 40 mg/kg PO as their first dose and 20 mg/kg daily for 7–9 days (Wallace *et al.*, 2002; Sellon, 2006). Protocols that have been used experimentally in cats include doses of 180 mg/kg PO every 12 h for 3 days, and 90 mg/kg PO every 12 h for 14 days (Webb *et al.*, 2003; Sellon, 2006).

There are reports of sodium sulfate use to treat acetaminophen toxicosis. This protocol decreased the plasma half-life of acetaminophen and increased the amount excreted as a sulfate conjugate, similar to *N*-acetylcysteine (Savides and Oehme, 1985). Severity and duration of clinical signs were decreased in treated animals, methemoglobin levels were decreased, and GSH concentrations rebounded faster than in untreated animals (Villar *et al.*, 1998). Sodium sulfate was given as a 1.6% solution at a dose of 50 mg/kg IV every 4 h for six treatments (Savides and Oehme, 1985; Villar *et al.*, 1998).

Ascorbic acid and methylene blue are the most common compounds used to reduce methemoglobin to hemoglobin. Ascorbic acid causes nonenzymatic reduction of methemoglobin, but is slow acting (Hjelle and Grauer, 1986; Rumbeiha *et al.*, 1995; Aronson and Drobatz, 1997). Ascorbic acid may be given at a dose of 30 mg/kg PO every 6 h for six or seven treatments (Hjelle and Grauer, 1986; Aronson and Drobatz, 1996; MacNaughton, 2003; Sellon, 2006). Methylene blue has a more rapid onset of action than ascorbic acid, but is known to induce hemolytic anemia in cats, thus its use may be risky. Still, methylene blue has been used successfully for short-term management of methemoglobinemia in felines (Rumbeiha *et al.*, 1995; Aronson and Drobatz, 1996; Sellon, 2006). Use of methylene blue concurrently with *N*-acetylcysteine in cats should probably be avoided, at least in males. Rumbeiha *et al.* (1995) reported that this combined therapy caused severe depletion of blood GSH and actually increased the half-life of methemoglobin in the male cats in his study.

Supportive and symptomatic therapy for acetaminophen toxicosis may include oxygen therapy for animals with methemoglobinemia (Aronson and Drobatz, 1997; Villar *et al.*, 1998; Sellon, 2006). Transfusions or hemoglobin replacements should be administered to animals with severe anemia if needed (Murphy, 1994; Sellon, 2006). Fluid therapy is aimed at improving hydration, electrolyte balance, and pH (MacNaughton, 2003).

Cimetidine has been recommended for use in dogs and cats to inhibit acetaminophen metabolism by inhibiting cytochrome P450 enzymes (Murphy, 1994; Schlesinger, 1995; Aronson and Drobatz, 1996; Wallace *et al.*, 2002; Sellon, 2006). However, the doses required for enzyme inhibition may be higher than doses used routinely (Sellon, 2006).

The time that elapses between exposure and treatment may be the most important factor when determining the prognosis for survival, according to Aronson and Drobatz (1996). The median dose of acetaminophen ingested by cats that died or were euthanized was 100 mg/kg with a range for 10–170 mg/kg, whereas the median dose for survivors was 170 mg/kg with a range of 10–400 mg/kg. Most cats that survived were treated within 14 h of exposure, though one was not treated for 24 h. Most of the cats that were treated 17 or more hours post exposure died. Cats with underlying disease may have a worse outcome than otherwise healthy cats. The survival rate in dogs is decreased if they are not treated within 72 h (Sellon, 2006). Animals that were treated and survived usually recovered within 48 h and were hospitalized for 3 days (Aronson and Drobatz, 1996).

#### *Postmortem findings*

Icterus is a common finding secondary to acute hemolysis in cats or chronic cholestasis in dogs (Sellon, 2006). Centrilobular necrosis occurs commonly in intoxicated dogs and has been described in cats, though necrosis is often more diffuse in cats (Hjelle and Grauer, 1986; Wallace *et al.*, 2002; Webb *et al.*, 2003; Roder, 2005a; Sellon, 2006). Bile duct proliferation, vacuolar degeneration of hepatocytes, and mononuclear cholangitis are all reported in dogs with chronic liver injury (Sellon, 2006). Subcutaneous (SC) edema may extend from the head along the fascial planes of the neck and thorax, and may affect the conjunctiva (Allen, 2003).

## Nonsteroidal anti-inflammatory drugs

NSAIDs are defined straightforwardly as "compounds that are not steroidal and that suppress inflammation" (Boothe, 2001a). These drugs have antithrombic actions when taken at low doses, relieve minor pain and pyrexia at higher doses, and have anti-inflammatory effects at

higher doses (Rubin and Papich, 1990; Boothe, 2001a). NSAIDs are the drugs most commonly prescribed by physicians and it is estimated that 30 million people worldwide take NSAIDs, including aspirin, each day (Verbeeck, 1990). NSAIDs are commonly used to treat orthopedic problems in dogs (Roder, 2005a). About 8% of all human- and veterinary-related calls to the Illinois Poison Control Center were reported to involve NSAIDs, most commonly aspirin, ibuprofen, naproxen, piroxicam, indomethacin, and phenylbutazone. Seventy percent of the nonhuman animal calls were for dogs and 25% for cats (Kore, 1990). NSAID toxicosis may be related to a single large dose or multiple smaller doses (Albretsen, 2002).

There are more than 30 commercially available NSAIDs (Mazué *et al.*, 1982). They are classified based on their structure into the carboxylic acid group, which includes salicylic acid derivatives, acetic acid derivatives, fenamates or anthranilic acid derivatives, and the proprionic acid derivatives, as well as the enolic acid groups. An incomplete list of NSAIDs may be found in Table 23.1.

**TABLE 23.1 An incomplete list of NSAIDs**

| Carboxylic acid group | Enolic group |
|---|---|
| *Salicylic acid derivatives* | *Pyrazolone derivatives* |
| Aspirin | Azapropazone |
| Diflunisal | Dipyrone |
| Salicylates | Isopyrin |
| *Indolacetic acids* | Oxyphenbutazone |
| Etodolac | Phenylbutazone |
| Indomethacin | *Oxicam derivatives* |
| Sulindac | Meloxicam |
| Tolmetin | Peroxicam |
| Zomepirac | Tenoxicam |
| *Fenamates/anthranilic acid derivatives* | |
| Alclofenac | |
| Diclofenac | |
| Ibufenac | |
| Meclofenamic acid | |
| Mefanamic Acid | |
| Niflumic | |
| Tolfenamic acid | |
| *Propionic acid derivatives* | |
| Benoxaprofen | |
| Carprofen | |
| Ibuprofen | |
| Fenbufren | |
| Fenoprofen | |
| Flurbiprofen | |
| Ketoprofen | |
| Suprofen | |
| Tiaprofenic acid | |
| *Aminonicotinic acid derivatives* | |
| Flunixin meglamine | |
| Clonixin | |
| *Quinolone derivatives* | |
| Cinchopen | |

A relative few of these drugs are available OTC, the most common being aspirin, which is discussed separately below, in addition to ibuprofen, ketoprofen, and naproxen. These drugs will be addressed in more detail here, and a few other important veterinary drugs such as phenylbutazone will be mentioned. The mechanism of action is similar for all drugs in this classification. However, toxicity and pharmacokinetic data vary significantly between different compounds and different species. Cats, for example, are more susceptible to salicylate toxicosis than other species (Roder, 2005a), and dogs are very sensitive to ibuprofen (Rubin and Papich, 1990). Attempts should not be made to extrapolate the therapeutic dose from one species to another (Lees *et al.*, 1991).

Individual factors also affect susceptibility to NSAIDs. Young and aged animals may not metabolize these drugs in the same way as older animals (Roder, 2005a). Drug excretion may be delayed in animals with poor hepatic or renal function (Lees *et al.*, 1991; Isaacs, 1996; Roder, 2005a). Dehydration or cardiac disease may decrease renal circulation, slowing drug excretion, and promoting renal damage (Isaacs, 1996; Roder, 2005a). Gastrointestinal disease may predispose to ulceration when NSAIDs are used (Isaacs, 1996).

Various drugs may interact with NSAIDs. Changes of bioavailability may be caused by inhibition of absorption, displacement from binding proteins in plasma and tissues, or interference with elimination through competition for active renal secretion. Drugs may enhance or diminish metabolism of other drugs by hepatic cytochrome P450 enzymes (Verbeeck, 1990). An incomplete list of possible interactions may be found in Table 23.2. NSAIDs may affect the bioavailability of other drugs. There have been reports of deaths in people using NSAIDs with methotrexate, and ibuprofen decreases renal clearance of digoxin and lithium (Verbeeck, 1990). The diuretic affects of furosemide and bumetanide are mediated by prostaglandins (PGs) and may be inhibited by NSAIDs (Verbeeck, 1990; Isaac, 1996; Talcott, 2006). NSAIDs do not influence response to thiazide diuretics in this way, but may decrease the hypotensive response to thiazides (Verbeeck, 1990). NSAIDs may decrease the effectiveness of angiotensin converting enzyme (ACE) inhibitors such as captopril (Verbeeck, 1990; Talcott, 2006). NSAIDs may decrease the hypotensive response to $\beta$-blockers, though naproxen does not interfere with the effects of propranolol in humans (Verbeeck, 1990). NSAIDs have additive effects when used together, with decreased rates of metabolism and clearance for each (Verbeeck, 1990; Lees *et al.*, 1991). NSAIDs also have additive effects when used with corticosteroids, and such mixing should be avoided (Lees *et al.*, 1991; Talcott, 2006).

### *Toxicity*

There are significant species difference in NSAID tolerance, as noted above. Dogs are more susceptible to ibuprofen

**TABLE 23.2 Some NSAID–drug interactions; consult Verbeeck (1990) for more detailed information**

| |
|---|
| **Drugs that decrease absorption of some NSAIDs** |
| Aluminum hydroxide |
| **Drugs that compete for protein binding with NSAIDs** |
| Acetazolamide |
| Corticosteroids |
| Coumarin |
| Digitoxin |
| Hydantoin |
| Methyltrexate |
| Phenylbutazone |
| Phenytoin |
| Salicylates |
| Sulfonamides |
| Tolbutamide |
| Valproic acid |
| **Drugs that increase metabolism rate of some NSAIDs** |
| Antihistamines |
| Phenobarbital |
| Phenytoin |
| Rifampicin |
| **Drugs that decrease metabolism rate of some NSAIDs** |
| Anabolic steroids |
| Chloramphenicol |
| **Drugs that increase excretion rate of some NSAIDs** |
| Aluminum hydroxide |
| Magnesium hydroxide |
| **Drugs that decrease excretion rate of some NSAIDs** |
| Probenecid |
| Methotrexate |

and naproxen toxicosis than many other species (Kore, 1990; McKellar *et al.*, 1991; Isaacs, 1996;). Use of these drugs in dogs should be avoided.

Ibuprofen doses greater than 5 mg/kg have been associated with adverse reactions in dogs (Villar *et al.*, 1998). Gastric lesions occurred in dogs when 8 mg/kg ibuprofen per day is given over a 30-day period, either PO or parenterally, though clinical signs were not evident (McKellar *et al.*, 1991; Godshalk *et al.*, 1992; Boothe, 2001a; Talcott, 2006). Clinical signs were evident in dogs dosed with 16 mg/kg/day for 8 weeks (Villar *et al.*, 1998). A single dose of 50–125 mg/kg may be associated with gastrointestinal disease (Jackson *et al.*, 1991, Murphy, 1994; Talcott, 2006) and a perforating gastric ulcer was reported in a dog dosed with 110 mg/kg ibuprofen over a period of 48 h (Godshalk *et al.*, 1992). Doses of 175–250 mg/kg ibuprofen may be associated with acute renal failure (Kore, 1990; Godshalk *et al.*, 1992; Villar *et al.*, 1998; Talcott, 2006). Serum ibuprofen concentrations less than 31 μg/ml have not been associated with clinical signs in dogs. Melena was noted with serum concentrations of 138 μg/ml. Serum ibuprofen concentrations of 10–50 μg/ml are considered therapeutic in humans (Jackson *et al.*, 1991).

Cats dosed with 50 mg/kg ibuprofen had gastrointestinal hemorrhage and irritation (Kore, 1990; Talcott, 2006). Doses of 200 mg/kg were associated with renal failure and over 600 mg/kg caused death (Kore, 1990). A ferret that had ingested at least 347 mg/kg ibuprofen died from respiratory failure and asystole approximately 12 h post exposure (Cathers *et al.*, 2000).

Naproxen induced toxicosis in dogs given 5 mg/kg/day (Daehler, 1986; Rubin and Papich, 1990; Boothe, 2001a). Plasma naproxen concentrations were greater than 50 μg/ml (Boothe, 2001a). Severe toxic effects were seen in a Samoyed given 5.6 mg naproxen per kg for 1 week, including anemia, melena, and renal and hepatic dysfunction (Kore, 1990). A perforating ulcer was described in a dog given 10–20 mg/kg naproxen per day sporadically over several weeks (Daehler, 1986). Doses of 15 mg/kg/day were lethal in some dogs (Daehler, 1986; Gfeller and Sandors, 1991).

Horses do not appear to be sensitive to naproxen toxicosis. Horses given 3 times the recommended dose of naproxen for 6 weeks did not have clinical signs (Boothe, 2001). More than a quarter of humans given therapeutic doses of naproxen, on the other hand, had gastric lesions on endoscopy and fatalities from gastrointestinal hemorrhage have been reported (Daehler *et al.*, 1986; Carson and Strom, 1988).

Phenylbutazone is commonly used in horses. The recommended oral dose is 4 mg/kg/day (Plumb, 2002). A dose of 4.4 mg/kg every 12 h over a 2-week period was associated with changes of mineralization patterns in cortical bone in growing horses from 18 to 30 months of age. This effect was reversible (Brumbaugh, 2001). Doses of 4.4 mg/kg twice per day have been associated with clinical signs including central nervous system (CNS) depression and protein losing enteropathy. Shock was reported in one of nine horses after 5 days of dosing (Collins and Tyler, 1985). Therapeutic plasma concentrations for phenylbutazone in a horse range from 10–30 μg/ml. The therapeutic range in humans, 100–150 μg/ml, would be lethal in a horse (Lees *et al.*, 1991). Phenylbutazone doses of 100 mg/kg/day did not cause gastrointestinal ulcers in dogs (Mazué *et al.*, 1982).

### *Toxicokinetics*

NSAIDs are small, weakly acidic molecules with p$K_a$ values less than 4.5 and thus are well absorbed in the stomach (Mazué *et al.*, 1982; Kore, 1990; Rubin and Papich, 1990; Verbeeck, 1990; Isaac, 1996; Boothe, 2001a). Peak plasma concentrations for most NSAIDs occur within 3 h of oral dosing (Verbeeck, 1990). Some absorption may occur in the proximal small intestine (Roder, 2005a).

Ibuprofen is 60–80% bioavailable in dogs (McKellar *et al.*, 1991; Boothe, 2001a). Naproxen is 68–100% bioavailable in dogs (McKellar *et al.*, 1991; Boothe, 2001a) and blood levels are almost identical whether dosing is PO or IV (Runkel *et al.*, 1972). Naproxen is 50% bioavailable in

horses (Boothe, 2001a) and nearly 100% bioavailable in pigs (Runkel *et al.*, 1972).

NSAID distribution is variable between individual drugs and species (Boothe, 2001a; Talcott, 2006). Because they are highly protein bound in the circulation, mostly to albumin, NSAIDs generally have a low volume of distribution (Brater, 1988; Verbeeck, 1990; Boothe, 2001a; Talcott, 2006). Protein binding in humans is 99% for ibuprofen (Brater, 1988), greater than 99% for naproxen (Runkel *et al.*, 1972; Rubin and Papich, 1990), and 98.7% for ketoprofen (Brater, 1988). The remaining unbound fraction becomes distributed in the extracellular fluid and is responsible for the clinical effects (Brater, 1988; Kore, 1990; Boothe, 2001a; Talcott, 2006). Hypoalbuminemia or displacement from binding sites on albumen by other drugs leads to a transient increase in the unbound fraction. Though this unbound drug will be excreted rapidly in most cases, clinical effects may be evident (Boothe, 2001a). Age effects protein binding in humans, and the unbound fraction of a drug in an elderly patient is approximately twice that expected in a younger individual (Brater, 1988). NSAIDs can partition into lipid, allowing them to cross cell membranes (Boothe, 2001a; Talcott 2006).

Metabolism of NSAIDs takes place primarily in the liver (Kore, 1990; Verbeeck, 1990; Boothe, 2001a). They often undergo cytochrome P450 mediated oxidation to increase water solubility (Verbeeck, 1990; Talcott, 2006). Phenylbutazone is converted to oxyphenbutazone, which is similarly potent, via phase I hepatic metabolism (Lees *et al.*, 1991). Phase I metabolites and, in the case of carboxylic acid group NSAIDs, the parent compound can undergo phase II reactions such as glucuronide conjugation, sulfate conjugation, and GSH conjugation, which further increases water solubility and usually inactivate the drug (Talcott, 2006). Acyl-glucuronidation of propionic acid NSAIDs such as ibuprofen and naproxen is reversible. Drugs may deconjugate if excretion is delayed (Brater, 1988; Verbeeck, 1990).

Drug elimination is dependent on compound and species (Lees *et al.*, 1991; Boothe, 2001a). This may be the basis for many adverse reactions (Boothe, 2001a). Animals less than 6 weeks old and aged animals may metabolize drugs more slowly, and hepatic or renal failure may delay elimination (Mazué *et al.*, 1982; Boothe, 2001a; Talcott, 2006). High plasma protein binding slows excretion (Runkel *et al.*, 1972; Talcott 2006).

Plasma clearance is biphasic, with a rapid initial decline as the drug is distributed to the tissues and a slower decline as it is metabolized and excreted, terminating drug activity (Lees *et al.*, 1991). Urinary excretion is the most important rout of elimination for metabolites of NSAIDs in humans (Verbeeck, 1990). Less than 1% of a dose of naproxen or ketoprofen is eliminated unchanged, and approximately 1% of a given dose of ibuprofen is excreted in the urine as the parent compound (Brater, 1988). Urinary excretion is pH dependent and tends to be more rapid in alkali urine due to ion trapping (Kore, 1990; Talcott, 2006).

Fecal elimination through biliary excretion is important for ibuprofen and naproxen in dogs (Runkel *et al.*, 1972; Gfeller and Sandors, 1991; Daehler, 1996; Isaacs, 1996; Talcott, 2006). Half of a given dose of naproxen is eliminated in the bile of dogs. Only 1–2% is eliminated in the bile in humans and 94% is found in the urine within 5 days of administration (Runkel *et al.*, 1972). Drugs eliminated in the bile, such as ibuprofen and naproxen, as well as indomethacin, piroxicam, flunixin, tolfenamic acid, meclofenamic acid, and diclofenac, undergo enterohepatic circulation in dogs (Lees *et al.*, 1991; Isaacs, 1996; Talcott, 2006). This may increase the susceptibility of dogs to these compounds by prolonging their plasma half-life.

The plasma half-life of ibuprofen is 2.5–6 h in dogs and cats, 1 h in rats, and 2–3 h in humans (Mazué *et al.*, 1982; Lees *et al.*, 1991; Boothe, 2001a). The half-life for naproxen in plasma is 35 h in beagles (Runkel *et al.*, 1972) and 74 h in mixed-breed dogs (Isaacs, 1996), 5 h in horses (Lees *et al.*, 1991; Isaacs, 1996), 4.8 h in cows (Lees *et al.*, 1991), 4.8 h in minipigs, 1.9 h in Rhesus monkeys, 8.7 h in guinea pigs, and about 14 h in humans (Runkel *et al.*, 1972; Lees *et al.*, 1991; Isaacs, 1996). Elimination of phenylbutazone in dogs may follow zero-order kinetics (Lees *et al.*, 1991). The plasma half-life is between 5 and 8 h in horses, 37 h in cattle, 4 h in swine, and 72 h in humans (Lees *et al.*, 1991).

### *Mechanism of action*

The basic mechanism of action of NSAIDs is through inhibition of COX enzymes. These enzymes are found in all cells except mature erythrocytes (Kore, 1990; Boothe, 2001a). Arachidonic acid, a 20-carbon unsaturated fatty acid, is released from the cell membrane by phospholipase $A_2$ and phospholipase C when a cell is damaged (Mazué *et al.*, 1982; Kore, 1990; Lees *et al.*, 1991; Boynton *et al.*, 1988; Boothe, 2001a). Carprofen causes moderate inhibition of phospholipases (Lees *et al.*, 1991; McKellar *et al.*, 1991). Though arachidonic acid itself has little activity, it can enter two pathways, the COX pathway which produces eicosanoids, or the lipoxygenase pathway which produces leukotrienes (LTs) (Lees *et al.*, 1991).

Oxidation of arachidonic acid by COX and further metabolism by other enzymes leads to the production of various PGs and release of oxygen free radicals (Boynton *et al.*, 1988; Lees *et al.*, 1991). These PGs include $PGH_2$, $PGE_2$, and $PGI_2$. With the addition of prostacycline synthase $PGF_{2\alpha}$ is formed, and thromboxane synthase is needed for production of thromboxane $A_2$ (Kore, 1990; Lees *et al.*, 1991; Boynton *et al.*, 1988).

$PGE_2$ and $PGI_2$ have similar actions, though the effects of $PGI_2$ tend to be of shorter duration (Lees *et al.*, 1991). $PGE_2$, which is secreted by the gastrointestinal mucosa and at other sites, causes smooth muscle relaxation, vasodilation

which enhances blood flow to the kidneys and gastric mucosa, and increased vascular permeability. It inhibits gastric acid production and pepsin production, increases gastric mucus synthesis, and is believed to mediate repair and turnover of gastric epithelium (Collins and Tyler, 1985; Boynton *et al.*, 1988; Kore, 1990; Rubin and Papich, 1990; Wallace *et al.*, 1990; Lees *et al.*, 1991). $PGE_2$ is also found in inflammatory exudate and enhances pain response due to bradykinin and histamine (Kore, 1990; Lees *et al.*, 1991). $PGI_2$ also inhibits platelet aggregation (Lees *et al.*, 1991). $TXA_2$ is produced in platelets, where it stimulates aggregation and promotes vasoconstriction through its action on vascular smooth muscle. $PGF_{2\alpha}$ causes smooth muscle constriction (Boynton *et al.*, 1988; Kore, 1990; Rubin and Papich, 1990).

NSAIDs bind the active site of COX, usually through competitive inhibition, though aspirin binds platelet COX irreversibly (Rubin and Papich, 1990; Talcott, 2006). There are two isoforms of COX, conveniently designated $COX_1$ and $COX_2$. $COX_1$ is found in almost all tissues, including the gastrointestinal tract, platelets, endothelium, and kidneys, is continuously produced, and functions in tissue homeostasis. Most of the adverse affects associated with NSAID use are due to inhibition of $COX_1$ (Boothe, 2001a; Roder, 2005a; Talcott, 2006). Inhibition of $PGE_2$ promotes production of gastric acid and pepsin, decreases the ability of the mucosa to secrete mucus glycoproteins and bicarbonate, and to respond to injury (Mazué *et al.*, 1982; Collins and Tyler, 1985; Kore, 1990; Rubin and Papich, 1990; Isaacs, 1996; Talcott, 2006). Impairments to mucosal circulation due to loss of PG activity may cause mucosal hypoxia and thrombosis (Mazué *et al.*, 1982; Talcott, 2006).

Loss of the vasodilative actions of $PGE_2$ and $PGI_2$ produced by the kidneys may lead to hypoxic renal injury (Rubin and Papich, 1990; Isaacs, 1996; Talcott, 2006). Production of PGs by the kidneys is relatively low, and the renal effects of acute NSAID toxicosis are seldom reported in the veterinary literature (Rubin and Papich, 1990; Talcott 2006). Renal pathology is more often associated with chronic NSAID use (Boothe, 2001a; Talcott, 2006). Renal papillary necrosis has been described frequently in horses, and is often associated with chronic phenylbutazone use (Gunson, 1983; Rubin and Papich, 1990). This condition has also been reported in cats, dogs, mice, rats, gerbils, hamsters, rabbits, desert mice, primates, and pigs (Brix, 2002). The traditional theory for pathogenesis of this lesion holds that decreased PG production leads to redistribution of blood away from the renal medulla, causing endothelial cell necrosis (Kore, 1990; Roder, 2005a). $COX_1$ is present in high concentrations within renal collecting ducts and vasculature. Recent evidence suggests that low doses of NSAIDs cause degeneration of medullary interstitial cells and later damage to vascular endothelium, leading to microvascular thrombosis and hypoxia. Higher doses cause early endothelial damage (Brix, 2002). Dehydration is a major predisposing factor for renal papillary necrosis (Gunson, 1983; Rubin and Papich, 1990; Villar *et al.*, 1998; Isaacs, 1996; Boothe, 2001a; Talcott, 2006). Dehydration may be secondary to gastrointestinal disease, diuretic use, anesthesia, surgical stress, hemorrhagic shock, or sepsis (Kore, 1990; Isaacs, 1996; Talcott, 2006). Other risk factors described for dogs include advancing age, congestive heart failure, hepatic cirrhosis, preexisting renal problems, hypotension, and concurrent administration of nephrotoxic drugs such as gentamicin or amphotericin (Boynton *et al.*, 1988; Kore, 1990; Rubin and Papich, 1990; Isaac, 1996; Boothe, 2001a; Talcott, 2006).

Inhibition of platelet $COX_1$ activity leads to decreased $TXA_2$ production and prolonged bleeding times (Kore, 1990; Rubin and Papich, 1990; Talcott, 2006). Predisposing factors in dogs include concurrent use of anticoagulants and preexisting bleeding disorders such as von Willebrands' disease (Kore, 1990; Rubin and Papich, 1990; Talcott, 2006).

$COX_2$ is produced by macrophages, fibroblasts, chondrocytes, endothelial cells, and some other cell types (Roder, 2005a). This isoform only functions intermittently and is induced by cytokines in areas of inflammation (Isaacs, 1996; Roder, 2005a; Talcott, 2006). Inhibition of this enzyme produces antipyretic, analgesic, and anti-inflammatory effects of NSAIDs (Roder, 2005a; Talcott, 2006).

Most NSAIDs inhibit both isoforms of COX, but some new drugs have been developed that are specific for human $COX_2$ enzymes. These drugs include nimesulide, meloxicam, flosulid, and etodolac. Selectivity is species specific, and thus these new drugs are likely to cause $COX_1$ inhibition in domestic species (Talcott, 2006). $COX_2$ selective drugs will cause $COX_1$ inhibition in humans if given in very high doses (Albretsen, 2002; Talcott, 2006). Interestingly, caprofen is $COX_2$ specific in dogs, though not in humans (Talcott, 2006).

LTs are also produced from arachidonic acid via the lipoxygenase pathway; 5-lipoxygenase produces LTA4 which is converted to LTB4, LTC4, LTD4, LTE4, and others. LTB4 is an important chemoattractant for neutrophils. Various LTs cause vasoconstriction, bronchospasm, and increased vascular permeability (Boynton *et al.*, 1988; Strøm and Krogsgaard-Thomsen, 1990; Lees *et al.*, 1991). Some NSAIDs inhibit lipoxygenase. It has been found that in dogs, flunixin is a more potent inhibitor of LTB4 mediated neutrophil migration than phenylbutazone, which is more potent than indomethacin (Strøm and Krogsgaard-Thomsen, 1990). Alternately, some NSAIDs may increase LT production due to increased availability of arachidonic acid not entering the COX pathway (Boothe, 2001a).

NSAIDs may inhibit phosphodiesterase. Phosphodiesterase breaks down cyclic AMP (cAMP). Increased intracellular cAMP levels may stabilize lysosomal membranes in polymorphonuclear leukocyte, inhibiting release of inflammatory products (Kore, 1990).

NSAIDs may have topical irritant properties. They are weak acids and may partition to the gastric mucosa. This leads to decreased hydrophobicity of mucus and thinning of the mucus barrier, allowing gastric acid to penetrate to the epithelial layer (Mazué *et al.*, 1982; Gfeller and Sandors, 1991; Roder, 2005a; Talcott, 2006). Enterohepatic recycling may enhance these toxic effects (Mazué *et al.*, 1982; Isaacs, 1996; Boothe, 2001a; Talcott, 2006).

NSAIDs frequently cause mild and transient liver damage, which may lead to cholestasis and increased liver enzymes (Boynton *et al.*, 1988; Isaacs, 1996; Roder, 2005a). More severe problems, such as hepatic necrosis, are rare (Boynton *et al.*, 1988). Four percent of people with ibuprofen toxicosis have hepatotoxicosis, but lethal hepatic effects have not been reported and hepatic toxicosis is uncommon in dogs (Talcott, 2006).

Cartilage degeneration has been reported in mice given aspirin, phenylbutazone, indomethacin, ibuprofen, and naproxen (Isaac, 1996). Anaphylactic reactions have been reported, but hypersensitivity to NSAIDs is rare (Kore, 1990; Isaacs, 1996). Agranulocytosis and aplastic anemia, though rare, is a major cause of NSAID-related death in humans (Boynton *et al.*, 1988). This problem is even less common in domestic animals, but has been documented in dogs due to phenylbutazone administration (Rubin and Papich, 1990; Lees *et al.*, 1991; Isaacs, 1996;). More commonly reported in dogs is idiosyncratic hepatotoxicosis due to carprofen (Albretsen, 2002; Roder, 2005a; Talcott, 2006). Approximately one-third of these cases were in Labrador retrievers (Talcott, 2006).

### *Clinical signs*

The most common clinical signs of NSAID overdose in all species are due to gastric mucosal irritation and ulceration. All NSAIDs tested have produced peptic ulcers in animal models (Carson and Strom, 1988). Clinical signs do not always correlate well with the severity of the lesion, possibly due to the analgesic effect of NSAIDs. The asymptomatic ulcer rate among NSAID users is estimated at 50% (Isaacs, 1996). Emesis is frequently reported and the vomitus may contain blood (Wallace *et al.*, 1990; Gfeller and Sandors, 1991; Lees *et al.*, 1991; Jones *et al.*, 1992; Murphy, 1994; Villar *et al.*, 1998; Boothe, 2001a; Roder, 2005a). Anorexia, diarrhea, melena, and abdominal pain or colic may be noted (Wallace *et al.*, 1990; Gfeller and Sandors, 1991; Daehler, 1986; Isaacs, 1996; Roder, 2005a). Animals with gastric perforations will often present with a distended abdomen due to effusion, dehydration, pyrexia, collapse, pallor, and abdominal pain (Godshalk *et al.*, 1992; Talcott, 2006).

The lower gastrointestinal tract may be affected, leading to protein losing enteropathy, most common in horses, or stricture formation (Collins and Tyler, 1984, 1985; Isaacs, 1996). Horses with hypoproteinemia may show secondary edema (Collins and Tyler, 1984, 1985). Endotoxemia and shock have been noted in horses secondary to gastrointestinal ulceration (Collins and Tyler, 1984, 1985).

Analgesic nephropathy occurs in humans but is uncommon in domestic animals, though it has been reported in dogs given naproxen (Isaacs, 1996; Boothe, 2001a). Chronic use of NSAIDs and high doses may be predisposing factors (Lees *et al.*, 1991; Boothe, 2001a). Clinical signs may be evident after 2 weeks or many months of chronic dosing (Kore, 1990) or after single very high doses (Mazué *et al.*, 1982; Murphy, 1994). Acute onset of oliguric renal failure has been described (Kore, 1990; Villar *et al.*, 1998; Roder, 2005a). Animals may present with polyuria and polydipsia, dehydration, oral ulceration, and uremic coma (Mazué *et al.*, 1982).

People in early stages of renal papillary necrosis may have headaches. Later clinical signs include nocutria, dysuria, polyuria with the presence of bacteria, ureteral colic, and lower back pain. Later in the disease process, they may experience hypertension, cardiovascular disease, urolithiasis, and pyelonephritis (Brix, 2002).

Hepatotoxicosis is not common in NSAID intoxicated small animals, though it has been reported in a dog given naproxen (Kore, 1990; Lees *et al.*, 1991; Isaacs, 1996). Clinical signs may include anorexia and weight loss, vomiting, lethargy, icterus, ascites, and coagulopathy (Albretsen, 2002; Roder, 2005a). Increased bleeding times are rarely reported in domestic animals (Murphy, 1994). Prolonged gestation and teratogenesis have been seen experimentally (Lees *et al.*, 1991).

### *Clinical chemistries*

Approximately one-quarter of gastroduodenal ulceration cases in dogs present with microcytic, hypochromic anemia due to chronic blood loss (Stanton and Bright, 1989). Acute blood loss is uncommon. Individuals with acute blood loss present with rapid deterioration, normocytic and normochromic anemia, a normal albumin to globulin ratio, and evidence of regeneration (Stanton and Bright, 1989; Wallace *et al.*, 1990). Peracute hemorrhage is rare and may occur if a mesenteric blood vessel is eroded in the area of ulceration. Such a complication would be rapidly lethal and no regenerative response would occur (Stanton and Bright, 1989; Wallace *et al.*, 1990). Increased PCV may be seen in horses with diarrhea due to dehydration (Collins and Tyler, 1985).

Fecal occult blood is unreliable. It may be negative for low-level blood loss or positive if red meat was fed within 72 h of testing (Talcott, 2006). Hypoalbuminemia has been seen with gastrointestinal ulcers and protein loosing eneteropathy secondary to NSAID use (Collins and Tyler, 1984; Roder 2005a; Talcott, 2006). Both albumin and globulin concentrations will be decreased (Collins and Tyler, 1984; Talcott, 2006). Coagulation profiles may reveal poor

platelet aggregation, thrombocytopenia, and increased bleeding times (Talcott, 2006).

Serum chemistry may reveal increased ALT, AST, ALP, and bilirubin (Albretsen, 2001; Roder, 2005a). A mild, transient rise in liver enzymes frequently occurs during the 1st week of NSAID administration (Boynton *et al.*, 1988; Isaacs, 1996). Elevated blood urea nitrogen (BUN) may indicate nephropathy (Roder, 2005a; Talcott, 2006). Phosphorus, calcium, and potassium may also be elevated (Kore, 1990; Villar *et al.*, 1998; Talcott, 2006).

Animals with renal damage due to NSAIDs may present with isosthenuria, hematuria, and proteinuriea (Kore, 1990; Talcott, 2006). Ketonuria and polyuria have also been reported (Mazué *et al.*, 1982; Kore, 1990).

Horses on chronic high-dose phenylbutazone therapy had normal lymphocyte counts with a left shift due to neutrophil loss into the gastrointestinal tract. Increased serum glucose levels were attributed to endotoxemia. Hypokalemia and hyponatremia may be seen in horses with diarrhea (Collins and Tyler, 1985).

### *Diagnosis and management*

Diagnosis of NSAID toxicosis is often based on history and clinical signs. Serum or plasma testing is available at many veterinary laboratories and human hospitals (Roder, 2005a; Talcott, 2006). Serum drug concentrations are unlikely to be useful in management, but some reference values are listed in the toxicokinetics portion of this section.

Endoscopy is the most sensitive test for gastric ulceration, though occasionally the ulcer will not be visible. Anesthesia is required, thus the patient must be stabilized (Wallace *et al.*, 1990; Talcott, 2006). Survey radiographs are not usually useful (Stanton and Bright, 1989; Wallace *et al.*, 1990). If there is gastrointestinal perforation, then poor visualization of serosal surfaces, peritoneal effusion, free gas in the peritoneal cavity, and intestinal ileus may be noted (Godshalk *et al.*, 1992; Talcott, 2006). Large ulcerations may be identified on contrast radiographs (Wallace *et al.*, 1990). However, if perforation is present, barium sulfate will complicate peritonitis, thus a water-soluble agent is preferred (Talcott, 2006). Ulcers may be detected using ultrasound in an animal given water by stomach tube. The gastric wall is thickened with a loss of the normal five-layer structure, disruption of the mucosa, and gas bubble formation. Increased fluid in the abdomen will be seen in the case of perforation due to peritonitis (Talcott, 2006).

Renal papillary damage can be diagnosed in humans, horses, and foals using ultrasound (Brix, 2002; Roder, 2005a). Computed tomography has been used to diagnose renal papillary necrosis in humans, and magnetic resonance microscopy has been used experimentally (Brix, 2002).

Animals on NSAID therapy should be closely monitored for adverse effects. Attitude and appetite, hydration, electrolyte status, urine concentrating ability, urine protein concentrations, BUN, creatinine, and liver enzymes should be examined (Villar *et al.*, 1998; Roder, 2005a).

When animals present with adverse effects attributable to an NSAID, administration of the drug should be discontinued (Wallace *et al.*, 1990; Albretsen, 2001; Talcott, 2006). Gastric emptying is appropriate for animals that have ingested large quantities of NSAIDs but are not yet showing clinical signs (Kore, 1990). Emetics may be used in some species, though they are contraindicated in animals with severe CNS suppression (Albretsen, 2001; Roder 2005a; Talcott, 2006). Gastric lavage may be used in the anesthetized, intubated animal (Cathers *et al.*, 2000). Activated charcoal and cathartics should be given after gastric emptying or if the animal presents late (Kore, 1990; Cathers *et al.*, 2000; Albretsen, 2001; Roder, 2005a; Talcott, 2006). Repeated activated charcoal administration is recommended for various reasons. Some tablets are sustained release. Regular tablets may form concretions in the stomach and have delayed absorption. Furthermore, enterohepatic recycling is believed to cause the prolonged half-life of ibuprofen and other NSAIDs in dogs (Kore, 1990; Cathers *et al.*, 2000). Animals should be monitored for hydration, electrolyte imbalances, acid–base imbalances, blood glucose, liver enzymes, BUN and creatinine, body temperature, and blood pressure. Deviations from expected values should be corrected as needed (Cathers *et al.*, 2000; Albretsen, 2001). Fluid therapy may be needed to correct dehydration, hypotension, electrolyte imbalances, and improve renal perfusion (Kore, 1990; Villar *et al.*, 1998; Talcott, 2006). It has been suggested that fluids be given at twice maintenance rates. Villar *et al.* (1998) recommend 0.9% saline, or 0.45% saline plus 2.5% dextrose in hypoglycemic animals, given at a rate of 120 ml/kg/day plus estimated fluid losses over a 48–72 h period. Lactated ringers solution or 0.9% saline may contribute to hypernatremia (Albretsen, 2001). Dopamine at a dose of 2.5 μg/kg/min or dubutamine at 2.5 μg/kg/min may be given to increase renal perfusion (Kore, 1990). Diuretics may predispose animals to dehydration and subsequent renal hypoperfusion, thus should be avoided (Albretsen, 2001). Sodium bicarbonate may be used to treat acidosis (Murphy, 1994; Roder, 2005a). Urine alkalinization for ion trapping may be useful with salicylates, aspirin, and ibuprofen, but urinary pH must exceed 7.5–8 to increase naproxen secretion (Runkel *et al.*, 1972; Villar *et al.*, 1998; Roder, 2005a). Urine alkalinization must be used with caution to prevent metabolic alkalosis. Forced diuresis and dialysis are of limited use to enhance elimination of NSAIDs due to the high level of protein binding (Kore, 1990; Cathers *et al.*, 2000). If perforation has occurred, parenteral fluids can become sequestered into the abdomen (Talcott, 2006).

Gastric lavage with iced saline or water has been recommended for severe and continued gastric bleeding, but the efficacy of this treatment is questionable and surgical

correction may be required in cases of refractory bleeding (Wallace *et al.*, 1990). Transfusions or use of blood substitutes may be needed to treat severe anemia (Murphy, 1994; Sellon, 2006). Acute loss of greater than 30% of the blood volume, a PCV less than 20%, plasma protein less than 3.5 g/dl, or continued blood loss are possible indications for transfusion (Talcott, 2006). Vitamin $K_1$ may be given to aid in coagulation (Kore, 1990). Perforating ulcers must be managed surgically (Godshalk *et al.*, 1992; Talcott, 2006).

Gastrointestinal protectants are typically used in NSAID overdose cases to prevent or heal gastric ulcerations. Healing of gastric ulcers is highly correlated with gastric pH in humans. Gastric pH must be maintained above 3 for 18–20 h a day for effective healing, and above 4 in critically ill patients. Gastric pH above 4 inhibits fibrinolysis and the activity of pepsin. Gastric pH must be above 6 to promote platelet aggregation and prevent clot dissolution, therefore allowing hemostasis. Gastric secretion is variable in beagles, with a median pH of 4.44 in fasted dogs and of 1.30 in dogs with fixed feeding times (Bersenas *et al.*, 2005). Therefore, simply withholding food has the benefit of decreasing gastric acid secretion as well as minimizing emesis (Wallace *et al*. 1990; Talcott, 2006).

Treatment with antacids such as magnesium hydroxide (milk of magnesia) or aluminum hydroxide may be adequate for animals exposed to low NSAID doses (Kore, 1990; Jackson *et al.*, 1991; Isaacs, 1996; Villar *et al.*, 1998; Cathers *et al.*, 2000). Antacids require dosing every 2–4 h and may cause changes in fecal consistency (Wallace *et al.*, 1990). Products containing bismuth subsalicylate (Pepto Bismol and Kaopectate) should be avoided because salicylate is an NSAID (Kore, 1990).

Misoprostol is a long-lasting synthetic analog of $PGE_1$. It is the only agent that consistently prevents and treats NSAID-induced gastric and duodenal ulcers (Isaac, 1996). When given concurrently with aspirin, misoprostol prevented gastric ulcers in dogs (Villar *et al.*, 1998). Dogs given NSAIDs and misoprostol had less gastrointestinal hemorrhage, less vomiting, and less mucosal ulceration on endoscopy than dogs given NSAIDs only (Talcott, 2006). Misoprostol may protect renal function in domestic animals and may be chondroprotective in pigs (Isaacs, 1996). This drug has been used safely for up to a year in humans (Talcott, 2006). However, at high doses misoprostol may cause diarrhea (Villar *et al.*, 1998) and use of this synthetic PG is contraindicated in pregnant animals (Villar *et al.*, 1998; Talcott, 2006). Dogs may be given 1–5 μg/kg misoprostol PO every 8 h (Plumb, 2002).

Proton pump inhibitors suppress gastric acid secretion by inhibiting the $Na^+/K^+$ ATPase pump in gastric parietal cells (Walan *et al.*, 1989; Villar *et al.*, 1998). Omeprazole and pantoprazole are proton pump inhibitors (Bersenas *et al.*, 2005). Omeprazole has been used successfully in humans and dogs to treat gastric ulcer (Walan *et al.*, 1989; Villar *et al.*, 1998; Roder, 2005a; Talcott, 2006). Eighty percent of people treated with omeprazole at a dose of 40 mg for 4 weeks had gastric ulcer healing compared to 69% given a dose of 20 mg and 59% given ranitidine. After 8 weeks, 96% of the 40 mg dose group had ulcer healing, compared to 89% of the 20 mg group and 85% of the ranitidine group. Healing rates were also higher for duodenal ulcers in patients treated with omeprazole versus ranitidine (Walan *et al.*, 1989). Bersenas *et al.* (2005) found 2 mg/kg omeprazole PO given to dogs twice per day kept their gastric pH from going below 3 for 90.9% of the day, and kept the gastric pH above 4 for 78.3% of the day, though one in six dogs vomited. Omeprazole remains biologically active for nearly 24 h and only need be given once per day. Plumb (2002) lists the canine daily dose as 0.5–1.0 mg/kg PO, and the feline dose as 0.7 mg/kg. Villar *et al.* (1998) recommend a 3–4-week treatment protocol. Rebound acid secretion has been reported at discontinuation of omeprazole therapy (Driman *et al.*, 1995).

Sucralfate is an aluminum salt of sucrose sulfate. This drug complexes with exposed proteins on the surface of gastric ulcers, specifically fibrinogen and albumin, and acts as a direct mucosal protectant (Kore, 1990; Wallace *et al.*, 1990; Talcott, 2006). Sucralfate has 5 times as much affinity for damaged mucosa as it does for normal mucosa (Villar *et al.*, 1998). Sucralfate also adsorbs pepsin and bile acids (Kore, 1990; Wallace *et al.*, 1990; Talcott, 2006). This drug may promote bicarbonate and mucus secretion by the gastric mucosa (Kore, 1990). Sucralfate is equally as effective as cimetidine at reducing discomfort and promoting ulcer healing (Villar *et al.*, 1998). Dogs given sucralfate to treat gastric ulcers, with and without concurrent cimetidine treatment, had complete healing of the gastric mucosa within 9 days of treatment (Talcott, 2006). Sucralfate may be used alone if gastric hyperacidity is not suspected (Wallace *et al.*, 1990). If used with cimetidine, dosing should be staggered or cimetidine should be given by a parenteral route. Sucralfate may interfere with absorption of cimetidine from the gastrointestinal tract (Wallace *et al.*, 1990). Cimetidine should be given 1 h before a meal (Murphy, 1994; Villar *et al.*, 1998). Dogs are dosed with sucralfate every 8 h. Dogs weighing more than 20 kg can be given 1 g per dose, and smaller dogs can be dosed with 0.5 g (Murphy, 1994; Albretsen, 2002; Plumb, 2002). Cats may be given a total of 0.25–0.5 g every 8–12 h and ferrets may be given 75 mg/kg 4–6 times a day, preferably 10 min before feeding. Foals may be given 1–2 g PO 2–3 times daily (Plumb, 2002).

$H_2$ histamine receptor inhibitors, including cimetidine, ranitidine, and famotidine, are routinely used to treat NSAID-induced gastric ulcers. Histamine stimulates gastric parietal cells to secrete hydrogen ions through an $H^+/K^+$ ATPase pump. Acetylcholine, gastrin, and histamine stimulate this proton pump. $H_2$ inhibitors only block the histaminic effect on parietal cells, but cause significant inhibition of gastric acid secretion nonetheless

(Bersenas *et al.*, 2005). Human studies showed healing of 40–88% of NSAID-induced ulcers with 4 weeks of $H_2$ inhibitor treatment, and 90% healing after 3 months. There was 67–100% healing of duodenal ulcers, despite continued NSAID therapy. $H_2$ inhibitors may suppress clinical signs associated with unhealed ulcers (Talcott, 2006).

Cimetidine has been commonly used to treat and prevent NSAID-induced ulcers, though research on its efficacy has had conflicting results (Verbeeck, 1990). Cimetidine may decrease gastrointestinal discomfort and does decrease gastric acid secretion (Boulay *et al.*, 1986; Isaacs, 1996). Cimetidine has not been proven to be effective with continued NSAID use and did not prevent gastric ulcer formation when used concurrently with aspirin (Kore, 1990; Wallace *et al.*, 1990; Isaac, 1996; Talcott, 2006). Cimetidine is more useful when NSAID administration has ended and may be helpful with single large doses (Kore, 1990). Gastric acid production is dependent on local circulation, which provides oxygen and energy. Cimetidine decreases gastric blood flow, which contributes to decreased mucosal alkalinity (Cheung and Sonnenschein, 1983). Reduced circulation may contribute to tissue hypoxia. Cimetidine is known to inhibit hepatic cytochrome P450 enzymes and may decrease the rate of metabolism of NSAIDs (Wallace *et al.*, 1990; Verbeeck, 1990; Talcott, 2006). Cimetidine given at a dose of 6 mg/kg every 6 h caused decrease basal acid output in dogs by 30–50% and food-induced output by 63–71%. When 12 mg/kg was used every 6 h, there was a 70–80% decrease in basal acid output and complete suppression of food-induced output (Boulay *et al.*, 1986). Cimetidine can be given to dogs at a dose of 5–10 mg/kg PO, SC, or slow IV every 6–8 h (Villar *et al.*, 1999; Plumb, 2002). Similar dosing protocols are used in cats and ferrets (Plumb, 2002).

Ranitidine does not inhibit microsomal enzymes like cimetidine (Wallace *et al.*, 1990; Talcott, 2006). However, Bersenas *et al.* (2005) found no significant effect on gastric acid secretion in dogs dosed with ranitidine. Ranitidine also decreases gastric blood flow (Wallace *et al.*, 1990; Talcott, 2006). Ranitidine is given at a dose of 0.5–2.0 mg/kg PO, IV, or IM every 8–12 h in dogs, and 2.5 mg/kg IV or 3.5 mg/kg PO in cats every 12 h (Plumb, 2002). Villar *et al.* (1998) recommend continuous treatment for 3–6 weeks.

Famotidine increases intragastric pH in dogs better than ranitidine. Famotidine given at high doses prevents gastric lesion formation in human patients when given concurrently with NSAIDs. Gastric pH must remain above 3 for 18 out of every 24 h for optimal ulcer healing in humans. A 0.5 mg/kg IV dose of famotidine given 2–3 times a day was not able to accomplish this prolonged pH increase in dogs (Bersenas *et al.*, 2005). Famotidine was found protective against reduced gastric perfusion when used at a dose of 0.5 mg/kg IV in dogs given diclofenac (Hata *et al.*, 2005). Dogs may be given 0.5–1 mg/kg famotidine PO or slow IV every 12 h. The dose for cats is 0.5 mg/kg and 0.25–0.5 mg/kg may be given to ferrets. Horses are given 0.23 mg/kg IV or 1.88 mg/kg PO every 8 h, 0.35 mg/kg IV or 2.8 mg/kg PO every 12 h. Rapid IV infusion has been associated with bradycardia (Plumb, 2002).

The prognosis for NSAID toxicosis is dependent on chronicity, dose, and clinical signs (Talcott, 2006). A study by Wallace *et al.* (1990) found that seven of seven dogs treated for chronic NSAID toxicosis recovered after 2–9 days of hospitalization. The mean hospital stay was 6 days. Gastrointestinal irritation and ulceration are reversible, but perforation and peritonitis require intensive surgical and medical management and have a guarded prognosis (Roder, 2005a; Talcott, 2006). Renal effects such as nephropathy are often reversible, but papillary necrosis is a permanent change (Kore, 1990; Roder, 2005a). Loss of the long loops of Henle decreases urine concentrating ability, but horses with renal papillary necrosis may appear clinically normal (Gunson, 1983; Roder, 2005a). Severe acute cortical necrosis is associated with irreversible renal failure (Jones *et al.*, 1992). NSAID-induced coagulopathies are reversible once the NSAID has been eliminated (Talcott, 2006). However, with aspirin and salicylates, coagulopathy is prolonged as discussed below. Dogs with idiosyncratic hepatic injury usually recover within 4 weeks (Albretsen, 2002).

#### *Postmortem findings*

Gastrointestinal lesions are most commonly associated with NSAID toxicosis. Mild lesions may include mucosal edema, irritation, and petechiation. Erosive lesions, which do not extend through the mucosa, may progress to ulcerations through the mucosal layer (Collins and Tyler, 1985; Murphy, 1994; Isaacs, 1996; Roder, 2005a; Talcott, 2006). The location for gastric ulceration in the canine stomach is variable according to the literature. Lesions may be near the pylorus, lesser curvature of the fundus, or diffuse (Daehler, 1986; Stanton and Bright, 1989; Wallace *et al.*, 1990; Godshalk *et al.*, 1992; Talcott, 2006). Duodenal ulcers and perforations have been reported in dogs, but other predisposing factors may have been present (Stanton and Bright, 1989; Gfeller and Sandors, 1991). Perforations may occur in the stomach, small intestine, or colon (Roder, 2005a). Ulcers that erode mesenteric vasculature are rapidly lethal (Stanton and Bright, 1989).

Gastric ulcerations in the horse usually occur in the glandular mucosa and may be adjacent to the margo plicatus (Collins and Tyler, 1984, 1985; Roder, 2005a). Linear circular erosions were reported in the duodenum of horses dosed with phenylbutazone, and ulcers and erosions were found throughout the small intestine (Collins and Tyler, 1985). Ulcerative colitis of the right dorsal colon is commonly seen in horses with NSAID toxicosis (Roder, 2005a). Fibrinonecrotic typhlocolitis was seen in the Collins and Tyler study (1985).

Papillary necrosis occurs with long-term NSAID administration (Mazué *et al.*, 1982). The lesion is bilateral and the papillae are cavitated, yellow-green to orange, and demarcated from the medulla by hemorrhage. The lesion is most severe at the poles. Histologically, the papilla has undergone coagulative necrosis with dilation of the collecting ducts and loops of Henle. Interstitial fibrosis may extend through the medulla and cortex in chronic cases (Gunson, 1983). This lesion has been reported in horses, dogs, cats, mice, rats, gerbils, hamsters, rabbits, desert mice, primates, and pigs (Brix, 2002). The lesion is common in horses given phenylbutazone, but has also been reported with flunixin, aspirin, and dipyrone (Gunson, 1983). Papillary necrosis occurred in two of five dogs dosed with peroxicam (Talcott, 2006).

Interstitial nephritis, with multifocal or diffuse infiltrates of lymphocytes, has been reported. There may be vacuolar degeneration of proximal and distal convoluted tubules (Kore, 1990). Tubular nephritis with epithelial necrosis and regeneration, in addition to interstitial inflammation and fibrosis, has been described (Mazué *et al.*, 1982). Acute cortical necrosis due to NSAID toxicosis has been documented (Jones *et al.*, 1982).

Lymphoid necrosis has been noted in dogs and a ferret. Mild necrosis in the white pulp of the spleen was described in the ferret (Cathers *et al.*, 2000). Depletion and necrosis of germinal centers was discovered in a dog, though circulating lymphocytes were within the reference range (Collins and Tyler, 1985).

## Aspirin and salicylates

This group, which constitutes the most extensively used OTC drugs (Kore, 1990), includes acetylsalicylic acid or aspirin, sodium salicylate, bismuth subsalicylate, and diflunisal. Aspirin and salicylates are NSAIDs with many characteristics similar to those of other NSAIDs, as described above. Aspirin and salicylate also have certain unique properties. Clinical toxicosis of products containing bismuth subsalicylate, an OTC drug commonly used for gastrointestinal distress, are similar to those associated with other sources of salicylate, thus these products will be further discussed in this section.

Salicylates have been used since ancient times by Greek and Roman physicians Hippocrates and Galen, by South African tribes, and by North American Indians (Boynton *et al.*, 1988; Lees *et al.*, 1991). Reverand Edmund Stone wrote of the use of *Salix alba* bark in the journal of the Royal Philosophical Society in London in 1763. Salicylic acid was first isolated from the bark of willows (*Salix* spp.) and beech trees (*Fagus* spp.) in the early 19th century (Lees *et al.*, 1991). Bayer Pharmaceutical Company synthesized acetylsalicylic acid in 1893 and aspirin has been marketed since 1899 (Boynton *et al.*, 1988; Lees *et al.*, 1991).

Contraindications for aspirin use include coagulation disorders, recent (within 1 week) surgery, and concurrent use with certain drugs (Brater, 1988; Verbeeck, 1990; Talcott, 2006). See Table 23.2 for a list of drugs that may interact with aspirin and other NSAIDs. Aspirin inhibits the diuretic effects of spironolactone (Verbeeck, 1990).

### *Toxicity*

The efficacy of aspirin is dose dependent. Circulating aspirin concentrations of 20–60 μg/ml are associated with analgesic and antipyretic effects in humans (Papich *et al.*, 1987; Boothe, 2001). Plasma levels in excess of 50 μg/ml are associated with anti-inflammatory effects and concentrations of 200 μg/ml occur in the treatment of rheumatic disorders (Lees *et al.*, 1991; Boothe, 2001a). Fecal blood loss is reported at concentrations between 120 and 350 μg/ml (Papich *et al.*, 1987). However, toxic changes are most commonly associated with levels greater than 300 μg/ml (Papich *et al.*, 1987; Boothe, 2001a). About half of people receiving therapeutic doses of aspirin that were tested had gastric lesions visible on endoscopy (Carson and Strom, 1988). Neonates less than 1 month old may be more susceptible to the adverse effects of aspirin because of the prolonged plasma half-life in this group (Davis, 1980).

Aspirin is used in dogs at doses of 10–25 mg/kg every 8–12 h for analgesia (Kore, 1990; Rubin and Papich, 1990; Plumb, 2002). Dogs dosed with 25 mg/kg aspirin every 8 h for up to 3 weeks had no severe adverse effects (Lees *et al.*, 1991). Dogs dosed in this way have peak plasma concentrations were greater than 50 μg/ml but peak concentrations were lower in pups 12–16 weeks old. The half-life may be increased in pups less than 30 days old, who have a decreased metabolic capability (Waters *et al.*, 1993). A study of dogs dosed with 25–35 mg/kg aspirin every 8 h found increased fecal hemoglobin and gastric ulceration on endoscopy (McKellar *et al.*, 1991). Seizures were reported on a dog given 37 mg/kg aspirin every 12 h for 2 weeks (Schubert, 1984); 50 mg/kg aspirin given to dogs every 12 h was associated with emesis (McKellar *et al.*, 1991). Similar daily doses were associated with perforating gastric ulcers within 4 weeks (Kore, 1990). One dose of 400 mg/kg will cause hemorrhage, and daily dosing caused pyloric ulcers within 2 weeks (Mazué *et al.*, 1982).

Cats are more susceptible to the toxic effects of aspirin than dogs and humans (Papich, 1990; Roder, 2005a). Doses of 25 mg/kg every 2–3 days are recommended to control thromboembolic disease and 10 mg/kg every other day may be used for analgesia and antipyresis (Plumb, 2002). A dose of 10.5 mg/kg aspirin every 52 h produced plasma concentrations ranging from 50 to 20 μg/ml. No clinical signs were seen in cats dosed with 25 mg/kg aspirin every 48 h (Boothe, 2001a). No severe signs were seen in cats dosed with 25 mg/kg aspirin every 24 h for 2–3 weeks (Lees *et al.*, 1991). Aspirin doses between 100 and 110 mg/kg daily

may cause death in cats within 7 days (McKellar *et al.*, 1991). Young cats, old cats, or cats with renal or hepatic disease may be particularly sensitive to the effects of aspirin (Kore, 1990).

Aspirin may be used in cattle and goats, but they require larger oral doses to reach appropriate plasma concentrations. A dose of 100 mg/kg PO given every 12 h in cattle maintains a therapeutic concentration of 30 μg/ml (Davis, 1980; Boothe, 2001a). IV dosing with 6.5 mg/kg hourly is associated with plasma concentrations of 50–20 μg/ml, 26 mg every 1.6 h yields plasma concentrations of 200–50 μg/ml, and 39 mg every 2 h yields levels of 300–50 μg/ml in the plasma (Davis, 1980).

Horses may be given 35 mg/kg aspirin IV according to Davis (1980). A single oral dose of 20 mg/kg was associated with prolonged bleeding times (Boothe, 2001a).

Liquid bismuth subsalicylate preparations contain 8.77 mg salicylate per ml (Papich *et al.*, 1987). Ingestion of greater than 7 ml/kg daily has been associated with toxicosis in humans (Papich *et al.*, 1987; Papich, 1990). Tablets contain 102 mg available salicylate (Sainsbury, 1991).

### *Toxicokinetics*

Aspirin is lipophilic at acid pH, thus it is readily absorbed from the stomach and duodenum in dogs and cats (Rubin and Papich, 1990; Talcott, 2006). Aspirin is 68–76% bioavailable in dogs, with some variation based on formulation (Boothe, 2001a). Enteric-coated and buffered products are used in humans and dogs (McKellar *et al.*, 1991; Murtaugh *et al.*, 1993; Boothe, 2001a). Acid-resistant forms are more readily absorbed at the relatively alkaline pH range of the duodenum and absorption may be delayed up to 12 h (Verbeeck, 1990). Buffered aspirin is more soluble and less ionized, thus slowing absorption and decreasing gastric irritation (Rubin and Papich, 1990; Boothe, 2001a). Ingestion of a fatty meal can reduce aspirin bioavailability by 30% (Mazué *et al.*, 1982). Aspirin is 70% bioavailable in cattle, but absorption is relatively slow (Boothe, 2001a). When humans were given bismuth subsalicylate, 95% of the salicylate was recovered in the urine. Salicylates in bismuth subsalicylate are 97.5% absorbed in dogs and 85.4% absorbed in cats (Papich *et al.*, 1987).

Peak serum concentrations occur 4 h after oral dosing with buffered aspirin. Peak serum concentrations are lower in immature dogs than in mature dogs after PO or IV dosing (Waters *et al.*, 1993). Circulating salicylate is 72% protein bound in humans and 45% protein bound in dogs (Waters *et al.*, 1993). There is more free, and therefore bioavailable, salicylate in the hypoalbuminemic animal, but the excess is rapidly eliminated (Boothe, 2001a). Aspirin is rapidly distributed to most tissues, including synovial fluid, peritoneal fluid, saliva, and milk (Boothe, 2001a; Talcott, 2006). Aspirin has a lower volume of distribution in cattle compared to other domestic species (Davis, 1980).

Aspirin is rapidly hydrolyzed to salicylate by esterases in the gastrointestinal tract, liver, and erythrocytes. These enzymes are less efficient in immature dogs (Waters *et al.*, 1993). Most of the metabolism takes place in the liver (Talcott, 2006). Salicylate is bioactive and responsible for most of the clinical effects of aspirin (Lees *et al.*, 1991; Talcott, 2006). Salicylate may be conjugated to glucuronide or glycine. The glucuronide pathway is deficient in cats, limiting their ability to eliminate salicylate (Rubin and Papich, 1990; Jones *et al.*, 1992; Isaac, 1996; Boothe, 2001a; Talcott, 2006). Bismuth subsalicylate is metabolized to bismuth and salicylate in the gastrointestinal tract (Boothe, 2001a).

The kidneys eliminate salicylate and its glycine conjugate (Boothe, 2001a). Two to 30% of a dose of aspirin is excreted as unconjugated salicylate (Verbeeck, 1990). The rate of elimination for aspirin varies with species and age (McKellar *et al.*, 1991). The elimination half-life in dogs is dependent on the dose form. The half-life for enteric-coated products ranges from 7.5 to 12.2 h. The elimination half-life for aspirin given IV is 2.2–8.7 h (Boothe, 2001a). The elimination half-life in cats is long and increases with dose. If low doses of 5–12 mg/kg are given, the elimination half-life is 22–27 h, but the half-life for the much higher dose of 25 mg/kg is 45 h (Boothe, 2001a). Salicylate may have zero-order kinetics in cats (Lees *et al.*, 1991). The elimination half-life in horses is about an hour due to ion trapping in alkaline urine. Salicylates are a normally component equine urine (Boothe, 2001a). The elimination half-life in cattle is about half an hour (Lees *et al.*, 1991; Boothe, 2001a). The half-life in humans is 3 h (Lees *et al.*, 1991; Isaacs, 1996). Young animals metabolize aspirin more slowly, and the elimination half-life may be prolonged in puppies less than 30 days old, piglets, kids, and foals (McKellar *et al.*, 1991).

### *Mechanism of action*

The effects attributed to aspirin are mostly caused by the active metabolite salicylate (McKellar *et al.*, 1991). Salicylate inhibits COX to block PG synthesis, as described for NSAIDs (Boulay *et al.*, 1986; Carson and Strom, 1988; Jones *et al.*, 1992; Murtaugh *et al.*, 1993). Other NSAIDs competitively inhibit COX, but salicylates can permanently inactivate the enzyme by acetylating a serine residue (Kore, 1990; Rubin and Papich, 1990; Boothe, 2001a; Talcott, 2006). Platelets are unable to synthesize COX (Rubin and Papich, 1990; Lees *et al.*, 1991; McKellar *et al.*, 1991). Unlike other NSAIDs, where coagulopathy resolves once the drug is eliminated, it takes about a week to resolve aspirin-induced coagulopathy (Boothe, 2001a).

Aspirin has a direct irritant effect on the gastric mucosa. Aspirin is an acidic drug and is taken up by the mucosal epithelium, where it becomes concentrated (Carson and Strom, 1988; Rubin and Papich, 1990; Isaacs, 1996). Aspirin inhibits oxidative phosphorylation of the gastric mucosal

epithelium, thus decreasing ATP production and $Na^+/K^+$ movement across the cell membrane, leading to cell swelling and necrosis (Rubin and Papich, 1990). Disruption of ion transport allows increased $H^+$ ion back-diffusion into the mucosa (Boulay *et al.*, 1986; Carson and Strom, 1988; Rubin and Papich, 1990). Injury may extend into submucosal capillaries, leading to hemorrhage, inflammation, and ulceration (Boulay *et al.*, 1986; Rubin and Papich, 1990). IV dosing of cats produced gastric erosions and ulcers, confirming that there is also a role for COX inhibition and reduced PG synthesis in the pathophysiology of gastric mucosal damage (Villar *et al.*, 1998).

Aspirin uncouples oxidative phosphorylation, as noted above. Aspirin allows penetration of $H^+$ across the mitochondrial membrane, thus disrupting the proton gradient. The result is inhibition of the enzyme ATP synthetase. Energy that would go into ATP production is dissipated as heat. When oxidative phosphorylation is inhibited systemically, the result is elevated body temperature (Roder, 2005a).

Salicylates may produce acidosis and increase the anion gap. Salicylate and its metabolites increase the anion gap directly (Schubert, 1984; Kore, 1990). Anaerobic metabolism compensates for reduced ATP production due to inhibition of oxidative phosphorylation. Metabolites of anaerobic metabolism include lactic acid, pyruvic acid, and ketones. These substances cause acidemia (Kore, 1990; Roder, 2005a). Aspirin may also directly stimulate respiratory centers in the CNS, causing hyperventilation and respiratory alkalosis, which promotes renal secretion of bicarbonate (Kore, 1990; Boothe, 2001a). Later in the progression of the toxicosis, CNS suppression may decrease the respiratory rate, resulting in acidosis (Schubert, 1984; Kore, 1990).

### Clinical signs

Similar to other NSAIDs, gastric irritation is the most common side effect of aspirin (Talcott, 2006). Vomiting, gastric ulceration, and hemorrhage are often reported (Davis, 1980; Papich, 1990; McKellar *et al.*, 1991).

Depression is often seen in aspirin toxicosis in dogs and cats (Kore, 1990; Jones *et al.*, 1992). Profound depression has been reported in dogs given high doses of aspirin (McKellar *et al.*, 1991). Dogs collapse or appear too weak to stand (Wallace *et al.*, 1990). Restlessness progressing to tremors, seizures, and eventually coma have been reported in dogs (Schubert, 1984; Kore, 1990). One dog had a progressive increase in seizure duration from 5 to 45 min (Schubert, 1984). Seizures and coma have also been reported in cats (Jones *et al.*, 1992; Isaacs, 1996; Talcott, 2006). Seizures have been attributed to hypoventilation, acidosis, and hypoglycemia (Schubert, 1984; Talcott, 2006). Anemia due to bone marrow suppression has also been reported in cats (Kore, 1990; McKellar *et al.*, 1991).

Aspirin toxicosis may cause hyperthermia in children and cats. Hyperthermia is uncommon in adults and carries a poor prognosis (Talcott, 2006). There may be an initial hyperpnea in acute aspirin toxicosis due to stimulation of respiratory centers (Kore, 1990; Boothe, 2001a; Talcott, 2006). Fatal acidosis may occur within 4 h of aspirin overdose in humans (Talcott, 2006).

Bismuth subsalicylate toxicosis has not been described in the veterinary literature (Papich *et al.*, 1987). Overdose described in humans is similar to aspirin toxicosis. One lethally affected patient presented with dehydration, progressive CNS depression, coagulopathy and anemia, and pulmonary edema (Sainsbury, 1991).

### Clinical chemistries and postmortem findings

Metabolic acidosis and increased anion gap have been noted in acute aspirin toxicosis (Roder, 2005a). Other changes are similar to those described with other NSAIDs.

Lesions similar to other NSAIDs are expected on necropsy. Gastric mucosal lesions have been documented in the pyloric antrum, body, and cardiac region of the stomach of dogs (Boulay *et al.*, 1986). Pulmonary edema has been found in humans that died from acute aspirin toxicosis (Talcott, 2006).

### Management

Diagnosis and treatment is similar to that described above for NSAIDs. Urine and plasma testing is available at many veterinary and hospital laboratories. There is a poor correlation between plasma salicylate levels and with clinical signs (Talcott, 2006). The presence of salicylate in horse urine is expected, even in horses not given aspirin, and not a significant finding (Boothe, 2001a).

Salicylates may inhibit gastric emptying and gastrointestinal absorption is prolonged with enteric-coated tablets, therefore gastric lavage may be useful up to 12 h after exposure. Activated charcoal should be administered, and is most useful within 2 h of aspirin ingestion. Cathartic use has not been shown to decrease absorption (Talcott, 2006).

Forced alkaline diuresis is more useful with aspirin than with other NSAIDs, and is frequently used by physicians. Low urinary pH promotes salicylate elimination by trapping the ionic form in the urine (Kore, 1990; Roder, 2005a; Talcott, 2006). Alkaline peritoneal dialysis has been recommended for severe overdose (Davis, 1980; Kore, 1990). Acetazolamide, a carbonic anhydrase inhibitor, has been used to alkalize urine, but may exacerbate metabolic acidosis (Talcott, 2006). Acid–base status must be closely monitored in any animal undergoing alkaline diuresis.

Monitoring and supportive care for changes in hydration, electrolyte balance, and body temperature are warranted. Use of NSAIDs to treat hyperthermia is to be avoided (Kore, 1990). Diazepam may be used for seizure management (Schubert, 1984). Gastric protectants should be used, as described with NSAIDs.

## COLD, COUGH, AND ALLERGY MEDICATIONS

Medications for the symptomatic relief of cold, flu, and allergies are common and often contain multiple active ingredients. Preparations frequently contain analgesics, decongestants, antihistamines, expectorants, and antitussives. Other ingredients may include ethanol and caffeine (Papich, 1990).

Detailed information on analgesic toxicosis can be found in the previous section of this chapter. Toxic effects of decongestants and antihistamines will be discussed below. Toxic effects of alcohol and caffeine are addressed elsewhere in this book.

Dextromethorphan is a centrally active antitussive. Structurally similar to opioids, it acts on receptors in medullary cough centers. Toxic effects are rare in small animals. Sedation is the most common side effect, but respiratory suppression is unlikely. If an animal is overdosed with dextromethorphan, activated charcoal may be given early. Treatment consists of observation and supportive and symptomatic care as needed (Papich, 1990).

Guafenasin or glyceryl guaicolate, is the most common expectorant. It is used as a sedative and muscle relaxant at high doses and may cause gastritis and vomiting, but is present in relatively small concentrations in OTC products. Treatment for guafenasin overdose would include routine detoxification early, observation, and symptomatic and supportive care (Papich, 1990).

### Decongestants

Sympathomimetic amines are used as decongestants because of their vasoconstricting effects. Common decongestants include pseudoephedrine, ephedrine, phenylephrine, and phenylpropanolamine. Pseudoephedrine is the most common decongestant associated with toxicosis in small animals, more commonly dogs than cats (Papich, 1990). Pseudoephedrine has been commonly used in cold and allergy preparations (Means, 2005). Due to its illicit use in the manufacture of methamphetamine, some US states now regulate its sale. Pseudoephedrine is a steroisomer of the plant alkaloid ephedrine. Ephedrine is found in *Ephedra* sp. and *Sida cordifolia*. *Ephedra* sp. is used to produce ma huang, an herbal drug used in asthma, allergy, and cold formulations, diet pills, and in other supplements (Means, 1999; Ooms and Khan, 2001; Means, 2005). Phenylephrine is found in nasal sprays and hemorrhoid creams (Means, 2005). Oxymetazolin is found in ophthalmic drugs (Papich, 1990). Phenylpropanolamine was used in cold and allergy products and diet pills (Papich, 1990; Means, 2005). It is no longer sold OTC in the United States (Crandell and Ware, 2005).

Sympathomimetic amines may interact with digoxin, monoamine oxidase (MAO) inhibitors, halothane, and methylxanthines (Means, 1999; Ooms and Khan, 2001; Means, 2005). Certain conditions may predispose animals to adverse reactions when given sympathomimetic amines. These include diabetes, hypothyroidism, hyperthyroidism, cardiac disease, hypertension, seizure disorders, renal disease, and glaucoma (Means, 2005).

#### *Toxicity*

Pseudoephedrine is used to improve urethral sphincter tone as a treatment for urinary incontinence in dogs, and is given at a dose of 1–2 mg every 12 h (Means, 2005). Clinical toxicosis has been reported in dogs given 5–6 mg/kg, and death was documented in dogs given 10–12 mg/kg (Means, 1999; Ooms and Khan, 2001; Means, 2005).

Drugs containing ephedrine, in the form of ma huang, are often combined with caffeine, in the form of guarana, and this combination of drugs may act synergistically and enhance the toxicity of this product. Doses of 1.3–88.9 mg/kg ma huang given concurrently with 4.4–296.2 mg/kg guarana have been associated with clinical toxicosis in dogs. One dog given a dose of 5.8 mg/kg ma huang and 19.1 mg/kg guarana died. There seems to be great individual difference in sensitivity to this combination of drugs, and prognosis may be dependent on the time that elapses between exposure and treatment (Ooms and Khan, 2001).

The therapeutic dose of phenylpropanolamine in dogs is 1.1 mg/kg (Crandell and Ware, 2005; Means, 2005). Elevated blood pressure was seen in beagles given 3.1 mg/kg PO every 8 h, and myocardial damage was noted in a dog given 48 mg/kg (Crandell and Ware, 2005).

#### *Pharmacokinetics*

Decongestants are rapidly absorbed by the gastrointestinal tract (Papich, 1990; Means, 2005). Ephedrine is absorbed within 2 h of ingestion (Ooms and Khan, 2001). The onset of action is usually within 30 min, though it may be delayed up to 8 h with extended release products. Sympathomimetic amines are believed to cross the blood–brain barrier and the placenta, and are secreted into milk. Metabolism occurs primarily in the liver (Means, 2005). Most of the dose of ephedrine is excreted unchanged in the urine, and other decongestants may be excreted 55–75% as the parent compound. The elimination half-life of pseudoephedrine is 2–21 h and that for phenylpropanolamine is 2–4 h (Means, 2005). Urinary excretion is accelerated at low urine pH (Ooms and Khan, 2001; Means, 2005).

#### *Mechanism of action*

The effects produced by decongestants are from stimulation of adrenergic receptors (Papich, 1990; Means, 1999;

Ooms and Khan, 2001; Crandell and Ware, 2005; Means, 2005). Stimulation of α-1 receptors causes vasoconstriction and subsequent drying of the mucous membranes of the nasal mucosa and sinuses. Ophthalmic solutions decrease eye redness by constriction of conjunctival and scleral vasculature (Papich, 1990). Peripheral vasoconstriction leads to increased systemic vascular resistance and hypertension (Papich, 1990; Means, 1999; Ooms and Khan, 2001; Means, 2005). α-1 receptor stimulation may cause vasospasm of the coronary artery and myocardial necrosis (Crandell and Ware, 2005). Other α-adrenergic effects may include appetite suppression, CNS stimulation, and mydriasis (Papich, 1990; Means, 1999; Ooms and Khan, 2001; Means, 2005). The effects of phenylephrine and oxymetazole are α-receptor specific (Papich, 1990). Stimulation of α-receptors by phenylpropanolamine is known to cause release of endogenous catecholamines in the brain and heart, and may inhibit MAO at high doses (Means, 1999; Crandell and Ware, 2005).

Stimulation of β-receptors is the cause of the cardiac effects attributed to decongestants, which include increased contractility and output, increased heart rate and tachyarrhythmia (Papich, 1990; Means, 1999; Means, 2005). Reflex bradycardia may occur. Bronchodilation is also mediated β-receptors (Papich, 1990).

### *Clinical signs*

Stimulation of the CNS is common in decongestant overdose. Hyperactivity, restlessness, agitation, pacing, and vocalization may be observed (Papich, 1990; Means, 1999; Means, 2005). Hallucinatory behaviors in dogs may include staring into a corner or at unseen objects, perhaps even biting at them. Tremors, seizures, and head bobbing have been reported (Means, 1999; Means, 2005). Hyperthermia may be secondary to increased activity, and disseminated intravascular coagulation (DIC) or rhabdomyolysis with associated renal failure are possible outcomes (Papich, 1990; Means, 2005). Cardiovascular changes include tachycardia, reflex bradycardia, and hypertension (Means, 1999; Papich, 1990; Means, 2005). Hypertension was described in beagles 30–60 min after dosing. Blood pressure in some dogs remained elevated for 6 h (Crandell and Ware, 2005). Animals may die from cardiovascular collapse (Means, 1999; Means, 2005). Postmortem lesions are nonspecific (Means, 2005).

Vomiting, diarrhea, dehydration, and anorexia were reported in a dog with phenylpropanolamine toxicosis. This dog was ataxic, lethargic, tachycardic, and tachypneic. Eyes were bilaterally dilated with vertical nystagmus and loss of pupillary light reflex. This dog was not hypertensive, possibly because it did not present to the veterinarian until 12 h after ingestion (Crandell and Ware, 2005).

Ooms and Khan (2001) studied dogs accidentally overdosed with a combination of ma huang and guarana herbal preparations. They found that the onset of clinical signs could be as early as 30 min after ingestion, but was usually within 8 h. Duration of signs ranged from 10 to 48 h. Vomiting was seen in 47% of dogs and 5% were anorexic. Tachycardia occurred in 30%, tachypnea in 6%. Mydriasis was reported in 21% of dogs, tremors in 27%, and behavioral changes such as snapping, pacing, and head shaking in 6%, and seizures in 6%. Incidence of depression, weakness, and apprehension were each 5%. Hyperthermia was reported in 28% of these dogs.

### *Clinical chemistries*

Hypokalemia, hyperglycemia, and hyperinsulinemia are usually reported in dogs with decongestant toxicosis (Means, 1999; Means, 2005). Hypoglycemia was seen in a dog that had ingested phenylpropanolamine. Other findings with phenylpropanolamine overdose included elevated ALT and ALP, mild hyperbilirubinemia, elevated BUN and creatinine, and increased CK. Blood pH and calcium levels were elevated. Polycythemia and thrombocytopenia were noted. A urine sample contained blood, hemoglobin or myoglobin, and protein. Serum troponin was elevated, indicating myocardial damage (Crandell and Ware, 2005).

### *Management*

Diagnosis of decongestant toxicosis is usually based on history and clinical signs (Ooms and Khan, 2001; Means, 2005). Some laboratories are able to test for pseudoephedrine, ephedrine, and phenylpropanolamine in plasma or urine.

Treatment consists of detoxification, symptomatic, and supportive care. Emetics should only be used in the asymptomatic animal due to the risk of aspiration (Papich, 1990; Means, 1999; Means, 2005). Onset of signs is often rapid (Means, 2005). Gastric lavage may be performed in the stabilized, anesthetized, and intubated patient after a large ingestion (Ooms and Khan, 2001). Activated charcoal and cathartic should be given (Papich, 1990; Means, 1999; Ooms and Khan, 2001; Means, 2005). Blood pressure, ECG, and body temperature should be monitored closely. Complete blood count (CBC), serum chemistry, and acid–base status should be monitored every 1–3 days.

Tachycardia is treated with β-blockers. Propranolol may be administered at a dose of 0.02–0.06 mg/kg slowly by IV (Means, 1999; Ooms and Khan, 2001; Means, 2005). Propranolol therapy may stabilize hypokalemia (Means, 2005). Lidocaine has been used at a dose of 2 mg/kg IV by intermittent bolus or by continuous infusion at a rate of 80 μg/kg/min in dogs (Crandell and Ware, 2005). Crandell and Ware (2005) recommended atenolol at 0.2 mg/kg every 12 h and enalapril 0.5 mg/kg every 12 h, both given orally, to support myocardial function for phenylpropanolamine toxicosis. Papich (1990) recommended atropine at a dose of 0.04 mg/kg SC or IV. High peripheral

vascular resistance and hypertension may be treated with α-adrenergic receptor blocking agents. Prazocin may be given at 1–2 mg PO every 8 h. Phentolamine may be given at 0.1 mg/kg IV as needed (Papich, 1990; Ooms and 2001).

Severe CNS stimulation may require treatment with more than one anticonvulsant. Though acepromazine and chlorpromazine are known to decrease the seizure threshold, they can be used to treat pseudoephedrine or ephedrine toxicosis, which induce seizures by a different mechanism (Means, 1999; Ooms and Khan, 2001). Acepromazine is given at a dose of 0.05–1.0 mg/kg IM, IV, or SC in dogs, starting with a low dose and adding more as needed. Chlorpromazine is given IM or IV at a dose of 0.5–1.0 mg/kg, starting with a low dose and increasing as needed. Phenobarbital may be given at a starting dose of 3 mg/kg to effect (Means, 1999; Means, 2005). Isofluorane anesthesia may be used for severe clinical signs. Use of diazepam or other benzodiazepines may be contraindicated because the dissociative effects of this drug class may exacerbate clinical signs of sympathomimetic amines (Means, 1999; Ooms and Khan, 2001; Means, 2005).

Fluid therapy and urinary acidification may promote excretion. Glucose is added to IV fluids to treat hypoglycemia. Overhydration must be avoided to prevent pulmonary edema of the hypertensive patient (Means, 1999, 2005). Urinary acidifiers enhance excretion of pseudoephedrine in humans and may be used in dogs. Acid–base status must be monitored closely when acidifiers are used. The dose for ascorbic acid is 20–30 mg/kg IM or IV every 8 h. Ammonium chloride is given 50 mg/kg PO every 6 h (Means, 2005).

Adverse clinical signs associated with decongestants may last for 24–72 h (Means, 2005). One dog with severe clinical signs after phenylpropanolamine ingestion recovered after 6 days of hospitalization (Crandell and Ware, 2005). Most animals respond to treatment. Clinical signs that have been associated with an unfavorable outcome include uncontrollable seizures, DIC, myoglobinuria, and head bobbing (Means, 2005). Ooms and Khan (2001) reported that 26 of 34 dogs recovered, usually within 10–48 h, with treatment for toxicosis after ingestion of a ma huang and guarana combination drug. The remaining 8 dogs died or were euthanized.

## Antihistamines

Antihistamines act by competitive inhibition of histamine at histamine receptors. Compounds referred to as antihistamines in this section are the $H_1$ histamine receptor inhibitors. $H_2$ receptor antagonists are also sold OTC and are covered later, with drugs affecting the gastrointestinal system. They will be referred to as $H_2$ antagonists.

Many antihistamines, including brompheniramine, chlorpheniramine, clemastine, diphenhydramine, loratidine, and triprolidine, are found in allergy, cold, and flu formulations. Others are used as antiemetics in drugs to treat motion sickness. Examples of these include dimenhydrinate and meclizine. Doxylamine is used as a sedative in sleep aids. Hydroxyzine is used by veterinarians to treat canine atopy (Tegzes *et al.*, 2002). Amitriptyline is a prescription tricyclic antidepressant.

There were 23,000 human antihistamine overdoses reported to poison control centers in 2002. Drugs that were implicated in these reports included diphenhydramine, brompheniramine, chlorpheniramine, doxylamine, hydroxyzine, carbinoxamine, promethazine, and meclizine (Nine and Rund, 2006). An incomplete list of antihistamines can be found in Table 23.3.

**TABLE 23.3 Antihistamines ($H_1$ histamine receptor inhibitors); see Gwaltney-Brant (2006) for more detailed information**

| First generation | Second generation |
|---|---|
| *Alkylamines* | *Piperadines* |
| Brompheniramine | Terfenadine |
| Chlorpheniramine | Astemizole |
| Dexbrompheniramine | Levocabastine |
| Dexchlorpheniramine | Loratidine |
| Dimethindene | *Piperazines* |
| Pheniramine | Cetirizine |
| Pyrrobutamine | |
| Triprolidine | **Third generation** |
| *Ethanolamines* | Desloratidine |
| Clemastine | Fexofenadine |
| Dimenhydrinate | Levocetirizine |
| Diphenhydramine | |
| Bromodiphenhydramine | |
| Carbinoxamine | |
| Doxylamine | |
| Phenyltoxamine | |
| *Ethylenediamines* | |
| Antazoline | |
| Methapyriline | |
| Pyrilamine | |
| Thenyldiamine | |
| Tripelennamine | |
| *Phenothiazines* | |
| Methdilazine | |
| Trimeprazine | |
| *Piperazines* | |
| Hydroxyzine | |
| Cyproheptadine | |
| Meclizine | |
| Cyclizine | |
| Buclizine | |
| Chlorcyclizine | |
| Niaprazine | |
| *Tricyclics* | |
| Amitriptyline | |
| Promethazine | |

### *Toxicity*

Chlorpheniramine is the antihistamine most commonly associated with adverse effects in dogs (Papich, 1990; Gwaltney-Brant, 2006). The oral $LD_{50}$ for clemastine in dogs is 175 mg/kg (Gwaltney-Brant, 2006). The therapeutic dose for hydroxyzine is 2.2 mg/kg, and 111 mg/kg is reported as toxic in dogs (Tegzes *et al.*, 2002). Terfenidine, which is no longer sold in the United States, caused clinical signs of toxicosis in a dog at a dose of 6.6 mg/kg, but there is considerable individual difference in sensitivity to this drug (Otto and Greentree, 1994; Gwaltney-Brant, 2006). Electroencephalogram changes were noted in dogs dosed with 30 mg/kg. Clinical signs were noted in dogs given in 100 mg/kg/day for 2–3 weeks. Vomiting was a consistent finding in dogs dosed with 150 mg/kg (Otto and Greentree, 1994).

### *Pharmacokinetics*

Antihistamines are well absorbed by the monogastric gastrointestinal tract, but oral doses are poorly absorbed in ruminants (Adams, 2001; Gwaltney-Brant, 2005). The anticholinergic effects of antihistamines may slow absorption by delaying gastric emptying (Tegzes *et al.*, 2002; Gwaltney-Brant, 2005). Peak plasma concentrations usually occur within 2–4 h of ingestion and the onset of clinical effects tends to be 20–45 min after ingestion. Therapeutic effects may last from 3 to 12 h (Adams, 2001; Gwaltney-Brant, 2005). Antihistamines are highly protein bound (Gwaltney-Brant, 2005). First-generation antihistamines are able to freely cross the blood–brain barrier and are more likely to cause CNS effects than second-generation products, which do not normally enter the CNS (Table 23.3). Second-generation antihistamines may cross the blood–brain barrier if given at very high doses. Terfenidine is known to cross into the cerebrospinal fluid in overdose situations (Otto and Greentree, 1994).

Metabolism of antihistamines takes place predominantly in the liver (Gwaltney-Brant, 2005). Hydroxyzine is metabolized to the active product cetirizine, which does not cross the blood–brain barrier (Tegzes *et al.*, 2002). The elimination half-life of antihistamines is dependent on the individual compound. Most metabolites are excreted in the urine, though there is some biliary excretion of terfenadine (Otto and Greentree, 1994; Gwaltney-Brant, 2006). Antihistamines excreted into the bile may undergo enterohepatic recycling.

### *Mechanism of action*

Antihistamines act by competitive inhibition of histamine at $H_1$ receptors (Papich, 1990; Gwaltney-Brant, 2005). Binding is reversible, but may become irreversible or slow to dissociate at high doses, as with terfenadine (Otto and Greentree, 1994; Gwaltney-Brant, 2005).

$H_1$ receptors are found on mast cells in the skin, smooth muscle myocytes, capillary endothelium, and in the CNS. Histamine produces dermal itching and allergic responses in the skin (Gwaltney-Brant, 2005). Histamine causes contraction of smooth muscle in the bronchial tree and intestine (Adams, 2001; Gwaltney-Brant, 2005). Low doses of histamine produce a rapid onset of vascular dilation. Histamine causes a wheal-and-flair reaction due to increased vascular permeability (Gwaltney-Brant, 2005). Histamine in the CNS modulates sleep–wake cycles (Tegzes *et al.*, 2002).

Antahistamines block allergic response and reduce itching (Adams, 2001; Gwaltney-Brant, 2005). Antihistamines block smooth muscle contraction, reducing bronchoconstriction, and affecting vascular and uterine smooth muscle. Effects on intestinal smooth muscle may cause gastrointestinal disturbances (Adams, 2001). Antihistamines prevent increases in vascular permeability associated with histamine release. CNS effects produced by antihistamines include both sedation and excitement (Adams, 2001; Gwaltney-Brant, 2005).

Muscarinic stimulation is believed to be involved in motion-sickness-induced vomiting. The antimuscarinic actions of antihistamines decrease nausea and vomiting (Papich, 1990). Gastrointestinal motility is decreased, and there may be respiratory suppression (Tegzes *et al.*, 2002). Phenothiazine type antihistamines also block $\alpha$-adrenergic receptors (Gwaltney-Brant, 2005). Allergic reactions to antihistamines have been documented (Adams, 2001).

### *Clinical signs*

Clinical signs of antihistamine overdose are usually evident within half an hour of dosing. Signs of CNS depression may occur with therapeutic doses of first-generation antihistamines and include sedation, ataxia, and drowsiness (Papich, 1990; Adams, 2001; Gwaltney-Brant, 2005). More severe clinical signs such as profound depression, coma, and respiratory suppression incompatible with life have been described (Gwaltney-Brant, 2005). A dog with hydroxyzine toxicosis presented with tachycardia and weakness progressing to stupor, coma, loss of gag reflex, and apnea (Tegzes *et al.*, 2002).

Higher doses of antihistamines may have a stimulatory effect on the CNS, particularly in children and young animals. These effects are less commonly reported in adults (Otto and Greentree, 1994; Gwaltney-Brant, 2005). Overdosed individuals may appear to experience hallucinations, lack of coordination, disorientation, irritability, anxiety, aggression, seizures, and pyrexia.

Vomiting and diarrhea have been associated with first-generation antihistamines (Gwaltney-Brant, 2005). Anticholinergic effects include dry mucous membranes, fixed and dilated pupils, tachycardia and arrhythmia, and animals may be hypertensive or hypotensive (Adams,

2001; Gwaltney-Brant, 2005). Cardiac abnormalities were documented in humans and dogs that ingested terfenadine (Otto and Greentree, 1994; Gwaltney-Brant, 2005).

Diphenhydramine has been associated with 75 human fatalities in the literature. Cardiac arrhythmias are commonly reported and may include bradycardia, tachycardia, ventricular fibrillation, and asystole. Hyperexcitability, seizures, respiratory suppression, apnea, and cyanosis also contributed to deaths (Nine and Rund, 2006).

Animals may have allergic reactions to topical or oral antihistamines. A list of associated clinical signs would include dermatitis, pyrexia, and photosensitization (Gwaltney-Brant, 2005). Teratogenic effects have been detected in experimental animals treated with piperazine (Gwaltney-Brant, 2005).

Metabolic acidosis and electrolyte abnormalities may be documented based on serum chemistries of animals overdosed with antihistamines, but overall changes are nonspecific. Changes reported on postmortem examination also tend to be nonspecific. Rhabdomyolysis and associated renal lesions or DIC may be complications of antihistamine toxicosis (Gwaltney-Brant, 2005). Pulmonary congestion has been reported with diphenhydramine toxicosis in humans (Nine and Rund, 2006).

#### *Management*

Diagnosis of antihistamine toxicosis may be based on history and clinical signs. Laboratory testing of urine or plasma may be helpful to confirm exposure, but quantitation is unlikely to be of value (Gwaltney-Brant, 2005).

Emetics are appropriate to promote gastric emptying in asymptomatic animals that recently ingested large doses of antihistamines, but onset of clinical signs may be rapid (Papich, 1990; Gwaltney-Brant, 2005). Gastric lavage of the anesthetized, intubated animal is more appropriate in the treatment of symptomatic animals (Otto and Greentree, 1994). Activated charcoal and a cathartic are instilled after lavage or given to the stable patient. Multiple doses of activated charcoal may interrupt enterohepatic circulation (Otto and Greentree, 1994; Staley and Staley, 1995; Tegzes *et al.*, 2002; Gwaltney-Brant, 2005).

Drugs such as penicillin G and NSAIDs have been recommended to reduce protein binding and enhance excretion (Tegzes *et al.*, 2002). However, this may worsen clinical signs in the short term by making the antihistamine more bioavailable.

Serum chemistries should be assessed and monitored for hydration, electrolyte balance, acid–base status, and liver and kidney function. Animals with poor hepatic or renal function may have a decreased rate of antihistamine elimination (Gwaltney-Brant, 2005). Cardiac function, blood pressure, and body temperature should be monitored as well (Otto and Greentree, 1994; Gwaltney-Brant, 2005). Respiratory function should be closely monitored, as intubation may be required to support the comatose patient (Tegzes *et al.*, 2002; Gwaltney-Brant, 2005).

Fluid therapy is useful to maintain hydration and for cardiac support and diuresis (Staley and Staley, 1995; Tegzes *et al.*, 2002; Gwaltney-Brant, 2005). Imbalances in electrolytes and pH are corrected as needed. Animals rarely require treatment for hypotension and mild to moderate cardiac arrhythmias, which often respond to fluid therapy. Epinephrine should not be used to treat promethazine overdose. Promethazine inhibits adrenergic receptors and addition of epinephrine may lead to further decrease in blood pressure (Staley and Staley, 1995). Atropine should not be used, as it may potentiate the anticholinergic effects of antihistamines (Gwaltney-Brant, 2005).

Seizures may be treated cautiously with benzodiazepines or short-acting barbiturates (Otto and Greentree, 1994; Adams, 2001; Gwaltney-Brant, 2005). The depressive effects of antihistamines may be additive with the effects of sedatives, causing "rebound depression" (Gwaltney-Brant, 2005).

Though cholinergic signs may be present, treatment with physostigmine is contraindicated as it may potentiate cardiovascular dysfunction and seizures (Gwaltney-Brant, 2005).

Animals overdosed with antihistamines usually improve within 24 h, though signs sometimes persist for 3 days. Prognosis is dependent on the severity of signs and is guarded in animals presenting with seizures or coma (Gwaltney-Brant, 2005). Tegzes *et al.*, (2002) reported recovery of a comatose dog that had been overdosed with hydroxyzine, though the authors noted that 1 or 2 weeks of supportive care are required in such cases.

## DRUGS USED TO TREAT GASTROINTESTINAL SYMPTOMS

This classification includes antacids, laxatives, and antidiarrheal drugs.

### Antacids

$H_2$ histamine receptor inhibitors and mineral antacids are used to increase gastric pH and associated discomfort. Use of these products to aide in healing of gastroduodenal ulcers secondary to NSAID use was discussed noted in a previous section. Few adverse effects have been reported with these drugs.

#### *$H_2$ histamine receptor inhibitors*

$H_2$ histamine receptor inhibitors include burimamide, cimetidine, famotidine, metamide, nizatidine, and

ranitidine. Cimetidine may impair gastrointestinal absorption of some drugs and may prolong the effects of others by inhibiting their metabolism by microsomal enzymes (Boothe, 2001).

*Pharmacokinetics*

Cimetidine is rapidly absorbed and 70% of a given dose is bioavailable. Absorption may be slowed by the presence of food in the stomach. The mean absorption time for ranitidine was approximately 1 h and absorption was not impaired by the presence of food in the stomach. Ranitidine is 73% bioavailable in dogs and 27% available in horses after oral dosing. Famotidine is poorly absorbed and only 37% bioavailable. Nizatidine is rapidly and almost completely absorbed (Boothe, 2001b). $H_2$ receptor inhibitors are less lipid soluble than $H_1$ receptor inhibitors and are less likely to cross the blood–brain barrier well (Adams, 2001). Ranitidine is about 15% protein bound (Boothe, 2001b).

Cimetidine and ranitidine are metabolized predominantly in the liver. Seventy-three percent of an oral dose of ranitidine undergoes hepatic metabolism in people, compared to 40% in dogs. Cimetidine is mostly eliminated as the parent compound. Unchanged cimetidine, famotidine, and nizatidine are excreted in the urine. The plasma half-life for cimetidine is about an hour, but may be prolonged in individuals with renal or hepatic insufficiency. The elimination half-life of ranitidine is 2.5 h in humans and 4 h in beagles (Boothe, 2001b).

*Mechanism of action*

Histamine inhibitors block histamine receptors by competitive inhibition. $H_2$ receptor inhibitors act specifically at $H_2$ receptors. $H_2$ receptors are present in the enterochromaffin cells of the gastric mucosa and histamine stimulates acid secretion and, to a limited extent, pepsin secretion (Adams, 2001; Tegzes *et al.*, 2002). High doses of histamine produce a slow onset, prolonged vasodilatory response (Adams, 2001). $H_2$ receptor inhibitors decrease acid and pepsin secretion (Boothe, 2001b). They also block some of the cardiovascular effects of histamine. Famotidine is 9 times more potent as a $H_2$ receptor inhibitor than ranitidine, which is 5–12 times more potent than cimetidine. Famotidine also has the longest duration of clinical effects (Boothe, 2001b).

Cimetidine reduces hepatic blood flow by around 20% and inhibits microsomal cytochrome P450 enzymes, thus it may interfere with metabolism of other drugs (Boothe, 2001b).

*Clinical signs*

Minor side effects have been reported with cimetidine, but Krenzelock *et al*. (1987) reported that 79% of large ingestions in humans did not produce adverse effects. Signs that were reported included CNS depression and dizziness within 90 min of ingestion, slurred speech, mental confusion, xerostomia, epigastric pain, sinus tachycardia, and mydriasis. None of the signs were deemed life threatening (Krenzelock *et al*., 1987). When $H_2$ receptor inhibitor drugs are discontinued, “rebound gastric acid hypersecretion” may occur. This is more common with cimetidine than famotidine or nazitidine (Boothe, 2001b).

Idiosyncratic effects have been reported with $H_2$ receptor inhibitors. Idiosyncratic hepatitis has been reported in humans, and is often asymptomatic. The incidence of this response in people dosed with ranitidine is 1/100,000, it is 1/600,000 in patients on cimetidine, and rarely reported with famotidine (Jiménez-Sáenz *et al*., 2000). Idiosyncratic dermatosis has also been reported with famotidine (Scheinfeld *et al*., 2000). Muscle spasms of the face and neck have been reported in people given therapeutic doses of cimetidine (Peiris and Peckler, 2001). Thrombocytopenia has been associated with $H_2$ receptor inhibitors in people (Wade *et al*., 2002).

### Mineral antacids

A list of common active ingredients in mineral antacids can be found in Table 23.4. Products may contain more than one active ingredient (Papich, 1990; Boothe, 2001b). Sodium bicarbonate is present in baking soda and in effervescent antacid products. These products are common in many households, and may be accidentally ingested by pets or administered by pet owners (Papich, 1990). They are used in veterinary medicine to decrease gastric hyperacidity and treat peptic ulcers, uremic ulcers, reflux esophagitis, and rumen acidosis secondary to grain overload (Boothe, 2001b). Few adverse effects are associated with mineral antacids (Papich, 1990), but severe clinical signs have been reported due to sodium bicarbonate and calcium carbonate use in humans (Gonzalez and Hogg, 1981; Okada *et al*., 1996; Fitzgibbons and Snoey, 1999).

Sodium bicarbonate and calcium carbonate are absorbed after ingestion. Transcutaneous absorption of sodium

**TABLE 23.4 An incomplete list of mineral antacids**

| |
|---|
| *Aluminum salts* |
| Aluminum hydroxide |
| Aluminum magnesium silicate |
| Aluminum phosphate |
| *Magnesium salts* |
| Magnesium hydroxide |
| Magnesium oxide |
| Magnesium silicate |
| *Others* |
| Calcium carbonate |
| Sodium bicarbonate |

bicarbonate across damaged skin may occur (Gonzalez and Hogg, 1981). Some of the magnesium in magnesium salt-containing antacids is absorbed, and hypermagnesemia has been reported with repeated dosing (Boothe, 2001b).

Mineral antacids increase gastric pH. Rebound acid secretion may occur when dosing is discontinued. Some mineral antacids inactivate pepsin and some bind bile salts. Magnesium-containing salts have a laxative effect, and aluminum-containing salts have a constipating effect, thus these active ingredients are often used in combination. Aluminum complexes phosphate in the intestine and aluminum hydroxide has been used to decrease phosphate absorption in patients with renal disease (Boothe, 2001b).

Carbon dioxide is produced rapidly when sodium bicarbonate is introduced to an acid, and this reaction may cause gastric distension (Boothe, 2001b). Metabolic acidosis and secondary hypokalemia have been reported in humans after sodium bicarbonate ingestion (Okada *et al.*, 1996). Renal dysfunction may predispose to the adverse effects associated with bicarbonate ingestion. Clinical signs that have been documented in human patients with bicarbonate overdose include weakness, fatigue, dizziness, syncope, seizures, muscle pain, tachycardia and arrhythmia, volume depletion, and hypoxia (Fitzgibbons and Snoey, 1999). Patients have systemic alkalosis, hypokalemia, and hypernatremia (Gonzalez and Hogg, 1981; Papich, 1990; Okada *et al.*, 1996). Treatment consists of fluid and electrolyte therapy, and produces rapid resolution of symptoms. Electrolyte and pH abnormalities resolve over a few days (Gonzalez and Hogg, 1981; Okada *et al.*, 1996).

Adverse effects attributed to calcium carbonate in humans include nausea and vomiting, dehydration, and progressive renal insufficiency. Chronic use of calcium carbonate antacids may produce metabolic acidosis, hypercalcemia, hypophosphatemia, and calciuria with urolithiasis and metastatic calcification (Fitzgibbons and Snoey, 1999; Boothe, 2001b).

Antacids may interfere with absorption of other drugs, including digoxin, prednisolone, ranitidine, and cimetidine (Papich, 1990).

## Laxatives, cathartics, and enemas

Drugs in this category may be designated as bulk forming laxatives, lubricant laxatives, irritant laxatives, hyperosmotic cathartics, and enemas.

### *Bulk forming laxatives*

Nonabsorbable polysaccharide cellulose derivatives are used as bulk forming laxatives (Papich, 1990). Natural sources include psyllium or plantago seed, wheat bran, and fruits such as prunes. Synthetic forms include methylcellulose and carboxymethylcellulose. These products act by absorbing water in the gastrointestinal tract and swelling the softened fecal mass. This causes intestinal distension and reflex peristaltic contractions. Intestinal bacteria act on cellulose and hemicellulose, and produce volatile fatty acids, enhancing the osmotic effect. Fluid feces and tympany may result (Boothe, 2001b). No serious adverse effects have been reported in companion animals, though these products may cause fluid and electrolyte loss secondary to diarrhea. Papich (1990) suggested that subsequent dehydration may lead to intestinal impaction.

Animals treated with bulk forming laxatives should have fresh water available. If diarrhea occurs, monitor hydration and electrolyte status and correct as needed.

### *Lubricant laxatives*

Lubricant laxatives, including mineral oil and white petrolatum, are hydrocarbon mixtures derived from petroleum. Mineral oil is frequently used in large animals and white petrolatum products are often used to treat trichobezoars in cats (Papich, 1990). These large hydrocarbons are minimally absorbed and act by coating feces with a film that entraps moisture and lubricates passage. Hydrocarbon laxatives reduce absorption of fat-soluble vitamins and possibly other nutrients, thus chronic use may be associated with deficiencies. Intestinal irritability may decrease over time with repeated use, leading to chronic constipation. The small amount of absorbed hydrocarbons may provide a nidus for granuloma formation in the intestinal mucosa, mesenteric lymph nodes, or liver (Papich, 1990; Boothe, 2001b). Adverse effects, however, are rarely reported with lubricant laxatives (Papich, 1990).

### *Irritant laxatives*

This group accounts for most of the common OTC laxatives. Table 23.5 lists common types of irritant laxative. Phenophthalein laxatives may be chocolate flavored, increasing palatability, but are only effective in primates and swine (Papich, 1990; Boothe, 2001b). Vegetable oil

**TABLE 23.5 Irritant cathartics**

| |
|---|
| *Diphenylmethanes* |
| Phenophthalein |
| Biscodyl |
| *Vegetable oils* |
| Castor oil |
| Raw linseed oil |
| Olive oil |
| *Anthraquinones* |
| Danthron |
| Cascara sagrada |
| Senna |
| Aloin |

products contain irritant fatty acids such as ricinoleic acid in castor oil, linoleates in linseed oil, and oliveates in olive oil. Common anthraquinone type cathartics are derived from plants: cascara sagrada from *Rhamnus* spp., senna from *Cassia* spp., and aloins from *Aloe* spp. Danthron (1,8-dihydroxyanthroquinone) is synthetic, but is considered the prototype for the anthraquinone laxative group.

Five percent of an oral dose of bisacodyl is absorbed. There is some absorption of anthraquinone glycosides, and delayed transit through the small intestine decreases effectiveness. Anthraquinones may be secreted in the milk, causing clinical effects in the nursing young (Boothe, 2001b).

As suggested by the term irritant laxative, these compounds cause contact irritation of gastrointestinal mucosa and increase fluid secretion into the lumen. Stimulation of intramural nerve plexi promotes intestinal transit (Papich, 1990; Boothe, 2001b).

Diarrhea occurs 6–8 h after diphenylmethane cathartics are administered and may be severe if large doses are given (Boothe, 2001b). Pink discoloration of alkaline urine may be seen with phenolphthalein. Acidic urine will turn pink with addition of sodium hydroxide or sodium bicarbonate (Papich, 1990).

Vegetable oil laxatives are hydrolyzed by lipase in the small intestine and form sodium and potassium salts which act as soaps, producing irritation. Ricinoleic acid is the most potent of these compounds, and initiates rapid and complete colonic emptying (Papich, 1990; Boothe, 2001b). This clinical effect is seen 4–8 h after administration of castor oil in small animals, and 12–18 h post dosing in large animals. Animals treated with castor oil should be fed moist, bulky material afterward.

Anthraquinones are hydrolyzed by bacteria in the large intestine to emodins which stimulate the myenteric plexus. Anthraquinones produce catharsis after 6–12 h in small animals and 12–36 h in large animals. Catharsis may be accompanied by reduced hydration and electrolyte loss. Abdominal pain or colic may produced by large doses (Papich, 1990; Boothe, 2001b). Changes in urine color are reported with anthraquinones. With chronic use of these laxatives, the mesenteric plexus will degenerate causing a loss in intestinal motility (Boothe, 2001b).

Animals treated with irritant cathartics should be monitored for hydration and electrolyte status with correction as needed. Some irritant cathartics may increase PG synthesis, and NSAIDs would minimize this effect (Papich, 1990).

### *Hyperosmotic cathartics*

Magnesium sulfate, or Epsom salt, is a common osmotic cathartic and is used in 6% isotonic solution. Other magnesium-containing cathartics include magnesium hydroxide (milk of magnesia), magnesium oxide, and magnesium citrate. Sodium sulfate (Glauber's salt), sodium phosphate, potassium sodium tartrate, and sodium tartrate (Rochelle salt) are used as cathartics and ingestion of large quantities of sodium chloride will produce catharsis. The sugar alcohols, mannitol and sorbitol, and synthetic disaccharides such as lactulose are also used as cathartics (Boothe, 2001b).

Osmotic cathartic use should be avoided in dehydrated animals, and water should be freely available. Other contraindications for use may include congestive heart failure, gastrointestinal stasis, and hepatic or renal impairment (Henninger and Horst, 1997; Ezri *et al.*, 2006). Hyperosmotic cathartics draw water into the intestinal tract via osmosis (Papich, 1990; Boothe, 2001b; Ezri *et al.*, 2006). Intestinal distension promotes motility. Effects of cathartics are usually evident 3–12 h after dosing in monogastrics and within 18 h in ruminants (Boothe, 2001b).

There is minimal slow absorption of most cathartics, but up to 20% of the magnesium in a dose of magnesium sulfate is absorbed in the small intestine (Henninger and Horst, 1997; Boothe, 2001b). Renal failure may enhance systemic magnesium accumulation, leading to hypermagnesemia (Papich, 1990; Boothe, 2001b). Administration of magnesium sulfate with dioctyl sodium sulfosuccinate in horses may lead to increased magnesium absorption due to mucosal damage (Henninger and Horst, 1997). Magnesium ions promote the release of cholecystokinin which enhances peristalsis (Boothe, 2001b). Systemic effects of magnesium include inhibition of calcium ion release at neuromuscular junctions, inhibition of acetylcholine release, decreased sensitivity of motor endplates, and decreased excitability of myocyte membranes, leading to paralysis (Henninger and Horst, 1997). Clinical signs of hypermagnesia secondary to magnesium sulfate administration in the horse occurred 1–6 h post administration and included perspiration, progressive tremors, recumbence, severe tachycardia, tachypnea, pale mucous membranes, prolonged capillary refill time, flaccid paralysis of the head and neck, and loss of flexor and perineal reflexes. Horses remained alert. Similar signs are reported in humans, but bradycardia is more common than tachycardia in people. Clinical signs of hypermagnesemia in humans were associated with serum magnesium concentrations greater than 3 mg/dl. Paralysis of respiratory muscles and cardiac arrest occurred at concentrations above 18 mg/dl. Severe clinical signs in horses were associated with serum magnesium levels of 14.7 and 15.8 mg/dl (Henninger and Horst, 1997).

Absorbed phosphate from phosphate-containing cathartics may deplete intracellular potassium ions and induce hypokalemia. Clinical signs reported in humans dosed with sodium phosphate include coma, hypovolemia, hypocalcemia and tetany, acute renal failure, and death. Increased serum phosphate and sodium, and decreased potassium, magnesium, and calcium, as well as lactic acidemia were reported in people given phosphate salts (Ezri *et al.*, 2006).

Lactulose reaches the colon largely intact and is degraded into lactic acid and acetic acid by large intestinal microflora. Gas may be generated, causing tympany. The associated decrease in pH has been used for ion trapping of ammonium to prevent hepatic encephalopathy in animals with liver failure (Boothe, 2001b).

Dehydration and electrolyte imbalances are the most common changes associated with cathartics. Management is aimed at correcting these imbalances. Calcium is used to treat hypermagnesemia. Calcium ions displace magnesium ions from cell membranes. Response to therapy is usually rapid, but repeated dosing with calcium gluconate may be needed. Diuresis with IV fluids and furosemide may be used to promote renal excretion. Henninger and Horst (1997) reported that horses treated for hypermagnesemia had serum magnesium concentrations within reference ranges the next day. Electrolyte changes in asymptomatic patients given oral sodium phosphate for pre-surgical catharsis returned to normal within 24 h (Ezri *et al.*, 2006).

### *Enemas*

An enema is a material given intrarectally to induce defecation. Commonly used enemas include soft anionic soap in water, isotonic or hypertonic sodium chloride, sorbitol, glycerol, sodium lauryl sulfate, sulfoacetate, mineral oil, olive oil, and phosphate salts.

Toxicosis has been reported in people and small animals administered hypertonic sodium phosphate solutions, or fleet enemas. Debilitated cats are commonly affected (Papich, 1990). Predisposing factors may include administration of a full-strength fleet enema (intended for human use) to a small animal, dehydration, preexisting electrolyte abnormalities, renal or hepatic dysfunction, colonic dilation, and colonic ulceration (Jorgensen *et al.*, 1985; Papich, 1990; Roder, 2005b). A 60 ml hypertonic phosphate enema may cause toxicosis in a cat (Roder, 2005b).

Sodium and phosphate are absorbed in the colon, and absorption may be increased with disruption of the mucosa or in animals with chronic constipation (Jorgensen *et al.*, 1985). Uptake of phosphate promotes cellular uptake of calcium and phosphate may directly bind serum calcium, both contributing to hypocalcemia. Adverse effects associated with sodium phosphate enemas usually occur within an hour of dosing (Jorgensen *et al.*, 1985; Papich, 1990). Vomiting and bloody diarrhea may be seen. Nervous signs that have been documented include depression, ataxia, anxiety, neuromuscular irritability, and convulsions. Dehydration, weak pulse, tachycardia, hypothermia, shock, and death have also been reported (Jorgensen *et al.*, 1985; Papich, 1990). Serum chemistry in these patients often reveals hyperphosphatemia, hypernatremia, hypocalcemia, metabolic acidosis, and hyperglycemia. Hyperkalemia and hypokalemia have both been documented (Jorgensen *et al.*, 1985).

Intensive fluid therapy is required in acute sodium phosphate enema overdose situations. The purpose is to correct dehydration, electrolyte imbalances, acid–base imbalances, and hypoglycemia, and treat circulatory shock. Isotonic saline solution may increase renal calcium loss and thus should be avoided. Saline solutions containing 0.45% sodium chloride and 2.5% dextrose, or 2.5–5.0% dextrose solutions have been recommended for hypoglycemic patients (Jorgensen *et al.*, 1985; Papich, 1990). Lactated ringers solution may be used in hyperglycemic patients. Fluid therapy may be needed for 4 days. Jorgensen *et al.*, (1985) suggested instillation of aluminum carbonate or aluminum hydroxide to bind phosphate in the intestine. Animals should be treated for hypothermia if needed. Prophylactic antibiotics are given for compromise of the colonic mucosa. Intoxicated cats showed improvement within 6 h of initiation of therapy, and electrolyte imbalances resolved within 2 days (Jorgensen *et al.*, 1985).

## Antidiarrheal drugs

Active ingredients in products used to treat diarrhea include bismuth subsalicylate, kaolin–pectin, and opioid receptor agonists. Most of the toxic effects associated with bismuth subsalicylate are due to the salicylate component, therefore this formulation is discussed in more detail with aspirin and salicylates. Bismuth is believed to adsorb bacterial endotoxin and may have a direct antimicrobial effect (Papich *et al.*, 1987).

Kaolin and pectin were previously the active ingredients in the popular OTC product Kaopectate®, but bismuth subsalicylate is now used. Kaolin is hydrated aluminum silicate and acts as an adsorbent. Pectin is a carbohydrate extracted from citrus fruit that acts as an adsorbed and intestinal protectant. Toxicosis attributed to kaolin–pectin preparations has not been observed, though these products may decrease absorption of other drugs.

### *Opioid receptor agonists*

The OTC drug Imodium® and the prescription drug Lomotil® contain the opioid receptor agonists loperamide and diphenoxylate, respectively. Loperamide is sold in 2 mg capsules and liquids at a concentration of 0.20 mg/ml. The effective dose for loperamide is 0.08 ml/kg PO every 12 h in small dogs and cats. These products are poorly absorbed. They function to decrease gastrointestinal secretion and motility. Side effects may include constipation, abdominal pain, vomiting, and drowsiness, but severe toxicosis is unlikely. Management consists of monitoring and supportive and symptomatic care. Activated charcoal may be given to decrease intestinal absorption if large doses were ingested. The opioid antagonist naloxone may be used to manage severe clinical signs, but this is unlikely to be necessary. Naloxone has a short half-life, requiring repeated dosing as needed (Papich, 1990).

## TOPICAL DRUGS

Toxicosis secondary to topical preparations may occur through dermal absorption or ingestion. Cats in particular are fastidious groomers and any product that is used *on* a cat may end up *in* the cat for this reason. Animals, dogs in particular, may chew through a tube containing a topical drug (Papich, 1990).

Like other OTC drugs, topical products frequently contain more than one active ingredient. Antibiotic preparations may contain bacitracin, neomycin, and polymyxin. Though rare topical hypersensitivities have been reported, these antimicrobials are poorly absorbed from the normal gastrointestinal tract, limiting their systemic effects (Papich, 1990). However, if large quantities are ingested, damage may occur to the resident gastrointestinal microflora leading to diarrhea and discomfort. Use of these products in hamsters or guinea pigs, which are predisposed to develop antibiotic-induced enterocolitis, should probably be avoided.

Benzoyle peroxide is present in some topical antiseptics and acne medications. It acts by releasing hydrogen peroxide, which has antibacterial and keratinolytic effects and promotes epithelial cell turnover. Hyperemia and blistering have been reported with overuse of products containing benzoyle peroxide. Ingestion may lead to intragastrointestinal gas production and distension. Emetics are contraindicated if this should happen. Symptomatic treatment includes gastric decompression using a stomach tube (Papich, 1990).

## REFERENCES

Adams RH (2001) Autocoids and anti-inflammatory drugs. In *Veterinary Pharmacology and Therapeutics*, 8th edn, Adams R (ed.). Iowa State University Press, Ames, IA, pp. 403–12.

Albretsen JC (2002) Oral medications. *Vet Clin Small Anim* **32**: 421–42.

Allen AL (2003) The diagnosis of acetaminophen toxicosis in a cat. *Can Vet J* **44**: 509–10.

Aronson LR, Drobatz K (1996) Acetaminophen toxicosis in 17 cats. *J Vet Emerg Crit Care* **6**: 65–9.

Bersenas AME, Mathews KA, Allen DG, Conlon PD (2005) Effects of ranitidine, famotidine, pantoprazoline, and omeprazole on intragastric pH in dogs. *Am J Vet Res* **66**: 425.

Boothe DM (2001a) The analgesic, antipyretic, anti-inflammatory drugs. In *Veterinary Pharmacology and Therapeutics*, 8th edn, Adams R (ed.). Iowa State University Press, Ames, IA, pp. 433–51.

Booth PM (2001b) Histamine, seratonin, and their antagonists. In *Veterinary Pharmacology and Therapeutics*, 8th edn, Adams R (ed.). Iowa State University Press, Ames, IA, pp. 403–12.

Boulay JP, Lipowitz AJ, Klausner JS (1986) Effects of cimetidine on aspirin-induced gastric hemorrhage in dogs. *Am J Vet Res* **47**: 1744–6.

Boynton CS, Dick CF, Mayor GF (1988) NSAIDs: an overview. *J Clin Pharmacol* **28**: 512–17.

Brater DC (1988) Clinical pharmacology of NSAIDs. *J Clin Pharmacol* **28**: 518–23.

Brix AE (2002) Renal papillary necrosis. *Toxicol Pathol* **30**: 672–4.

Brumbaugh GW (2001) Adverse drug reactions and interactions in the horse. *Vet Clin N Am Equine Pract* **17**: 445–53.

Carson JL, Strom BL (1988) The gastrointestinal side effects of the nonsteroidal anti-inflammatory drugs. *J Clin Pharmacol* **28**: 554–9.

Cathers ATE, Isaza R, Oehme F (2000) Acute ibuprofen toxicosis in a ferret. *J Am Vet Med Assoc* **216**: 1246–8.

Cheung LY, Sonnenschein LA (1983) Effect of cimetidine on canine gastric mucosal pH and blood flow. *Am J Surg* **145**: 24–8.

Collins LG, Tyler DE (1984) Phenylbutazone toxicosis in a horse: a clinical study. *J Am Vet Med Assoc* **184**: 699–703.

Collins LG, Tyler DE (1985) Experimentally induced phenylbutazone toxicosis in ponies: description of the syndrome and its prevention with synthetic prostaglandin $E_2$. *Am J Vet Res* **46**: 1605.

Court MH, Greenblatt DJ (1997) Molecular basis for deficient acetaminophen glucuronidation in cats. *Biochem Pharmacol* **5**: 1041–7.

Crandell JM, Ware WA (2005) Cardiac toxicity from phenylpropanolamine overdose in a dog. *J Am Anim Hosp Assoc* **41**: 413–20.

Daehler MH (1986) Transmural pyloric perforation associated with naproxen administration in a dog. *J Am Vet Med Assoc* **189**: 694–5.

Dahm LJ, Jones DP (1996) Mechanisms of chemically induced liver disease. In *Hepatology A Textbook of Liver Disease* Zakim D, Boyer TD (eds). W.B. Saunders Company, Philadelphia, PA, pp. 875–90.

Davis LE (1980) Clinical pharmacology of salicylates. *J Am Vet Med Assoc* **176**: 65–6.

Driman D, Wright C, Tougas G, Riddell R (1995) Omeprazole produces parietal cell hypertrophy and hyperplasia in humans. *Gastroenterol* **108**: A87.

Ezri T, Lerner E, Muggia-Sullam M, Medalion B, Tzivian A, Cherniak A, Szmuck P, Shimonov M (2006) Phosphate salt bowel preparation regimens alter perioperative acid-base and electrolyte balance. *Can J Anesth* **53**: 153–8.

Fitzgibbons LJ, Snoey ER (1999) Severe metabolic acidosis due to baking soda ingestion: case reports of two patients with unsuspected antacid overdose. *J Emerg Med* **17**: 57–61.

Gfeller RW, Sandors AD (1991) Naproxen-associated duodenal ulcer complicated by perforation and bacteria-and barium sulfate-induced peritonitis in a dog. *J Am Vet Med Assoc* **198**: 644–6.

Godshalk CP, Roush JK, Fingland RB, Sujjena D, Vorhies MW (1992) Gastric perforation associated with administration of ibuprofen in a dog. *J Am Vet Med Assoc* **201**: 1734–6.

Gonzalez J, Hogg RJ (1981) Metabolic acidosis secondary to baking soda treatment of diaper rash. *Pediatrics* **67**: 820–2.

Gunson DE (1983) Renal papillary necrosis in horses. *J Am Vet Med Assoc* **182**: 263–6.

Gwaltney-Brant S (2005) Antihistamines. In *Clinical Veterinary Toxicology*, Plumlee K (ed.). Mosby, St. Louis, MO, pp. 291–3.

Hashimoto F, Davis RL, Egli D (1994) Hepatitis following treatments with famotidine and then cimetidine. *Ann Pharmacother* **28**: 37–9.

Hata J, Kamada T, Manabe N, Kusunoki H, Kamino D, Nakao M, Fukumoto A, Yamaguchi T, Sato M, Haruma K (2005) Famotidine prevents canine gastric blood flow reduction by NSAIDs. *Aliment Pharmacol Ther* **21**: 55–9.

Henninger RW, Horst J (1997) Magnesium toxicosis in two horses. *J Am Vet Med Assoc* **211**: 82–5.

Hjelle JJ, Grauer GF (1986) Acetaminophen-induced toxicosis in dogs and cats. *J Am Vet Med Assoc* **188**: 742–6.

Isaacs JP (1996) Adverse effects of non-steroidal anti-inflammatory drugs in the dog and cat. *Aust Vet Pract* **26**: 180–6.

Jackson TW, Costin C, Link K, Heule M, Murphy MJ (1991) Correlation of serum ibuprofen concentration with clinical signs of toxicity in three canine exposures. *Vet Hum Toxicol* **33**: 486–8.

Jiménez-Sáenz M, Argüelles-Arias F, Herrerías-Gutiérrez JM, Durán-Quintana JA (2000) Acute cholestatic hepatitis in a child treated with femotidine. *Am J Gastroenterol* **95**: 3665–6.

Jones RD, Baynes RE, Nimitz CT (1992) Nonsteroidal anti-inflammatory drug toxicosis in dogs and cats: 240 cases (1989–1990). *J Am Vet Med Assoc* **201**: 475–7.

Jorgensen LS, Center SA, Randolph JF, Brum D (1985) Electrolyte abnormalities included by hypertonic phosphate enemas in two cats. *J Am Vet Med Assoc* **187**: 136–7.

Kore AM (1990) Toxicology of nonsteroidal anti-inflammatory drugs. *Vet Clin N Am Small Anim Pract* **20**: 419–30.

Krenzelock EP, Litovitz T, Lippold KP, McNalley CF (1987) Cimetidine toxicity: an assessment of 881 cases. *Ann Emerg Med* **16**: 43–7.

Lees R, May SA, McKellar QA (1991) Pharmacology and therapeutics of non-steroidal anti-inflammatory drugs in the dog and cat: 1 general pharmacology. *J Small Anim Pract* **32**: 183–93.

MacNaughton SM (2003) Acetaminophen toxicosis in a dalmation. *Can Vet J* **44**: 142–4.

Mazué G, Richez P, Berthe J (1982) Pharmacology and comparative toxicology of non-steroidal anti-inflammatory agents. In *Veterinary Pharmacology and Toxicology*, Ruckebush Y, Toutain P, Koritz GD (eds). MTP, Boston, MA, pp. 321–31.

McKellar QA, May SA, Lees P (1991) Pharmacology and therapeutics of non-steroidal anti-inflammatory drugs in the dog and cat: 2 individual agents. *J Small Anim Pract* **32**: 225–35.

Means C (1999) Ma huang: all natural but not always innocuous. *Vet Med* **94**: 511–12.

Means C (2005) Decongestants. In *Clinical Veterinary Toxicology*, Plumlee K (ed.). Mosby, St. Louis, MO, pp. 309–11.

Murphy MJ (1994) Toxin exposures in dogs and cats: drugs and household products. *J Am Vet Med Assoc* **205**: 557–60.

Murtaugh RJ, Matz ME, Labato MA, Boudrieau RJ (1993). Use of synthetic prostaglandin E1 (misoprostol) for prevention of aspirin-induced gastroduodenal ulceration in arthritic dogs. *J Am Vet Med Assoc* **202**: 251–6.

Nine JS, Rund CR (2006) Fatality from diphenhydramine monointoxication a case report and review of the infant, pediatric, and adult literature. *Am J Forensic Med Pathol* **27**: 36–41.

Okada K, Kono N, Kobayashi S, Yamaguchi S (1996) Metabolic acidosis and myoclonus from antacid ingestion. *Internal Med* **35**: 515–16.

Ooms TG, Khan S (2001) Suspected caffeine and ephedrine toxicosis resulting from ingestion of an herbal supplement containing guarana and ma huang in dogs: 47 cases (1997–1999). *J Am Vet Med Assoc* **218**: 225–9.

Otto CM, Greentree WF (1994) Terfenadine toxicosis in dogs. *J Am Vet Med Assoc* **205**: 1004–6.

Papich MG (1990) Toxicosis from over-the-counter human drugs. *Vet Clin N Am Small Anim Pract* **20**: 431–51.

Papich MG, Davis CA, Davis LE (1987) Absorption of salicylate from an antidiarrheal preparation in dogs and cats. *J Am Anim Hosp Assoc* **23**: 221–6.

Peiris R, Peckler BF (2001) Cimetidine-induced dystonic reaction. *J Emerg Med* **21**: 27–9.

Plumb DC (2002) *Veterinary Drug Handbook*. Iowa State Press, Ames, IA.

Roder JD (2005a). Nonsteroidal anti-inflammatory agents. In *Clinical Veterinary Toxicology* Plumlee K (ed.). Mosby, St. Louis, MO, pp. 282–4.

Roder JD (2005b). Hypertonic phosphate enema. In *Clinical Veterinary Toxicology* Plumlee K (ed.). Mosby, St. Louis, MO, p. 319.

Rubin SL, Papich MG (1990) Clinical uses of nonsteroidal anti-inflammatory drugs in companion animal practice. Part II. Drugs, therapeutic uses and adverse effects. *Canine Pract*. **15**: 27–32.

Rumbeiha WK, Lin Y, Oehme FW (1995) Comparison of *N*-acetylcysteine and methylene blue, alone or in combination, for treatment of acetaminophen toxicosis in cats. *Am J Vet Res* **56**: 1529–33.

Runkel R, Chaplin M, Boost G, Segre E, Forchielle E (1972) Absorption, distribution, metabolism, and excretion of naproxen in various laboratory animal and human subjects. *J Pharmaceut Sci* **61**: 703–8.

Sainsbury SJ (1991) Fatal salicylate toxicity from bismuth subsalicylate. *West J Med* **155**: 637–9.

Savides MC, Oehme FW (1985) Effects of various antidotal treatments on acetaminophen toxicosis and biotransformation in cats. *Am J Vet Res* **46**: 1485–9.

Scheinfeld N, Wesson K, Perry P, Weinberg J (2000) Acute generalized exanthematous pustulosis resembling toxic epidermal necrolysis caused by famotidine. *Acta Dermatol Venereol* **83**: 76–7.

Schlesinger DP (1995) Methemoglobinemia in a dog with acetaminophen toxicity. *Can Vet J* **36**: 515–17.

Schubert TA (1984) Salicylate-induced seizure in a dog. *J Am Vet Med Assoc* **185**: 1000–1.

Sellon RK (2006) Acetaminophen. In *Small Animal Toxicology*, 2nd edn, Petersen ME, Talcott PA (eds.). Saunders, Philadelphia, PA, pp. 550–8.

Staley EC, Staley EE (1995) Promethazine toxicity in a seven-month-old Doberman pinscher. *Vet Hum Toxicol* **37**: 243–4.

Stanton ME, Bright RM (1989) Gastroduodenal ulceration in dogs retrospective study of 43 cases and literature review. *J Vet Int Med* **3**: 238–44.

Strøm H, Krogsgaard Thomsen M (1990) Effects of non-steroidal anti-inflammatory drugs on canine neutrophil chemotaxis. *J Vet Pharmacol Ther* **13**: 186–91.

Sturgill MG, Lambert GH (1997) Xenobiotic-induced hepatotoxicity: mechanisms of liver injury and methods of monitoring hepatic function. *Clin Chem* **43**: 1512–26.

Talcott PA (2006) Nonsteroidal antiinflammatories. In *Small Animal Toxicology*, 2nd edn, Petersen ME, Talcott PA (eds.). Saunders, Philadelphia, PA, pp. 902–33.

Tegzes JH, Smarick SD, Puschner B (2002) Coma and apnea in a dog with hydroxyzine toxicosis. *Vet Hum Toxicol* **44**: 24–6.

Treinen-Moslen M (2001) Toxic responses of the liver. In *Casarett Doull's Toxicology: The Basic Science of Poisons*, Klaassen CD (ed.). McGraw Hill, New York, NY, pp. 471–89.

Verbeeck RK (1990) Pharmacokinetic drug interactions with nonsteroidal anti-inflammatory drugs. *Clin Pharmacokinet* **19**: 44–66.

Villar D, Buck WB, Gonzalez JM (1998) Ibuprofen, aspirin, acetaminophen toxicosis and treatment in dogs and cats. *Vet Hum Toxicol* **40**: 156–61.

Wade EE, Rebuck JA, Healy MA, Rogers FB (2002) H2 antagonist-induced thrombocytopenia: is this a real phenomenon? *Intens Care Med* **28**: 459–65.

Walan A, Bader J, Classen M, Lamers CBHW, Piper DW, Rutgersson K, Eriksson S (1989) Effect of omeprazole and ranitidine on ulcer healing and relapse rates in patients with benign gastric ulcer. *N Engl J Med* **320**: 69–75.

Wallace KP, Center SA, Hickford FH, Warner KL, Smith S (2002) S-adenosyl-L-methionine (SAMe) for treatment of acetaminophen toxicity in a dog. *J Am Anim Hosp Assoc* **38**: 246–54.

Wallace MS, Zawie DA, Garvey MS (1990) Gastric ulceration in the dog secondary to use of nonsteroidal anti-inflammatory drugs. *J Am Anim Hosp Assoc* **26**: 467–72.

Waters DJ, Bowers LD, Cipolle RJ, Caywood DD, Bills RL (1993) Plasma salicylate concentrations in immature dogs following aspirin administration: comparison with adult dogs. *J Vet Pharmacol Ther* **16**: 275–82.

Webb CB, Twedt DC, Fettman MJ, Mason G (2003) S-adenosylmethionine (SAMe) in a feline acetaminophen model of oxidative injury. *J Feline Med Surg* **5**: 69–75.

Zimmerman HJ (1999) Drug induced liver disease. In *Schiff's Diseases of the Liver*, Schiff ER, Sorrell MF, Maddrey WF (eds). Lippincott-Raven Publishers, Philadelphia, PA, pp. 973–1064.

CHAPTER

# 24

# Toxicity of drugs of abuse

*Karyn Bischoff*

## INTRODUCTION

Potential for exposure to illegal drugs may exist for many companion animals, horses, and even livestock on occasion; 46% of Americans have used illegal drugs in one form or another (Compton *et al.*, 2005). The illegal drug trade in the United States peaked in the late 1970s, declined in the 1980s and early 1990s, and increased again through the mid- to late 1990s to stabilize in recent years (Compton *et al.*, 2005).

Marijuana is one of the most prevalent recreational drugs in the world after legal substances ethanol and nicotine (Janczyk *et al.*, 2004; Compton *et al.*, 2005; Johnson *et al.*, 2005; Vitale and van de Mheen, 2005). Cocaine may be second in popularity among illegal substances in the United States and parts of Europe (Kisseberth and Trammel, 1990; Dumonceaux, 1995; Queiroz-Neto *et al.*, 2002; Vitale and van de Mheen, 2005). Since the 1980s, the term "club drugs" has come into use, representing drugs frequently found at nightclubs and all-night "rave" parties. Club drugs are a continuing trend (Smith *et al.*, 2002; Banken, 2004). Many are stimulants, though depressants may be used to counter the effects of the stimulants or given secretly to sedate a victim for the purpose of theft or assault. Hallucinogens are also used. Some of the most common club drugs include ketamine, 3,4-methylenedioxymethamphetamine (MDMA) commonly called "ecstasy," flunitrazepam (Rohypnol®), the "date rape drug," and $\gamma$-hydroxybutanoic acid (GHB).

### Illicit drugs and small animals

Among companion animals, dogs are the most susceptible to poisoning with illicit substances though problems occasionally arise in cats, ferrets, birds, or other animals in the household. Exposure may be through voluntary ingestion or malicious poisoning and other forms of animal abuse (Kisseberth and Trammel, 1990). There are reports of adolescents entertaining themselves by intoxicating dogs, cats, and birds with second-hand marijuana smoke (Schwartz and Riddle, 1985; Buchta, 1988).

Police dogs are at particular risk for ingestion of illegal drugs. They may come into contact with large quantities of the high-purity chemicals in the line of duty (Dumonceaux and Beasley, 1990; Kisseberth and Trammel, 1990). They may ingest whole bags of drugs which must be removed surgically or via endoscopy to prevent rupture and massive exposure (Dumonceaux and Beasley, 1990). Police dogs may be at increased risk for malicious poisonings. Recently news reports document the use of dogs as "drug mules" to move bags of heroin, which were surgically implanted. Deaths in several of these dogs were attributed to secondary infections (McLaughlin, *CNN News* February 2, 2006).

Illegal drug ingestion in small animals presents a diagnostic challenge, and often an ethical challenge, to the clinician. Pet owners may not be aware of what the animal was exposed to, as in the case of animal intoxication by adolescents (Schwartz and Riddle, 1985; Buchta, 1988). Jones in 1978 reported that a dog ingested illicit substances from a neighbor's garbage can. Violence is intrinsic to the drug culture in poor neighborhoods (Johnson and Myron, 1995). It could take the form of malicious poisoning or other forms of animal abuse. Suspected malicious poisoning with illicit drugs has been reported (Bischoff *et al.*, 1998).

The pet owner who *is* aware that the animal ingested an illegal substance may be reluctant to admit it (Godbold *et al.*, 1979; Kisseberth and Trammel, 1990; Dumonceaux, 1995; Welshman, 1986; Frazier, 1998; Janczyk *et al.*, 2004; Volmer, 2005). Sadly, animals may occasionally be euthanized in part due to an incomplete history (Smith, 1988). The owner should be aware that a proper history is

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required if appropriate treatment is to be administered (Welshman, 1986). Diagnostic laboratories have screens available to detect the presence of many illegal drugs, but these screens take time and may be expensive (Janczyk *et al.*, 2004). Awareness of the animal's home situation, including the neighborhood or the presence or adolescent children in the household may be helpful in determining the potential for exposure to illegal substances. The astute clinician may want to ask if there was a party in the home when the pet became ill (Kisseberth and Trammel, 1990).

A veterinarian may have to balance client confidentiality with legal obligation. The client bringing the pet to a veterinary clinic is aware that it requires medical attention, but the threat of legal action may discourage them from disclosing critical information. It is prudent to be aware of the local laws concerning animal abuse, drug possession, and their responsibility toward reporting illegal activities.

Pet owners who do admit that their pet ingested illegal drugs may not be fully aware of what was ingested, or know only the street name of the drug. Drug dealers may combined drugs or make substitutions and many drugs are very similar in appearance (Kisseberth and Trammel, 1990).

## Illicit drugs and large animals

Horses may be "doped" to improve athletic performance, or to hide soundness or temperament problems. Cocaine has been detected in urine samples from horses at athletic events (Queiroz-Neto *et al.*, 2002; Kollias-Baker *et al.*, 2003). It is expected that such drugs would be given in relatively small doses, but dose miscalculations may occur. Exposure to plants grown for illicit drug manufacture may occur in herbivores. There are reports of lethal marijuana exposure in cattle fed hay and horses on pasture (Cardassis, 1951; Driemeier, 1997).

Anhydrous ammonia is an ingredient in illegal methamphetamine production. It is also used to instill nitrogen into the soil for fertilization and tanks may be stored near animal facilities. Unscrupulous manufacturers of the drug often steal anhydrous ammonia from farm tanks and may fail to close the tank valves before leaving the premises. The *Iowa State Daily* on September 7, 2004 reported 21 cattle dead from anhydrous ammonia intoxication blamed on the methamphetamine trade. The *Huron Daily Tribune* reported more than 30 cattle dead and at least 40 with severe ocular lesions in Michigan after a similar incident.

# THE VETERINARIAN AND THE DRUG ENFORCEMENT ADMINISTRATION (DEA)

Title 21 of the US Code of Federal Regulations establishes the Drug Enforcement Administration (DEA), which classifies drugs into five categories as listed in Table 24.1.

**TABLE 24.1 DEA drug schedules**

| Classification | Description | Examples |
|---|---|---|
| Schedule I | 1. High potential for abuse.<br>2. No accepted medical use in Unites States.<br>3. Lack of accepted safety for use under medical supervision. | Heroin<br>LSD<br>Marijuana<br>MDMA<br>Mescaline<br>Psilocybin |
| Schedule II | 1. High potential for abuse.<br>2. Currently accepted medical uses, may have restrictions.<br>3. Severe physical or psychological dependence potential with abuse. | Amphetamine<br>Cocaine<br>Methamphetamine<br>Morphine<br>Opium<br>Pentobarbital<br>Phencyclidine |
| Schedule III | 1. Less potential for abuse than schedule I or II drugs.<br>2. Currently accepted medical uses.<br>3. Moderate or low potential for physical or psychological dependence with abuse. | Ketamine<br>LSA<br>Thiopental |
| Schedule IV | 1. Low potential for abuse relative to schedule III.<br>2. Currently accepted medical use.<br>3. Limited physical or psychological dependence potential with abuse. | Butorphanol<br>Diazepam<br>Flunitrazepam |
| Schedule V | 1. Low potential for abuse compared to schedule IV.<br>2. Currently accepted medical use.<br>3. Limited potential for physical or psychological dependence with abuse compared to schedule IV. | Low-dose codeine preparations<br>Low-dose opium preparations |

Because some veterinary drugs, such as ketamine and phenobarbital, are coveted on the illegal drug market, failure of the veterinarian to prescribe drugs appropriately may lead to loss of license or risk of imprisonment (Gloyd, 1982). A veterinarian was fined and lost his license in the year 2000 for selling steroids illegally (*AVMA News*, October 15, 2000) and a few years later, an Internet pharmacy was fined more than $40,000 for contracting veterinarians to write prescriptions without examining animals and for dispensing drugs not approved by the Food and Drug Administration (FDA) (*AVMA News*, April 19, 2002).

## MAJOR CNS DEPRESSANTS

Substances that have a depressant effect on the central nervous system (CNS) include marijuana, barbiturates, opioids, and club drugs flunitrazepam (Rohypnol®), and GHB. Ketamine, a sedative-hypnotic, will be discussed with related compound phencyclidine under the category of hallucinogens. Depressants act at a variety of receptor sites within the CNS. Some receptors are named for the drugs that bind to them, including opioid receptors and cannabinoid receptors.

### Marijuana

*Cannabis sativa* has been used for over 4000 years for its psychotropic effects (Di Marzo and De Petrocellis, 2006). Common names include marijuana, hemp or Indian hemp, pot, ganja, dagga, hashish, and kief (Burrows and Tyrl, 2001). The crude product, usually called marijuana in the United States, is produced from dried chopped leaves and the female inflorescences (Kisseberth and Trammel, 1990; Frohne and Pfänder, 2004; Volmer, 2005). Sinsemilla, Spanish for "without seeds," accounts for approximately 85% of the US marijuana production due to the absence of seeds and the very high content of the active ingredient (Anonymous, 2005; Volmer, 2005). Hashish is a dried resin and compressed resin made from the *C. Sativa* often formed into balls, sheets, and cakes and used for smoking. Hashish oil even more concentrated and may be mixed with tobacco or marijuana and smoked.

Cannabinoids are a variety of related compounds found in *C. sativa*. More than 60 are known, the most important being $\Delta^9$-tetrahydrocannabinol (THC). THC is a lipid-soluble monoterpene present in all parts of the plant, with highest levels in the flowers and leaves (Ashton, 2001; Burrows and Tyrl, 2001). Common cultivars grown in 1974 contained approximately 1% THC, whereas recent cultivars of sinsemilla may contain more than 6% THC (Anonymous, 2005). The average marijuana cigarette ("joint" or "reefer") used today contains about 150 mg of THC, up from 10 mg in the 1960s and 1970s (Ashton, 2001). Hashish contains 5% THC on average, and hashish oil averages 15% THC and may be added to a marijuana cigarette to double the potency (Ashton, 2001; Anonymous, 2005).

There are a variety of accepted and controversial medical uses for marijuana and its active compound. Some states allow possession and use of marijuana for medical treatment under certain conditions. Though laws change frequently, states with medical marijuana laws currently include Alaska, California, Colorado, Maine, Maryland, Nevada, Oregon, Rhode Island, Vermont, and Washington. Prescription products include dronabinol (Marinol®) capsules, which contain THC in sesame oil and are currently classified as a schedule I drug by the DEA, and Nabilone (Cesamet®), a synthetic form of THC classified as schedule II. These drugs are used to treat nausea in cancer patients and may be superior to other antiemetics such as metaclopramide. They improve weight gain among patients with AIDS, cancer, or Alzheimer's disease and may have uses in the treatment of glaucoma, multiple sclerosis, chronic pain, epilepsy, and various psychiatric disorders (Di Marzo and De Petrocellis, 2006).

Illegal marijuana is most commonly used in the form of marijuana cigarettes called "joints" or "reefer." Alternately, marijuana may be rolled in a tobacco leaf and called a "blunt" or smoked using a water pipe ("bong") or other type of pipe. Marijuana may be brewed into a tea, or more commonly baked into brownies, cookies, or cakes (Ashton, 2001; Volmer, 2005). Many veterinary exposures come from the ingestion of tainted baked goods. Marijuana cigarettes are sometimes dipped in a mixture of phencyclidine, methanol, and formaldehyde. Ketamine, opium, cocaine, and heroin may be added to a marijuana cigarette or used with marijuana (Volmer, 2005). Street names for marijuana include "grass," "hemp," "Mary Jane" or "MJ," "pot," "puff," and "weed."

Dogs less than 1 year of age are the most likely companion animal to ingest marijuana (Kisseberth and Trammel, 1990; Janczyk *et al.*, 2004). Free marijuana leaves or marijuana cigarettes were recovered from 203 of 213 canine ingestions in a report by Janczyk *et al.* 2004. Baked goods, as noted, may be attractive to dogs (Jones, 1978; Godbold *et al.*, 1979; Janczyk *et al.*, 2004). Fresh plants or the refined resins may also be available to animals (Burrows and Tyrl, 2001). Intentional intoxication of small animals using second-hand smoke has been reported (Schwartz and Riddle, 1985; Buchta, 1988; Frohne and Pfänder, 2004).

Large animals have been exposed through grazing marijuana or ingesting marijuana bales as hay (Cardassis, 1951; Driemeier, 1997). Horses have been bedded on hemp fiber, which may present an impaction risk (Green, 1996; Smith and Papworth, 1996).

#### *Toxicity*

No deaths from marijuana intoxication have been reported in humans (Ashton, 2001). Few veterinary deaths have

been reported. Marijuana has a very wide safety margin in that the lethal dose is approximately 1000 times the effective dose (Volmer, 2005). No deaths were reported in dogs and monkeys ingesting 3–9 g marijuana per kg of body weight (Burrows and Tyrl, 2001). Janczyk *et al.* (2004) documented the survival of a dog that ingested 26.8 g marijuana/kg body weight. The $LD_{50}$ for oral marijuana exposure in rats is 666–1000 mg/kg (Kisseberth and Trammel, 1990; Burrows and Tyrl, 2001). Driemeier (1997) reports that four of five debilitated cattle died after the group ingested 35 kg. Horses and mules have died after ingesting large quantities of fresh marijuana of the species *Cannabis indica* (Cardassis, 1951).

### Toxicokinetics

Absorption of inhaled THC may approach 50% (Burrows and Tyrl, 2001; Janczyk *et al.*, 2004). Gastrointestinal absorption is erratic in humans and dogs. Blood levels obtained by ingestion are 25–30% those obtained by smoking in humans (Ashton, 2001). Onset of clinical signs is delayed from the 6–12 min associated with the respiratory route to 30–60 minutes or longer after ingestion (Janczyk *et al.*, 2004).

Circulating THC is up to 99% protein bound in humans (Volmer, 2005). Plasma levels peak within 2–3 h. This lipid-soluble compound is rapidly distributed to the brain and other tissues. Within the brain, THC accumulates in the neocortical, limbic, sensory, and motor areas. Distribution is blood flow dependent and peak accumulation in adipose tissue occurs in 4–5 days in humans (Ashton, 2001). The plasma half-life of THC is short due to the rapid tissue distribution (Volmer, 2005).

THC is rapidly metabolized by the mixed-function oxidase system of the liver (Burrows and Tyrl, 2001). The significant first-pass effect may account for the lower blood levels associated with ingestion versus inhalation (Ashton, 2001, Janczyk *et al.*, 2004). 11-Hydroxy-$\Delta^9$-THC is the physiologically active major metabolite of THC (Volmer, 2005). There are more than 20 other known metabolites.

Between 65% and 90% of a dose of THC is excreted as the parent compound or conjugated metabolites through the feces and there may be significant enterohepatic cycling (Kisseberth and Trammel, 1990; Ashton, 2001; Volmer, 2005). Ten to twenty-five percent of THC is excreted as the parent compound, metabolites, and conjugates in the urine. Complete elimination in humans is expected to take 30 days.

### Mechanism of action

CB1 and CB2 are the two cannabinoid receptors that have been identified in rats, guinea pigs, dogs, monkeys, pigs, and humans (Ashton, 2001). CB1 receptors are widely distributed in certain areas of the brain: in the cerebral cortex, they regulate cognitive function. The CB1 receptors in the hippocampus and amygdala are important in emotional status. Cerebellar CB1 receptors influence dopaminergic signaling, movement, and postural reflexes. CB1 receptors are also present in the basal ganglia, brain stem, and autonomic nervous system (ANS) where they regulate pain perception and cardiovascular and gastrointestinal function (Ashton, 2001; Di Marzo and De Petrocellis, 2006). CB1 receptors are located within lipid membranes of presynaptic neurons and coupled to G-proteins. They inhibit cAMP and stimulate mitogen-activated protein kinases to modulate control of ion channels, particularly voltage-activated calcium ion channels and potassium channels (Janczyk *et al.*, 2004; Ashton, 2001; Di Marzo and De Petrocellis, 2006). The end result is inhibition of release of neurotransmitters, both excitatory and inhibitory. CB1 receptors also activate phosopholipase C and PI-3-kinase. The endogenous ligands for cannabinoid receptors, known as endocannabinoids, are derived from arachidonic acids and closely related to prostaglandins.

CB2 receptors are absent in the CNS but found in the peripheral nervous system (PNS) and immune system where they play a part in inflammation and pain regulation (Volmer, 2005; Di Marzo and De Petrocellis, 2006). CB2 receptors regulate ceramide biosynthesis (Di Marzo and De Petrocellis, 2006).

### Clinical signs

Clinical signs of marijuana intoxication in dogs are similar to those in humans (Dumonceaux and Beasley, 1990; Dumonceaux, 1995). The signs attributable to the CNS include depression, ataxia, mydriasis, disorientation, behavioral disturbances, hyperesthesia, and recumbence, or less commonly stupor, tremors, or seizures. Ingestion exposures may cause mild gastrointestinal irritation and vomiting. Other signs that have been reported include hypothermia or less commonly hyperthermia, bradycardia, vocalization, and compulsive eating (Burrows and Tyrl, 2001; Janczyk *et al.*, 2004; Volmer, 2005). Severe clinical signs described in a ferret included ataxia with rapid onset of coma, muscle twitching, hypotension, and hypothermia (Smith, 1998). A case of atopic dermatitis was reported in a dog living in a home where *C. Sativa* was grown previously (Evans, 1989).

Onset of clinical signs in cattle began 20 h after ingesting dried plant material and included muscle tremors, hypersalivation, and mydriasis. Animals were reluctant to move and lacked coordination. Four of the five exposed animals died within 3 days and one recovered with no treatment. These animals were already debilitated at the time of exposure (Driemeier, 1997). Rapid onset of clinical signs was described in eight horses and seven mules ingesting fresh plant material, including dyspnea, tremors, hypothermia, hypersalivation, sweating, recumbence, and death within 30 min (Cardassis, 1951).

### *Management*

The prognosis for full recovery in small animals exposed to marijuana is excellent with proper treatment. Janczyk *et al.* (2004) reported 100% survival in 213 cases. Rate of recovery is dependent on dose and route of exposure. Most animals exposed to second-hand smoke recover within a few hours. Dogs who ingest a small dose of plant material may recover within 24 h, but those ingesting large doses may show clinical signs for several days (Kisseberth and Trammel, 1990; Dumonceaux, 1995; Burrows and Tyrl, 2001; Volmer, 2005).

Treatment for THC exposure includes decontamination, supportive, and symptomatic care. Mild intoxication may only require observation (Burrows and Tyrl, 2001). Animals ingesting relatively large quantities may require gastrointestinal decontamination to decrease THC absorption. Emesis may be initiated in the asymptomatic patient within an hour of ingestion (Dumonceaux and Beasley, 1990; Dumonceaux, 1995). Animals showing signs such as CNS depression should not be given emetics, but repeated dosing with activated charcoal and cathartics may prevent absorption and enterohepatic cycling and thus decrease the duration of clinical signs.

Observation of the patient includes monitoring heart rate and rhythm, body temperature, and respiration. Stuperous or comatose dogs may be at risk for severe respiratory suppression or hypothermia and must be treated appropriately. Central nervous stimulation that is severe or dangerous can be treated with diazepam ( Janczyk *et al.*, 2003).

There is inadequate information in the literature concerning treatment of large animals for marijuana ingestion. Treatment was not attempted in the few cases presented. The rapid onset of clinical signs in the horse case did not allowed much time for veterinary intervention. Basic treatment procedures in large animals should parallel those used in small animals. Gastrointestinal decontamination for large ingestions may involve gastric lavage or, in cattle, rumenotomy, and intragastric or intraruminal instillation of activated charcoal and cathartics. Monitoring and symptomatic and supportive care should proceed as above. Veterinary laboratories may test blood or plasma for THC using thin-layer chromatography or gas chromatography/mass spectroscopy (GC/MS). Drug testing kits may be available form pharmacies. There is a poor correlation between blood and plasma THC levels and clinical signs (Ashton, 2001).

### *Pathology*

There is little information on lesions associated with marijuana overdose in small animals because so few have died. Pulmonary edema was noted in a cow (Driemeier, 1997). Plant material was identified in the stomach of the horses as *C. indica.* There was edema and petechiation of the gastric wall and myocardial hemorrhage (Cardassis, 1951).

## Barbiturates

Barbiturates are derived from the non-sedative barbituric acid. Purified barbiturates are bitter tasting white powders. They are frequently available as a sodium salt and are weakly acidic in aqueous solution. Barbiturates have been used in anesthesia and sedation and seizures control, and though still commonly used by veterinarians are becoming more uncommon in human medicine (Kisseberth and Trammel, 1990). There are four classifications of barbiturates based on the duration of their activities. The duration of ultra-short-acting barbiturates generally is approximately 20 min and these drugs are given intravenously (IV) to effect. Examples include thiamylal sodium and thiopental sodium, both schedule III and methohexital sodium which is schedule IV. The duration of short-acting is approximately 3 h and they are given IV for anesthesia. Common examples of short-acting barbiturates are pentobarbital sodium and secobarbital sodium, both schedule II. The duration of an intermediate-acting barbiturate such as butabarbital or amobarbital, both schedule III, is 3–6 h. Effects of long-acting barbiturates may last for 12 h and these drugs have use in sedation and anticonvulsant therapy. Phenobarbital, methylphenobarbital, and barbital sodium are examples of long-acting barbiturates and are schedule IV drugs (Kisseberth and Trammel, 1990; Branson, 2001; Volmer, 2005). Barbiturates are known as downers, reds, Christmas trees, and dolls on the illegal market (Anonymous, 2005).

Barbiturate overdose may be iatrogenic or due to accidental ingestion of prescription or illicit drugs. A common problem in veterinary medicine is exposure to carcasses of animals that were euthanized with barbiturates. This problem has been reported in dogs and in wildlife (Humphreys *et al.*, 1980; Branson, 2001; *AVMA News*, 2002; Volmer, 2005). According to the *AVMA News*, at least 34 eagles have died from pentobarbital poisoning. Veterinarians and animal owners are responsible for proper carcass disposal and may be liable for wildlife poisonings.

### *Toxicity*

The $LD_{50}$ for pentobarbital in the dog is 40–60 mg/kg by the IV route or 85 mg/kg per os (PO) (Branson, 2001). The oral $LD_{50}$ for cats is 125 mg/kg (Volmer, 2005). The margin of safety for barbiturates is low, the therapeutic dose may be 50–70% the $LD_{50}$ (Kisseberth and Trammel, 1990; Branson, 2001).

### *Toxicokinetics*

Gastrointestinal absorption of barbiturates is variable, with rapid absorption of short-acting compounds and slower absorption of long-acting barbiturates (Branson, 2001). Barbiturates are rapidly distributed throughout the body and readily cross the blood–brain barrier. Short-acting

thiobarbiturates are highly lipid soluble and cross rapidly from the circulation to the brain, causing a rapid onset of CNS depression. This is followed by rapid redistribution to tissues with less perfusion and rapid recovery from the clinical effects. Longer-acting barbiturates are less lipophilic, therefore they enter and leave the brain more slowly and thus have a more gradual onset and longer duration (Branson, 2001; Volmer, 2005). Barbiturates cross the placenta and fetal concentrations equilibrate with those of the dam within minute (Branson, 2001).

There is significant variation in barbiturate metabolism and excretion based on the barbituric acid derivative, the species of animal, and the individual. Barbiturates are metabolized by microsomal P450 enzymes in the liver. Barbiturates may interfere with metabolism of other compounds either by binding to the P450 enzymes to block metabolism or, in chronic exposures, through induction of P450 enzymes to increase the rate of metabolism of substances including endogenous steroids (Volmer, 2005). Some barbiturates, in particular the short-acting thiobarbiturates, undergo significant oxidation in the tissue. Phenobarbital is metabolized very rapidly in ruminants and horses, relatively rapidly in dogs, more slowly in humans, and slower still in cats (Branson, 2001).

Barbiturates are excreted as the parent compound and metabolites in the urine. Excretion of some barbiturates is dependent on urine pH and may be increased 5–10 times in alkaline urine through ion trapping (Volmer, 2005). Ion trapping is less effective with short-acting barbiturates which are highly metabolized, highly protein bound, and have high p$K_a$s (Kisseberth and Trammel, 1990; Volmer, 2005). Barbital is excreted very slowly in birds (Branson, 2001).

### *Mechanism of action*

Barbiturates bind to the $\gamma$-aminobutyric acid (GABA) receptor complex and decrease the rate of GABA dissociation (Branson, 2001). These actions increase permeability of the postsynaptic membrane to chloride and lead to membrane hyperpolarization and reduced excitability. GABA receptors are found in motor and sensory area of the cerebral cortex, and may be present in the thalamus where barbiturates may act to control seizures and induce anesthesia. Barbiturates may also act to inhibit glutamate receptors and decrease norepinephrine (NE) release. Actions on the PNS include inhibition of depolarization of acetylcholine-sensitive nerves at postsynaptic junctions and motor end plates (Branson, 2001).

Respiratory depression is caused by suppressing chemoreceptors by barbiturates (Branson, 2001; Volmer, 2005). The effect is more severe in cats, where the reticular formation governs medullary control of respiration. Control of respiratory activity is believed to be more complex in other species (Branson, 2001). Barbiturates may cause hypotension and secondary anuria. Increased heart rate is believed to be governed by arterial pressoreceptors. The effects of barbiturates on levels of sodium, potassium, and calcium in cardiac myocytes affect cardiac contractility. Hypothermic animals are predisposed to ventricular fibrillation with barbiturates. Fibrillation is reported in 100% of hypothermic animals given pentobarbital and 50% of those given thiopental.

### *Clinical signs*

Onset of clinical signs depends on the route of expose, the barbiturate involved, and the presence or absence of food in the stomach. Animals that have ingested short-acting barbiturates may show clinical signs within 10–30 min. If long-acting barbiturates were ingested, the first effects may be not been seen for an hour (Kisseberth and Trammel, 1990). The time to onset of clinical anesthesia is doubled if phenobarbital is given to an animal with food in the stomach versus an empty stomach. Duration of effects is also variable, as alluded to previously, and dependent on the agent involved, species of animal, nutritional status, age, sex, and weight. The duration of a given barbiturate may be prolonged in greyhounds, which have less adipose tissue for the drug to partition (Branson, 2001).

The predominant signs of barbiturate intoxication are profound CNS depression and anesthesia (Humphreys *et al.*, 1980; Kisseberth and Trammel, 1990; Volmer, 2005). Animals may prevent with severe ataxia, weakness, disorientation, and loss of deep tendon reflexes. Hypothermia is common and is associated with cardiac arrhythmia in humans and dogs. The pulse is rapid and weak and severe respiratory depression may lead to cyanosis and death (Branson, 2001; Kisseberth and Trammel, 1990).

Certain preexisting conditions may enhance the effects of barbiturates such as renal failure and uremia, which may decrease plasma protein damage as well as rate of excretion. Barbiturates may accelerate liver damage in individuals with liver disease (Branson, 2001). Barbiturates are also known to cause allergic reactions.

### *Management*

Decontamination for recent exposures, monitoring, and symptomatic and supportive therapy are the basis of treatment for barbiturate overdose. Respiratory function, cardiac function, and body temperature must be monitored closely. Emetics may be given to the *asymptomatic* animal within several hours of ingestion. Intubation and gastric lavage are more appropriate in the animal with CNS depression. Repeated doses of activated charcoal function to decrease the biological half-life of barbiturates. A cathartic such as sorbitol may be added, but magnesium-containing cathartics such as magnesium sulfate (Epsom salt) should be avoided as they may enhance CNS depression. Monitoring and control of normal body temperature is essential to prevent ventricular fibrillation and decrease the duration of the clinical signs (Branson, 2001). Intubation and assisted ventilation may be required in cases of severe

respiratory depression. Fluid therapy may be needed to maintain cardiac and renal function. Alkaline diuresis has been used to increase the rate of excretion of some barbiturates but is ineffective with short-acting barbiturates (Kisseberth and Trammel, 1990; Volmer, 2005). Because tissue partitioning attenuates the effects of barbiturates, it is possible for the patient to relapse as the drug repartitions from the tissues back into plasma (Kisseberth and Trammel, 1990).

The use of certain drugs may be contraindicated in the barbiturate overdose patient. Dextrose, fructose, lactate, pyruvate, and glutamate may increase partitioning of barbiturates into the CNS. Epinephrine and isoproterenol cause reanesthetization after thiopental exposure. Sulfonamides, salicylates, and doxycycline increase bioavailability of barbiturates by displacing them on plasma proteins (Branson, 2001).

Many laboratories can analyze sample such as urine, blood products, or tissues for barbiturates. Common techniques include thin-layer chromatography and GC/MS techniques.

## Opioids

Opium is produced from *Papaver somniferum*, a poppy, and its use was recorded in the Ebers Papyrus, one of the oldest known medical texts dated around 1500 BC. The unripe seed capsule is incised after the petals have fallen. Material that exudes from the capsule is dried, collected, and dried further to produce opium. Powdered opium is 75% inert ingredients, about 10% morphine, the major active alkaloid, 0.5% codeine, and the third major alkaloids is dimethylmorphine or thebaine, which acts as a convulsant (Branson and Gross, 2001). Laudanum is deodorized tincture of opium and paregoric is camphorated tincture of opium.

Morphine sulfate is a schedule II drug commonly used medicinally for pain control and sedation. Oxymorphone is a schedule II morphine derivative approved for use in dogs and cats in the United States. Oxomorphone is approximately 10 times more potent than morphine (Branson and Gross, 2001). Other morphine derivatives include codeine and the more potent hydromorphone, both schedule II drugs. Heroin is a schedule I morphine derivative.

Synthetic opioids include methadone, propoxyphene, meperidine, pentazocine, all schedule III drugs. Butorphanol is schedule IV drug. Oxycodone and hydrocodone are common prescription synthetic opioids classified as schedule II. Fentanyl is formulated into injectable solutions, slow-dissolving sticks for oral transmucosal absorption, and patches that release doses of 25, 50, 75, or 100 μg/dl/h for transdermal absorption. Fentanyl is about 80 times more potent than morphine (Branson and Gross, 2001). Other potent synthetic opioids are alfentayl, sufentanil citrate, and etorphine HCl, which is 10,000 times as potent as morphine and used in wildlife capture. These potent drugs are classified in schedule II.

The morphine derivative heroin is the most abused of the opioids (Dumonceaux, 1995; Anonymous, 2005). A white to dark brown powder, heroin often contains additives such as sugar, starch, powdered milk, quinine, or strychnine. Heroin is usually injected but may be insufflated (snorted) or smoked. Oxycodone, sold as OxyContin®, Percocet® and in a generic form, is commonly used recreationally. Pills are time released but may be crushed and injected, or insufflated. Hydrocodone, sold under various trade names and in a formulations with acetaminophen (Vicodin®), is also commonly sold on the illegal drug market. Fentanyl may be produced in clandestine laboratories, and is used IV. Small animal exposure to opioids may be due to accidental ingestion of pharmaceutical drugs, illegal drugs, or occasionally oral or parenteral dosing with malicious intention.

### *Toxicity*

With such a wide variety of opioids, there is much variation in toxicity. The minimum lethal dose of morphine in dogs is between 110 and 210 mg/kg given parenterally (Kisseberth and Trammel, 1990; Branson, 2001; Volmer, 2005). The minimal lethal dose for subcutaneous (SC) injection of morphine in the cat is 40 mg/kg (Volmer, 2005). Heroine doses of approximately 0.20 mg/kg caused clinical signs in dogs, including sedation and respiratory depression, whereas a dose of 0.58 mg/kg caused increased duration of effects, respiratory difficulty, and aggressive behavior with clinical signs lasting up to 8 h (Garret and Gürkan, 1980). The minimum lethal dose of heroin for the dog is 25 mg/kg given SC (Volmer, 2005). The minimum oral lethal dose of heroin for the cat is 20 mg/kg (Volmer, 2005). Meperidine causes clonic convulsions in cats at a dose of 30 mg/kg (Branson and Gross, 2001).

### *Toxicokinetics*

Opioids are weakly acidic and absorption after oral dosing is predominantly in the small intestine. There is rapid absorption from the subcutis (Branson and Gross, 2001). Distribution is somewhat variable. Heroin is more lipophilic than morphine and more readily crosses the blood–brain barrier (Garrett and Gürkan, 1980). Opioids are also distributed to skeletal muscle, kidney, liver, intestine, lungs, spleen, brain, and placenta (Kisseberth and Trammel, 1990).

Opioids are readily metabolized in the liver and, when ingested, there is a significant first-pass effect (Kisseberth and Trammel, 1990; Volmer, 2005). Phase I metabolism includes hydrolysis, oxidation, and *N*-dealkylation. Approximately 50% of a morphine dose is conjugated to glucuronide in most species. A notable exception is the cat, accounting in part for the increased sensitivity of felines (Kisseberth and Trammel, 1990; Branson and Gross, 2001; Volmer, 2005). The primary conjugate is morphine-3-glucuronide, though the metabolically active morphine-6-glucuronide is also produced. Heroin is metabolized in the

liver and other tissues and is cleared more rapidly than morphine. Deacetylation produces 6-*o*-acetylmorphine and morphine, which appear in the plasma of dogs within minutes (Garrett and Gürkan, 1980).

Opioids are excreted predominantly as metabolites in the urine (Volmer, 2005). Metabolites may be detected horse urine for up to 6 days (Branson and Gross, 2001). There is some biliary excretion and enterohepatic cycling (Garrett and Gürkan, 1980; Branson and Gross, 2001; Volmer, 2005). The biological half-life of morphine in cats is about 3 h (Branson and Gross, 2001). The initial plasma half-life of heroin in dogs is 8 min, but the terminal half-life is 80 min due to repartitioning from the tissues (Garrett and Gürkan, 1980).

### *Mechanism of action*

Opioids may act as agonists or antagonists. Morphine and related drugs act as agonists, particularly at $\mu$ receptors. Naloxone is an antagonist with a high affinity for the $\mu$ receptors and a low affinity for $\delta$ receptors (Volmer, 2005). An opioid is classified as a partial agonist if it acts as an agonist at one receptor but an antagonist at another. Major opioid receptors are designated $\mu$, $\delta$, and $\kappa$ and are believed to be stimulated by endogenous endorphins. The $\mu$ receptors are activated by endogenous enkephalins. These receptors may be further differentiated into $\mu_1$ and $\mu_2$. Activation of the $\mu_1$ receptor induces supraspinal analgesia and $\mu_2$ receptor activation causes spinal analgesia, suppression of respiration, and inhibition of gastrointestinal motility. The $\delta$ receptors are more selective for enkephalins than the $\mu$ opioid receptors and are involved in spinal analgesia. Activation of $\kappa$ receptors produces spinal and supraspinal analgesia, sedation, and dysphoria (Branson and Gross, 2001). The $\sigma$ receptor, previously classified as an opioid receptor, does not appear to mediate analgesia.

Opioid receptors are found in the CNS, ANS, gastrointestinal tract, heart, kidney, pancreas, adrenal glands, vas deferens, and on the surface of lymphocytes and adipocyte (Volmer, 2005). Opioid receptors in the CNS are concentrated in the amygdala and frontal cortex in the dog, monkey, and human where stimulation is associated with CNS depression. Activation in the cat, swine, goat, sheep, horse, or ox may alter dopaminergic or noradrenergic function, leading to excitation. Activation of opioid centers in the chemoreceptor trigger zone in the fourth ventricle of the brain produces emesis. Opioid receptors are present in the brain stem, including the cough centers where they suppress the cough reflex, and the respiratory centers, though initial increases in respiration are followed by respiratory suppression due to generalized CNS depression. Stimulation of gastrointestinal opioid receptors decreased motility, leading eventually to delayed passage of ingesta, increased water absorption, and constipation.

### *Clinical signs*

The clinical effects of opioids are dependent on age (neonates may have an incomplete blood–brain barrier), species, and the compound involved. Clinical signs in dogs are similar to those described in humans. Early clinical signs in dogs exposed to narcotics may include transient excitation and rapid respiration, drowsiness and ataxia, decreased pain perception, hypersalivation, vomiting, defecation, and urination. Animals may progress to stupor or coma with hypothermia, hypotension, respiratory depression, and death (Kisseberth and Trammel, 1990; Dumonceaux, 1995; Branson and Gross, 2001; Volmer, 2005). Other signs may include decreased urination and constipation. Signs were similar in dogs given low doses of heroin and included early aggressive behavior, brief unconsciousness, and a period of weakness, hypersalivation, and respiratory difficulty that lasted up to 8 h (Garret and Gürkan, 1980). Propyoxyphine may induce tremors and convulsions in dogs.

Cats may present with excitation, aggression, insomnia, and hyperthermia as well as increased pain threshold. Cats do not vomit at doses less than 700 times the emetic dose for dogs. Horses, ruminants, and swine show similar CNS stimulation. Clinical signs in rabbits depend on the drug used, e.g. morphine induces hypothermia whereas apomorphine induces hypothermia (Branson and Gross, 2001).

### *Management*

The basis of treatment for opioid exposure is early decontamination, symptomatic and supportive care, and the judicious use of an opioid antagonist. Animals presenting immediately after ingestion of narcotics should be given emetics to induce vomiting. If large doses were ingested and contraindications against emetic use are present, enterogastric lavage on the anesthetized and intubated animal may be performed to evacuate the stomach and instill activated charcoal and cathartics. Decontamination may be effective for several hours after ingestion due to decreased gastrointestinal motility (Dumonceaux and Beasley, 1990; Kisseberth and Trammel, 1990; Dumonceaux, 1995; Volmer, 2005).

Animals must be monitored closely for respiratory dysfunction, the most common cause of death in opioid overdoses. Assisted ventilation may be necessary. Body temperature should be monitored and maintained. Seizures can be treated with diazepam. The opioid antagonist Naloxone may be used to treat severe CNS depression or respiratory depression. Naloxone is given parenterally at a dose of 0.01–0.02 mg/kg and repeated as necessary. This drug has a very short half-life. Patients that fail to respond to a 10 mg dose of Naloxone are unlikely to respond to a higher dose.

## OTHER CNS DEPRESSANTS

Other drugs may have depressant activities on the CNS. Two commonly used "club drugs" are discussed below: flunitrazepam and GHB.

### Flunitrazepam

Flunitrazepam is not sold in the United States but available in more than 60 countries, including many in Europe and Latin America, as Rohypnol®, Narcozep®, and other trade names, as a sedative. Rohypnol® has gained notoriety as the "date rape drug" and is sold as tablets imprinted "Roche 1" and "Roche 2" designating their manufacturer and mg dosage. Tablets may be crushed and insufflated, or dissolved in a drink intended for an unsuspecting victim (Smith, 2002; Anonymous, 2005). Blue coloring has been added to these pills to reduce the potential for this use (Smith *et al.*, 1996; Rimsza and Moses, 2005). Street names include "roofies" or "rophies," "Roche" or "la rocha," "Mexican valium," "forget me pills," "Rope," and "R2." Injectable forms of flunitrazepam are also available (Anonymous, 2005).

The estimated human lethal dose of flunitrazepam is 415 mg/kg, though death has been reported in an elderly patient given 28 mg PO. This drug is 85% bioavailable after ingestion with peak plasma concentrations within an hour or two. Flunitrazepam is metabolized to two active products, which are excreted with the parent compound in human urine for a few days (Smith *et al.*, 2002; Gable, 2004; Anonymous, 2005). Clinical signs of sedation and amnesia occur within 20 min of oral dosing and may persist for 12 h. Other signs include muscle relaxation, visual disturbances, and confusion. Deaths attributed to this drug are rare (Smith *et al.*, 2002; Anonymous, 2005; Rimsza and Moses, 2005).

Animals that have ingested flunitrazepam should be monitored closely for respiratory and CNS depression and changes in heart rate and body temperature. If ingestion was recent and there is no evidence of clinical signs, emetics can be given. Activated charcoal and cathartics may be given to minimize absorption. Gastric lavage may be performed on the anesthetized and intubated patient who has ingested a large dose. Human overdoses have been treated with the flunitrazepam antagonist flumazenil, but repeated dosing may be needed due to the short half-life of this drug. Flumazenil treatment has been reported to cause seizures in some people (Smith *et al.*, 2002). Analyses are available at some laboratories for flunitrazepam, but doses are often quite low and urine must be collected soon after ingestion.

## GHB

Another popular "club drug" that has been associated with date rape is GHB. This drug is a derivative of GABA and is present in the body under normal conditions. Synthetic GHB is used medicinally in Europe and produced in clandestine laboratories. It was sold as a nutritional supplement for body builders at one time, and is currently used to treat narcolepsy. Industrial solvents $\gamma$-butyrolactone (GBL) and 1,4-butanediol (BD) are metabolized to GHB. BD is available in printer ink cartridges and has been sold as a supplement at health food stores. GHB is most commonly sold as a clear liquid in small vials and added to bottled water (Smith *et al.*, 2002; Rimsza and Moses, 2005). Street names include "Liquid X" or "liquid ecstasy," "soap," or "salty water" due to the flavor of the product, "easy lay," "Georgia homeboy," "grievous bodily harm," "G," "goop," "gib," or "scoop."

GHB receptors are present in the hippocampus, cortex, and other areas of the brain (McDonough *et al.*, 2004). GHB is involved in regulation of sleep cycles, body temperature, memory, glucose metabolism, and dopamine levels (Smith *et al.*, 2002). GHB may also be converted back to GABA (McDonough *et al.*, 2004). Human death has been reported at a GHB dose of 5.4 g taken orally, but other patients have survived doses of 29 g. The $LD_{50}$ for rodents and rabbits is approximately 2 g/kg. GHB is rapidly absorbed from the gastrointestinal tract. This highly lipophilic drug readily crosses the blood–brain barrier. GHB is rapidly metabolized to carbon dioxide though 2–4% of a given dose may be excreted in the urine (Smith *et al.*, 2002; Gable, 2004). Half-life is dose dependent.

Clinical signs attributed to GHB usually occur within half an hour of ingestion and include euphoria, reduced anxiety, and drowsiness. CNS depression may progress to loss of motor control, unconsciousness, and respiratory depression (Smith *et al.*, 2002; McDonough *et al.*, 2004; Rimsza and Moses, 2005). Seizures, bradycardia, and hypothermia are noted in nearly a third of overdose patients (Smith *et al.*, 2002).

Treatment consists of decontamination with gastric lavage for large ingestion or activated charcoal, *emetics are contraindicated* due to the rapid onset of this drug, and close monitoring of the respiratory, cardiovascular, CNS, and body temperature, and supportive care as needed. Seizures have been treated with benzodiazepines. Patients who appear stable should be monitored for at least 8 h. GHB is difficult to analyze for as it is rapidly metabolized and normally present in the body (Smith *et al.*, 2002; Gable, 2004).

## STIMULANTS

The major illicit drugs classified as stimulants are cocaine and amphetamines. While the cocaine is a plant alkaloid, amphetamines are a large group of compounds, including prescription drugs such as methylphenidate, sold as Ritalin®, methamphetamine, which is frequently produced in clandestine laboratories. Stimulants in general

act on the ANS, usually on adrenergic receptors. Popular "club drug" MDMA, or ecstasy, has significant action on serotonin receptors as well.

Various legally available compounds act as stimulants through various mechanisms. These include nicotine, caffeine, and related compounds, discussed elsewhere in the text. Areca alkaloids, described below, are commonly used as stimulants across much of Asia and are available in some parts of the Unites States and Europe.

## MAJOR STIMULANTS

### Cocaine

Cocaine is the natural alkaloid of the shrubs *Erythroxylon coca* and *Erythroxylon monogynum*, originally from the Andes Mountains in South America but currently grown in Mexico, the West Indies, and Indonesia as well (Queiroz-Neto *et al.*, 2002; Anonymous, 2005; Volmer, 2005). Traditionally, *E. coca* leaves are brewed into tea by those native to the Andes Mountains, who may still share this delightful beverage with unsuspecting tourists to counter the effects of the high altitude. Cocaine is a schedule II drug used for topical anesthesia and vasoconstriction of mucous membranes (Kisseberth and Trammel, 1990; Volmer, 2005). It may be second only to marijuana in illegal consumption in the Unites States. According to surveys in the 1980s, approximately 15% of the US population had tried cocaine (Kabas *et al.*, 1990).

Cocaine is sold in the powdered white salt form, cocaine HCl, ranging in purity from 12% to more than 60%. It may be diluted with inert ingredients such as lactose, inositol, mannitol, cornstarch, or sucrose, or with active compounds including procaine, lidocaine, tetracaine, caffeine, amphetamine, or quinine (Kisseberth and Trammel, 1990). The water-soluble salt may be injected, ingested, or insufflated (Anonymous, 2005; Rimsza and Moses, 2005). Common street names for cocaine HCl include "bernies," "blow," "C" or "big c," "coke," "girl" or "white girl," "gold dust" or "star dust," "her," "lady" or "white lady," "nose candy," "snow," or "toot."

Cocaine HCl is converted to the free alkaloid by dissolving it in a basic solution which is boiled to precipitate the alkaloid. The dry precipitate is broken into "rocks" which are 75–90% pure, but may be diluted with inert ingredients or active compounds such as procaine, lidocaine, amphetamines, heroin, caffeine, phencyclidine, ergot alkaloids, or strychnine. The free base readily vaporizes with heat and the smoke is inhaled, though it may be taken orally. This form of cocaine is termed "crack" because of the sound produced when it is heated, "bedrock," "beamers," "BJ's," "bolo," "crank," "crystal," "flake," "ice," "jelly beans," "rock," "rooster," "space," "tornado" or "24/7."

Exposure to illicit cocaine is most likely to effect dogs, particularly police dogs, but athletic horses may be dosed with cocaine to improve performance (Dumonceaux and Beasley, 1990; Kisseberth and Trammel, 1990; Frazier *et al.*, 1998; Queiroz-Neto *et al.*, 2002; Kollias-Baker *et al.*, 2003; Volmer, 2005).

#### *Toxicity*

The $LD_{50}$ for cocaine in dogs is 3 mg/kg IV, and 20 mg/kg IV is the $LD_{99}$ (Kisseberth and Trammel, 1990; Volmer, 2005). Dogs can tolerate 2–4 times the above doses given PO (Kisseberth and Trammel, 1990). The minimum lethal dose in cats is approximately 7.5 mg/kg IV or 16 mg/kg SC (Volmer, 2005). Horses given 50 mg of cocaine IV showed no clinical signs, but performance was enhanced at 200 mg (Kollias-Baker *et al.*, 2003).

#### *Toxicokinetics*

Cocaine is highly lipophilic and readily absorbed from all mucosal surfaces, including those of the nose, oral cavity, gastrointestinal tract, and alveoli. Approximately 20% of an ingested dose is absorbed (Kisseberth and Trammel, 1990). Peak plasma concentrations appear between 15 min and 2 h after ingestion and cocaine readily crosses the blood–brain barrier (Volmer, 2005). The neurological effects of cocaine and crack last for 15–20 min after insufflating or 5–10 min after smoking recreational doses. Cocaine undergoes hydrolysis by plasma esterases to water-soluble metabolites benzoylecgonine, ecgonine methyl ester, and others (Queiroz-Neto *et al.*, 2002). Hepatic esterases and demethylating enzymes play a role in cocaine metabolism.

Up to 20% of a dose of cocaine may be excreted unchanged in the urine (Volmer, 2005). Benzoylecgonine and ecgonine methyl ester are the primary metabolites excreted in the urine of most mammals in both conjugated and unconjugated forms (Kollias-Baker *et al.*, 2003). Other metabolites include norcocaine, benzolynorcgonine, norecgonine, and ecgonine. Kollias-Baker *et al.* (2003) found cocaine in the urine for up to 24 h when horses are dosed sublingually with 2.5 mg. Larger doses are detectable for 2–3 days.

#### *Mechanism of action*

Cocaine increases release of catecholamines and blocks reuptake of NE, serotonin, and dopamine, leading to increased neurotransmitter concentrations at synaptic junctions (Kisseberth and Trammel, 1990; Volmer, 2005; Queiroz-Neto *et al.*, 2002). NE regulates thalamic effects on appetite, body temperature, and sleep (Queiroz-Neto *et al.*, 2002). Cardiac effects are often associated with IV dosing (Kabas *et al.*, 1990; Kisseberth and Trammel, 1990). Cocaine has direct effects on the myocardium, where it acts by blocking sodium ion channels, thus causing conduction disturbances and prolonged R waves. Cocaine increases calcium concentrations within cardiac myocytes and may promote depolarization during the diastolic interval and ventricular fibrillation. Cocaine slows conductance at the

bundle of His. Oxygen demand is increased within the myocardium and there may be constriction of the coronary vasculature, which may cause infarction.

### *Clinical signs*

Clinical signs of cocaine toxicosis are associated with CNS stimulation, sometimes followed by depression. Signs in dogs include hyperactivity, hyperesthesia, tremors and seizures, and death. Other signs are mydriasis, hypersalivation, and vomiting. Cardiac changes are consistently reported. Dogs dosed IV with cocaine had increased heart rate, cardiac output, and mean arterial pressure (Catravas and Waters, 1981). Death was attributed to hyperthermia, which could be secondary to increased muscular activity and peripheral vasoconstriction. Frazier *et al.* (1998) reported a body temperature of 105 °F (40.56°C) in one dog. Respiratory and cardiac arrest, the latter attributed to coronary vasospasm, have also been implicated as the cause of death. Lactic acidosis, hypoglycemia or hyperglycemia, and elevated creatinine phosphokinase have been reported (Kisseberth and Trammel, 1990; Volmer, 2005).

Queiroz-Neto *et al.* (2002) reported mild clinical signs in horses given low IV doses of cocaine, such as increased alertness, irritability, muscle tremors, vocalization, and stereotypical behaviors like head bobbing and pawing at the ground. Cribbing was reported in at higher doses. Most horses defecated within 10 min of dosing and recovered within 20 min.

Lesions reported in dogs include subendocardial and epicardial hemorrhage, degeneration of cardiac myofibers, coronary vasoconstriction, pericardial effusion, and pulmonary hemorrhage (Kisseberth and Trammel, 1990; Frazier *et al.*, 1998).

### *Management*

Early decontamination of dogs suspected of recently ingesting cocaine has been recommended, but may have limited effect as the drug is absorbed extremely rapidly. Emesis may initiate seizures. Gastric lavage in the patient who has ingested a large quantity of cocaine may be safer. Police dogs that have ingested bags of cocaine require cautious endoscopic or surgical retrieval to prevent rupture or obstruction. Surgically implanted bags must be removed with equal caution and secondary infection treated as necessary. Symptomatic and supportive care includes maintaining body temperature, acid–base, and electrolyte status, and monitoring cardiac and respiratory function. Body temperature may be maintained by use of a cool environment, cool fluids, cool bath, wet towels, fans, or cool water enemas (Dumonceaux, 1995; Volmer, 2005). Decreased stress is recommended to prevent hyperthermia. Physical restraint has been associated with death in humans (Pestaner and Southall, 2003).

Seizure control may also prevent hyperthermia. Diazepam has been used to decrease seizure activity, however two of six animals that had an apparent favorable response to diazepam died within 72 h (Catravas *et al.*, 1977). Barbiturates have been recommended to treat refractory seizures (Volmer, 2005). Chlorpromazine given before cocaine dosing has been effective experimentally to reduce severity of seizures, maintain blood pH, maintain body temperature, decrease heart rate and blood pressure, and prevent arrhythmias (Catravas and Waters, 1981). However, phenothiazine tranquilizers are known to lower the seizure threshold (Dumonceaux and Beasley, 1990; Volmer, 2005).

Animals may require respiratory support, including intubation and mechanical ventilation, for severe or prolonged respiratory depression. Cardiac signs such as tachycardia may be short lived. Treatment of life-threatening cardiac arrhythmia with $\beta$-blockers such as propranolol has been recommended. Pretreatment of dogs with propranolol before cocaine injection did not increase survival (Catravas and Waters, 1981).

Urine and plasma are routinely tested for cocaine at many laboratories. Thin-layer chromatography and immunoassays may be used with confirmation by GC/MS.

## Amphetamines

The term "amphetamine" refers specifically to $\alpha$-methylphenylethylamine, a schedule II drug, but the term is often used to describe various derivatives. The term "amphetamines" (plural) will be used here to describe a group of related compounds unless specified otherwise. Common amphetamines include schedule II drug methamphetamine and schedule I drugs 2,5-dimethoxy-4-methylamphetamine ("DOM," "STP"), 2,5-dimethoxy-4-bromoamphetamine ("DOB"), and methylphenidate, 4-methylaminorex (4MA), and 3,4-methylenedioxy-*N*-ethylamphetamine (MDEA). The "designer drug" MDMA ("ecstasy") has some unique characteristics which are discussed separately.

Historically, amphetamines were used by veterinarians to the stimulation of medullary respiratory centers in order to increase respiratory rate and depth in animals (Adams, 2001). These drugs are not currently used in veterinary medicine. Physicians have used amphetamines to control appetite in obese patients, to treat narcolepsy, depression, alcoholism, and, counter intuitively, to control hyperkinetic behavior in children. Most are sold as tablets or capsules, which may be sustained release.

Amphetamines on the illegal market are sold as "bennies," "dex" or "dexies", "speed," or "uppers." Prescription products may find their way to the illegal market, but many "designer" amphetamines such as methamphetamine are created in clandestine laboratories. Crystal methamphetamine, called "ice" or "glass," may be smoked, powdered methamphetamine, termed "crank," or "meth," may be dosed IV, orally, or insufflated. "Yaba" may also contain caffeine. Other "designer" amphetamines include 4MA, sold as "euphoria," "U4EUH," or occasionally "ice," MDEA,

sold as "Eve," and MDMA. Drug dealers may combine amphetamine with inert or other active ingredients, or may substitute other drugs such as heroin, cocaine, or phenylethylamine. Accidental ingestion of prescription amphetamines is the most likely exposure risk for companion animals (Volmer, 2005). Potential exists for exposure of companion animals to illegal drugs. Illegal doping may occur in horses.

### *Toxicity*

Catravas *et al.* (1977) found that 10 mg/kg IV amphetamine killed dogs within 3 h. $LD_{50}$s for orally administered amphetamine sulfate and methamphetamine in the dog are 20–27 and 9–100 mg/kg, respectively (Diniz *et al.*, 2003; Volmer, 2005).

### *Toxicokinetics*

Absorption of amphetamines through the gastrointestinal system is usually rapid, though it is slower with sustained release products. Methamphetamine absorption is more rapid by insufflation with onset of clinical in 2–5 min, whereas the onset after PO dosing is 15–20 min (Anonymous, 2005). Peak plasma concentrations of amphetamine occur 1–3 h after ingestion, unless a sustained release product was ingested. Amphetamines are highly lipid soluble and readily cross the blood–brain barrier (Volmer, 2005). Concentrations in the cerebral spinal fluid may be 80% those found in the plasma (Kisseberth and Trammel, 1990). Methamphetamine has increased partitioning to the CNS compared to other amphetamines (Rimsza and Moses, 2005). Amphetamine is also distributed to the kidneys, liver, and lungs, with negligible storage in the adipose tissue (Baggot and Davis, 1972).

There is significant hepatic metabolism of amphetamines. The two major pathways are hydroxylation and deamination. Deaminated products may be oxidized and conjugated to glycine (Baggot, 1972). Active metabolites may be produced (Volmer, 2005). Amphetamine and its metabolites are excreted primarily in the urine and minimally in the bile. About 8% of an amphetamine sulfate dose is excreted unchanged in the urine in swine and 30% in dogs. Rate of excretion is significantly increased as urine pH declines. Amphetamine is almost completely eliminated within about 6 h in dogs with an average urinary pH of 7.5, and in 3⅓ hours if the urinary pH averages around 6.0 (Volmer, 2005).

### *Mechanism of action*

Questions remain concerning the mechanism of action of amphetamines. Sympathetic central and peripheral effects are due to direct actions on $\alpha$ and $\beta$ adrenergic receptors, increased release of catecholamines, particularly NE, inhibition of monoamine oxidase (MAO), and inhibition of catecholamine reuptake (Adams, 2001; Diniz *et al.*, 2003). Amphetamines may promote serotonin and dopamine release, and act directly on dopamine receptors (Volmer, 2005).

### *Clinical signs*

Common clinical signs of amphetamine toxicosis in animals include hyperactivity, restlessness, tremors, and seizures, though occasionally ataxia and depression. Hyperthermia may be secondary to seizures and peripheral vasoconstriction. Tachycardia and ventricular premature contractions, hypertension, or occasionally hypotension can also occur. Other signs include mydriasis, hypersalivation, and vocalization. The cause of death in amphetamine overdosed dogs could be disseminated intravascular coagulation secondary to hyperthermia and respiratory failure (Davis *et al.*, 1978; Diniz *et al.*, 2003). Cerebrovascular hemorrhages due to hypertension, hypoglycemia, lactic acidosis, and cardiac failure have all been implicated as the cause of death after amphetamine overdose (Catravas *et al.*, 1977). Liver failure, possibly secondary to hyperthermia inducing heat-shock proteins and hypoxia due to reduced hepatic blood flow, has been described in humans (Diniz *et al.*, 2003).

Serum chemistry abnormalities in addition to lactic acidosis and hypoglycemia that have been reported with amphetamine overdose include hyperkalemia, hyperphosphatemia, and elevated liver enzymes such as alanine transaminase, alkaline phosphatase, and aspartate transaminase (Catravas *et al.*, 1977; Diniz *et al.*, 2003). Rhabdomyolysis may be evident based on increased creatine kinase, myoglobinuria, and evidence of renal failure. Lesions reported in experimental dogs dosed with amphetamines include subendocardial and epicardial hemorrhage and myocardial necrosis.

Low doses of amphetamine given to horses caused increases in heart rate during rest and exercise, increased blood pressure, second-degree block at the atrioventricular (AV) node, and premature ventricular contractions (Smetzer *et al.*, 1972).

### *Management*

The prognosis for animals that have ingested amphetamines depends on the dose, time between exposure and presentation, and severity of clinical signs. Gastrointestinal decontamination should be initiated in animals that present within 2 h of ingestion (Dumonceaux, 1995; Kisseberth and Trammel, 1990). Rapid onset of clinical signs may preclude the use of emetics. Gastric lavage of the sedated animal may be warranted if large doses are ingested. Activated charcoal and a cathartic may help prevent absorption. Repeat dosing may be necessary for sustained release products.

Animals should be monitored closely for neurological signs, hyperthermia, cardiac arrhythmias, or respiratory insufficiency. Baseline serum chemistries should be taken and animals monitored for hypoglycemia, electrolyte abnormalities, lactic acidosis, myoglobinuria, and liver or kidney damage, etc.

Minimal external stimulation may prevent seizure activity. Diazepam may paradoxically exacerbate the clinical signs of many amphetamines (a possible exception is MDMA, see below) and is thus contraindicated (Volmer, 2005). Chlorpromazine given at 10–18 mg/kg IV was determined by Catravas *et al.* (1977) to prevent death in experimental dogs dosed with amphetamine when given early in the progression of clinical signs. Treatment decreased hyperthermia, convulsions, hypertension, and heart rate. Normal respiration was maintained. However, chlorpromazine may lower the seizure threshold. Haloperidol at 1 mg/kg IV also decreased the clinical effects of amphetamines on body temperature, blood pressure, heart rate, respiration, and decrease convulsions in experimental dogs (Catravas *et al.*, 1977). Short-acting barbiturates have also been recommended for treatment of amphetamine-induced seizures (Dumonceaux and Beasley, 1990).

Cool IV fluids, ice packs, fans, cool water baths or cool moist towels, or gastric lavage with cool water may be used to treat hypothermia. The shivering response may increase body temperature (Smith *et al.*, 2002).

Cardiac arrhythmias in the amphetamine overdose patient may resolve with treatment of central nervous signs (Smith *et al.*, 2002; Diniz *et al.*, 2003). Propranolol has been found to decrease the heart rate and blood pressure in experimental dogs given amphetamines, but did not improve survival (Catravas *et al.*, 1977). The use of $\beta$-blockers may lead to $\alpha$-receptor-mediated vasoconstriction and spasms of the coronary artery (Diniz *et al.*, 2003). Lidocaine, procainamide, or amiodarone have been recommended to treat ventricular arrhythmias.

Urinary acidification with ascorbic acid or ammonium chloride may increase amphetamine excretion but should not be attempted in the presence of acidosis or rhabdomyolysis, or if acid–base status cannot be monitored. Treatment of acidosis and rhabdomyolysis involves fluid diuresis and alkalinization. Respiratory support may be required (Liechti *et al.*, 2005).

Urine or plasma samples may be tested for amphetamines at many diagnostic laboratories. Thin-layer chromatography is commonly used, and immunological assays are available for some compounds (Smith *et al.*, 2002). GC/MS may be used for confirmation.

## OTHER STIMULANTS

MDMA, sold as "ecstasy," is a common illegal club drug and will be discussed in some detail. Many legally available compounds are commonly used as stimulants. These include nicotine from tobacco products, the methylxanthines caffeine, theobromine, and theophyllin found in common food and drink products, and alkaloids of the areca nut, present in betel quid. The latter compounds will be addressed briefly.

## MDMA

MDMA, more frequently known as "ecstasy," is a schedule I drug. MDMA was used as an appetite suppressant and in psychotherapy (Smith *et al.*, 2002). The drug has shown some promise in the treatment of Parkinson's disease and post-traumatic stress disorder, but there are no accepted medical uses at this time (Morton, 2005). Currently, MDMA is one of the most popular club drugs in the Unites States and use increased 70% worldwide between 1995 and 2001 (Anonymous, 2005).

MDMA has been sold under numerous different names, a short list of which includes "Adam," "Batman," "bibs," "blue kisses," "blue Nile," "charity," "clarity," "Debs," "decadence," "E," "Eve," "go," "happy pill," "hug drug," "lover's speed," "M," "roll" or "rolling," "Scooby snacks," "X," and "XTC." Tablets containing 50–150 mg MDMA are often brightly colored with pressed images, often of product logos, butterflies, smiley faces, numbers, or letters. Less commonly encountered are the powdered or capsule forms. "Ecstasy" tablets tested in Europe during the 1990s only contained 50% MDMA, on average (Libiseller *et al.*, 2005). Other common ingredients included 3,4-methylenedioxyamphetamine (MDA), MDEA, caffeine, dextromethorphan, ephedra, and phenylpropanolamine.

The estimated lethal oral dose of MDMA for humans is 2 g. Based on this and the average recreational dose of 125 mg, the safety ratio (or therapeutic index) is estimated to be approximately 15 (Gable, 2004). The $LD_{50}$ of MDMA in rats is between 160 and 325 mg/kg PO. Dogs given 15 mg/kg MDMA showed severe clinical signs and one of six dogs died (Frith *et al.*, 1987). MDMA is metabolized in the liver by *N*-demethylation to the active metabolite MDA (Smith *et al.*, 2002).

MDMA is structurally similar to amphetamines and mescaline, which may explain some of the clinical effects (Lyles and Cadet, 2003). MDMA increases neurotransmitter release, including serotonin, dopamine, and NE (Smith *et al.*, 2002; Morton, 2005; Rimsza and Moses, 2005). Hallucinogenic effects have been attributed to serotonin release and inhibition of reuptake. This drug also inhibits MAO. MDMA directly binds to certain receptors, including 5-$HT_2$ receptors, $\alpha_2$-adrenergic receptors, $M_1$ muscarinic receptors, and $H_1$ histamine receptors, with less affinity for 5-$HT_1$, dopamine, $M_2$, $\alpha_1$, and $\beta$ receptors (Lyles and Cadet, 2003; Morton, 2005). Frith *et al.* (1987) described the effects of MDMA given to dogs at increasing oral doses. Clinical

signs began after about 45 min continued for 6–8 h. Dogs given low doses (3 mg/kg) most frequently showed signs of hyperactivity and mydriasis. Dogs given 9 mg/kg also became tachypneic with hypersalivation and circling behavior. One of six dogs given 15 mg/kg MDMA died after showing clinical sings that included vocalization, aggression, convulsions, and front limb paralysis.

Onset and duration of clinical signs are similar in humans, though onset is more rapid with insufflation (Smith *et al.*, 2002; Rimsza and Moses, 2005). Mydriasis, delirium confusion, agitation, bruxism, tremors, seizures, and loss of consciousness have been noted in MDMA users, as have hyperthermia and rhabdomyolysis. Cardiovascular changes may include tachycardia or less commonly bradycardia, AV block, and hypertension. A few deaths have been attributed to cardiopulmonary arrest. Liver failure has been reported to cause a few deaths after ingestion MDMA (Andreu *et al.*, 1998; Gable, 2004; Liechti *et al.*, 2005; Rimsza and Moses, 2005). Onset may occur days to weeks after exposure and the mechanism is not understood, though a hypersensitivity reaction has been suggested in some cases.

Treatment of MDMA exposure consists of appropriate gastrointestinal detoxification, as with amphetamines, and symptomatic and supportive care. Though not recommended for treatment of other amphetamine-type drugs, diazepam has been recommended to reduce agitation and anxiety in humans that have ingested MDMA (Smith *et al.*, 2002).

### Betel quid

Though not common in most areas of the Unites States, alkaloids of the areca nut are believed to be the fourth most commonly used psychoactive substances in the world after caffeine, ethanol, and nicotine. It is estimated that 10–20% of the world population use "betel quid" containing these alkaloids, mostly in South and Southeast Asia and the Asia Pacific regions (Gupta and Ray, 2004). However, these products can be purchased in the US cities with large populations of immigrants from these areas.

Palm trees of the genus *Areca* are cultivated in tropical and subtropical areas of the world to produce betel quid (Deng *et al.*, 2001). The nut of this palm tree may be ingested green, ripe and raw, baked, roasted, boiled, fermented, or processed with various sweeteners (Gupta and Ray, 2004). It is most commonly processed with leaves from the betel shrub, *Piper betle.* Calcium hydroxide is usually added (Deng *et al.*, 2001; Gupta and Ray, 2004). Tobacco and various spices may be added. The betel quid industry is worth hundreds of millions of dollars. Betel quid is produced locally or under trade names such as Supari, Mainpuri tobacco, mawa, pan masala, and Gutka. Gutka is illegal in some parts of India (Gupta and Ray, 2004). Alkaloids present in areca nut include arecoline, arecaidine, guvacine, and guvacoline. These alkaloids bind to muscarinic receptors, induce coronary vasospasms, and have weak activity on nicotinic receptors in ganglia.

Oral submucosal fibrosis is a preneoplastic disease that is common in young people who use betel quid (Gupta and Ray, 2004). Clinical signs seen in inexperienced users and overdoses are more likely to be of interest to the veterinarian and may include mucosal irritation, nausea and vomiting, bronchoconstriction, hypersalivation, lacrimation, urinary incontinence, diarrhea, hypertension or hypotension, tachycardia, acute myocardial infarction, and extrapyramidal signs (Deng *et al.*, 2001). Most patients given supportive care recover within 24 h, and atropine has not been effective as a treatment in humans.

## HALLUCINOGENS AND DISSOCIATIVE DRUGS

The clinical effect of this type of drug is relatively unpredictable and dependent very much on the individual and their environment (Nichols, 2004). Many compounds that alter consciousness have been termed "hallucinogens," though they may only induce hallucinations under specific circumstances or at very high doses, like marijuana or MDMA. "Psychomimetic," is a term used to describe drugs that cause behavioral changes mimicking psychosis, but the value of this term in veterinary medicine is questionable. "Psychedelic" is also used commonly by the media.

Lysergic acid diethylamide (LSD), a synthetic product similar to natural ergot alkaloids, is a common hallucinogen that acts on serotonin receptors. Hallucinogens with a similar mechanism of action include mescaline and *N*,*N*-dimethyltryptamine (DMT), described below. *Psilocybe* spp. and related mushroom species contain the hallucinogen psilocybin. Vomiting was reported after ingestion in a dog (Spoerke, 2005). Behavioral changes in a horse suspected of ingesting *Psilocybe* spp. included fear and extreme aggression with recovery within 48 h (Hyde, 1990). Severe toxicosis in a colt included hyperexcitability, tremors, mydriasis, and bruxism, and was eventually euthanized due to traumatic injuries and weakness (Jones, 1990). Signs of psilocybin in cats are similar to those described below with LSD (Jacobs *et al.*, 1977). DMT is produced by several plants including *Psychotria viridis*, used to produce a tea known as Ayahuasca in the traditional practices of the indigenous people of the Amazon, *Anadenanthera peregrina* seeds, called yopo by native people of the Amazon rain forest, and *Phalaris* spp. of grass which grow around the world. Leaves of the plant *Salvia divinorum* are legal to possess and are commonly used to produce hallucinogenic effects through a unique mechanism described below.

Dissociative anesthetics are so-called because they uncouple functions of the brain such as sensory and motor

activity, emotion, and consciousness, sometimes producing a cataleptic-type state (Branson, 2001). Affected individuals become disconnected from their environment and there is an absence of response to nociceptive stimuli (Branson, 2001; Volmer, 2005). The synthetic drug phencyclidine is an example of a dissociative agent. Ketamine, a familiar therapeutic drug to most veterinarians, is a dissociative anesthetic and also a popular "club drug." These drugs will be discussed at some length.

Several naturally occurring substances are used to produce hallucinogenic effects by various mechanisms. Certain species of mushrooms in the genus *Amanita*, specifically *A. muscaria and A. pantherina*, contain ibotenic acid and muscimol, which bind glutamate receptors and may be intentionally ingested. Various plants containing atropine and scopolamine, including *Datura stramonium, Atropa belladonna, Mandragora officinarum*, and *Hyoscyamus niger*, are routinely smoked or ingested (Halpern, 2004). The drug dextromethorphan is used recreationally for its dissociative effects. These substances will not be addressed further in this section.

## Lysergic acid diethylamide

LSD is the most powerful known hallucinogen (Nichols, 2004; O'Shea and Fagan, 2006). The D isomer of LSD is responsible for the molecule's effect on the CNS. The story of Albert Hoffman's synthesis and subsequent exposure to LSD is well documented. This drug was marketed under the trade name Delysid and used in psychotherapy and for experimental purposes. Though there are no current medical uses for this drug, it has shown some promise for use in the treatment of alcoholism, drug addiction, and obsessive–compulsive disorder. After becoming a popular recreational drug, LSD use was banned by the government in 1966, and it is currently a Schedule I drug (Nichols, 2004; Volmer, 2005).

LSD is a colorless, odorless, and flavorless white powder that is usually dissolved in water. It is applied to other substances such as blotter paper, microdots, tiny tablets, gelatin squares (termed "window pane" or "window glass"), stamps, and sugar cubes. Street names for LSD reflect these applications and include "acid," as in "spiked with acid," "blotter" or "blotter acid," "cubes," "dots" or "microdot," "L," "sugar" or "sugar cubes," "trip," or "wedding bells." Use of LSD is declining in the Unites States (Banken, 2004).

### *Toxicity*

The human effective dose of LSD is between 0.05 and 0.20 mg. Products sold currently usually contain 0.04–0.06 mg and carry less likelihood of an adverse reaction than the pills sold in the 1960s, which could contain up to 0.25 mg LSD. Increasing the dose may produce both quantitative and qualitative differences in the response (Nichols, 2004). The IV $LD_{50}$ for rats is 16 mg/kg. The minimal toxic dose in humans is between 0.70 and 2.80 mg/kg (Volmer, 2005). No human deaths have been attributed to the direct effects of LSD (Nichols, 2004). Some cats given intraperitoneal (IP) injections of 2.5 μg LSD/kg body weight showed mild clinical signs, and a dose of 50 μg/kg produced significant clinical signs in all cats tested (Jacobs *et al.*, 1977).

### *Toxicokinetics*

LSD is rapidly absorbed after ingestion in humans (Riordan *et al.*, 2002; Volmer, 2005). Peak plasma concentrations occur within 6 h and LSD is approximately 80% protein bound. Metabolism occurs primarily in the liver by hydroxylation and glucuronide conjugation to an inactive metabolite. Eighty percent of a dose of LSD is excreted in the feces and the elimination half-life is between 2 and 5 h (Volmer, 2005). Clinical effects may persist for 12 h (Nichols, 2004).

### *Mechanism of action*

Many "recreational" hallucinogens act primarily as antagonists at serotonin receptors (Volmer, 2005; O'Shea and Fagan, 2006). LSD is structurally similar to serotonin. Actions at the $5\text{-HT}_{2A}$ receptor are believed to be responsible for the hallucinogenic effects, though the signaling pathways involved have not been completely elucidated. Affected $5\text{-HT}_{2A}$ receptors are located in the pyramidal cells of the prefrontal cortex, the reticular nucleus of the thalamus, and possibly the locus coeruleus, where the effect is to alter sensory processing. LSD and some other hallucinogens also have a strong affinity for the $5\text{-HT}_{1A}$, $5\text{-HT}_{2C}$, and other serotonin receptors, but the significance of this is not understood (Nichols, 2004). LSD causes increased release of glutamate in the prefrontal cortex, has a high affinity for dopamine receptors $D_1$ and $D_2$, and shows some affinity for $\alpha_1$ and $\alpha_2$ adrenergic receptors.

### *Clinical signs*

Clinical signs in humans begin within an hour and a half of ingestion and usually last a few hours, though if large quantities are ingested effects may linger for up to 12 h (Anonymous, 2005). Signs in humans include euphoria, distraction, hallucinations, synesthesia, disorientation, incoordination, mydriasis, and sometimes severe anxiety. Malignant hyperthermia has also been reported in humans after LSD use (Riordan *et al.*, 2002). General signs reported in nonhuman animals include disorientation, mydriasis, depression or excitation, and vocalization. Clinical signs reported in cats given IP injections of LSD included paw flicking, head and body shaking, yawning, chops licking, bizarre sitting and standing positions, falling from their perch, leaping about, compulsive scratching at the litter,

pawing at the water, biting objects in the cage, grooming behavior including rubbing the head with paws, licking, biting and scratching, play behaviors including pawing and sniffing objects, tail chasing, and "hallucinatory behavior" which involved tracking, staring at, batting at, or pouncing on objects that were not apparent to the observer, frequent defecation, and occasionally emesis. Grooming behaviors were often not completed, the cat would lick or bite the air or stick her tongue out. Rage behavior and vocalization were not seen in these cats (Jacobs *et al.*, 1977). Based on this study, one can only imagine the presenting complaint in a companion animal after accidental exposure to LSD.

#### *Management*

Deaths have not been ascribed directly to LSD toxicosis humans, though fatal accidents have been reported due to behavioral changes and impaired judgment (Nichols, 2004). Treatment is based on close observation with supportive and symptomatic care. Because absorption of LSD is relatively rapid and effects are self-limiting, gastrointestinal decontamination is considered to have limited, if any, benefit. Clinical signs may persist for up to 12 h after ingestion of a large dose.

Animals should be kept in a quiet, dark room to minimize sensory stimulation. Minimal restraint may prevent hyperthermia (Volmer, 2005). Diazepam may be used to control severe agitation (Volmer, 2005; O'Shea and Fagan, 2006). Phenothiazine tranquilizers may lower seizure threshold. Haloperidol has been recommended to treat psychotic behavior secondary to LSD exposure in humans.

Laboratory techniques that have been employed in the detection of LSD include immunoassays, thin-layer chromatography, high-pressure liquid chromatography (HPLC), and liquid chromatography/mass spectrometry (LC/MS).

#### *Lysergic acid amide*

The seeds of *Ipomoea violacea*, the morning glory, contain lysergic acid amide (LSA), which is approximately 1/10th as potent as LSD, at a concentration of 0.02% dry matter (Halpern, 2004). Ingestion of 150–300 seeds may cause clinical effects in humans. The seed coat may be protective, thus seeds must be crushed, germinated, or soaked in water for ingestion to be effective. A word of caution to the adventurous, emetics may be added to commercial morning glory seeds. Other sources of LSA include seeds of the Hawaiian baby woodrose, *Argyreia nervosa*, at a concentration of 0.14% dry matter, and endophyte-infected sleepy grass, *Stipa robusta. S. robusta* is present in the Southwestern Unites States.

## Phencyclidine and ketamine

Phencyclidine, 1-(1-phenylcyclohexyl)piperidine, is a schedule II synthetic drug with more than 80 known analogs. One such analog, ketamine or 2((-*o*-chlorophenyl)-2-methylamino)-cyclohexanone HCl, has less than 1/10th the potency of phencyclidine and is a schedule III drug (Volmer, 2005). Phencyclidine was originally used as an anesthetic under the trade names Sernyl for human patients and later Sernylan for veterinary patients. Sernyl, given IV, was decreased response to nociceptive stimuli with insignificant respiratory and cardiac depression and no loss of corneal, papillary, or other reflexes (Branson, 2001). However, Sernyl was associated with postoperative psychosis, dysphoria, delirium, violent behavior, and hallucinations (Kisseberth and Trammel, 1990; Pestaner and Southall, 2003; Anonymous, 2005; Volmer, 2005). Sernylan was used in dogs, nonhuman primates, and was the preferred anesthetic for crocodilians (Ortega, 1967; Stunkard and Miller, 1974). Phencyclidine was last used commercially in the United States in 1978.

Phencyclidine is easily synthesized in clandestine laboratories. It is available in powdered or crystal forms which may be dissolved in liquids such as water or "embalming fluid," which contains formaldehyde and methanol, or it may be sold in tablets or capsules. Street products range from 5% to 90% purity and phencyclidine may be substituted for THC, mescaline, LSD, amphetamine, or cocaine (Kisseberth and Trammel, 1990). Phencyclidine is said to have a distinctive odor. This drug is frequently sold under the name "PCP" but other terms include "angel dust" or "angel hair," "boat" or "love boat," "dummy dust," "CJ," "hog" or "hog dust," "PeaCe Pill," "rocket fuel," "stardust," "whack," and "zombie dust." "Embalming fluid" is added to tobacco, marijuana, or other leafy material such as parsley, mint, or oregano for smoking. Names for this type of product include "supergrass." "amp," "happy sticks," "sherm," and "wet sticks."

Ketamine is not as readily produced by the amateur chemist but is used in veterinary and human medicine. Ketamine is labeled for use in cats and nonhuman primates. It may be used by physicians in pediatrics and in emergency and critical care situations where maintenance of blood pressure and respiration are crucial (Smith *et al.*, 2002; Volmer, 2005). Ketamine is a flavorless, odorless liquid which may be dried and crystallized then powdered for illicit use. Ketamine may be ingested and used to "spike" drinks, injected, insufflated, or placed into tobacco or marijuana cigarettes and smoked. Street names for ketamine include "cat valium," "green," "jet," "K," "special K," or "vitamin K," "keets," "kit-kat," "super acid," and "super C."

#### *Toxicity*

Phencyclidine given orally at doses of 2.5–10 mg/kg produces clinical effects in dogs and doses of 25 mg/kg were lethal in all of six dogs treated (Kisseberth and Trammel, 1990; Volmer, 2005). Given via intramuscular (IM) injection, 2 mg/kg caused muscular incoordination, 5 mg/kg caused immobilization and convulsions in one of five dogs, and

15 mg/kg caused convulsions in both of two dogs treated (Ortega, 1967).

Ketamine has a high therapeutic index in domestic mammals. The sedative dose in cats is 20 mg/kg IM. Doses of 5–10 mg/kg IM have produced convulsive seizures in dogs. IV injections of 2 mg/kg produce ataxia in sheep and dissociative analgesia in cattle. Doses of 5–15 mg/kg IV may cause struggling and vocalization in goats (Branson, 2001). The approximate $LD_{50}$ for ketamine in rodents is 600 mg/kg PO (Gable, 2004). Large doses of ketamine do not appear to produce analgesia in chickens or pigeons and pigeons may go into respiratory failure with doses of 0.11 mg/g; 0.05–0.1 mg/g produces anesthesia in parakeets, but 0.5 mg/g IM may be lethal (Branson, 2001).

### *Toxicokinetics*

Phencyclidine is ionized at gastric pH, thus little is absorbed in the stomach, but there is significant intestinal absorption (Kisseberth and Trammel, 1990; Volmer, 2005). Inhaled phencyclidine is well absorbed. The drug is lipophilic, with a wide tissue distribution that includes the CNS, adipose tissue, and gastric secretions. The latter leads to recycling and increases the biological half-life. Metabolism is variable between species. Approximately 68% of a given dose undergoes hepatic metabolism in the dog to a monohydroxyl form which is then conjugated for excretion. The remaining 32% is excreted unchanged in the urine. Approximately 88% of a dose of phencyclidine is excreted unchanged in the urine in cats.

Ketamine is well absorbed by parenteral routes, but little is absorbed after oral dosing in humans. There is a significant first-pass effect after ingestion of ketamine (Smith *et al.*, 2002). Peak plasma levels in cats occur 10 min after IM injection (Branson, 2001; Volmer, 2005). Ketamine is about 50% bound to plasma proteins in horses. It is distributed to the brain, adipose tissue, liver, lung, and other tissues. Ketamine undergoes hepatic metabolism via *N*-demethylation or hydroxylation and glucuronide conjugation to a water-soluble metabolite for excretion in the urine. The elimination half-life is approximately 67 min in the cat after parenteral dosing, 60 min in the calf, 42 min in the horse after IV dosing, and 2–3 h in the human.

### *Mechanism of action*

How compounds like phencyclidine and ketamine produce dissociative effects is not entirely understood. It is known that these drugs act on sites in the cerebral cortex, thalamus, and limbic system (Branson, 2001; Pal *et al.*, 2002; Volmer, 2005). The effects on behavior and cognition in humans may mimic schizophrenia. Phencyclidine and ketamine bind to *N*-methyl-D-aspartate (NMDA) receptors at a different site than glutamate, the excitatory neurotransmitter, and therefore act as noncompetitive inhibitors. Reuptake of NE, dopamine, and serotonin is decreased through inhibition of the biogenic amine reuptake complex (Smith *et al.*, 2002; Volmer, 2005). The sympathomimetic effects are associated with changes in heart rate, blood pressure, and cardiac output and increased myocardial oxygen consumption. Cardiovascular effects are more prominent with ketamine than phencyclidine. Ketamine induces stage I and stage II anesthesia but not stage III (Branson, 2001). Ketamine acts on non-NMDA glutamate receptors, dopaminergic receptors, nicotinic receptors, muscarinic receptors, and opioid receptors. Analgesic properties may be attributed to the actions of ketamine on opioid receptors. Ketamine binds dopamine receptors in dogs.

### *Clinical signs*

Dogs dosed with phencyclidine appear depressed at low doses and stimulated at high doses, with the potential for convulsive seizures (Branson, 2001). Onset of clinical effects is within 2 min of IV dosing (Ortega, 1967). Signs reported in dogs include muscular rigidity, grimacing facial expression, increased motor activity, head weaving, stereotyped sniffing behaviors, blank staring, incoordination, hypersalivation, nystagmus, and opisthotonus, ascending loss of motor function, coma, tonic–clonic convulsions, and hyperthermia. Cardiovascular effects include tachycardia, hypertension, and cardiac arrhythmia and deaths occur due to respiratory failure. Signs of phencyclidine in swine include muscle tremors and hypersalivation (Frost, 1972). Behavioral changes have been noted in neonates that were exposed during gestation (Branson, 2001).

Reported changes in clinical chemistry parameters include acidosis, hypoglycemia, electrolyte imbalances, and increased creatine phosphokinase and aspartate transaminase. Postmortem lesions associated with phencyclidine include epicardial and subendocardial hemorrhage and pulmonary congestion and hemorrhage (Kisseberth and Trammel, 1990). Clinical signs attributed to low doses of phencyclidine in humans include ataxia, slurred speech, hyperesthesia, hostility, sweating, drooling, hallucinations, incoordination, and disorientation. Overdose may cause coma, seizures, hypotension, muscle rigidity, and hyperthermia with the potential for rhabdomyolysis and renal failure. The clinical effects may last for days.

Pineal, pedal, photic, corneal, papillary, laryngeal and pharyngeal, and other reflexes are maintained during ketamine anesthesia (Branson, 2001; Volmer, 2005). There are significant species differences in the effects of ketamine. Ketamine increases muscle tone in cats, sometimes causing forelimb extensor rigidity or opisthotonus. Other signs in cats include mydriasis and fixed staring. Hypersalivation and chops licking are associated with oral dosing. Most cats can sit within 2 h and recover completely within 10 h. Ketamine causes excitation in dogs and may produce tonic–clonic seizures. Pulmonary edema was reported in a dog 2 days after anesthesia with ketamine and xylazine. Hypersalivation, apneustic breathing, and increased pulse

are reported in sheep. Goats hypersalivate, struggle, and vocalize but usually recover within 20 min of dosing. Nonhuman primates have increased heart and respiratory rates and hypertension (Branson, 2001).

Ketamine in humans has a rapid onset after ingestion or insufflation. Low doses produce analgesia and higher doses producing amnesia. Effects usually last about 45 min, but some users report hallucinations or "out of body experiences" lasting up to 1 h and full recovery may take up to 5 h (Smith *et al*., 2002; Gable, 2004; Anonymous, 2005). Other signs include mydriasis, perspiration, slurred speech, disorientation, nausea and emesis, paranoia, hypertension, and respiratory depression. Human deaths due to ketamine are quite rare and may be due to a combination of factors.

#### *Treatment*

Prognosis for an animal overdosed with phencyclidine or ketamine is generally good with early intervention, but self-induced trauma or rhabdomyolysis may complicate treatment. Cats that have undergone ketamine anesthesia usually recover rapidly and are able to sit up within about 2 h (Branson, 2001). Dogs injected with low doses of phencyclidine (1 mg/kg IM) recovered almost completely in a little over an hour, and at higher doses (5 mg/kg) were able to sit up within 2 h (Ortega, 1967).

Treatment of phencyclidine or ketamine overdose is generally symptomatic and supportive. Animals must be kept in a dark and quite room (Volmer, 2005). Use of restraint on human phencyclidine users has been associated with deaths, possibly due to catecholamine release and hypertensive crisis, or respiratory compromise (Pestaner and Southall, 2003).

Inducing emesis is of limited use due to the rapid absorption of phencyclidine, but may be attempted in very recent ingestions of large doses if no contraindications exist. Activated charcoal binds phencyclidine, prevents recycling, and is known to reduce mortality in dogs and rats. Forced diuresis with mannitol or furosemide may increase the rate of clearance. Urinary acidification enhances excretion but must be pursued with caution to prevent exacerbation of acidosis or, less commonly, myoglobinuric renal failure due to rhabdomyolysis. Electrolyte abnormalities and hypoglycemia should be corrected as necessary (Kisseberth and Trammel, 1990; Volmer, 2005).

Body temperature of the patient should be monitored. Cardiorespiratory abnormalities have been reported in some species. Muscle rigidity, hyperactivity, and seizures associated with ketamine can be successfully treated with diazepam (Branson, 2001). Severe seizures may require barbiturates or general anesthesia. Phenothiazine tranquilizers may decrease the seizure threshold, exacerbate anticholinergic effects, and produce hypotension, and therefore are contraindicated (Volmer, 2005).

## Other compounds

Mescaline, the active compound in peyote, is a well studied hallucinogen due to its significance both culturally and in the illicit drug trade. DMT is also another culturally significant compound that is familiar to some in the illegal drug trade. Salvinorin A is an agent hallucinogenic potential that is found in the legal herb *S. divinorum*.

#### *Mescaline*

Use of mescaline dates back to approximately 8500 BC (Bruhn *et al*., 2002). It is derived from several species of cactus including *Lephophora williamsii*, the peyote, *Trichocereus pachanoi*, the San Pedro cactus, and *Trichocereus peruvianus*, the Peruvian torch cactus. The latter two cacti are native to South America and are common ornamental plants. Both may be boiled into a soup for ingestion. Peyote grows in the Southwestern Unites States and Mexico, and is used legally by indigenous people. A tuberous root grows into the ground and is capped by crowns or "buttons" which are removed. The buttons may contain 1.5% mescaline on a dry matter basis. Buttons may be eaten fresh but are often dried. Dried buttons may be chewed, powdered, reconstituted, or steeped into a tea. Pickled buttons have caused botulism (Halpern, 2004). Though traditional uses are permitted, mescaline is a schedule I drug.

Mescaline is well absorbed by oral and parenteral routes in the dog with maximum plasma levels detected within an hour of ingestion. Mescaline levels detected in the brain are similar to blood levels. Renal, hepatic, and spleenic concentrations are 3–6 times blood levels. Mescaline can be detected in the urine within 30 min of exposure and remains detectable for up to 24 h. Between 28% and 46% of a given dose is excreted unchanged in the urine (Cochin, 1950). The effective dose of mescaline in humans is 0.3–0.5 g (Anonymous, 2005). The mescaline molecule is structurally similar to serotonin and acts on the 5-$HT_{2A}$ receptor (Nichols, 2004).

Clinical signs of mescaline intoxication in dogs include vomiting, mydriasis, injection of the conjunctiva, hyperreflexia, chewing motions of the jaw, and either excitation and disorientation or profound depression. Dogs are reported to recover from severe depression within 10 h. Signs in humans are fairly similar and may include emesis, especially after ingestion of *Trichocereus* spp. (Halpern, 2004). Other signs may include mydriasis, sweating, hallucinations, synesthesia, disorientation, incoordination, increased heart rate and blood pressure, and hyperthermia. People usually recover after 12 h.

#### *S. divinorum*

*S. divinorum*, known as salvia, is a perennial sage closely related to mint. The plant is native to Mexico and grows in humid, semitropical climates. It is grown in the United

States. The plant is of cultural significance to the Mazatec Indians of Oaxaca, Mexico, where it is used to treat diarrhea, headaches, rheumatism, and semi-magical diseases (Prisinzano, 2005). These indigenous Mexicans either chew the fresh leaves or extract juice. Salvia may also be smoked (Frohne and Pfänder, 2004; Prisinzano, 2005). Use of salvia is currently legal in the Unites States, though it is considered a "drug of concern" by the DEA. Salvia is a controlled substance in Denmark, Austria, and Italy.

Salvia contains various diterpenes, including salvinorums A through F and divinatorins A through C. Biological effects are attributed to salvinorin A. Similar compounds are present in plants of the genus *Coleus*. Salvinorin A is absorbed through the oral mucosa (Halpern, 2004). Little is known about the metabolism of this compound, but the elimination half-life in nonhuman primates averages 55.6 min (Prisinzano, 2005). Unlike other traditional hallucinogens, salvinorin A is an agonist at the $\kappa$ receptor and has no known effect on the $5\text{-HT}_{2A}$ receptor (Halpern, 2004; Prisinzano, 2005). No toxic effects were seen in mice given high doses of salvia (Prisinzano, 2005). Clinical signs associated with salvia use in humans are brief and self-limiting. They may include hallucinations, and at high doses loss of consciousness, and short-term memory. Most users recover within an hour. Treatment should be based on observation with symptomatic and supportive care as needed.

## CONCLUSION

Toxicoses due to ingestion of illegal substances are infrequently reported in the veterinary literature, but with the high incidence of "recreational" drug use in the United States, occasional companion animal exposures are likely. Exposure may be through accidental ingestion of the owner or neighbor's private "stash" or intentional exposure, either with lethal intent or as an attempt to get the pet "stoned." Though companion animals are far more likely to ingest "recreational" drugs, there are documented exposures of cattle and horses to illegally cultivated marijuana and illegal drugs have been used to enhance the performance of horses.

There are a large variety of drugs used for recreational purposes, and their actions vary mechanistically and produce a broad range of clinical signs. Treatment for most is through detoxification – though often severe CNS signs preclude use of emetics – and symptomatic and supportive therapy. Rarely are specific treatments available, though antagonists are available for opioid drugs. Quantity of the drug ingested and severity of clinical signs are important for determining prognosis with many drugs. Some drugs, however, such as marijuana, may cause severe CNS signs but affected animals have an excellent prognosis with early and appropriate intervention.

## REFERENCES

Adams HR (2001) Adrenergic agonists and antagonists. In *Veterinary Pharmacology and Therapeutics*, 8th edn, Adams R (ed.). Iowa State University Press, Ames, IA, pp. 91–116.

Andreu V, Mas A, Bruguera M, *et al.* (1998) Ecstasy: a common cause of severe acute hepatotoxicity. *J Hepatol* **29**: 394–7.

Anonymous (2005) *Street Drugs A Drug Identification Guide*. Publishers Group, LLC, Plymouth, MN.

Ashton CH (2001) Pharmacology and effects of cannabis: a brief review. *Br J Psychiatr* **178**: 101–86.

Baggot JD, Davis LE (1972) Pharmacokinetic study of amphetamine elimination in dogs and swine. *Biochem Pharmacol* **21**: 1967–76.

Banken JA (2004) Drug abuse trends among youth in the United States. *Ann NY Acad Sci USA* **1025**: 465–71.

Bischoff K, Beier E, Edwards WC (1998) Methamphetamine poisoning in three Oklahoma dogs. *Vet Hum Toxicol* **40**: 19–20.

Branson KR (2001) Injectable anesthetics. In *Veterinary Pharmacology and Therapeutics*, 8th edn, Adams R (ed.). Iowa State University Press, Ames, IA, pp. 213–67.

Branson KR, Gross ME (2001) Opioid agonists and antagonists. In *Veterinary Pharmacology and Therapeutics*, 8th edn, Adams R (ed.). Iowa State University Press, Ames, Iowa, pp. 213–67.

Bruhn JG, *et al.* (2002) Mescaline use for 5700 years. *Lancet* **359**: 1866.

Buchta R (1988) Deliberate intoxication of young children and pets with drugs: a survey of an adolescent population in a private practice. *Am J Dis Child* **142**: 701–2.

Burrows GE, Tyrl RJ (2001) *Toxic Plants of North America*. Iowa State University Press, Ames, IA, pp. 316–19.

Cardassis J (1951) Intoxication des équidés par. *Cannabis indica. Rec Méd Vét* **127**: 971–3.

Catravas JD, Waters IW (1981) Acute cocaine intoxication in the conscious dog: studies on the mechanism of lethality. *J Pharmacol Exp Ther* **217**: 350–6.

Catravas JD, Waters IW, Hickenbottom JP, *et al.* (1977) The effects of haloperidol, chlorpromazine, and propranolol on acute amphetamine poisoning in the conscious dog. *J Pharmacol Exp Ther* **202**: 230–43.

Cochin J, Woods LA, Seevers MH (1950) The absorption, distribution, and urinary excretion of mescaline in the dog. *J Pharmacol Exp Ther* **101**: 205–9.

Compton WM, Thomas YF, Conway KP, *et al.* (2005) Developments in the epidemiology of drug use and drug use disorders. *Am J Psychiatr* **162**: 1492–502.

Davis WM, Bedford JA, Buelke JL, *et al.* (1978) Acute toxicity and gross behavioral effects of amphetamine, 4-methoxyamphetamines, and mescaline in rodents, dogs, and monkeys. *Toxicol Appl Pharmacol* **45**: 49–62.

Deng JF, Ger J, Tsai WJ, *et al.* (2001) Acute toxicities of betel nut: rare but probably overlooked events. *Clin Toxicol* **39**: 355–60.

Di Marzo V, De Petrocellis L (2006) Plant, synthetic, and endogenous cannabinoids in medicine. *Annu Rev Med* **57**: 17.1–17.22.

Diniz PP, Sousa MG, Gerardi DG, *et al.* (2003) Amphetamine poisoning in a dog: case report, literature review, and veterinary medical perspectives. *Vet Hum Toxicol* **45**: 315–17.

Driemeier D (1997) Marijuana (*Cannabis sativa*) toxicosis in cattle. *Vet Hum Toxicol* **39**: 351–2.

Dumonceaux GA (1995) Illicit drug intoxication in dogs. In *Current Veterinary Therapy XII: Small Animal Practice*, Kirk RW (ed.). W.B. Saunders Co, Philadelphia, PA, pp. 250–2.

Dumonceaux GA, Beasley VR (1990) Emergency treatment for police dogs used for illicit drug detection. *J Am Vet Med Assoc* **197**: 185–7.

Evans AG (1989) Allergic inhalant dermatitis attributed to marijuana exposure in a dog. *J Am Vet Med Assoc* **195**: 1588–90.

Frazier K, Colvin B, Hullinger G (1998) Postmortem diagnosis of accidental cocaine intoxication in a dog. *Vet Hum Toxicol* **40**: 154–5.

Frith CH, Chang LW, Lattin DL, *et al.* (1987) Toxicity of methylenedioxymethamphetamine (MDMA) in the dog and rat. *Fundam Appl Toxicol* **9**: 110–19.

Frohne D, Pfänder HJ (2004) *Poisonous Plants*, 2nd edn. Timber Press Inc, Portland, pp. 118–19, 239–41.

Gable RS (2004) Acute toxic effects of club drugs. *J Psychoactive Drug* **36**: 303–13.

Garrett ER, Gürkan T (1980) Pharmacokinetics of morphine and its surrogates IV: pharmacokinetics of heroin and its derived metabolites in dogs. *J Pharm Sci* **69**: 1116–34.

Gloyd JS (1982) Abused drugs, street drugs, and drug misuse. *J Am Vet Med Assoc* **181**: 880–1.

Godbold JC, Hawkins J, Woodward MG (1979) Acute oral marijuana poisoning in the dog. *J Am Vet Med Assoc* **175**: 1101–2.

Green P (1996) Intestinal obstruction with hemp bedding. *Vet Rec* **138**: 71–2.

Gupta PC, Ray CS (2004) Epidemiology of betel quid usage. Ann Acad Med Singapore **33**: 31–36.

Halpern JH (2004) Hallucinogens and dissociative agents naturally growing in the United States. *Pharmacol Ther* **102**: 131–8.

Humphreys DJ, Longstaffe JA, Stodulski JB, *et al.* (1980) Barbiturate poisoning from pet shop meat: possible associated with perivascular injection. *Vet Rec* **107**: 517.

Hyde PN (1990) High horse? *Vet Rec* **22**: 554.

Jacobs BL, Trulson ME, Stern WC (1977) Behavioral effects of LSD in the cat: proposal of an animal behavior model for studying the actions of hallucinogenic drugs. *Brain Res* **132**: 301–14.

Janczyk P, Donaldson CW, Gwaltney S (2004) Two hundred and thirteen cases of marijuana toxicosis in dogs. *Vet Hum Toxicol* **46**: 19–21.

Johnson EM, Myron LB (1995) Substance abuse and violence: cause and consequence. *J Health Care Poor Underserved* **6**: 113–21.

Johnson LD, O'Malley PM, Bachman JG, *et al.* (2005) Teen Drug Use Down but Progress Halts Among Youngest Teens. University of Michigan News and Information Service, Ann Arbor, MI.

Jones DL (1978) A case of canine cannabis ingestion. *NZ Vet J* **26**: 135–6.

Jones J (1990) Magic mushroom poisoning in a colt. *Vet Rec* **24**: 603.

Jones RS (1972) A review of tranquilisation and sedation in large animals. *Vet Rec* **90**: 613–17.

Kabas JS, Blancahrd SM, Matsuyama Y, *et al.* (1990) Cocaine-mediated impairment of cardiac conduction in the dog: a potential mechanism for sudden death after cocaine. *J Pharmacol Exp Ther* **252**: 185–91.

Kisseberth WC, Trammel HL (1990) Illicit and abused drugs. *Vet Clin N Am Small Anim Pract* **20**: 405–18.

Kollias-Baker C, Maxewell L, Stanley S, *et al.* (2003) Detection and quantification of cocaine metabolites in urine samples from horses administered cocaine. *J Vet Pharmacol Ther* **26**: 429–34.

Libiseller K, Pavlic M, Rabl W, *et al.* (2005) An announced suicide with ecstasy. *Int J Leg Med* **21**: 1–4.

Liechti ME, Kunz I, Kupferschmidt H (2005) Acute medical problems due to ecstasy use. *Swiss Med Wkly* **135**: 652–7.

Lyles J, Cadet JL (2003) Methylenedioxymethamphetamine (MDMA, Exstacy) neurotoxicity: cellular and molecular mechanisms. *Brain Res Rev* **42**: 155–68.

McDonough M, Kennedy N, Glasper A, *et al.* (2004) Clinical features and management of gamma-hydroxybutyrate (GHB) withdrawal: a review. *Drug Alcohol Depend* **75**: 3–9.

Morton J (2005) Ecstasy: pharmacology and neurotoxicity. *Curr Opin Pharmacol* **5**: 79–86.

Nichols DE (2004) Hallucinogens. *Pharmacol Ther* **101**: 131–81.

Ortega JJZ (1967) Phencyclidine for capture of stray dogs. *J Am Vet Med Assoc* **150**: 772–6.

O'Shea B, Fagan J (2006) Lysergic acid diethylamide. *Irish Med J* **94**: 217.

Pal HR, Berry N, Kumar R, *et al.* (2002) Ketamine dependence. *Anaesth Intens Cure* **30**: 382–4.

Pestaner JP, Southall PE (2003) Sudden death during arrest and phencyclidine intoxication. *Am J Forensic Med Pathol* **24**: 119–22.

Prisinzano TE (2005) Psychopharmacology of the hallucinogenic sage *Salvia divinorum. Life Sci* **78**: 527–31.

Queiroz-Neto A, Zamur G, Lacerda-Neto JC, *et al.* (2002) Determination of the highest no-effect dose (HNED) and of the elimination pattern for cocaine in horses. *J Appl Toxicol* **22**: 117–21.

Rimsza ME, Moses KS (2005) Substance abuse on the college campus. *Pediatr Clin N Am* **52**: 307–19.

Riordan M, Rylance G, Berry K (2002) Poisoning in children 5: rare and dangerous poisons. *Arc Dis Child* **87**: 407–10.

Schwartz RH, Riddle M (1985) Marijuana intoxication in pets. *J Am Vet Med Assoc* **187**: 206.

Smetzer DL, Senta T, Hensel JD (1972) Cardiovascular effects of amphetamine in the horse. *Can J Comp Med* **36**: 185–94.

Smith KM, Karvube KL, Romanelli F (2002) Club drugs: methylenedioxymethamphetamine, flunitrazepam, ketamine HCl, and $\gamma$-hydroxybutyrate. *Am J Health-Syst Pharm* **59**: 1067–76.

Smith RA (1988) Coma in a ferret after ingestion of cannabis. *Vet Hum Toxicol* **31**: 262.

Smith RK, Papworth S (1996) Intestinal obstruction with hemp bedding. *Vet Rec* **138**: 71–2.

Spoerke D (2005) Mushrooms. In *Small Animal Toxicology*, 2nd edn, Peterson ME, Talcott PA (eds). Saunders, Philadelphia, PA, pp. 273–311.

Stunkard JA, Miller JC (1974) An outline guide to general anesthesia in exotic species. *Vet Med Small Anim Clin* **69**: 1181–6.

Vitale S, van de Mheen D (2005) Illicit drug use and injuries: a review of emergency room studies. *Drug Alcohol Depend* **82**: 1–9.

Volmer PA (2005) Recreational drugs. In *Small Animal Toxicology*, 2nd edn, Peterson ME, Talcott PA (eds). Saunders, Philadelphia, PA, pp. 273–311.

Welshman MD (1986) Doped Doberman. *Vet Rec* **119**: 512.

## USEFUL WEBSITES

McLaughlin EC. Narcs nab drug smuggling puppies.
www.cnn.com/2006/WORLD/americas/02/02/drug.pup/index.html, accessed December 9, 2006.

Neihause R. Additive may stop theft.
www.iowastatedaily.com/storage/paper818/news/2004/09/07/Agriculture/Additive.May.Stop.Teft-1100869.shtml?norewrite200612092001&sourcedomain=222.iowastatedaily.com, accessed December 9, 2006.

Langley S. Police looking for anhydrous thief.
www.michigansthumb.com/site/news.cfm?BRD=461&f:=9

Anonymous. Convicted Kansas veterinarian loses license.
www.avma/.org/onlnews/default_001015.asp, accessed December 9, 2006

Anonymous. Florida Board of Pharmacy disciplines Pet Med Express, Savemax.
www.avma.org/onlnews/javma/june02/S020601a.asp, accessed December 9, 2006.

Anonymous. Euthanized animals can poison wildlife: veterinarians receive fines.
www.avma.org/onlnews/javma/Jan02/S011502d.asp, accessed December 9, 2006.

# Part 5

## Metals and Micronutrients

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C H A P T E R

# 25

# Aluminum

*Ramesh C. Gupta*

## INTRODUCTION

Aluminum (Al) is the third most abundant element that naturally occurs in the earth crust. As a result, it is present in water, soil, and air. Small amounts of Al are released into the environment from coal-fired power plants, incinerators, and roofing and siding materials. In addition, Al and Al-based compounds are used for a variety of purposes, from medicines to explosives. Under some circumstances exposure of animals and humans to Al is inevitable. Poisoning in animals by Al is rare, but in several incidents the outcome has been very serious or deadly. Among all Al compounds, Al phosphide is of major concern to animals, because at low stomach pH, phosphide converts to toxic phosphine gas. Al has been widely studied for its toxicological effects, especially for neurotoxicity and developmental toxicity in laboratory animals. This chapter focuses on Al toxicity in animals.

## BACKGROUND

Aluminum (Al) metal is obtained from Al-containing minerals, primarily bauxite. Al metal is silvery white in appearance and light in weight. Al is a component of beverage cans, pots and pans, siding and roofing, and foil. It is also found in the products such as antacids, astringents, buffered aspirin, and food additives. In the powdered form, Al is used in fireworks and explosives (ATSDR, 1999). Often, Al complexes with other elements and forms Al oxide, Al chlorhydrate, Al hydroxide, Al chloride, Al lactate, Al phosphate, and Al nitrate.

The implication of Al in the etiology of neurodegenerative disease like Alzheimer's disease has been controversial; its role has clearly been established in encephalopathy and amyotrophic sclerosis (Deloncle and Pages, 1997). From a toxicological perspective, Al phosphide, which is commonly used as a pesticide, has been encountered in poisoning of cows. In these cases, the majority of toxicity comes from phosphine ($PH_3$) gas which is generated from phosphide in the gastrointestinal (GI) tract. Acute toxicity of Al in animals is rare, but following subacute or chronic exposure, Al can produce a variety of toxicological effects. Since Al readily crosses the blood–brain barrier (BBB) and the placental barrier, it appears that neurotoxicity and developmental toxicity are of particular concern in relation to Al toxicity.

## TOXICOKINETICS

Aluminum (Al) is poorly absorbed following either oral or inhalation exposure, and practically none is absorbed following dermal exposure. Bioavailability of Al depends on its form. For example, Al nitrate has shown to be twice as bioavailable as Al chloride in rats (Yokel and McNamara, 1988). In Wistar rats receiving a single gavage dose of Al hydroxide, Al citrate, Al citrate with added sodium citrate, or Al maltonate, the fractional intestinal absorptions were 0.1%, 0.7%, 5.1%, and 0.1%, respectively. In rabbits, following a single oral dose of the water-soluble compounds, Al chloride (333 mg Al/kg), Al nitrate (934 mg Al/kg), Al citrate (1081 mg Al/kg), and Al lactate (2942 mg Al/kg), Al absorption was 0.57%, 1.16%, 2.18%, and 6.3%, respectively (Yokel and McNamara, 1988). It appears that the oral absorption of Al can vary 10-fold based on chemical form alone, that is less absorption for water-insoluble forms and more for water-soluble forms. Furthermore, bioavailability can be influenced by other factors, such as variable

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amounts of essential and nonessential trace minerals, metal binding ligands, and other dietary constituents, that can enhance or inhibit Al absorption.

Evidence suggests that Al is primarily absorbed in the duodenum and jejunum. The acidic pH of the stomach may solubilize Al from insoluble species such as $Al(OH)_3$, facilitating absorption (Yokel, 1997). The main mechanism of absorption of Al is probably passive diffusion through paracellular pathways. Another proposed mechanism for absorption of Al is an energy-dependent process that involves calcium channels. Al may be taken up into mucosal cells, which may provide a barrier to its absorption. Small amounts of Al may then be slowly released into circulation (Van der Voet, 1992). Free Al ions occur in very low concentrations, because they complex with many molecules in the body, such as organic acids, amino acids, nucleotides, phosphates, carbohydrates, and macromolecules. Therefore, toxicokinetics of Al can vary depending on the nature of these complexes.

From the circulation, Al distributes to every organ, and the highest concentrations can be found in bone, brain, and lung tissues. It is well established that Al deposits in bones and from there it is slowly released. Long-term oral exposure to Al results in an increase in Al levels in the bone (Ahn *et al.*, 1995; Konishi *et al.*, 1996). Al also accumulates in the brain, kidneys, liver, lung, and in hematopoietic tissue. Slow elimination coupled with continued exposure may produce an increasing body burden of Al. Furthermore, Al levels are increased with age. Within cells, Al accumulates in the lysosome, cell nucleus, and chromatin. In the blood, about 80–90% of Al binds to plasma proteins (Wilhelm *et al.*, 1990). Evidence suggests that Al primarily binds to transferrin, and some amounts to albumin. It is important to note that binding of Al to albumin is nonspecific and much weaker than to transferrin.

In general, brain Al concentrations are lower than many other tissues. Al is known to cross the BBB and enter the brain by transferrin receptor-mediated endocytosis. Following inhalation exposure, Al can enter the brain by two mechanisms: (1) via the olfactory tract and (2) via nasal epithelium and axonal transport (Perl and Good, 1987; Zatta *et al.*, 1993). It is noteworthy that the cells that accumulate the most Al are long-lived postmitotic cells, such as neurons (Ganrot, 1986). In rabbits, increases of 4- to 10-fold and 10- to 20-fold Al concentrations in brain are associated with neurotoxicity and death, respectively. Al is actively removed from the brain by means of an energy-dependent process.

Regardless of the route of exposure, from the circulation Al primarily excretes in the urine and very little in the bile. Renal elimination of Al depends on the Al complex. For example, Al bound in a low-molecular-weight complex could be filtered at the renal glomeruli and excreted, while Al in a high-molecular-weight complex would not. Animal studies suggest that following a single exposure Al levels in urine can elevate as much as 14-fold. Al is primarily excreted in urine during the first 24 h period, and returns to normal levels 5 days post exposure (Ittel *et al.*, 1987). The rate of Al clearance is consistent with glomerular filtration rate (GFR), although proximal tubular Al reabsorption and Al excretion in the distal nephron have been suggested. Several animal studies have revealed a decrease in Al clearance and an increase in $t_{1/2}$ with increased Al concentration. This may be due to the high Al concentrations that probably formed unfilterable Al complexes, thereby reducing the plasma filterable Al fraction. Because of the limited GI tract absorption of Al, only a limited amount of Al excretes in the milk.

Following oral ingestion, unabsorbed Al excretes in the feces.

## MECHANISM OF ACTION

The central nervous system (CNS) and bone appear to be the two major target organs for Al toxicity. It has been known for a while that Al is involved in neurodegenerative diseases like Alzheimer's, encephalopathy, and amyotrophic sclerosis (Deloncle and Pages, 1997). However, the exact mechanism by which Al- induces neurotoxicity remains to be established. Al mainly deposits in the hippocampus, cortex, and amygdala, which are the areas of brain that are also rich in glutamatergic neurons as well as in transferrin receptors. Upon entering the brain, Al displaces physiological cations, such as magnesium, calcium, or iron, and modulates their metabolism. Studies suggest that Al neurotoxicity can be modulated by the levels of tissue and cytoplasmic calcium. Al, by replacing calcium in the synaptic area, perturbs neurotransmitter release and alters neurotransmitter systems. High Al levels appear to modify cholinergic neurotransmission. Al is known to associate with many epithelia and endothelia, including the BBB, and may be responsible for compromising the properties and integrity of these membranes (Wen and Wisniewski, 1985; Exley, 1996).

Al competes with magnesium in the biological system despite an oxidation state difference, and binds to transferrin and citrate in the bloodstream (Ganrot, 1986; McDonald and Martin, 1988). Al may also affect second messenger systems and calcium availability (Birchall and Chappell, 1988), and irreversibly bind to cell nucleus components (Dryssen *et al.*, 1987; Crapper-McLachlan, 1989). In addition, Al has been shown to inhibit neuronal microtubule formation.

It has been demonstrated that, in some conditions, Al can cross the BBB without altering the functional characteristics of the membrane, while in other conditions Al interacts with the BBB with subsequent effects on its barrier function (Vorbrodt *et al.*, 1994). Al-related toxicological

effects are noticed on both sides of the BBB (Deloncle and Pages, 1997). In neurons, glutamic acid in the form of a stable Al–glutamate complex is unable to detoxify cellular ammonia, which leads to neuronal death (Harris, 1992). Important events such as accumulation of Al in lysosomes (protease-rich vacuoles) and the hyperphosphorylation of neurofilaments (NFs) are involved in the molecular mechanism of Al-induced neurotoxicity (Bizzi and Gambetti, 1986; Ganrot, 1986; Delamarche, 1993).

## TOXICITY

Al is a toxic metal to animals. Although acute toxicity is rarely encountered, a high acute dose or repeated long-term exposure often leads to serious toxicological effects. Toxicity of Al depends on its form or complexes. Oral $LD_{50}$ values for Al nitrate in Sprague–Dawley rats and Swiss Webster mice are reported to be 261 and 286 mg Al/kg, respectively (Llobet *et al.*, 1987). For Al bromide, these values are 162 and 164 mg Al/kg, respectively. $LD_{50}$ values for Al chloride in Sprague–Dawley rats, Swiss Webster mice, and male Dobra Voda mice are 370, 222, and 770 mg Al/kg, respectively (Ondreicka *et al.*, 1966; Llobet *et al.*, 1987). The $LD_{50}$ values for Al sulfate in male Dobra Voda mice are reported to be 980 mg Al/kg (Ondreicka *et al.*, 1966). A single gavage exposure to 540 mg/kg as Al lactate was found to be lethal in female New Zealand rabbits (Yokel and McNamara, 1985). In subchronic and chronic studies, Al has been found to cause lethality in mice (Golub *et al.*, 1987), but not in rats (Dixon *et al.*, 1979) and dogs (Pettersen *et al.*, 1990).

Toxic effects of Al depend on the target organ. Such effects may in part be related to Al deposition and substitution of physiological elements, such as calcium, magnesium, and iron. Alterations by Al deposits can occur in: (1) the bone, interfering in heme synthesis leading to anemia, and (2) the myocardium, leading to myocardiac infarction, and (3) the brain, leading to neurotoxicity.

There is conclusive evidence that Al compounds are neurotoxic to laboratory animals following oral exposure. Marked signs of neurotoxicity, including ataxia, splaying, and dragging of hind limbs, and paralysis occurred in maternal mice that were exposed during gestation and lactation to Al with estimated doses of 184 mg Al/kg/day (Golub *et al.*, 1987) or 250 mg Al/kg/day (Golub *et al.*, 1992) as Al lactate. Adult mice that consumed 195 mg Al/kg/day as Al chloride for 5–7 weeks in a diet that also contained 3.5% sodium citrate showed neurotoxic effects (Oteiza *et al.*, 1993). The citrate is likely to have enhanced the responses of Al neurotoxicity. Changes in brain biogenic amines (decreased dopamine and 5-hydroxytryptamine, and increased norepinephrine) occurred in rats that were treated with 21.4 mg Al/kg/day as Al nitrate by gavage for 6 weeks (Flora *et al.*, 1991).

Evidence suggests that more serious disturbances occur in the brain following Al exposure. An alteration of the BBB would be necessary for Al brain transfer, but the mechanism leading to this alteration is still completely unknown, even though Al-induced peroxidation is perhaps a process involved therein. Elevated Al levels have been related to impaired motor function and to a number of cognitive deficiencies in both humans and experimental animals (Sturman and Wisniewski, 1988). Neurofibrillary degeneration has been observed in brain regions that exhibit elevated Al levels (Krishnan *et al.*, 1988).

Histopathological changes in the brain of rats, receiving 92 mg Al/kg/day as Al chloride and a high level of citrate (598 mg/kg/day) for 6 months, showed extensive vacuolization in astrocytes, swelling of astrocytic processes, neuronal vacuolization, and nuclear inclusions (Florence *et al.*, 1994). In other studies, increased Al levels and histological alterations in the brain (particularly increased numbers of abnormal and damaged neurons and reductions in cell density in areas of the hippocampus and neocortex) also occurred in rats that received an estimated 12 mg Al/kg/day as Al fluoride in drinking water and base diet for 45–52 weeks (Varner *et al.*, 1993, 1998).

There is some evidence that neurotoxic agents given prenatally induce subtle neurobehavioral impairment and delayed development of nervous system functions without any morphological malformation (Vorhees *et al.*, 1979; Adams and Buelke-Sam, 1981). The entry of Al into the developing nervous system is enhanced by the immaturity of the BBB (Thomas *et al.*, 1989), and the increased neuronal expression of transferrin receptors (Mollgard *et al.*, 1987). Al has been widely studied for reproductive/developmental/placental toxicity and teratogenecity in laboratory animals. These effects have been discussed in detail in the "Placental Toxicity" chapter of this book (chapter 15). In brief, Al crosses the placental barrier, accumulates in fetal tissues, and produces adverse/birth defects (Golub and Domingo, 1998).

It is important to mention that the acute toxicity associated with Al phosphide ingestion is primarily due to the formation of the highly toxic gas phosphine, instead of Al. There are incidences in which cows died by inhaling and ingesting grains treated with Al phosphide that was used for fumigation.

Although long-term oral exposure to Al results in an increase in Al levels in bones, there is no evidence that the accumulation of Al alters the bone structures histologically (Konishi *et al.*, 1996).

### Diagnosis

Diagnosis of Al poisoning can be based on history of Al exposure, clinical signs, and testing of Al in animal tissues.

Using atomic absorption spectrometer or inductively coupled plasma, Al can be measured in the blood, urine, feces, and hair. Only the urine measurement can indicate whether recent exposure to excess levels of Al has occurred. High concentrations in the lung usually reflect inhalation exposure and elevated levels in bone, liver, and spleen reflect sequestration (Yokel, 1977). In the diagnostic setting, liver and kidney are tested for Al and stomach/rumen content for phosphene ($PH_3$) when aluminum sulfide poisoning is suspected. Aluminum levels in the range of 6–11 ppm in the liver and 4–5 ppm in the kidney of cows and sheep are regarded as toxic levels. In dog liver, an Al level greater than 1.2 ppm is considered high (Puls, 1994). Aluminum levels greater than 1200 ppm in the diet are considered toxic to cattle and sheep.

## TREATMENT

There is no specific antidote for Al toxicity. So, treatment relies on symptomatic and supportive therapies. Use of activated charcoal and cathartics can be rewarding. In the case of Al phosphide, sodium bicarbonate (5% solution) can be administered to stop conversion of phosphide to phosphine gas.

## CONCLUSIONS

Aluminum is a commonly occurring metal in the environment, and as a result a small amount is normally present in the body. Single exposure or repeat exposure to higher levels of Al or its compounds (particularly Al phosphide) often leads to serious toxic effects or death. Since Al crosses the BBB and placental barrier, it produces neurotoxicity and developmental toxicity.

There is no specific antidote for Al toxicity. There is evidence that dietary Si can reduce GI Al absorption and increase its elimination. Dietary Si can also reduce brain Al accumulation. Avoid using desferrioxamine, as it can increase the Al concentration in the brain. Instead, use of an orally effective Al chelator, such as 3-hydroxypyridine-4-one, can be recommended.

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## REFERENCES

Adams J, Buelke-Sam J (1981) Behavioral assessment of the postnatal animal: testing and methods of development. In *Developmental Toxicology*, Kimmel CA, Buelke-Sam J (eds). Raven Press, New York, pp. 233–58.

Ahn HW, Fulton B, Moxon D, *et al.* (1995) Interactive effects of fluoride and aluminum uptake and accumulation in bones of rabbits administered both agents in their drinking water. *J Toxicol Environ Health* **44**: 337–50.

ATSDR (1999) *Toxicological Profile for Aluminum*. Agency for Toxic Substances and Disease Registry. US Department of Health and Human Services, Atlanta, GA.

Birchall JD, Chappell JS (1988) The chemistry of aluminum and silicon in relation to Alzheimer's disease. *Clin Chem* **34**: 265–7.

Bizzi A, Gambetti P (1986) Phosphorylation of neurofilaments is altered in aluminum intoxication. *Acta Neuropathol (Berlin)* **71**: 154–8.

Crapper-McLachlan DR (1989) Aluminum neurotoxicity: criteria for assigning a role in Alzheimer's disease. In *Environmental Chemistry and Toxicology of Aluminum*, Lewis TE (ed.). Lewis Publishers, Inc., Chelsea, MI, pp. 299–315.

Delamarche C (1993) A molecular mechanism of aluminum neurotoxicity. *J Neurochem* **60**: 384–5.

Deloncle R, Pages N (1997) Aluminum: on both sides of the blood–brain barrier. In *Mineral and Metal Neurotoxicology*, Yasui M, Strong MJ, Ota K, Verity AM (eds). CRC Press, Boca Raton, FL, pp. 91–7.

Dixon RL, Sherins RJ, Lee IP (1979) Assessment of environmental factors affecting male fertility. *Environ Health Perspect* **30**: 53–68.

Dryssen D, Haraldson C, Nyberg E, *et al*. (1987) Complexation of aluminum with DNA. *J Inorg Biochem* **29**: 67–75.

Exley C (1996) Aluminum in the brain and heart of the rainbow trout. *J Fish Biol* **48**: 706–13.

Flora SJS, Dhawan M, Tandon SK (1991) Effects of combined exposure to aluminum and ethanol on aluminum body burden and some neuronal, hepatic, and hematopoietic biochemical variable in the rat. *Hum Exp Toxicol* **10**: 45–8.

Florence AL, Gauthier A, Ponsar C, *et al*. (1994) An experimental animal model of aluminum overload. *Neurodegeneration* **3**: 315–23.

Ganrot PO (1986) Metabolism and possible health effects of aluminum. *Environ Health Perspect* **65**: 363–441.

Golub MS, Domingo JL (1998) Fetal aluminum accumulation. *Teratology* **58**: 225–6

Golub MS, Gershwin ME, Donald JM, *et al*. (1987) Maternal and developmental toxicity of chronic aluminum exposure in mice. *Fund Appl Toxicol* **8**: 346–57.

Golub MS, Keen CL, Gershwin ME (1992) Neurodevelopmental effects of aluminum in mice: fostering studies. *Neurotoxicol Teratol* **14**: 177–82.

Harris WR (1992) Equilibrium model for speciation of aluminum in serum. *Clin Chem* **38/39**: 1809–18.

Ittel TH, Buddington B, Miller NL, *et al*. (1987) Enhanced gastrointestinal absorption of aluminum in uremic rats. *Kidney Intl* **32**: 821–6.

Konishi Y, Yagyu Y, Kinebuchi H, *et al*. (1996) Chronic effect of aluminum ingestion on bone in calcium-deficient rats. *Pharmacol Toxicol* **78**: 429–34.

Krishnan SS, McLachlan DR, Dalton AJ, Krishnan B, Fenton SSA, Harrison JE, Kruck T. (1988) Aluminum toxicity in humans. In *Essential and Toxic Trace Elements in Human Health and Disease*. Alan R. Liss, New York, pp. 645–59.

Llobet JM, Domingo JL, Gomez M, *et al*. (1987) Acute toxicity studies of aluminum compounds: antidotal efficacy of several chelating agents. *Pharmacol Toxicol* **60**: 280–3.

McDonald TL, Martin RB (1988) Aluminum ion in biological systems. *Trend Biochem Sci* **13**: 15–19.

Mollgard K, Stagaard M, Saunders NR (1987) Cellular distribution of transferrin-immunoreactivity in the developing rat brain. *Neurosci Lett* **768**: 35–40.

Ondreicka R, Ginder E, Kortus J (1966) Chronic toxicity of aluminum in rats and mice and its effects on phosphorus metabolism. *Br J Ind Med* **23**: 305–17.

Oteiza PI, Keen CL, Han B, *et al*. (1993) Aluminum accumulation and neurotoxicity in Swiss–Webster mice after long-term dietary exposure to aluminum and citrate. *Metabolism* **42**: 1296–1300.

Perl DP, Good PF (1987) Uptake of aluminum into central nervous system along nasal olfactory pathways. *Lancet* **1**: 1028.

Pettersen JC, Hackett DS, Zwicker GM, *et al*. (1990) Twenty-six week toxicity study with KASAL (basic sodium aluminum phosphate) in beagle dogs. *Environ Geochem Health* **12**: 121–3.

Puls R (1994) *Mineral Levels in Animal Health: Diagnostic Data*. Sherpa International, BC, Canada, pp. 15–16.

Sturman JA, Wisniewski HM (1988) Aluminum. In *Metal Neurotoxicity*, Bondy SC, Prasad KN (eds). CRC Press, Boca Raton, FL, pp. 62–80.

Thomas T, Schreiber G, Jaworoski A (1989) Developmental patterns of gene expression of secreted proteins in brain and choroid plexus. *Develop Biol* **134**: 38–47.

Van der Voet GB (1992) Intestinal absorption of aluminum. Relation to neurotoxicity. In *The Vulnerable Brain and Environmental Risks. Vol. 2. Toxins in Food*, Isaacson RL, Jenson KF (eds). Plenum Press, New York, pp. 35–47.

Varner JA, Huie C, Horvath W, *et al*. (1993). Chronic $AlF_3$ administration. II. Selected histological observations. *Neurosci Res Commun* **13**: 99–104.

Varner JA, Jensen KF, Horvath W, *et al*. (1998) Chronic administration of aluminum fluoride or sodium fluoride to rats in drinking water: alterations in neuronal and cerebrovascular integrity. *Brain Res* **784**: 284–98.

Vorbrodt AW, Dobrogowska DH, Lossinsky AS (1994) Ultracytochemical studies of the effects of aluminum on the blood–brain barrier of mice. *J Histochem Cytochem* **42**: 203–212.

Vorhees CV, Butcher RJ, Brunner RJ, Sobotka TJ (1979) A developmental test battery for neurobehavioral toxicity in rats. A preliminary analysis using monosodium glutamate, calcium carrageenan and hydroxylurea. *Toxicol Appl Pharmacol* **50**: 267–82.

Wen GY, Wisniewski HM (1985) Histochemical localization of aluminum in the rabbit CNS. *Acta Neuropathol* **68**: 175–84.

Wilhelm M, Jager DE, Ohnesorge FK (1990) Aluminum toxicokinetics. *Pharmacol Toxicol* **66**: 4–9.

Yokel RA (1997) The metabolism and toxicokinetics of aluminum relevant to neurotoxicity. In *Mineral and Metal Neurotoxicology*, Yasui M, Strong MJ, Ota K, Verity AM (eds). CRC Press, Boca Raton, FL, pp. 81–9.

Yokel RA, McNamara PJ (1985) Aluminum bioavailability and disposition in adult and immature rabbits. *Toxicol Appl Pharmacol* **77**: 344–52.

Yokel RA, McNamara PJ (1988) Influence of renal impairment, chemical form, and serum protein binding on intravenous and oral aluminum kinetics in the rabbit. *Toxicol Appl Pharmacol* **95**: 32–43.

Zatta P, Favarato M, Nicolini M (1993) Deposition of aluminum in brain tissues of rats exposed to inhalation of aluminum acetylacetonate. *NeuroReport* **4**: 1119–22.

CHAPTER

# 26

# Arsenic

*Tam Garland*

## INTRODUCTION

The ubiquitous element arsenic (As) is a non-metal or metalloid in group V of the period chart. Frequently it is referred to as arsenic metal and is classified for many toxicological purposes as a metal. It exists in several forms and has a long history of various uses. It has been used in preparations from insecticides for animal to wood preservatives, herbicides and even has some medicinal uses. It is responsible for many poisonings in people and animals, both large and small.

## BACKGROUND

Arsenic is a ubiquitous element with several different forms. The form may determine the toxicity. The prevalent valences are the +3 and the +5 forms. Arsenic is found in both organic and inorganic forms with valet numbers ranging from +3 to +5. $As^{+3}$ or arsenite is more toxic than arsenate or $As^{+5}$. The toxicity of arsenic is determined by its form.

It is found as different ores and rocks being mined, then smelted resulting in elemental arsenic and arsenic trioxide. In the environment, arsenic usually exists as the pentavalent form and soil microorganisms may methylate it. Since it is a ubiquitous in many forms it is not likely that complete avoidance is possible.

Arsenic has a long and varied history of its sources and uses. A partial list is available in Table 26.1.

## PHARMACOKINETICS/ TOXICOKINETICS

Different toxic disease syndromes are caused by the different forms of arsenic. Inorganic arsenicals and trivalent organics cause a disease syndromes characterized by an effect on the gastrointestinal (GI) tract and the capillaries. In extremely low doses it is possible the body will develop a tolerance to the arsenic. Pentavalent organic arsenicals produce a neurological syndrome.

There are many factors influencing the absorption of arsenicals. Among those variables are the form of the metal, the particle size, the purity, the solubility, the species affected, and the physical condition of the animal exposed. Susceptibility to inorganic arsenicals varies among species, being highest in humans, followed by dogs, rats, and mice (Harrison *et al.*, 1958; Hays, 1982). So clearly there are many variable affecting the absorption and toxicity of this metal, which increases the difficulty of making accurate predictions of lethal amounts.

Pentavalent organic arsenicals are better absorbed than are the trivalent arsenicals, especially through the GI tract. Small amounts of either form may be absorbed via the intact skin, but it usually remains locally within the skin. However, absorption is limited by the size of the arsenical particle size. If the particle size is too large, it is not absorbed. Hence, a more toxic arsenical that is not absorbed because of large particle size may effectively be less toxic.

Once arsenicals are absorbed, the distribution is through the blood to all the organs of the body. Arsenic accumulates in the liver and is slowly distributed to the

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TABLE 26.1 Sources and uses of arsenic

| Sources | Valence/form | Uses |
|---|---|---|
| Commercial uses and products | Inorganic arsenic trioxide (+3) | Insecticide, cattle dip (0.18%) |
| | Inorganic sodium arsenite (+3) | Defoliant (highly toxic) |
| | Inorganic copper acetoarsenite (+3) | Paris green – insecticide (emerald green) |
| | Inorganic arsenic trioxide (+3) | Smelters |
| | Inorganic sodium arsenate (+5) | Herbicide |
| | Inorganic chromated copper arsenate (+5) | Wood preservative |
| | Inorganic lead arsenate (+5) | Insecticide and medicinal |
| | Organic pentavalent (+5) | Monosodium methylarsenate (MSMA) and disodium methylarsenate (DSMA) (highly toxic to cattle) |
| | | Ant bait |
| | | Leaded gasoline |
| | | Ant baits |
| Natural sources | | Ores, minerals, volcanoes |
| | | Ground water and soil |
| Medicinals | Potassium arsenite (+3) | Fowler's solution tonic/conditioner |
| | Organic trivalent arsenical | Thiacetarsamide – heartworm treatment in dogs |
| | Organic pentavalent arsenical | Tryparsamide – trypanosomiasis – old |
| | Organic trivalent arsenical | Melarsoprol – trypanocidal |
| | Organic pentavalent arsenical | Arsenicals feed additives (arsanilic acid, sodium arsanilate, 3-nitro, 4-hydroxyphenylarsonic acid) |

other tissues. The spleen, kidneys, and lungs are able to accumulate large amounts of arsenic. Arsenic has been shown to cross into the placenta barrier, particularly in monkeys, hamsters, and gerbils. Chronic doses are stored in the bone, the skin, and other keratinized tissues, such as skin, hair, hooves, and nails (Agency for Toxic Substances and Disease Registry, 1990).

The biotransformation of the arsenicals is poorly understood. There is some conversion from the +5 state to the +3 state, but the redox equilibrium favors the +3.

Methylation occurs by microorganism in the soil, but inorganic arsenicals are also methylated *in vivo*. The *in vivo* process may aid in the detoxification process. The kidneys may reduce a small amount of pentavalent arsenic to the more toxic trivalent form.

Arsenicals are excreted through many processes. In most species, between 40% and 70% of the absorbed amount of pentavalent arsenicals are excreted through the urine within 48 h (Vahter, 1983). It may also be excreted in much smaller quantities through the sweat. Trivalent forms of arsenic are excreted more slowly and through the bile into the fees.

## MECHANISM OF ACTION

Arsenite (+3) react with sulfhydryl groups (—SH) of proteins and inhibits the enzymes by blocking the active groups. The arsenite inhibits alpha-keto oxidases which contain dithiol groups and are involved in oxidation of pyruvate. Lipoic acid, an essential co-enzyme for pyruvic acid oxidase and alpha-oxyglutaric acid oxidase are inhibited by the arsenite. These play an essential role in the tricarboxylic acid cycle. Actively dividing cells that have a high oxidative energy requirement are most susceptible to the effects of arsenicals.

Arsenites induce vasodilation and can cause capillary damage. The cellular integrity of the capillary is affected by an unknown mechanism. Evidence of vascular instability is seen by the presence of congestion, edema, and hemorrhage in most of the visceral organs of animal with acute poisoning. This same mechanism of action occurs with inorganic arsenicals and with organic trivalent arsenicals, and they may be considered as "vascular poisons" (Hann and McHugo, 1960; Agency for Toxic Substances and Disease Registry, 1990; Jubb and Huxtable, 1993).

Arsenates (+5) are a little different. They are uncouplers of oxidative phosphorylation. The inorganic pentavalents may substitute of phosphate in this reaction. The result is an increase in body temperature. Organic pentavalents have an unknown mechanism of action. There is some thought that they may interfere with vitamins B6 and B1, which may allow for the demyelination and subsequent axonal degeneration that occurs.

Although arsenicals have been classified as carcinogens in people, this has not been the case in animals. Experimentally there have attempts to document arsenic-related

cancer in animals but the experiments have been unsuccessful (Agency for Toxic Substances and Disease Registry, 1990; Chan and Huff, 1997).

## TOXICITY

Inorganic arsenicals are up to 10 times more toxic than pentavalent arsenicals. The order of toxicity from greatest to least follows this schematic: inorganic $As^{+3}$ (arsenite) > inorganic $As^{+5}$ (arsenate) > trivalent organics > pentavalent organics ($I\,As^{+3} > I\,As^{+5} > O\,As^{+3} > O\,As^{+5}$). In other metal toxicities, the organics are more toxic but with arsenicals the inorganics are the more toxic.

Toxicity is also influenced by many factors, including particle size. The more finely ground, the more surface area there is for reactions. Solutions, such as dips and defoliants are the most dangerous. However the causes of the poisonings are varied. Debilitated animals are more sensitive. Since arsenic is not biodegradable, the soil and the old corrals around old dipping vats are still a source for arsenic poisoning. The area around smelters are also a source of poisoning, similar to that of dipping vats. Human mistakes and carelessness are the largest contributing factors to toxic events. For example, feeding a product known as gin trash instead of cotton seed hulls has resulted in acts numerous animals being poisoned.

Clinical sings caused by either inorganic or trivalent aliphatic arsenicals are similar. Peracute toxicities often result in sudden death within minutes to a few hours, if the dose is high, of dissolved arsenic ingestion. Acute poisonings have more clinical signs: abdominal pain or colic, vomiting, a staggering gait and weakness, clear incoordination, rapid weak pulse and shock, diarrhea, followed collapse, and death. If the acute poisoning is through dermal contact, then the arsenic will also be systemic. The skin will have blisters, edema, and may be cracked and bleeding, leaving the skin susceptible for secondary infection (National Academy of Science, 1977; Evinger and Blakemaore, 1984) Those receiving a lower dose over a period of time may have subacute poisonings will likely live several days, developing depression and anorexia. Movements may be difficult, stiff, and incoordinated. Diarrhea is dark and possibly hemorrhagic and very fluid. Hematuria may be present, or the urine may contain protein and casts (National Academy of Science, 1977; Osweiler *et al*., 1985). However, those suffering chronic poisoning are easily fatigued and have dyspnea when they are moved. These animals display intense thirst and have a rough dry hair coat as well as dry, brick-red mucous membranes. Cattle are described as having enlarged joints.

Clinical signs of phenylarsenic poisoning occur within 3 days of a high dose or after chronic exposure. Most noticeable are the neurological signs. The animal is generally bright and alert but uncoordinated. The animal may or may not be blind, and these animals may have erythema in the skin. Some of the neurological damage may be reversible unless the nerves are damaged.

Lesions are often dependent upon the dose and survival times. There may be no lesions at all in animals that die from peracute poisoning. However, even these animals have some GI irritation. With the exception of peracute deaths, most of the other animals that die from some form of arsenic poisoning may have excess fluid in the GI tract. In cattle there is hyperemia of the abomasum and this may be the only finding. If there are other lesions in cattle, it is often necrosis of the rumen mucosal epithelium. Ruminants have gelatinous serosal edema in the rumen, reticulum, omasum, and abomasum. The GI tract may have indication of irritation and be hemorrhagic. Lesions are indicative of capillary damage and the liver is usually soft and yellow.

The phenylarsonics (+5) are used in feed additives and lesions would be expected to be associated with overdoses in the feed mixture. A "downer pig" may have severe abrasions with muscle atrophy. Microscopic lesions indicate there is demyelination in the optic nerve and the posterior cord.

## TREATMENT

A diagnosis of arsenic poisoning is important and is based upon clinical history and signs. If more than one animal is involved, then lesions may also be important. Diagnostic arsenic levels in the kidney and liver are usually more than 8–10 parts per million (ppm), unless several days have lapsed since exposure, in which case it would likely be 2–4 ppm. Diagnostic levels of arsenic in the urine and feces are greater between 10 and 20 ppm. Arsenic should not be found in phenylarsonic acid intoxications.

Arsenic can poison to large and small animals. If small animals are not showing clinical signs then evacuation of the stomach followed by lavage with 1% of sodium bicarbonate solution is recommended treatment. As a general rule early treatment, within 4 h of exposure is best. In carnivorous small animals, emetics followed by gastric lavage is the best treatment. The lavage may be milk and egg whites or with 1–5 g of sodium thiosulfate. In herbivores treatment within 4 h of exposure is also best. A large saline purgative is effective, demulcents, 20–30 g of sodium thiosulfate orally followed by a 10% solution 3 times a day for the next several days.

Regardless of the carnivore or herbivore if treatment is more than 4 h after exposure, then dimercaprol (British anti-Lewisite, BAL) is recommended at 1.5–5 mg/kg,

intramuscularly, 2–4 times a day for 10 days or until recovery should be administered. Additionally, sodium thiosulfate at 30–40 mg/kg administered intravenously 2–4 times daily until recovery, which if recovery occurs will be within 2–4 days.

If the diagnosis is a phenylarsenic compound, there is no effective treatment for nerve damage caused by these compounds. BAL is not effective for these intoxications.

## CONCLUSION

Determining the diagnosis early and initiating treatment early will determine the prognosis. Acutely poisoned animals have a poor prognosis without early intervention. Understanding the differentials is important to institute the appropriate treatment for the appropriate condition. Arsenic produces signs of severe gastroenteritis, similar to those of pancreatitis, viral or bacterial gastroenteritis, irritating plants, caustic agents, and zinc phosphide poisoning. Likewise, other heavy metals will produce similar clinical signs. However, poisoning with phenylarsonics, used most frequently in feed additives, have a high morbidity rate but are associated with a low mortality rate. Recovery generally requires 2–4 weeks.

## REFERENCES

Agency for Toxic Substances and Disease Registry (ATSDR) (1990) *Arsenic Toxicity, Case Studies in Environmental Medicine*. US Department of Health and Human Services, Washington, DC.

Chan PC, Huff J (1997) Arsenic carcinogenesis in animals and in humans: mechanistic, experimental, and epidemiological evidence. *Environ Carcino Ecotox Rev* **15**(2): 83–122.

Evinger JF, Blakemaore JC (1984) Dermititis in a dog associated with exposure to an arsenic compound. *J Am Vet Med Assoc* **184**: 1281–2.

Hann C, McHugo PB (1960) Studies on the capillary and cardiovascular actions of intravenous sodium arsenate and arsenite. *Toxicol Appl Pharmacol* **2**: 674–82.

Harrison WE, Packman EW, Abbott DD (1958) Acute oral toxicity and chemical and physical properties of arsenic trioxides. *AMA Arch Ind Health* **17**: 118–23.

Hays WJ (1982) *Pesticides Studied in Man*. Williams and Wilkins, Baltimore, MD.

Jubb KVF, Huxtable CR (1993) The nervous system. In *Pathology of Domestic Animals*, 4th edn, vol. 1, Jubb KVF, Kennedy PC, Palmer N (eds). Academic Press, New York.

National Academy of Science (NAS) (1977) *Arsenic*. National Academy of Science, Washington, DC.

Osweiler GD, Carson TL, Buck WB, Van Gelder GA (1985) *Clinical and Diagnostic Veterinary Toxicology*. Kendall/Hunt, Dubuque, IA.

Vahter M (1983) Metabolism of arsenic. In *Biological and Environmental Effects of Arsenic*, Fowler BA (ed.). Elsevier, New York.

CHAPTER

# 27

# Cadmium

*Stephen B. Hooser*

## INTRODUCTION

Cadmium accumulation in plants and animals is increasing from several sources of environmental exposure. The application of rock phosphate (which contains varying amounts of cadmium depending on the source) and sewage sludge fertilizers results in cadmium deposition in the soil of pastures (Piscator, 1985; ATSDR, 1999). In addition to direct ingestion of soil containing cadmium, some forage plants extract cadmium from the soil. Although cadmium accumulation in the soft tissues of livestock has been demonstrated and there is ample experimental documentation of the toxicity of cadmium in animals, under natural conditions, documented cases of direct toxic or carcinogenic effects of cadmium in livestock have been uncommon (Dorn, 1979).

## BACKGROUND

Pure cadmium is a soft, silver-white metal with an atomic number of 48 and a molecular weight of 112.41. It is a divalent transition metal with chemical properties that are similar to zinc and is usually found as a mineral in combination with other elements to form cadmium oxide, cadmium chloride, or cadmium sulfate (ATSDR, 1999). Numerous compounds are formed from cadmium and thus it is used in batteries, solders, semiconductors, solar cells, plastics stabilizers, and to plate iron and steel. All soil and rocks contain some cadmium. It can enter the environment from zinc smelting and refining, coal combustion, mine wastes, iron and steel production, and from the use of rock phosphate and sewage sludge as fertilizers (Klasing, 2005).

Cadmium accumulation in plants and animals is increasing from a variety of sources, being the most severe in the vicinity of zinc smelters. The use of cadmium-containing mineral supplements in feed (e.g. from calcium phosphate), the application on pastures and hay fields of phosphate fertilizers (which contain varying amounts of cadmium depending on the source) and sewage sludge results in cadmium deposition in the soil (Piscator, 1985). Some plants readily extract cadmium from the soil thereby making it available for consumption. For example, cadmium concentrations in clover grown in soil fertilized with high cadmium rock phosphate were significantly higher than the concentrations in clover grown in soils treated with low cadmium phosphate fertilizer (McLaughlin *et al.*, 1997). A New Zealand National Survey of soils and plants, and random testing of kidneys from grazing animals revealed that there was an approximately two-fold increase in soil cadmium while over a 3-year period, 14–20% of cattle kidneys exceeded the New Zealand maximum residue level of 1 μg Cd/g (Roberts *et al.*, 1994). In a study where cattle were allowed to graze pastures treated with anaerobically digested sewage sludge for up to 8 years, cadmium was the only metal to accumulate consistently in increased amounts in the tissues of the cattle (Fitzgerald *et al.*, 1985). It has been reported that cattle grazing on sewage sludge treated pastures consumed significantly more (up to 3 times) cadmium than cattle on control pastures (Reddy and Dorn, 1985). In addition, a Swedish study has found a direct correlation between cadmium in feed and pig kidneys (Grawe *et al.*, 1997). A recent study reporting the analysis of Wisconsin dairy feeds for heavy metals found that cadmium concentrations in complete dairy feed rations were the closest of the heavy metals to US maximum acceptable concentrations, suggesting that cadmium has the greatest potential to exceed those maximum standards if the amounts of

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cadmium in feeds increases in the future (Li *et al.*, 2005). However, several studies have failed to demonstrate any adverse clinical manifestations related to increased cadmium concentrations in the animals examined. In one study, although cattle on pasture fertilized with sewage sludge consumed increased amounts of cadmium and had increased fecal excretion and kidney accumulation of cadmium (Reddy *et al.*, 1985), there were no adverse health effects noted in these cattle (Dorn *et al.*, 1985). Similarly, when corn silage or corn that was grown on sewage sludge fertilized fields were fed to sheep or pigs, respectively, significant increases in kidney cadmium concentrations were measured, but no other adverse treatment-related effects were noted (Lisk *et al.*, 1982; Telford *et al.*, 1982). Although cadmium is of concern in the environment, and cattle grazing on cadmium-contaminated pastures have increased tissue concentrations of cadmium, two additional studies conclude that accumulation of cadmium in the liver and kidneys of cattle may be a moderately effective screen for the entry of cadmium into the human food chain, as long as liver and especially kidneys are not consumed (Sharma and Street, 1980; Johnson *et al.*, 1981). It has been reported that regardless of the concentrations of cadmium fed to livestock, the amount in meat, milk, and eggs is always lower than that in the diet that the animal was eating. Thus, foods derived from those products decrease human exposure (Klasing, 2005). This is fortunate as chronic cadmium poisoning has been documented in humans. In these cases, it has been associated with osteoporosis, renal lesions, tissue mineral imbalances, and death. In addition, the Department of Health and Human Services has determined that cadmium and cadmium compounds may be reasonably anticipated to be carcinogens.

## PHARMACOKINETICS/ TOXICOKINETICS

In animals, cadmium exposure is primarily through oral ingestion. Compared to other divalent cations such as zinc and iron, intestinal absorption of cadmium is relatively low, ranging from approximately 1% to 5% in most species, with up to as much as 16% in cattle, dependent on the dose (Klasing, 2005). Interestingly, cadmium bound to metallothionein in foods of animal origin is absorbed less efficiently than cadmium salts and therefore, may be less available for uptake (Groten *et al.*, 1990). After absorption, cadmium is transported in the plasma bound to albumin and in lesser amounts to other serum proteins. It distributes throughout the body with the highest concentrations in the liver and kidneys, which account for approximately one-half of the total cadmium in the body. Muscle and bone do not accumulate high concentrations of cadmium. Blood cadmium concentrations are indicators of recent exposure while urine cadmium is a better indicator of the body burden. Cadmium is not transported well into milk or eggs, or across the placental barrier (Klasing, 2005). In pregnant and lactating livestock, the toxicokinetics of cadmium have been compared. In this study, the kinetics of cadmium were measured in lactating versus non-lactating ewes after a single intravenous or oral administration of cadmium chloride. The non-lactating ewes exhibited a low cadmium bioavailability (0.12–0.22%), a large steady-state volume of distribution (23.8 ± 5.41/kg), and a low blood clearance (0.20 ± 0.031/kg/day) with a mean residence time of 113 ± 28 days. The lactating ewes had a higher bioavailability (0.33–1.7%), and the mean residence time was close to that of the non-lactating ewes despite a greater blood clearance (0.46 ± 0.0131/kg/day) because the volume of distribution of cadmium in the body was larger. The cadmium clearance in milk remained low in the lactating ewes (Houpert *et al.*, 1997).

In the body, cadmium is excreted very slowly, with daily losses of approximately 0.009% of the total via the urine and approximately 0.007% in the feces via the bile. Cadmium–protein complexes are excreted in the kidneys and then resorbed from the filtrate in the proximal tubules. This area of the renal cortex accumulates cadmium and is susceptible to damage and necrosis. Depending on the species, the biological half-life of cadmium can vary from months to years, which results in cadmium accumulating in animals as they age (Klasing, 2005). For example, several studies have documented age-related increases in cadmium in the kidneys of horses (Elinder *et al.*, 1981a; Anke *et al.*, 1989).

In mammals and birds, cadmium accumulates in the liver and kidneys at concentrations of 0.1–2.0 and 1–10 mg/kg wet weight, respectively. It has been discovered that animals with long-life spans, such as horses, can accumulate large amounts of cadmium in their organs, particularly in their kidneys. In samples of renal cortex from old horses, concentrations of up to 200 mg/kg have been reported (Elinder, 1992).

## MECHANISM OF ACTION

Experimentally, acute exposure to high doses of inorganic cadmium leads to its accumulation in many organs, eliciting liver and in some cases, testicular damage (Dixit *et al.*, 1975; Habeebu *et al.*, 1998; Klasing, 2005). Once inside the cell, free cadmium binds to protein sulfhydryl groups, disrupting the cellular redox cycle, depleting glutathione, and eliciting intracellular oxidant damage. In addition, its similarity to other divalent cations such as calcium interferes with their normal functioning (Klasing, 2005). Cadmium ions can displace zinc and other divalent metals from their

binding sites on metalloproteins. For example, in the testis, cadmium can interfere with zinc-proteins, leading to widespread apoptosis and necrosis (Xu *et al.*, 1999). In the liver, acute cadmium toxicity results in widespread hepatocyte apoptosis, followed by varying degrees of necrosis depending on the dose (Habeebu *et al.*, 1998). This is related, in part, to the effects of resident liver macrophages (Kupffer cells) to potentiate and increase the initial liver damage caused by cadmium alone. This has been demonstrated in several systems in which inhibition of Kupffer cells significantly decreases liver damage caused by a toxic dose of cadmium (Sauer *et al.*, 1997a, b).

Cadmium readily binds to, and induces the production of, metallothionein, a cysteine-rich, metal-binding protein. Binding to metallothionein does not have a major effect on the uptake of cadmium, but is, in part, responsible for retention of cadmium within cells and its long half-life (greater than 10 years in humans). Metallothionein does this by decreasing cadmium elimination, especially in bile. Within hepatocytes, metallothionein binds to cadmium, decreasing its hepatotoxicity. Experimentally, rats that have greater induction of metallothionein in the liver are somewhat protected from cadmium hepatotoxicity (Kuester *et al.*, 2002). However, in the kidneys the cadmium–metallothionein complex is nephrotoxic and it has been theorized that it may play a role in chronic poisoning in humans (Klaassen and Liu, 1997).

## TOXICITY

Increased exposure to cadmium in combination with zinc, lead, and/or other metals continues to occur in the vicinity of non-ferrous metal smelters and processing facilities. These exposures have resulted in toxicoses, although it can be difficult to separate the effects of cadmium from those of lead, zinc, and other metals. In one such case in the Netherlands, kidney cadmium concentrations were found to be twice those of cattle in control areas. However, although hemoglobin, blood iron concentrations, and iron-binding capacity were lower in the cadmium-exposed cattle compared to controls, no adverse clinical effects were observed (Wentink *et al.*, 1992). In an additional study in the Netherlands, bulls fed diets containing increased concentrations of cadmium, lead, mercury, and arsenic had increased concentrations of cadmium in the kidney and liver, but did not exhibit histological lesions related to the intake of heavy metals (Vreman *et al.*, 1988). However, more recently, deaths in horses exposed to cadmium, lead, and zinc from a non-ferrous metal processing plant in Eastern Europe were attributed to ingestion of these metals in their feed. Analysis of tissues from a number of these horses revealed extremely high concentrations of cadmium (40–100 times normal) and 3–6 times the normal concentrations of lead (Bianu and Nica, 2004). Toxicoses have also been reported in sheep and horses in the vicinity of non-ferrous metal smelters in China. Analysis of the tissues from these animals revealed lead and cadmium concentrations significantly higher than those of controls (Liu, 2003).

A survey of cadmium concentrations in tissues from healthy swine, cattle, dogs, and horses in the midwestern United States was conducted in the mid-1970s. While the median cadmium concentration was low, at or below 0.6 ppm in the kidneys of cattle, swine, and dogs, the median concentration in the kidneys was 4 times greater in horses (Penumarthy *et al.*, 1980). One study has indicated that horses may be more at risk for cadmium toxicity than other species. In this Swedish study, the cadmium concentrations in the kidney cortices of 69 otherwise normal horses were measured and correlated to any histological lesions that were noted. In that study, renal cadmium concentrations ranged from 11 to 186 μg Cd/g wet wt., with an average of 60 μg Cd/g. This study found a correlation between increased chronic interstitial nephritis and increasing cadmium concentrations in the renal cortex. There was no obvious relationship between the age and the frequency of renal lesions (Elinder *et al.*, 1981a). These same authors also found that cadmium concentrations in the kidney cortices were approximately 15 times greater than those in the liver of the same animals (Elinder *et al.*, 1981b). Age-dependent increases in kidney metallothionein and cadmium have also been reported in horses (Elinder *et al.*, 1981a; Jeffrey *et al.*, 1989; Plumlee *et al.*, 1996). However, these later studies and others (Holterman *et al.*, 1984) have not reported renal lesions similar to those reported by Elinder *et al.* (1981a). One diagnostic investigation has reported lameness and swollen joints, i.e lesions of osteochondrosis, in addition to osteoporosis and nephrocalcinosis in horses near a zinc smelter in Pennsylvania. In the horses examined, kidney zinc and cadmium concentrations were elevated. It this case, it was postulated that the osteoporosis that was observed in one foal and the nephrocalcinosis seen in the foal and its dam were related to the elevated renal cadmium (Gunson *et al.*, 1982). When ponies were raised near a similar zinc smelter for periods of time up to 18.5 months, there were significant elevations in tissue zinc and cadmium concentrations. Increases in tissue cadmium concentrations were correlated with increasing age, although increases in tissue zinc concentrations were not. Generalized osteochondrosis was present in joints of the limbs and cervical vertebrae, and there was lymphoid hyperplasia. From this study, it was concluded that the development of osteochondrosis was associated with increased exposure to zinc and possibly cadmium. However, other lesions of cadmium toxicosis, such as renal damage or osteomalacia, were not present (Kowalczyk *et al.*, 1986).

In wildlife, white-tailed deer (*Odocoileus virginianius*) harvested within 20 km of zinc smelters in Pennsylvania had very high kidney concentrations of cadmium and zinc. These deer were also reported to have had joint lesions similar to zinc-poisoned horses from the same area (Sileo and Beyer, 1985).

In humans, occupational exposure to cadmium has been associated with renal dysfunction and osteomalacia with osteoporosis. One of the earliest effects of chronic cadmium exposure is renal tubular damage with proteinuria (Bernard *et al*., 1992). Other chronic effects can include liver damage, emphysema (through inhalation), osteomalacia, neurological impairment, testicular, pancreatic, adrenal damage, and anemia. Tumorigenic effects have been reported in experimental animals (Lee and White, 1980). Historically in the 1940s, high environmental exposure in one area of Japan from eating cadmium-contaminated rice resulted in itai-itai (ouch-ouch) disease. This was manifested by intense bone pains and pathological bone fractures, mainly in elderly women, with osteoporosis and renal dysfunction (Kobayashi, 1978). In addition, studies in Europe and China have demonstrated that low to moderate exposure to cadmium from zinc smelters resulted in a decrease in bone density, and an increase in fractures in women. In one of these studies, cadmium concentrations in the blood and urine were taken as biomarkers of exposure (Katzantzis, 2004). Experimental studies in animals have confirmed the adverse effects of cadmium on bones. Six mechanisms have been theorized to explain these effects: (1) interference with parathyroid hormone stimulation of vitamin D production in the kidney, (2) reduced renal vitamin D activation, (3) increased urinary excretion of calcium, (4) reduced intestinal calcium absorption, (5) interference with calcium deposition in bones, and (6) interference with bone collagen production (Kjellstrom, 1992).

## TREATMENT

In animals, cadmium toxicosis is prevented by minimizing exposure in the environment and in feedstuffs.

## CONCLUDING REMARKS/FUTURE DIRECTIONS

Although toxicoses in domestic animals are uncommon, exposure is gradually increasing especially in grazing animals. Since chronic cadmium toxicity is of concern to humans, continued close observation and analysis of cadmium in animals is indicated to provide a system for environmental cadmium surveillance.

## REFERENCES


ATSDR (1999) *Toxicological Profile for Cadmium*. Agency for Toxic Substances and Disease Registry. US Department of Health and Human Services, Public Health Service, Atlanta, GA.

Anke M, Kosla T, Groppel B (1989) The cadmium status of horses from central Europe depending on breed, sex, age, and living area. *Arch Tierernahrung* **39**: 657–83.

Bernard A, Roels H, Buchet JP, Cardenas A, Lauwerys R (1992) *Cadmium and Health, the Belgian Experience*, vol. 118. IARC Scientific Publications, Lyon, France, pp. 15–33.

Bianu E, Nica D (2004) Chronic intoxication with cadmium in the horses at Copsa Mica area. *Revista Romana de Medicina Veterinara* **14**: 99–106.

Dixit VP, Lohiya NK, Agrawal M (1975) Effect of cadmium chloride on testis and epididymis of dog. A biochemical study. *Acta Biol* **26**: 97–103.

Dorn CR (1979) Cadmium and the food chain. *Cornell Vet* **69**: 323–44.

Dorn CR, Reddy CS, Lamphere DN, Gaeuman JV, Lanese R (1985) Municipal sewage sludge application on Ohio farms: health effects. *Environ Res* **38**: 332–59.

Elinder CG (1992) *Cadmium as an Environmental Hazard*, vol. 118. IARC Scientific Publications, Lyon, France, pp. 123–32.

Elinder CG, Jonsson L, Piscator M, Rahnster B (1981a) Histopathological changes in relation to cadmium concentration in horse kidneys. *Environ Res* **26**: 1–21.

Elinder CG, Nordberg M, Palm B, Piscator M (1981b) Cadmium, zinc, and copper in horse liver and in horse liver metallothionein: comparisons with kidney cortex. *Environ Res* **26**: 22–32.

Fitzgerald PR, Peterson J, Lue-Hing C (1985) Heavy metals in tissues of cattle exposed to sludge-treated pastures for eight years. *Am J Vet Res* **46**: 703–7.

Grawe KP, Thierfelder T, Jorhem L, Oskarsson A (1997) Cadmium levels in kidneys from Swedish pigs in relation to environmental factors – temporal and spatial trends. *Sci Total Environ* **208**: 111–22.

Groten JP, Sinkeldam EJ, Luten JB, van Bladeren PJ (1990) Comparison of the toxicity of inorganic and liver-incorporated cadmium: a 4-wk feeding study in rats. *Food Chem Toxicol* **28**: 435–41.

Gunson DE, Kowalczyk DF, Shoop CR, Ramberg CF (1982) Environmental zinc and cadmium pollution associated with generalized osteochondrosis, osteoporosis, and nephrocalcinosis in horses. *J Am Vet Med Assoc* **180**: 295–9.

Habeebu SSM, Liu J, Klaassen CD (1998) Cadmium-induced apoptosis in mouse liver. *Toxicol Appl Pharmacol* **149**: 203–9.

Holterman WF, de Voogt P, Peereboom-Stegeman JH (1984) Cadmium/zinc relationships in kidney cortex and metallothionein of horse and red deer: histopathological observations on horse kidneys. *Environ Res* **35**: 466–81.

Houpert P, Federspiel B, Milhaud G (1997) Toxicokinetics of cadmium in lactating and nonlactating ewes after oral and intravenous administration. *Environ Res* **72**: 140–50.

Jeffrey EH, Noseworthy R, Cherian MG (1989) Age dependent changes in metallothionein and accumulation of cadmium in horses. *Comp Biochem Physiol C Comp Pharm Toxicol* **93**: 327–32.

Johnson DE, Kienholz EW, Baxter JC, Spangler E, Ward GM (1981) Heavy metal retention in tissues of cattle fed high cadmium sewage sludge. *J Anim Sci* **52**: 108–14.

Katzantzis G (2004) Cadmium, osteoporosis, and calcium metabolism. *Biometals* **17**: 493–8.

Kjellstrom T (1992) *Mechanism and Epidemiology of Bone Effects of Cadmium*. IARC Scientific Publications, Lyon, France, pp. 301–10.

Klaassen CD, Liu J (1997) Role of metallothionein in cadmium-induced hepatotoxicity and nephrotoxicity. *Drug Metab Rev* **29**: 79–102.

Klasing KC (2005) Cadmium. In *Mineral Tolerances of Animals*. National Research Council, The National Academies Press, Washington, DC.

Kobayashi J (1978) Pollution by cadmium and the itai-itai disease in Japan. In *Toxicity of Heavy Metals in the Environment*, Oehme F (ed.), Marcel Dekker, Inc., New York, NY, pp. 199–259.

Kowalczyk DF, Gunson DE, Shoop CR, Ramberg CF (1986) The effects of natural exposure to high levels of zinc and cadmium in the immature pony as a function of age. *Environ Res* **40**: 285–300.

Kuester RK, Waalkes MP, Goering PL, Fisher BL, McCuskey RS, Sipes IG (2002) Differential hepatotoxicity induced by cadmium in Fischer 344 and Sprague–Dawley rats. *Toxicol Sci* **65**: 151–9.

Lee JS, White KL (1980) A review of the health effects of cadmium. *Am J Ind Med* **1**: 307–17.

Li Y, McCrory DF, Powell JM, Saam H, Jackson-Smith D (2005) A survey of selected heavy metal concentrations in Wisconsin dairy feeds. *J Dairy Sci* **88**: 2911–22.

Lisk DJ, Boyd RD, Telford JN, Babish JG, Stoewsand GS, Bache CA, Gutenman WH (1982) Toxicologic studies with swine fed corn grown on municipal sewage sludge-amended soil. *J Anim Sci* **55**: 613–19.

Liu ZP (2003) Lead poisoning combined with cadmium in sheep and horses in the vicinity of non-ferrous metal smelters. *Sci Total Environ* **309**: 117–26.

McLaughlin MJ, Simpson PG, Fleming N, Stevens DP, Cozens G, Smart MK (1997) Effect of fertilizer type on cadmium and fluorine concentrations in clover herbage. *Aust J Exp Agric* **37**: 1019–26.

Penumarthy L, Oehme FW, Hayes RH (1980) Lead, cadmium, and mercury tissue residues in healthy swine, cattle, dogs, and horses from the midwestern United States. *Arch Environ Contam Toxicol* **9**: 193–206.

Piscator M (1985) Dietary exposure to cadmium and health effects: impact of environmental change. *Environ Health Perspect* **63**: 127–32.

Plumlee KH, Johnson B, Gardner IA (1996) Heavy metal concentrations in injured racehorses. *Vet Hum Toxicol* **38**: 204–6.

Reddy CS, Dorn CR (1985) Municipal sewage sludge application on Ohio farms: estimation of cadmium intake. *Environ Res* **38**: 377–88.

Reddy CS, Dorn CR, Lamphere DN, Powers JD (1985) Municipal sewage sludge application on Ohio farms: tissue metal residues and infections. *Environ Res* **38**: 360–76.

Roberts AHC, Longhurst RD, Brown MW (1994) Cadmium status of soils, plants and grazing animals in New Zealand. *New Zeal J Agric Res* **37**: 119–29.

Sauer J-M, Waalkes MP, Hooser SB, Kuester RK, McQueen CA, Sipes IG (1997a) Suppression of Kupffer cell function prevents cadmium induced hepatocellular necrosis in the male Sprague–Dawley rat. *Toxicology* **121**: 155–64.

Sauer J-M, Waalkes MP, Hooser SB, Baines AT, Kuester RK, Sipes IG (1997b) Tolerance induced by all-trans-retinol to the hepatotoxic effects of cadmium in rats: role of metallothionein expression. *Toxicol Appl Pharmacol* **143**: 110–19.

Sharma RP, Street JC (1980) Public health aspects of toxic heavy metals in animal feeds. *J Am Vet Med Assoc* **177**: 149–53.

Sileo L, Beyer WN (1985) Heavy metals in white-tailed deer living near a zinc smelter in Pennsylvania. *J Wildl Dis* **21**: 289–96.

Telford JN, Thonney ML, Hogue DE, Stouffer JR, Bache CA, Gutenman WH, Lisk DJ, Babish JG, Stoewsand GS (1982) Toxicologic studies in growing sheep fed silage corn cultured on municipal sludge-amended acid subsoil. *J Toxicol Environ Health* **10**: 73–85.

Vreman K, van der Veen NG, van der Molen EJ, de Ruig WG (1988) Transfer of cadmium, lead, mercury and arsenic from feed into tissues of fattening bulls: chemical and pathological data. *Netherlands J Agric Sci* **36**: 327–38.

Wentink GH, Wensing T, Kessels BG (1992) Toxicity of cadmium in cattle. *Tijdschrift Diergeneeskunde* **117**: 548–50.

Xu G, Zhou G, Jin T, Zhou T, Hammarstrom S, Bergh A, Nordberg G (1999) Apoptosis and p53 gene expression in male reproductive tissues of cadmium exposed rats. *Biometals* **12**: 131–9.

CHAPTER

# 28

# Copper

*Larry J. Thompson*

## INTRODUCTION

Copper is a transition group metal with high electrical and thermal conductivity. The chemical abbreviation for copper is Cu, which comes from the Latin word, cuprum, which refers to the island of Cyprus, known in the ancient times for large deposits of copper ore. The Bronze Age followed the discovery that adding tin (and other metals) to copper made the resulting metal alloy much more durable. Copper has been shown to be an essential element for both animals and plants but can be toxic under certain conditions. This chapter describes the toxicity and deficiency of copper in animals.

## BACKGROUND

Toxic insults from copper occur in two main categories, acute and chronic. The acute overexposure of animals to copper can occur by both oral and parenteral routes (Galey, *et al.*, 1991) but is relatively uncommon. Sheep are very susceptible to chronic copper poisoning, where even a slight excess of copper in the diet can build up over weeks or months to cause problems. Cattle and other ruminants are less likely to suffer from chronic mild to moderate copper excesses in the diet but can be affected by relatively high levels (Gummow, 1996). Monogastric animals seem to tolerate excess dietary copper much better than ruminants and high dietary copper additions (up to 250 ppm) have been used for growth promotant effects, such as in pigs and poultry. Finally, certain breeds of dogs (e.g. Bedlington terriers) have a genetic predisposition for liver copper accumulation similar to Wilson's Disease in humans (Taboada and Thompson, 1997). Each of these may have a different clinical presentation with the main target organs for acute copper exposure generally being the gastrointestinal tract, liver and kidney with the target organs for chronic problems being the liver, red blood cells and kidney.

Dietary sources of copper include normal plant concentrations of copper, with forages and cereal grains usually containing less than 10 ppm copper on a dry matter (DM) basis. Some oilseed meals can contain up to 35 ppm copper in DM. By far the largest source of copper in the diet will be feed additives such as copper sulfate, copper chloride and copper oxide. Additional sources of copper for the animal may be chelated copper sources in the diet or the administration of boluses containing copper metal wires or other solid forms of copper that remain in the rumen/reticulum and slowly dissolve over time. Misformulation of rations or errors in the mixing of feed can result in high concentrations of copper. Problems can arise from the use of feed formulated for a copper-tolerant species when used for a copper-sensitive species such as sheep, or the use of trace mineralized salt in sheep diets. Other sources for large acute oral copper exposures would include copper sulfate foot baths and copper algaecides or fungicides. Most water sources have low copper concentrations but the use of copper piping with slightly acidic water can result in additional copper being dissolved.

## PHARMACOKINETICS

Copper is primarily absorbed in the small intestine and transported in the blood by transcuprein and albumin, which serve to reduce the oxidative effects of divalent copper. In the liver, copper can be stored in the lysosomes,

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excreted in the bile or incorporated into ceruloplasmin for use and transport to cells in other parts of the body. Biliary excretion is the major mechanism responsible for copper homeostasis and identified genetic disorders of copper accumulation are due to impaired biliary excretion. Copper is utilized by essentially every cell in the body and there are several important copper-dependent enzymes including cytochrome C oxidase, superoxide dismutase, lysyl oxidase and dopamine beta hydroxylase.

In the ruminant, copper has a complex interrelationship with dietary molybdenum and sulfur which, when present in excess, will both decrease copper absorption and inhibit copper utilization. In the reducing environment of the rumen, excess sulfur favors the formation of sulfides which can inhibit copper absorption from the gastrointestinal tract. Additionally, the formation of tri- and tetrathiomolybdates can also inhibit the absorption of copper or, if in high amounts, can cause systemic effects by holding copper in a non-biologically available form (NRC, 2005).

## MECHANISM OF ACTION

Acute exposure to excess copper causes gastrointestinal irritation and can cause erosions of the mucosa as well as a blue-green discoloration of the contents and wall. Normally, the free copper concentration in cells is kept very low by copper-binding proteins such as metallothionein, glutathione and copper chaperone proteins. An excess of copper can overwhelm these binding proteins and allow free copper ions to exist in the cell which can directly bind proteins and nucleic acids. Additionally, the free copper can form reactive oxygen species and hydroxyl radicals, causing lipid peroxidation of membranes and damage to nucleic acids and cellular proteins (NRC, 2005).

Chronic copper toxicosis in sheep is caused by the inability of the sheep to increase the biliary excretion of copper in response to dietary increases. Copper will then accumulate in the liver but during this accumulation phase there will be little or no evidence of negative effects (Bremner, 1998). If accumulation continues, the liver can suffer direct damage from the high concentrations of copper. When damage is severe, hepatic necrosis develops and copper is released into the bloodstream. Additionally, stress to the animal can also accentuate this mobilization of copper from the liver and into the bloodstream. Increasing copper concentrations in the bloodstream will overwhelm the protective transport actions of transcuprein and albumin, which can then result in the lysis of red blood cells due to the oxidation of the red blood cell membrane by ionic copper. As copper is mobilized from the liver, it can accumulate in the kidney. The kidney can be damaged both from the accumulation of copper as well as the direct toxic effects of hemoglobin following the hemolytic event. Cattle can also be affected by chronic copper toxicosis, with a strong suspicion of breed differences (Du *et al.*, 1996) but with hemolysis occurring with less frequency than in sheep.

Chronic copper toxicosis in dogs is primarily seen in the Bedlington terrier where genetic studies have shown it to be an autosomal recessive disorder (Forman *et al.*, 2005). Other breeds including West Highland White terriers, Skye terriers and Doberman pinscher (Speeti *et al.*, 1998) have breed related hepatic copper accumulation, the origin of which is less defined. Chronic copper toxicosis in dogs will also have a period of copper accumulation without clinical signs or detectable damage to the liver. As copper concentrations increase, the animal develops a chronic active hepatitis with necrosis and inflammation. As with sheep, excess free copper damages many cellular components of the liver, including the lipid peroxidation of mitochondrial membranes. A sudden release of copper with the resulting hemolytic crisis is much less likely to occur in dogs. The disease usually appears at 2–6 years of age, with animals often showing no problems before this time.

## TOXICITY

There is a paucity of information on the amount of copper needed for acute poisoning in various species, but the general range is given as 25–50 mg/kg of body weight. Copper sulfate is most often implicated in clinical cases as the copper source. The toxic dose of copper sulfate in cattle is 200– 800 mg/kg, with sheep more sensitive at 20–100 mg/kg. Affected animals show clinical signs of salivation, gastroenteritis and abdominal pain which may rapidly develop to dehydration, shock and death. Animals that survive longer than 24–48 h develop liver and kidney damage and the animal may have an acute hemolytic crisis. Postmortem findings include gastroenteritis and a blue-green discoloration to the gastrointestinal tract and contents. Varying degrees of liver and kidney lesions are present, with milder lesions in acute deaths and more dramatic lesions developing after 48 h. Diagnostic testing of the intestinal tract contents shows a high level of copper. In acute deaths, there are normal levels of copper in the liver and kidney. If the animal survives over 24 h, elevated copper levels are found in liver and kidney.

In sheep, even normal levels of copper in feeds (10–20 ppm) can cause hepatic copper accumulation when the molybdenum level in feed is low (less than 1 ppm). As the copper-to-molybdenum ratio (Cu:Mo) increases above 6:1, the risk of copper accumulation increases, with those diets whose ratio is above 20:1 being very dangerous for sheep. At the higher Cu:Mo ratios, toxic accumulation of copper in the liver can occur over a matter of weeks. In

affected animals, there may be an acute hemolytic crisis and the animals show clinical signs of weakness, anorexia, icterus, dyspnea and pale mucous membranes. There may be hemoglobinuria and death is common amongst severely affected animals. Postmortem findings include icterus, swollen liver, enlarged spleen and the kidneys appear dark, often referred to as gunmetal blue or black kidneys. Antemortem testing of serum copper often shows an elevation above normal, but this decreases over time with a rapid decrease following fluid therapy. Postmortem diagnostic testing for copper should be performed on both liver and kidney. Mobilization of copper from the liver may reduce copper concentrations to normal levels but the mobilized copper is then accumulated in the kidney. The ration should be tested for both copper and molybdenum. The close environment of the sheep should be inspected for extraneous sources of copper.

Young dogs with genetic susceptibility to chronic copper toxicosis may develop an acute syndrome of weakness, vomiting and anorexia. Older dogs may present with a more chronic syndrome of weight loss and anorexia, which may progress to ascites and neurological signs related to a developing hepatic encephalopathy. If untreated, the animal may succumb to liver dysfunction and postmortem findings may include a cirrhotic liver. Antemortem diagnostics would include an evaluation of liver enzymes. In suspect cases, a liver biopsy should be used to confirm elevated copper content (Taboada and Thompson, 1997).

## TREATMENT

Treatment of animals acutely poisoned with copper mainly consists of supportive treatment directed at the shock, dehydration and damage to the gastrointestinal tract.

Treatment of sheep with severe clinical signs following hemolytic crisis is often unrewarding. Supportive care should include fluid therapy and the consideration of a blood transfusion. Ammonium or sodium molybdate (50–500 mg) and sodium thiosulfate (0.3–1 g) should be used daily as a drench for up to 3 weeks not only in affected animals but also in other animals that have received the same diet. Ammonium tetrathiomolybdate has been suggested as a treatment but is difficult to obtain. It can be administered IV or SQ at 1.7–3.4 mg/kg on alternate days for three treatments. Molybdenum in the diet can be increased to 5 ppm and zinc can be supplemented at 100 ppm to reduce copper absorption.

Dogs affected with chronic copper toxicosis should be fed a low copper diet, e.g. avoiding organs meats which are usually higher in copper. The use of oral chelating agents is suggested to enhance urinary excretion of copper. The use of d-penicillamine at 10–15 mg/kg PO twice daily or the use of trientine hydrochloride at 10–15 mg/kg PO twice daily have been suggested. Liver enzymes should be monitored every 6 months and consideration be given to liver biopsy to assess liver copper concentrations. The addition of elemental zinc to the diet (100–200 mg/day as the acetate) should be considered to reduce copper absorption.

## CONCLUSION

In trace amounts, copper is an essential element, but in excess it is a toxicant. In general, sheep and certain breeds of dogs are more susceptible to copper poisoning. Copper produces toxicity by multiple mechanisms. Poisoned animals are usually treated with ammonium molybdate and sodium thiosulfate. In addition, supportive care including fluid therapy and blood transfusion are beneficial.

## REFERENCES

Bremner I (1998) Manifestations of copper excess. *Am J Clin Nutr* **67**(Suppl.): 1069S–73S.

Du Z, Hemken RW, Harmon RJ (1996) Copper metabolism of Holstein and Jersey cows and heifers fed diets high in cupric sulfate or copper proteinate. *J Dairy Sci* **79**: 1873–80.

Forman OP, Boursnell MEG, Dunmore BJ, Stendall N, van de Sluis B, Fretwell N, Jones C, Wijmenga C, Rothuizen J, van Oost BA, Holmes NG, Binns MM, Jones P (2005) Characterization of the COMMD1 (MURR1) mutation causing copper toxicosis in Bedlington terriers. *Anim Genet* **36**: 497–501.

Galey FD, Maas J, Tronstad RJ, Woods LW, Johnson BJ, Littlefield ES, Wallstrum R, Darius LC (1991) Copper toxicosis in two herds of beef calves following injection with copper disodium edetate. *J Vet Diagn Invest* **3**: 260–3.

Gummow B (1996) Experimentally induced chronic copper toxicity in cattle. *Onderstepoort J Vet Res* **63**: 277–88.

National Research Council (NRC) (2005) Copper. In *Mineral Tolerance of Animals*, 2nd Revised edn. The National Academies Press, Washington, DC, pp. 134–53.

Speeti M, Eriksson J, Saari S, Westermarck E (1998) Lesions of subclinical doberman hepatitis. *Vet Pathol* **35**: 361–9.

Taboada J, Thompson LJ (1997) Copper hepatopathy. In *The 5 Minute Veterinary Consult: Canine and Feline*, Tilley LP, Smith FWK MacMurray AC (eds). Williams and Wilkins, Baltimore, MD, pp. 478–9.

CHAPTER

# 29

# Fluoride

*Larry J. Thompson*

## INTRODUCTION

Fluorine is a member of the halogen group on the periodic table and is rarely found in elemental form in nature but instead exists as fluoride, the monovalent anion, combined with other elements. The most common mineral containing fluoride is fluorspar, also known as fluorite ($CaF_2$), and soils generally contain calcium fluoride ($CaF_2$). Although now rare, sodium fluoride and sodium fluorosilicate ($Na_2SiF_6$) had been used as insecticides and anthelminthics. Sodium fluoroacetate (compound 1080) is another formerly used rodenticide that is rarely seen in the United States today but may be found in other parts of the world (e.g. Australia). Fluoroacetate can also be found naturally in several species of plants. (*Gastrolobium* spp., *Oxylobium* spp. and others.) Sodium fluoride, sodium fluorosilicate and fluorosilicic acid have been used in the United States for the fluoridation of drinking water for humans to prevent the development of dental caries (CDC, 2001). When the gas hydrogen fluoride (HF) is dissolved in water it forms hydrofluoric acid, a very hazardous chemical that has industrial and laboratory uses such as purifying metals, etching glass and cleaning semiconductors due to its ability to dissolve silicates and metal oxides. This chapter will stress the chronic effects of fluoride excess, also called fluorosis or fluoride toxicosis, in large animals.

## BACKGROUND

Fluoride is present at low levels in virtually all feed and water sources, thus animals will have continuing exposure throughout their lifetime. While a small amount of fluoride in the diet has been shown to improve bone and teeth development, a chronic excess of fluoride can have adverse effects on teeth, bone and other body systems. Large animals have been exposed to excess fluorides through the ingestion of high-fluoride rock phosphates used as nutritional supplements, by the ingestion of forages contaminated with excess fluorides from industrial pollutants or volcanic emissions, or through water containing excess fluorides from industrial pollution or dissolved from natural sources (Shupe and Olson, 1971, 1983). Rock phosphates destined for animal diets must be defluorinated before use or have a phosphorus to fluorine ratio (P:F) of more than 100:1 in order to avoid exposing the animal to excess fluoride (Osweiler, 2004). Following a long history of problems, industrial contamination of forages and water with excess fluoride has decreased due to recognition of the problems caused by excess fluoride and increased regulatory controls on fluoride-emitting industries. Historical point sources for fluoride emissions have included the smelting industries (e.g. aluminum, copper and steel), brick or ceramic product factories, coal-fired power plants and the phosphate-processing industries. Plant uptake of fluoride by translocation from the soil is usually not an important source of fluoride for grazing animals. Much more significant sources include airborne fluoride that settles on plant surfaces and fluoride from soil that is ingested directly or contaminates the plant (NRC, 2005). Acute fluoride poisoning in large animals is rare but can occur following exposure to fluoride containing commercial products (Bischoff *et al*., 1999) or to ash and tephra following volcanic eruptions (Shanks, 1997).

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## PHARMACOKINETICS AND MECHANISM OF ACTION

Sodium fluoride is readily absorbed from the digestive tract and is several times more biologically available than fluoride compounds from feed or environmental sources. Fluoride is distributed to all parts of the body with approximately 50% of absorbed fluoride being excreted by the kidneys. The remainder will be incorporated into bone and teeth with very little accumulation in the soft tissue. Fluoride concentrations in the blood, urine and soft tissues may reflect recent ingestions but will also increase slowly over time with continuing excess fluoride exposure and accumulation in the bone. Greater than 95% of the body burden of fluoride will be contained in the bones with bone levels dependent upon the amount of fluoride ingested, duration of exposure, bioavailability, species, age and diet of the animal involved. If dietary fluoride exposure decreases, bone fluoride levels will decrease slowly over a long period of time. In cattle there appears to be a partial placental barrier to the movement of fluoride to the fetus as even high levels of fluoride in the diet of the dam did not adversely affect the health of the calves, even though higher fetal blood and bone fluoride concentrations resulted (NRC, 2005). Fluoride is excreted in the milk but this does not appear to be a significant source for the neonate.

The major adverse effects of chronic excess fluoride ingestion concern the teeth and bones of affected animals. Fluoride substitutes for hydroxyl groups in the hydroxyapatite of the bone matrix which alters the mineralization and crystal structure of the bone. Bone changes induced by excess fluoride ingestion, termed skeletal fluorosis or osteofluorosis, include the interference of the normal sequences of osteogenesis and bone remodeling with the resulting production of abnormal bone or the resorption of normal bone. The fluoride content of bone can increase over a period of time without other noticeable changes in the bone structure or function. Once lesions start to develop, they are usually bilateral and symmetric. The most consistent gross changes are abnormal bone formation on the periosteal surface with thickening of the cortex. In cattle, earliest clinical changes usually occur on the ribs and mandible as well as the medial surfaces of the metatarsal and metacarpal bones. Histologically, bones will have abnormal remodeling and mineralization with irregular collagenous fibers and excess osteoid tissues. While an excess of ingested fluorides can adversely affect the bones at any time in the animal's life, the bones in younger animals are more responsive to the excess fluoride.

Dental fluorosis develops when the period of excess fluoride intake occurs during the period of tooth development, in cattle this will generally be before 30–36 months of age. Teeth are affected during development with damage to ameloblasts and odontoblasts and the resulting abnormal matrix unable to mineralize normally (Shearer *et al.*, 1978). Both enamel and dentine are adversely affected. Affected teeth may erupt with mottling (alternating white opaque horizontal areas or striations in the enamel), hypoplasia, dysplasia (abnormal soft dull white chalky enamel or horizontal zones of constriction), erosion or pitting of enamel and affected teeth are prone to excessive abrasion and discoloration.

Acute fluoride toxicosis occurs when soluble forms of fluoride (e.g. sodium fluoride) are ingested in large doses. Absorption is rapid and clinical signs can appear within 30–60 min following ingestion. Although the exact mechanism of action is not known with certainty, fluoride concentrations in blood and soft tissues rapidly increase which leads to hypocalcemia. Sudden death from acute fluoride exposure is thought to involve the development of hyperkalemia or diminished $Na^+/K^+$-ATPas activity and the inhibition of glycolysis (NRC, 2005).

## TOXICITY

There are a number of factors that influence the amount of fluoride required to produce specific lesions and clinical signs including the amount of fluoride ingested, duration of exposure, bioavailability, species, age and diet of the animal involved. The point where fluoride ingestion becomes detrimental to the animal also varies from animal to animal. Clinical signs develop slowly and can be confused with other chronic problems. Animals often show non-specific intermittent stiffness and lameness, which appear to be associated with periosteal overgrowth leading to spurring and bridging near joints as well as ossification of ligaments, tendon sheaths and tendons. The clinical presentation may easily be confused with other conditions, such as degenerative arthritis, but the lesions associated with fluorosis are not primarily associated with articular surfaces. In severe cases, affected cattle may become progressively more lame and eventually may refuse to stand or may stand with rear legs upright and be on their knees to graze (Shupe and Olson, 1983). Lameness in cattle leads to abnormal hoof wear with elongated toes, especially in the rear legs. In long-term studies with cattle on varying levels of fluoride intake, skeletal neoplasms were not seen even in cattle with severe osteofluoritic lesions (Shupe *et al.*, 1992).

A great deal of effort has gone into the classification of dental lesions in cattle produced by excess fluoride ingestion. The incisor teeth are evaluated for enamel defects and abrasion pattern. The usual classification system ranges from a value of 0 for normal teeth to a value of 5 for severe fluoride effects (Shearer *et al.*, 1978; Shupe *et al.*,

1992). Because of the nature and complexity of the disease these dental lesion should not be the sole criterion for diagnosis. In general, severely affected teeth appear with brown or black discoloration, may have enamel defects and show increased wear including exposure of the pulp cavity, which causes pain while chewing roughage or swallowing extremely cold water. There will be a correlation between lesions on incisor teeth and those cheek teeth that form and mineralize at the same time. Cheek teeth that are abnormally worn cause improper mastication with roughage being difficult for the animal to utilize. The animal will have variable and decreased intake and the decreased production, slowed growth and general poor health associated with poor nutritional status. Animals with chronic exposure to excess fluorides have dry skin and hair coat.

Acute fluoride poisoning of cattle can result in clinical signs of depression, weakness and ataxia with postmortem findings of gastroenteritis (Bischoff *et al.*, 1999) and degenerative changes in the renal tubular epithelium.

## TREATMENT

Diagnosis of chronic fluoride toxicosis is based upon clinical signs, exposure history, dental lesions, evaluation of lameness and bony lesions and elevated urinary fluoride concentrations. Normal cattle urine contains less than 6 ppm fluoride. Animals having recent exposure or continuing release from fluorotic bone will have 15–20 ppm urinary fluoride (Osweiler, 2004). The biopsy of a rib or coccygeal vertebrae for fluoride analysis, as well as radiographic examination of teeth or bones, may also be helpful. A full postmortem examination should be performed, with attention to bone and teeth for both gross and histopathologic examination. Various bone samples should be submitted for fluoride analysis including metatarsal, metacarpal, rib, pelvis and mandible. Normal values for cattle are 400–1200 ppm fluoride on a dry, fat-free basis. Animals affected with chronic fluorosis can contain 3000–5000 ppm fluoride on a dry, fat-free basis. Analysis of feed, water or suspect material should also be included.

There is no specific antidote or treatment for chronic fluoride toxicosis. Sources of excess fluoride should be identified and removed from the diet. With reduction of dietary fluoride to background levels, mild to moderate bone changes may be reduced and normal bone laid down. Extensive bone lesions will not be remodeled to normal and teeth lesions are irreversible. Symptomatic and supportive care for animals with bone and teeth changes include providing high quality easily masticated feeds, limited grazing area and provisions to avoid cold or frozen water. For mild fluorotic changes, improving the diet and grazing may avoid excess wear on the teeth and reduce mastication problems.

## REFERENCES

Bischoff KL, Edwards WC, Fearer J (1999) Acute fluoride toxicosis in beef cattle. *Bovine Practition* **33**: 1–3.

Centers for Disease Control and Prevention (CDC) (2001) Recommendations for using fluoride to prevent and control dental caries in the United States. *MMWR Recomm Rep* **50**(RR-14): 1–42.

National Research Council (NRC) (2005) Fluorine. In *Mineral Tolerance of Animals*, 2nd Revised edn. The National Academies Press, Washington, DC, pp. 154–81.

Osweiler GD (2004) Fluoride. In *Clinical Veterinary Toxicology*, Plumlee KH (ed.). Mosby, St. Louis, MO, pp. 197–200.

Shanks DF (1997) Clinical implications of volcanic eruptions on livestock – case studies following the 1995 and 1996 eruptions of Mt. Ruapehu. *Proc of the Society of Sheep and Beef Cattle Veterinarians of the New Zealand Veterinary Association*. Massey University, Palmerston North, New Zealand, **27**(1): 1–13.

Shearer TR, Kolstad DL, Suttie JW (1978) Bovine dental fluorosis: histologic and physical characteristics. *Am J Vet Res* **39**: 597–602.

Shupe JL, Olson AE (1971) Clinical aspects of fluorosis in horses. *J Am Vet Med Assoc* **158**: 167–74.

Shupe JL, Olson AE (1983) Clinical and pathological aspects of fluoride toxicosis in animals. In *Fluorides: Effects on Vegetation, Animals and Humans*, Shupe JL, Peterson HB, Leone NC (eds). Paragon Press, Inc., Salt Lake City, UT, pp. 319–38.

Shupe JL, Bruner RH, Seymour JL, Alden CL (1992) The pathology of chronic bovine fluorosis: a review. *Toxicol Pathol* **20**: 274–85.

C H A P T E R

# 30

# Iron

*Stephen B. Hooser*

## INTRODUCTION

Iron is an essential element necessary for animal and plant life. It works as an oxygen ($O_2$) carrier in hemoglobin/myoglobin and is involved in numerous biological oxidation–reduction reactions, including photosynthesis. Iron is present in cytochrome P450 and is crucial for the metabolism of many chemicals in the liver, kidney, and other organs. Deficiencies in iron can result in anemia. Excess iron can cause iron overload and organ damage, while oxidation of ferrous to ferric iron in hemoglobin results in methemoglobinemia and an inability of red blood cells (RBCs) to carry $O_2$. Consequently, because of its importance, the uptake, distribution, storage, and excretion of iron is very tightly regulated in animals under normal conditions. Because of its reactivity, free iron compounds are sequestered with proteins to ensure that they do not initiate intracellular oxidative damage through electron donation and the formation of reactive oxygen species (ROS) such as hydroxyl radical. In this chapter, we will deal primarily with clinical animal exposures to excess iron through ingestion, parenteral administration, or genetic iron storage abnormalities. Nutritional deficiencies or exposure to chemicals causing methemoglobin formation such as nitrates in ruminants, or nitrites and chlorates in all species are discussed elsewhere.

## BACKGROUND

Iron is very abundant in the universe and is the fourth most abundant element on earth. Its atomic number is 26 and its atomic weight is 55.847. It has been recognized to be an essential nutrient for more than 100 years and is present in all the cells of the body. The largest amount of iron is incorporated into the proteins hemoglobin and myoglobin. Within RBCs (erythrocytes), hemoglobin transports $O_2$ from the lungs to cells throughout the body, while myoglobin binds $O_2$ for use in muscle cells (Klasing, 2005). Iron present in the serum is bound to the protein transferrin, and in milk is bound to lactoferrin. Iron-containing proteins in the mitochondrial electron transport chain are essential for oxidative phosphorylation and energy production. Iron is also contained in enzymes of the Krebs cycle and in cytochromes P450 which are necessary for the metabolism of chemicals (Fairbanks, 1994).

The iron content of feedstuffs can be highly variable depending on the components. Iron in plants is subject to wide variation depending on the type of plant and the amount of iron in the soil, while many animal-based feed components are often rich in usable iron. The iron content of water can also vary greatly (Klasing, 2005). Large amounts of iron in water give the water a rusty color and a metallic taste, but upper limits of iron in drinking water for livestock and poultry have not been established since experimental data are not sufficient to make definite recommendations (NRC, 1974).

Worldwide, iron-deficiency anemias affect large numbers of people, but nutritional iron deficiencies are much less of a problem in animals. However, there are groups of animals which are vulnerable to iron deficiency including newborn piglets, veal calves, and those animals with parasitic infestations (Underwood, 1977). For instance, newborn piglets have very low concentrations of liver iron (29 mg/kg) compared to newborn rabbits whose liver iron stores average 135 mg/kg.

There is accumulating evidence that excessive iron deposits in the brain, and alterations in iron metabolism play an important role in neurodegenerative diseases (Connor *et al*., 1995; Lan and Jiang, 1997; Fredriksson *et al*., 1999;

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Dal-Pizzol *et al.*, 2001; Qian and Shen, 2001; Arosio and Levi, 2002).

## PHARMACOKINETICS/ TOXICOKINETICS

The homeostatic regulation of iron in the body is complex and involves uptake, transport, utilization, storage, and loss. Under normal conditions, iron is poorly absorbed from most diets with approximately 5–15% absorbed from the gastrointestinal (GI) tract. This uptake can double in iron deficiency. The body has a very limited ability to excrete iron, therefore iron homeostasis is maintained by adjusting iron absorption to the body's needs. The amount of dietary iron that is absorbed through the GI tract is determined by the needs of the individual animal and is inversely related to serum ferritin concentrations (Bothwell *et al.*, 1979). There are four main factors influencing iron absorption in the GI tract: 1) individual factors including the animal's age, iron status, and health, 2) conditions in the GI tract, 3) the chemical form and amount of iron ingested, and 4) other components of the diet which can enhance or reduce intestinal absorption. Iron is absorbed by enterocytes of the small intestine in ferrous ($Fe^{2+}$) form and transferred to the serum where it is converted to the ferric ($Fe^{3+}$) form and bound to transferrin (Goyer and Clarkson, 2001). In normal animals, the majority of fecal iron comes from ingested iron which is not absorbed. Once absorbed, the body vigorously retains ingested iron unless bleeding occurs with daily iron loss limited to about 0.01% (of the body total) per day (Goyer and Clarkson, 2001). It has been found that even in the face of hemolytic anemia with destruction of erythrocytes, less than 1% of the iron is excreted in the urine and feces (Underwood, 1977).

In the bloodstream, serum iron is primarily bound to transferrin with lesser amounts bound to ferritin. Iron in the serum forms a pool from which it enters, is transported, leaves, and re-enters at a variable rate for the synthesis of hemoglobin, ferritin, cytochromes, and other iron-containing proteins. Of the total iron in the body, approximately two-thirds is bound to hemoglobin and 10% to myoglobin and iron-containing enzymes, with the remainder bound to the storage proteins ferritin and hemosiderin (Goyer and Clarkson, 2001). Ferritin and hemosiderin are found throughout the body with the main concentrations being in the liver, spleen, and bone marrow. They are protective in that they keep cellular iron in a bound form. Ferritin contains up to 20% iron while hemosiderin is up to 35% iron. In the normal animal, non-viable RBCs are removed from the circulation by cells of the reticuloendothelial system in the liver, spleen, and bone marrow. There, heme is broken down and the iron recycled for further use. In aged animals, when large amounts of iron are injected and rapidly cleared from the serum, or during chronic iron storage disease, the iron is preferentially deposited as hemosiderin thereby increasing intracellular concentrations and giving rise to the hemosiderosis that can be seen histologically (Underwood, 1977; Goyer and Clarkson, 2001).

## MECHANISM OF ACTION

For many functions, the body utilizes ferrous ($Fe^{2+}$) iron to bind molecular $O_2$. In this way, $O_2$ is transported by hemoglobin to cells in the peripheral tissues, myocytes bind $O_2$ for intracellular utilization, mitochondrial proteins of the electron transport chain bind $O_2$ for energy production, and P450 enzymes bind $O_2$ for its use in phase I metabolism of endogenous and xenobiotic chemicals. However, because of its reactivity, iron in its ferrous state must be carefully sequestered away to prevent the formation of highly ROS which can elicit severe cellular damage. Ferrous iron ($Fe^{2+}$) and other transition metal ions, $Cu^{+}$, $Cr^{5+}$, $Ni^{2+}$, or $Mn^{2+}$ can catalyze the formation of hydroxyl ion ($HO^{-}$) and the extremely reactive and dangerous hydroxyl radical ($HO^{\bullet}$) from the reduction of endogenous hydrogen peroxide (HOOH) via the *Fenton reaction*:

$$HOOH + Fe^{2+} \rightarrow Fe^{3+} + HO^{-} + HO^{\bullet} \qquad 30.1$$

The Fenton reaction causes site specific accumulation of free radicals and initiates biomolecular damage. Free radicals are molecules or molecular fragments that contain one or more unpaired electrons in their outer orbital shell. If produced in great enough quantities to overwhelm the cellular antioxidant and radical-quenching protective mechanisms, hydroxyl radicals promote the formation of more hydroxyl radicals and other ROS such as superoxide. Superoxide combines with nitric oxide and forms peroxynitrite, which is as detrimental as hydroxyl radical. These ROS/RNS damage and destroy proteins and DNA by causing cross-linking, which inhibits their normal functions, or by initiating extensive damage and spontaneous degeneration of molecules such as lipids. ROS/RNS not only cause DNA damage, but also inhibit repair activities. ROS induce lipid peroxidation which if not quenched, can initiate a chain reaction of lipid destruction and ROS formation destroying vital cell membranes in mitochondria, nuclei, and the cell periphery. Together, these effects can be of great enough magnitude to cause cell death, organ dysfunction, and death. However, it is also of interest to note that iron is necessary for the normal functioning of macrophages and other leucocytes during the respiratory burst in inflammation to catalyze the formation of bactericidal hydroxyl radical (Gregus and Klaassen, 2001).

# TOXICITY

## General

Iron poisoning is not common in animals although potentially it could occur in any species. Clinical cases of acute iron toxicosis have been reported in dogs, pigs, horses, cattle, and goats (Greentree and Hall, 1983; Ruhr *et al.*, 1983; Osweiler *et al.*, 1985; Holter *et al.*, 1990). Toxicity can occur through ingestion or parenteral administration. Because of their indiscriminate eating habits and close proximity to people and their nutritional supplements, dogs are the species most likely to ingest large quantities of iron-containing vitamins. Baby pigs are iron deficient at birth and require iron supplementation, which can result in peracute or acute toxicity. Limited cases of iron poisoning have occurred in horses and cattle through the use of iron supplements. In general, toxicity occurs in the GI mucosa (oral exposure), liver, myocardium, and other tissues when the iron binding capacity of the body is overwhelmed, and free iron causes oxidative damage. Genetic iron storage diseases are uncommon, but have been reported in mynah birds, toucans, Saler cattle, Egyptian fruit bats, and rarely in horses.

## Acute toxicity in dogs, cattle, pigs, and adult horses

Acute iron toxicity has been reported in dogs (Greentree and Hall, 1983), cattle (Ruhr *et al.*, 1983), pigs (Velasquez and Aranzazu, 2004), horses (Arnbjerg, 1981), and humans. In dogs, toxicosis primarily occurs through the accidental ingestion of large amounts of iron-containing vitamins or other iron supplements. In cattle, horses and pigs, it has occurred through accidental administration of excess amounts of iron supplements by oral or parenteral routes. Diagnosis is based on history, appropriate clinical signs, and radiography in small animals as iron-containing pills are radiodense. In general, if the animals remain asymptomatic for greater than 8 h following a single exposure, it is reported that they are unlikely to develop iron toxicity. In all species, ingestion of a toxic dose (roughly greater than 20 mg/kg in dogs) initially results in necrosis of the GI mucosal cells. This is followed by fluid loss, direct cardiotoxicity, and widespread organ damage through the mechanisms described above. Fluid loss and decreased cardiac output can lead to circulatory shock. Iron toxicity has been described as occurring in four stages. Stage I occurs 0–6 h post-ingestion and is characterized by vomiting, diarrhea, abdominal pain, and depression. Stage II occurs from 6 to 24 h post-ingestion and is characterized by apparent recovery. Stage III begins at 12–96 h with commencement of additional vomiting, diarrhea, abdominal pain, GI hemorrhage, weakness, shock, and possibly death. Stage IV, if it occurs, begins 2–6 weeks after ingestion and is characterized by GI fibrosis and obstruction (Greentree and Hall, 1983).

## Neonatal pigs

Paradoxically, iron toxicity in piglets resulting from oral supplementation or parenteral injection occurs because of the very low liver iron stores in newborn pigs and the need to supplement the small amounts of iron that they receive in sow's milk. Because of their low iron stores at birth, iron-deficiency anemia can occur in non-supplemented baby pigs within 2–4 weeks after birth. This is manifested clinically in the pigs as dyspnea, anorexia, increased infections, and poor growth with some deaths. It is prevented by oral or parenteral injection of iron-containing compounds (Underwood, 1977; Osweiler *et al.*, 1985). When comparing routes of administration of the same iron compound the potential for toxicity is the greatest after intravenous injection followed by intramuscular injection with oral administration being the least toxic. In acute iron toxicosis of pigs, two syndromes are recognized. The first is a peracute syndrome which is characterized by sudden death minutes to a few hours after iron injection. In some ways this resembles an anaphylactic reaction in its rapidity of onset, vascular collapse, and death, but the exact mechanism is not known. This peracute syndrome has also been reported in horses following administration of iron compounds (Lannek and Persson, 1972; Bergsjoe, 1974). The second syndrome described in pigs is a subacute to acute syndrome characterized by GI necrosis, severe depression, coma, and death which can occur in the four stages described above. Pigs born to sows deficient in vitamin E and selenium are reported to be more susceptible to iron toxicosis (Osweiler *et al.*, 1985; Velasquez and Aranzazu, 2004).

## Neonatal horses

Although reports of iron toxicosis in horses and ponies are rare, several cases in the 1980s are of note. Newborn foals, 2–5 days old, were given an oral nutritional supplement containing ferrous fumarate. They became ill, icteric, weak, and died of liver failure within 1–5 days following administration. The histologic lesions in the livers of these foals were remarkable in that they had the appearance of longer-term chronic lesions even though they were as little as 24 h old. These hepatic lesions consisted of prominent bile ductule proliferation, hepatic cell necrosis, and periportal fibrosis (Divers *et al.*, 1983; Acland *et al.*, 1984). Through experimental administration, it was determined that ferrous fumarate, administered orally to foals within the first few days after birth, caused the liver failure seen in the earlier clinical cases (Mullaney and Brown, 1988). The

increased sensitivity of the neonatal foals is thought to be from increased absorption in the GI tract and lower systemic iron binding capacity (Poppenga, 2002). The iron overload in these foals resulting from oral administration of ferrous fumarate caused hepatic necrosis with bile ductule proliferation and fibrosis. It is speculated that what was histologically interpreted as proliferation of bile ductules was, in fact, proliferation of hepatic stem cells (oval cells) in response to the severe hepatic damage (personal observation). Experimentally, adult ponies have been administered iron as ferrous sulfate at 50 mg/kg/day orally for up to 8 weeks. Through the study and for 20 weeks after the end of dosing, no adverse clinical signs or hepatic damage were reported (Pearson and Andreasen, 2001).

### Iron storage disease

Iron storage disease resulting in hemosiderosis and hemochromatosis has been reported in several different species of animals. Hemochromatosis is the pathologic accumulation of iron in tissues, while hemosiderosis is the non-pathologic accumulation of iron. In birds, iron storage disease has been reported in mynah birds, toucans, birds of paradise, and quetzals. The clinical signs are dyspnea, hepatic damage/insufficiency, and death in mynah birds. Sudden death has been reported in toucans. It has been shown that the cause in mynah birds is due to maintenance of iron uptake from the GI tract, despite excess hepatic iron accumulation (Mete *et al*., 2003). The treatment in birds can consist of a low iron diet with phlebotomy or the use of iron chelators (Rodenbusch *et al*., 2004). A genetic, inheritable defect resulting in hemochromatosis, clinical wasting, and skeletal defects can occur in Salers cattle beginning at 9–22 months of age (O'Toole *et al*., 2001; Norrdin *et al*., 2004). Egyptian fruit bats (*Rousettus aegyptiacus*) are also known to have genetic iron storage disease. In these bats, it was found that if the sum of transferrin saturation and serum iron was greater than 51, the individual bat had a high probability of having iron overload. When the sum was greater than 90, there was a high probability of having hemochromatosis (Farina *et al*., 2005). In addition, although rarely reported, hemochromatosis with liver damage has also been reported in horses (Pearson *et al*., 1994) and captive Northern fur seals (Mazzaro *et al*., 2004). Finally, one case of hemochromatosis secondary to repeated blood transfusions every 6–8 weeks for 3 years for the treatment of red cell aplasia has been reported in an aged miniature schnauzer (Sprague *et al*., 2003).

## TREATMENT

Treatment of iron toxicity varies with the inciting cause, dose, and duration of the disease. General therapy is to limit absorption (although activated charcoal is ineffective at binding iron), provide symptomatic and supportive care, remove gastric bezoars of sticky iron-containing pills, surgically if necessary, and increase excretion. Because the body has limited ability to excrete excess iron (other than through bleeding), urinary excretion can be enhanced through the use of a chelating agent. A specific chelator of iron, deferoxamine has been used in the treatment of iron toxicity. Deferoxamine has a strong affinity for iron, a low affinity for calcium, and competes effectively for iron in ferritin and hemosiderin, but not in transferrin, hemoglobin, or heme-containing enzymes. It is poorly absorbed orally, and so is given parenterally by slow intravenous drip. Since it is excreted primarily by the kidneys, it should be given carefully to patients with renal insufficiency (Osweiler *et al*., 1985; Goyer and Clarkson, 2001; Poppenga, 2002).

## CONCLUDING REMARKS

Iron is essential for normal physiological functioning. Iron-deficiency anemia is common in humans and also occurs in piglets, veal calves, and parasitized animals. Therefore, iron supplements are readily available and widely used. In addition, some species have a propensity for genetic iron storage diseases. Fortunately, both acute and chronic iron toxicoses are rare in animals. When toxicosis does occur, cellular damage is caused by the presence of free iron in excess of the body's capacity to bind and sequester it. The free iron then initiates the generation of ROS in excess of cellular oxidant defenses resulting in lipid peroxidation of membranes, protein and DNA crosslinking, and cell death.

## REFERENCES

Acland HM, Mann PC, Robertson JL, Divers TJ, Lichtensteiger CA, Whitlock RH (1984) Toxic hepatopathy in neonatal foals. *Vet Pathol* **21**: 3–9.

Arnbjerg J (1981) Poisoning in animals due to oral application of iron. With description of a case in a horse. *Nordisk Veterinaermedicin* **33**: 71–6.

Arosio P, Levi S (2002) Ferritin, homeostasis, and oxidative damage. *Free Rad Biol Med* 33: 457–63.

Bergsjoe T (1974) Death in association with parenteral administration of iron in horses. Also a short comparison with similar events in other species. *Norsk Veterinaer-Tidsskrift* **85**: 346–9.

Bothwell TH, Charlton RW, Cook JD, Finch CA (1979) Iron metabolism in man. Blackwell Scientific Publications, Oxford.

Connor JR, Pavlick G, Karli D, *et al.* (1995) A histochemical study of iron-positive cells in the developing rat brain. *J Comp Neurol* **355**: 111–23.

Dal-Pizzol F, Klamt F, Frota Jr MLC *et al.* (2001) Neonatal iron exposure induces oxidative stress in adult Wistar rat. *Develop Brain Res* 130: 109–14.

Divers TJ, Warner A, Vaala WE, Whitlock RH, Acland HA, Mansmann RA, Palmer JE (1983) Toxic hepatic failure in newborn foals. *J Am Vet Med Assoc* **183**: 1407–13.

Fairbanks VF (1994) Iron in medicine and nutrition. In *Modern Nutrition in Health and Disease*, Shils M, Olson J, Shike M (eds). Lea & Febiger, Philadelphia, pp. 185–213.

Farina LL, Heard DJ, LeBlanc DM, Hall JO, Stevens G, Wellehan JFX, Detrisac CJ (2005) Iron storage disease in captive Egyptian fruit bats (*Rousettus aegyptiacus*): relationship of blood iron parameters to hepatic iron concentrations and hepatic histopathology. *J Zoo Wild Med* **36**: 212–21.

Fredriksson A, Schröder N, Eriksson P, *et al*. (1999) Neonatal iron exposure induces neurobehavioural dysfunction in mice. *Toxicol Appl Pharmacol*, **155**: 25–30.

Goyer RA, Clarkson TW (2001) Toxic effects of metals. In *Toxicology, The Basic Science of Poisons*, Klaassen CD (ed.). McGraw-Hill Co., New York, pp. 811–67.

Greentree WF, Hall JO (1983) Iron toxicosis. In *Kirk's Current Veterinary Therapy XII*, Bonagura JD (ed.). pp. 240–2.

Gregus Z, Klaassen CD (2001) Mechanisms of toxicity. In *Toxicology, The Basic Science of Poisons*, Klaassen CD (ed.). McGraw-Hill Co., New York, pp. 35–81.

Holter JA, Carson TL, Witte ST (1990) Acute iron intoxication in a herd of young bulls. *J Vet Diag Invest* **2**: 229–30.

Klasing KC (2005) Iron. In *Mineral Tolerances of Animals*. National Research Council, The National Academies Press, Washington, DC.

Lan J, Jiang DH (1997) Excessive iron accumulation in the brain: a possible potential risk of neurodegeneration in Parkinson's disease. *J Neural Transm* 104: 649–60.

Lannek N, Persson S (1972) Shock following parenteral iron injections in horses. *Svensk Veterinartidning* **24**: 341–3.

Mazzaro LM, Dunn JL, St. Aubin DJ, Andrews GA, Chavey PS (2004) Serum indices of body stores of iron in northern fur seals (*Callorhinus ursinus*) and their relationship to hemochromatosis. *Zoo Biol* **23**: 205–18.

Mete A, Hendriks HG, Klaren PHM, Dorrestein GM, van Dijk JE, Marx JJM (2003) Iron metabolism in mynah birds (*Gracula religiosa*) resembles human hereditary haemochromatosis. *Avian Pathol* **32**: 625–32.

Mullaney TP, Brown CM (1988) Iron toxicity in neonatal foals. *Eq Vet J* **20**: 119–24.

Norrdin RW, Hoopes KJ, O'Toole D (2004) Skeletal changes in hemochromatosis of salers cattle. *Vet Pathol* **41**: 612–23.

NRC (National Research Council) (1974) *Nutrients and Toxic Substances in Water for Livestock and Poultry.* National Academy Press, Washington, DC.

Osweiler GD, Carson TL, Buck WB, van Gelder GA (1985) Iron. In *Clinical and Diagnostic Veterinary Toxicology*. Kendall/Hunt Publishing Co., Dubuque, pp. 104–6.

O'Toole D, Kelly EJ, McAllister MM, Layton AW, Norrdin RW, Russell WC, Saeb-Parsy K, Walker AP (2001) Hepatic failure and hemochromatosis of Salers and Salers-cross cattle. *Vet Pathol* **38**: 372–89.

Pearson EG, Andreasen CB (2001) Effect of oral administration of excessive iron in adult ponies. *J Am Vet Med Assoc* **218**: 400–4.

Pearson EG, Hedstrom OR, Poppenga RH (1994) Hepatic cirrhosis and hemochromatosis in three horses. *J Am Vet Med Assoc* **204**: 1053–6.

Poppenga R H (2002) Iron toxicosis. In *The 5-Minute Veterinary Consult – Equine*, Brown C, Bertone J (eds). Lippincott, Williams & Wilkins, Baltimore, pp. 590–1.

Qian ZM, Shen X (2001) Brain iron transport and neurodegeneration. *Trends Molec Med* **7**: 103–8.

Rodenbusch CR, Canal CW, dos Santos EO (2004) Hemosiderosis and hemochromatosis in wild birds – a review. *Clin Vet* **9**: 44–50.

Ruhr LP, Nicholson SS, Confer AW, Blakewood BW (1983) Acute intoxication from a hematinic in calves. *J Am Vet Med Assoc* **182**: 616–18.

Sprague WS, Hackett TB, Johnson JS, Swardson-Olver CJ (2003) Hemochromatosis secondary to repeated blood transfusions in a dog. *Vet Pathol* **40**: 334–7.

Underwood EJ (1977) Iron. In *Trace Elements in Human and Animal Nutrition*, Underwood EJ (ed.). Academic Press, New York, pp. 13–55.

Velasquez JI, Aranzazu DA (2004) An acute case of iron toxicity in newborn piglets from vitamin E/Se deficient sows. *Revista Colombiana de Ciencias Pecuarias* **17**: 60–2.

CHAPTER

# 31

# Lead

*Larry J. Thompson*

## INTRODUCTION

Lead is a bluish white to gray heavy metal that was probably the first toxic element recognized by man and yet still has great relevance today. The chemical symbol for lead, Pb, is short for the Latin word plumbum, meaning liquid silver. The main source of lead is the ore named galena, which contains lead sulfide. The main use of lead today is in lead–acid storage batteries but historically it has had widespread usage in paints (white, yellow and red pigments) and as a gasoline additive (tetraethyl lead) although these latter uses have essentially been phased out. A debate still continues as to the role lead played in the fall of the Roman Empire. Although lead was indeed used in some Roman water pipes (the word plumbum leading to our modern word, plumber) the main source of lead for the Romans was probably sapa, a syrup used to sweeten wine and preserve fruit, which was made by boiling grape juice in lead pots thereby adding lead acetate to the liquid. The use of lead continues in our modern world with lead-containing solder in our electronics as well as lead-containing glass, from cathode ray tube monitors to fine crystal. Among all the metals, lead poisoning is encountered with greatest frequency in certain species of animals and poses a serious concern to animal health. This chapter describes the toxicity of lead in mammalian and avian species.

## BACKGROUND

Lead is a toxic element and has not been shown to be an essential trace element for nutrition. The historical use of lead in gasoline, paint, construction materials and many other products has resulted in lead being one of the most significant environmental contaminants in the world. Additional sources of lead have included lead weights (e.g. for fishing or curtains), small lead trinkets and toys, lead shot and bullets for weapons, lead arsenate pesticides and many other products as well as single source environmental contamination from mining, smelting and recycling operations. As a result of increased regulation of lead and the decreasing use of lead-containing products, the overall incidence of lead poisoning (also called plumbism) in animals and humans has been decreasing. While environmental contamination with lead does not resolve readily, the overt poisoning of domestic animals from environmental sources (e.g. from contaminated forages, plants, water or other food sources) has been decreasing in number. Still lead poisoning in animals encountered with greatest frequency compared to any other metal.

## TOXICOKINETICS

The main route of entry of lead into the body is the digestive tract with absorption dependent upon the chemical form of lead and the physiological state of the animal. Organic lead compounds are, in general, more readily absorbed than either inorganic lead salts or the metallic form of lead. Dermal absorption of organolead compounds can be significant but the salt or metallic forms of lead are not absorbed dermally. The fumes from heated lead or very fine particles ($<0.5\,\mu m$) of lead can enter the lung alveoli and be absorbed with the larger particles lodging in the ciliated portion of the bronchial tree. These larger particles can be transported up by mucociliary action and then swallowed with absorption through the

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gastrointestinal tract. Fine particles of lead and lead salts can be solubilized in the acid environmental of the stomach and the small intestine is the site of most lead absorption. Absorption of lead from the alimentary tract is usually influenced by dietary factors and by the size of lead particles. High dietary fat and mineral deficiency can increase lead absorption by 7- and 20-fold, respectively. In an experimental study, dogs maintained on a high fat, low calcium diet absorbed significantly more lead than those kept on a balanced diet (Hamir *et al*., 1988). Young animals absorb a larger portion of the lead from the gastrointestinal tract than do adults. Animals with a calcium deficiency have increased absorption of lead. Pregnancy or lactation as well as deficiencies of iron, zinc or vitamin D can also enhance lead absorption. Lead crosses the placental barrier and the residue can be detected in significant amounts in fetal blood and organs. Among the fetal organs, the highest concentrations are found in the blood and liver (Kelman and Walter, 1980; O'Hara *et al*., 1995). Lead also passes through the milk.

Nonruminant animals absorb approximately 10% of dietary lead, and ruminants absorb less than 3%. Young animals can absorb up to 90% of the ingested lead. Following absorption, a large proportion of lead is carried on erythrocyte membranes (60–90%, species dependent) with most of the remainder of lead bound to protein or sulfhydryl compounds, with only a very small proportion found free in the serum. Lead is widely distributed in the body, including crossing the blood–brain barrier (Seimiya *et al*., 1991). In the soft tissues lead binds to various proteins as well as metallothionein but accumulates in the active bone matrix (about 90%) serving as a relatively inert reservoir of lead in the body. This reservoir can be mobilized by lactation, pregnancy or the action of chelating agents. Otherwise lead has a very slow turnover rate from the bone. Lead is normally very slowly excreted via the bile with very little in the urine. Chelation therapy greatly increases the urinary output of lead.

## MECHANISM OF ACTION

Lead interferes with several biochemical processes in the body by binding to sulfhydryl and other nucleophilic functional groups causing inhibition of several enzymes and changes in calcium/vitamin D metabolism. Lead also contributes to oxidative stress within the body. Lead inhibits the body's ability to make hemoglobin by interfering with several enzymatic steps in the heme pathway. Specifically, lead decreases heme biosynthesis by inhibiting delta-aminolevulinic acid dehydratase and ferrochelatase activity. These changes contribute to the anemia that develops in chronic lead poisoning. An increased fragility of red blood cells also contributes to the anemia.

From various experimental studies, biochemical and pathological evidence suggests that lead is neurotoxicant, as it significantly disrupts certain brain structures and functions. High dose exposure to lead (i.e. blood levels in excess of 4 $\mu$M) disrupts the blood–brain barrier. Molecules such as albumin that normally are excluded, freely enter the brain of immature animals exposed to these concentrations of lead (Clasen *et al*., 1973; Goldstein *et al*., 1974; Bressler and Goldstein, 1991). Ions and water follow and edema is produced. Intracranial pressure rises as edema accumulates in the brain because of the physical restraint of the skull. When the intracranial pressure approaches the systemic pressure, cerebral perfusion decreases and brain ischemia occurs.

Many of the neurotoxic effects of lead appear related to the ability of lead to mimic or in some cases inhibit the action of calcium as a regulator of cell function (Bressler and Goldstein, 1991). At a neuronal level, exposure to lead alters the release of neurotransmitters (dopamine, acetylcholine and $\gamma$-aminobutyric acid) from nerve endings. Spontaneous release is enhanced and evoked release is inhibited. The former may be due to activation of protein kinases in the nerve endings and the latter to blockade of voltage-dependent calcium channels.

Brain homeostatic mechanisms are disrupted by exposure to higher levels of lead. The final pathway appears to be a breakdown in the blood–brain barrier. Again, the ability of lead to mimic or mobilize calcium and activate protein kinases may alter the properties of endothelial cells, especially in immature brain, and disrupt the barrier. In addition to a direct toxic effect upon the endothelial cells, lead may alter indirectly the microvasculature by damaging the astrocytes that provide signals for the maintenance of blood–brain barrier integrity, and necrosis in neurons with shrunken cytoplasm, pyknotic nuclei and increased perineuronal space.

## TOXICITY

Mammals, birds and reptiles have all been found to develop lead poisoning. The toxic dose of lead has been determined for several species but is difficult to apply to clinical cases where the exposure history is unclear (Gwaltney-Brant, 2004). In general, young animals are more susceptible to lead toxicosis because they are more prone to lead pica and have a higher rate of absorption (about 90%) from the intestinal tract. Cattle have been most widely reported with lead toxicosis, probably due to their propensity to ingest discarded lead–acid batteries and construction materials including paints. Dogs are also commonly reported with lead toxicosis, probably due to their chewing habits and ingestion of small lead objects around the house. Both cats and dogs have been

exposed to lead by the renovations of older homes containing leaded paints. The main route seems to be the ingestion of fine dust by the grooming habits of indoor pets and their tendency to ingest small objects.

Clinical signs of lead toxicosis vary with the species involved, duration of exposure and amount of lead absorbed. The major systems affected by lead poisoning are gastrointestinal, central nervous system and hematological system. Abdominal pain and diarrhea can be common clinical signs in animal exposed to excess lead. Anorexia is common as well as vomiting in those species that are able. Neurological signs including depression, weakness and ataxia can progress to more severe clinical signs of muscle tremors or fasciculations, head pressing (especially in ruminants), blindness, seizure-like activity and death. Many animals with chronic lead poisoning will show subtle and non-specific clinical signs such as abdominal discomfort, vague gastrointestinal upsets, anorexia, lethargy, weight loss and behavior changes. Horses develop acute lead toxicosis and show clinical signs of laryngeal paralysis and "roaring", in addition to colic and seizure-like activity. Evidence suggests that horses may be more susceptible to chronic lead toxicosis than cattle. Horses exposed to daily intake as low as 1.7 mg/kg body weight (approximately 80 ppm Pb in forage dry matter) were poisoned (Aronson, 1972). Clinical signs of lead toxicosis in avians vary with waterfowl and raptors mainly displaying a chronic wasting disorder with apparent peripheral neuropathy. Psittacines are more likely to display gastrointestinal problems and neurological abnormalities.

Gross lesions in animals dying of lead poisoning are often minimal and non-specific, although lead-containing objects may be visible in the gastrointestinal tract. Histologically, there may be degeneration and necrosis of the renal tubular epithelium or the presence of acid-fast inclusion bodies (Hamir *et al.*, 1988; O'Hara *et al.*, 1995). Brain lesions in a calf poisoned with lead included multiple focal or laminar lesions of neuronal necrosis in the cerebral cortex, caudatum and medial nuclei of thalamus, predominantly at the tips of gyri in the occipital and parietal lobes. The lesions spread occasionally to the deeper region of the gyri along the sulci (Seimiya *et al.*, 1991). The affected neurons were shrunken and angular, sometimes triangular in outline with pale eosinophilic cytoplasm. The nuclei showed pyknosis and rhexis. Edematous dilation of perivascular and perineuronal spaces with spongiotic state of neuropil was observed from the molecular layer to outer zone of the white matter. Astrocytic proliferation was also observed. Blood capillaries were congested with enlarged and increased endothelial cells. Meningeal blood vessels were prominently congested with mild lymphocytic infiltration. Edema of Purkinje cell layer in the cerebellum and mild neuronal degeneration in the nucleus of mesencephalon were seen.

Diagnosis of lead poisoning in animals should be made with a combination of history, clinical or necropsy findings, and lead analysis of tissue. Basophilic stippling of erythrocytes and inhibition of hemoglobin synthesis are characteristic hematological features of lead poisoning. From a living animal, whole blood is the best sample for laboratory determination of lead. The normal background concentration of lead in the blood of mammals is below 0.1 ppm. With clinically affected animals, lead concentrations above 0.35 ppm are compatible with a diagnosis of lead toxicosis. Postmortem samples of choice are kidney and liver with lead concentrations above 10 ppm on a wet weight basis being diagnostic for lead toxicosis in domestic species.

## TREATMENT

Acute lead poisoning in animals is usually fatal if the animals are not treated promptly. Treatment approach for lead poisoning in animals includes stabilizing and supporting the animal especially if severe clinical signs are present, preventing additional exposure to lead and chelation therapy to quickly reduce the body burden of lead. The exposure history of the animal should be reviewed for potential sources of lead and the need for gastrointestinal decontamination. The use of chelating agents when large amounts of lead are present in the gastrointestinal tract may actually enhance the absorption of lead into the body. Physical removal of lead-containing objects by surgical means may be necessary with larger objects. The parenteral use of calcium disodium ethylenediaminetetraacetic acid (CaEDTA) has been commonly used for several decades as a chelation agent in domestic animals (Kowalczyk, 1984).

Although other chelators may be superior, CaEDTA is still widely used in veterinary medicine, especially in large animals. CaEDTA is given intravenously (IV) or subcutaneously (SQ) and chelates and mobilizes the lead from bone resulting in a transient increase in blood lead levels. This increase in blood lead can increase soft tissue lead levels leading to an exacerbation of clinical signs. Preceding CaEDTA usage with a chelator that specifically targets lead in the soft tissue (e.g. British Anti-Lewisite or BAL) has been recommended but is difficult to accomplish in most practice settings. CaEDTA can be nephrotoxic, especially in situations where the animal is dehydrated. Recommended treatment with CaEDTA for large animals is 73 mg/kg/day, divided into two or three doses given over the course of a day given by slow IV. For example, a 6.6% solution of CaEDTA (in normal saline or 5% dextrose) can be given IV at a rate of 1 ml per 2 pounds (0.9 kg) of body weight per day, in divided doses. Treatment should continue for 3–5 days. If additional treatment is needed, a rest period of 2 days with continued supported care is

suggested before the additional 3–5-day second treatment period. An alternative treatment regime is to administer CaEDTA at 110 mg/kg IV twice daily for 2 days. If additional treatment is needed first apply the 2-day rest period of supportive care before initiating the second treatment period of 2 days at 110 mg/kg twice daily. Thiamine has been shown to be a valuable adjunct to the treatment of lead poisoning in ruminants (Bratton *et al.*, 1981) and is recommended for other species as well. A dose of 2 mg/kg/day for calves and 250–2000 mg/day for adult cattle has been recommended.

If commercial CaEDTA is unavailable, a stock solution can be formulated for emergency antidotal usage. A 10% stock solution can be made by dissolving 101.1 g of tetrasodium EDTA ($Na_4EDTA$) plus 30 g of anhydrous calcium chloride ($CaCl_2$) in distilled water to a final volume of 1000 ml. From the stock solution a working 2.22% solution can be made by mixing 220 ml of the 10% stock solution with 780 ml distilled water. Using the 2.22% solution, the daily dosage of 73 mg/kg/day is equal to approximately 3.5 ml/kg of bodyweight. This should be divided into two or three separate administrations (Thompson, 1992). Tetrasodium EDTA should never be administered by itself as it may cause hypocalcemia.

Recommended treatment with CaEDTA for dogs is 100 mg/kg/day in four divided doses. Treatment should continue for 2–5 days and a second round of treatment is rarely needed but a 5-day rest period is recommended before applying additional treatment. CaEDTA concentration should be 10 mg/ml and may be administered by slow IV or by SQ route. Cats can be treated with 27.5 mg in 15 ml normal saline or 5% dextrose SQ every 6 h for 5 days or the same dose as a slow IV infusion.

Succimer (meso-2,3-dimercaptosuccinic or DMSA) is an orally administered chelating agent that is less likely to have adverse side effects associated with CaEDTA. The recommended treatment in dogs is an oral dose of succimer at 10 mg/kg, repeated 3 times daily for 10 days (Ramsey *et al.*, 1996). Succimer has also been used orally in caged birds at a dose of 25–35 mg/kg twice daily for 5 days. Several weeks of therapy may be needed in avians. In initial experimental work, succimer given to experimentally lead-poisoned calves IV at 25 mg/kg/day for 4 days was more effective than CaEDTA at decreasing lead concentrations in the liver and kidney (Meldrum and Ko, 2003).

## CONCLUDING REMARKS

While cases of lead toxicosis in animals have been decreasing, it should remain on the clinician's list of rule-outs for seizure-like activity, blindness, and vague neurological and gastrointestinal disorders. At a minimum, the environment of the animal should be reviewed for possible lead sources.

## REFERENCES

Aronson AL (1972) Lead poisoning in cattle and horses following long-term exposure to lead. *Am J Vet Res* **33**: 627.

Bratton GR, Zmudzki J, Kincaid N, Joyce J (1981) Thiamine as treatment of lead poisoning in ruminants. *Mod Vet Pract* **62**: 441–6.

Bressler JP, Goldstein GW (1991) Mechanisms of lead neurotoxicity. *Biochem Pharmacol* **41**: 479–84.

Clasen RA, Hartmann JF, Starr AJ, *et al*. (1973) Electron microscopic and chemical studies of the vascular changes and edema of lead encephalopathy. *Am J Pathol* **74**: 215–40.

Goldstein GW, Asbury AK, Diamond I (1974) Pathogenesis of lead encephalopathy. Uptake of lead and reaction of brain capillaries. *Arch Neurol* **31**: 382–9.

Gwaltney-Brant S (2004) Lead. In *Clinical Veterinary Toxicology*, Plumlee KH (ed.). Mosby, St. Louis, MO, pp. 204–10.

Hamir AN, Sullivan ND, Handson PD (1988) Tissue lead distribution and pathological findings in lead exposed dogs maintained on fat and calcium modified diets. *Br Vet J* **144**: 240–5.

Kelman BJ, Walter BK (1980) Transplacental movements of inorganic lead from mother to fetus. *Proc Soc Exp Biol Med* **163**: 278–82.

Kowalczyk DF (1984) Clinical management of lead poisoning *J Am Vet Med Assoc* **184**: 858–60.

Meldrum JB, Ko KW (2003) Effects of calcium disodium EDTA and meso-2,3-dimercaptosuccinic acid on tissue concentrations of lead for use in treatment of calves with experimentally induced lead toxicosis. *Am J Vet Res* **64**: 672–6.

O'Hara TM, Bennett L, McCoy PC, *et al.* (1995) Lead poisoning and toxicokinetics in a heifer and fetus treated with $CaNa_2EDTA$ and thiamine. *J Vet Diag Investig* **7**: 531–7.

Ramsey DT, Casteel SW, Faggella AM, Chastain CB, Nun JW, Schaeffer DJ (1996) Use of orally administered succimer (meso-2,3-dimercaptosuccinic acid) for treatment of lead poisoning in dogs. *J Am Vet Med Assoc* **208**: 371–5.

Seimiya Y, Itoh H, Ohshima K-I (1991). Brain lesions of lead poisoning in a calf. *J Vet Med Sci* **53**: 117–19.

Thompson LJ (1992) Heavy metal toxicosis. In *Current Therapy in Equine Medicine*, vol. 3, Robinson NE (ed.). W.B. Saunders Co., Philadelphia, PA, pp. 363–6.

CHAPTER

# 32

# Mercury

*Ramesh C. Gupta*

## INTRODUCTION

Mercury (Hg) is a naturally occurring element that is found in the environment. It exists in several forms, such as elemental (metallic), inorganic, and organic. About 80% of the mercury released in the environment is metallic mercury, and comes from human activities, such as fossil fuel combustion, mining, smelting, and from solid waste incineration (ATSDR, 1999). Human activity can lead to mercury levels in soil as much as 200,000 times higher than natural levels. Metallic mercury in a pure form looks like a shiny-white liquid substance at room temperature. It is commonly used in thermometers, barometers, blood pressure devices, batteries, electric switches, dental fillings (amalgams), etc. Inorganic mercury compounds, or mercury salts, occur when it combines with other elements, such as chlorine, sulfur, and oxygen. Most of these compounds are white, except mercuric sulfide or cinnabar ore (i.e. red, but it turns black after exposure to light). Some of mercury compounds are used as fungicides, while others are used for medicinal purposes, e.g. laxatives, deworming agents, antiseptics, and disinfectants. When mercury combines with carbon, it is called organic mercury (organomercurials). Methylmercury, ethylmercury, and phenylmercury are a few examples. In the environment (by bacteria and fungi) and mammalian systems, various forms of mercury are interchangeable. For example, inorganic mercury can be methylated to methylmercury, and methylmercury can change to inorganic or elemental mercury. The animals at the top of the food chain tend to bioaccumulate methylmercury in their bodies. Therefore, poisoning by mercury is due to consumption of meat or grain contaminated with mercury. Poisoning can also result from excessive exposure to inorganic and organic mercury compounds from misuse or overuse of mercury-containing products. Much of the information presented in this chapter is from experimental studies conducted in laboratory animals and poisoning incidences.

## BACKGROUND

Mercury exists naturally in the environment, and as a result everyone is exposed to very low levels. Aristotle named it "Quicksilver". Animal poisoning by mercury is rare because of strict federal, state, and local regulations. Most of the mercury found in the environment is in the form of metallic mercury and inorganic compounds. The most common natural forms of mercury found in the environment are metallic mercury, mercury sulfide (cinnabar ore), mercuric chloride, and methylmercury. Methylmercury is of particular concern because it can bioaccumulate in certain edible freshwater and saltwater fish and marine mammals to levels that are many times greater than levels in the surrounding water. As a result, the larger and older fish living in contaminated water build up levels of mercury in their bodies. Inorganic mercury does not bioaccumulate in the food chain to any extent. Cultivation of edible mushrooms, where waste as compost material with high levels of mercury is used, can also accumulate high levels of mercury (Bressa *et al.*, 1988). The release of methylmercury into an ocean bay (Minamata) in Japan in the 1950s led to a massive health disaster, and the clinical syndrome was named Minamata disease (Tsubaki and Krukuyama, 1977). Thousands of people were poisoned, and hundreds of them had severe brain damage.

The Food and Drug Administration (FDA) estimates that on average most people are exposed to about 50 ng mercury/kg of body weight/day in the food they eat. This level is not enough to cause any harmful effects. A large part of this mercury is in the form of methylmercury and the

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majority of that comes from eating fish. Fish for food consumption are not allowed to have more than 1 ppm. This level is below a level that can be associated with adverse effects. Foods other than fish that may contain higher levels of mercury include wild animals, birds, and mammals (bears) that eat large amounts of contaminated fish (ATSDR, 1999). Meat and/or fat from fish, marine mammals, fish-eating wildlife, and birds; and mercury-based fungicides-treated grains have the highest-mercury levels. Certain species of commercially available saltwater fish, such as shark, swordfish, kingfish, and tilefish, can contain high levels of methylmercury. In addition, edible mushrooms can have unsafe levels of mercury. These are the potential sources of mercury poisoning. In horses, mercury toxicity occurs from wound dressings (blisters) when dimethyl sulfoxide (DMSO) is applied simultaneously, because DMSO enhances absorption of mercury (Schuh *et al.*, 1988).

## TOXICOKINETICS

Absorption of mercury from oral ingestion depends on the form of mercury. Metallic mercury is maximally absorbed (about 80%) from the lungs, while very little is absorbed from the gastrointestinal (GI) tract. Once mercury enters the circulation, it is rapidly distributed to other tissues, but more so in the kidneys, where it accumulates. Metallic mercury can stay in the body for weeks and months. Due to its high lipophilicity, metallic mercury can readily cross the blood–brain barrier and placental barrier. When metallic mercury enters the brain, it is readily converted to an inorganic divalent mercury (oxidized by the hydrogen peroxidase–catalase pathway), and it gets trapped there for a long time. The inorganic divalent cation can, in turn, be reduced to metallic mercury. Most of the absorbed metallic mercury excretes in the urine and feces, some amount passes in the milk, and very little in the exhaled air.

Inorganic mercury compounds (e.g. mercurous chloride and mercuric chloride) are absorbed 10–40% from the GI tract on ingestion, distributed to different organs, and mainly accumulate in the kidneys. In an experimental study, female Sprague–Dawley rats given a single dose of mercuric chloride (7.4 or 9.2 mg Hg/kg, p.o.) showed 12.6 and 18.9 ppm mercury, respectively, in the kidneys when sacrificed 14 days post-exposure (Lecavalier *et al.*, 1994). Trace amounts were also detected in the liver, brain, and serum. These compounds do not readily cross the blood–brain barrier or placental barrier. Inorganic mercury excretes in the urine and feces, and only detectable levels pass through the milk.

Organic mercury (e.g. methylmercury) readily gets absorbed from the GI tract (about 95%). From circulation it gets distributed to other organs. The distribution of methylmercury is similar to that of metallic mercury, i.e. a relatively large amount of mercury can accumulate in the brain and fetus (compared to inorganic mercury) because of its ability to penetrate the blood–brain and placental barriers and its conversion in the brain and fetus to the inorganic divalent cation mercury (ATSDR, 1999). However, the extent of conversion is less than with metallic mercury. In the brain, methylmercury can be changed to inorganic mercury and can remain in this tissue for a long time. Organic mercury excretes in the form of inorganic mercury in the feces over a period of several months. Some organic mercury also excretes in the urine and milk.

Depending on the route of exposure, dose, and single versus repeat exposure, toxicokinetics of mercury can follow one- or two-compartment model. Studies have shown that repeat or continuous exposure to any form of mercury can result in the accumulation of mercury in the body. Retention of mercury in the brain may persist long after cessation of short- and long-term exposures. Blood levels of mercury are closely related to whole-body retention of mercury during the first 3 days after administration. After the initial 3 days, the amount of mercury in the blood declines more rapidly than the whole-body burden.

Evidence suggests that the metabolism of all forms of mercury is similar for humans and animals. Mercury is metabolized through the oxidation–reduction cycle that takes place in intestinal microflora and after absorption in many tissues and in the red blood cells (RBCs). Elimination rates for methylmercury appear to vary with species, dose, sex, and strain. The elimination half-life in the blood of monkeys receiving inorganic and organic mercury was found to be 26 days (Vahter *et al.*, 1994). In a study of organs from sled dogs fed methylmercury-laden meat and organs from predatory marine animals, the highest concentration of total mercury was found in the mesenterial lymph nodes, followed by liver and kidneys, indicating that the lymphatic system may play an important role in the transport of mercury to target organs (Hansen and Danscher, 1995). The tissue concentrations of mercury observed in this study were found to be age related, and the results suggest that demethylation takes place in all organs, except the skeletal muscles. Demethylation of methylmercury was found to be lower in the brain than in other organs.

## MECHANISM OF ACTION

Mechanism of action for the toxic effects of organic and inorganic mercury is similar. Toxicities of the different forms of mercury are related, in part, to its differential accumulation in sensitive tissues. This theory is supported by the observation that mercury rapidly accumulates in the kidneys and specific areas of the brain (the two

major target organs). High-affinity binding of the divalent cationic mercury to thiol or sulfhydryl (SH) groups of proteins is believed to be a major mechanism involved in the toxicity of mercury. As a result, mercury can cause inactivation of various enzymes, structural proteins, transport proteins, and alteration of cell membrane permeability by the formation of mercaptides. In addition, mercury may induce one or more of the following effects: increased oxidative stress, mitochondrial dysfunction, changes in heme metabolism, glutathione depletion, increased permeability of the blood–brain barrier; and disruption of microtubule formation, protein synthesis, DNA replication, DNA polymerase activity, calcium homeostasis, synaptic transmission, and immune response (ATSDR, 1999).

The nervous system is especially sensitive to mercury. The degree of damage depends on the form of mercury and its dose. Metallic mercury at high doses causes irreparable damage to the brain. In many poisoning incidents, permanent damage to the brain and kidneys occurred by methylmercury. Since inorganic mercury does not readily cross the blood–brain barrier, it is highly unlikely that inorganic mercury may cause any damage to the brain or nerves. Most of the information concerning neurotoxicity in humans following oral exposure to organic mercury comes from reports describing the effects of ingesting contaminated fish or fungicide-treated grains, or meat from animals fed such grains. Studies conducted in experimental animals strongly indicate that organic mercury is a potent neurotoxicant.

Evidence suggests that a single dose of mercuric chloride (0.74 mg/kg) caused disruption of the blood–brain barrier in rats (Chang and Hartman, 1972). These investigators also administered mercuric chloride to rats at the same dose daily for 11 weeks. Within 2 weeks, there were coagulative or lucid changes in cerebellar granule cells and fragmentation, vacuolation, and cytoplasmic lesions in the neurons of dorsal root ganglia. Neurological disturbances consisted of severe ataxia and sensory loss, with an accompanying loss in body weight. It is expected that mercuric chloride administered via subcutaneous route would be much more toxic than that administered orally because of the poor absorption of inorganic forms of mercury from the GI tract.

Neurotoxic effects seen in the Minamata (Japan) and Eraqi poisonings have been associated with neuronal degeneration and glial proliferation in the cortical and cerebellar gray matter and basal ganglia (Al-Saleem, 1976), and derangement of basic developmental processes such as neuronal migration (Matsumoto *et al.*, 1965; Choi *et al.*, 1978) and neuronal cell division (Sager *et al.*, 1983). In the brain, Purkinje, basket, and stellate cells were severely affected. In the brain, methylmercury selectively damages specific focal areas such as the granule cells of the cerebellum and the neurons in the interstices of the visual cortex.

Methylmercury selectively inhibits protein synthesis in the brain (reversibly in neurons from the cerebrum and Purkinje cells; and irreversibly in granule cell of cerebellum), and this effect usually precedes the appearance of clinical signs. This selective action on the brain may be due to the fact that certain cells are susceptible because they cannot repair damage from methylmercury. Cheung and Verity (1985) identified the most sensitive step in the protein synthesis, i.e. the peptide elongation can be affected by the high concentrations of mercury, but the first stage of synthesis associated with tRNA may be the most sensitive. Methylmercury inhibits one or more of the amino acyl tRNA synthetase enzymes. Microtubules are essential for cell division (main component of the mitotic spindle), and methylmercury reacts with the SH groups on tubulin monomers, and thereby disrupts the assembly process. The dissociation process continues, and that leads to depolymerization of the tubule.

In all forms, mercury accumulates in the kidneys, and thereby it causes greater damage to this organ. The kidney damage appears to be dose dependent, and that means recovery can occur if exposure is at low level. Following entry of the mercuric or methylmercuric ion into the proximal tubular epithelial cells via transport across the brush-border or basolateral membrane, mercury interacts with thiol-containing compounds, such as glutathione and metallothionein. This interaction initially produces alterations in membrane permeability to calcium ions and inhibition of mitochondrial function. Subsequently, by unknown signaling mechanisms, mercury induced the synthesis of glutathione, glutathione-dependent enzymes, metallothionein, and several stress proteins. Furthermore, in the kidney, epithelial cell damage occurs as a result of excess free radical formation and lipid peroxidation.

## TOXICITY

In general, toxic effects of mercury depend on the form of mercury, the amount and duration of exposure, and the route of exposure. Mercury, in all forms, has been found to be toxic to both man and animals. There are many similarities in the toxic effects of the various forms of mercury, but there are also differences. Exposure to mercury, however, does not necessarily mean that adverse health effects will occur. Each form and route leads to different effects. Practically, it is organic mercury, which is more toxic and often encountered in poisonings. In both humans and animals, exposure to mercury is more frequently encountered by oral ingestion. The major targets of toxicity following oral exposure to inorganic and organic mercury are the kidneys and the central nervous system (CNS), respectively.

In humans, deaths resulting from organic mercury ingestion have been documented following outbreaks of

poisoning (Minamata disease) after consumption of methylmercury-contaminated fish in Minamata Bay, Japan (Tsubaki and Takahashi, 1986) and after consumption of grains contaminated with methylmercury and ethylmercury in Iraq (Bakir *et al*., 1973; Al-Saleem, 1976). Noninflammatory damage to the brain was the primary cause of deaths, while pneumonia and nonischemic heart damage were reported as secondary cause of death.

The signs of mercury acute toxicity in animals are similar to those described in humans. Signs and symptoms associated with short-term exposure to metallic mercury may include nausea, vomiting, diarrhea, increase in blood pressure or heart rate, skin rashes, and eye irritation. Inorganic mercury, if swallowed in large quantities, may cause damage to the kidney, and also in stomach and intestine, including nausea, diarrhea, and ulcers. Animal studies revealed that long-term oral exposure to inorganic mercury salts causes kidney damage, increase in blood pressure and heart rate, and effects on the stomach. Studies also show that nervous system damage occurs after long-term exposure to high levels of inorganic mercury. Short-term, high-level exposure of laboratory animals to inorganic mercury has been shown to affect the developing fetus and may cause termination of the pregnancy.

Laboratory animals exposed to long term, high levels of methylmercury or phenylmercury showed damage to the kidneys, stomach, and large intestine, changes in blood pressure and heart rate, and adverse effects on the developing fetus, sperm, and male reproductive organs; and increases in the number of spontaneous abortions and stillbirths.

In livestock animals, clinical signs of mercury poisoning vary greatly. In cattle, toxicity signs include ataxia, neuromuscular incoordination, and renal failure, followed by convulsions and a moribund state. Average time from ingestion to death is reported to be about 20 days. Ingestion of phenylmercuric acetate may cause sudden death with massive internal hemorrhage, without other signs of toxicity (Puls, 1994). In horses, signs of acute toxicity include severe gastroenteritis and nephritis. In chronic cases, signs may include neurological dysfunction, laminitis, in addition to renal disease which is characterized by glycosuria, proteinuria, phosphaturia, reduced urine osmolarity, reduced glomerular filtration rate, azotemia, and elevated creatinine and blood urea nitrogen. In sheep, the poisoning is characterized by severe neurological symptoms, and tetraplegia. Pigs show incoordination, unstable gait, lameness, recumbency and death.

Some of the toxic effects are described in detail for each organ/system affected by mercury exposure.

## Nervous system

Adverse effects on the nervous system of animals occur at lower doses than do harmful effects to most other systems of the body. This difference indicates that the nervous system is more sensitive to mercury than are other organs in the body. Animal studies also provide evidence of damage to the nervous system from exposure to methylmercury during development, and evidence suggests that the effects worsen with age, even after the exposure stops. The reason for this greater susceptibility is that mercury affects processes unique to the developing nervous system, namely cell migration and cell division (Clarkson, 1987).

Both human epidemiology and experimental animal studies indicate that organic mercury is a potent neurotoxicant. Studies suggest that cats and monkeys are more sensitive than rodents to the neurotoxic effects of mercury (especially methylmercury). In several animal species, the major effects that are seen across the studies include motor disturbances, such as ataxia and tremors, as well as signs of sensory dysfunction, such as impaired vision. The predominant pathological feature is degenerative changes in the cerebellum, which is likely to be the mechanism involved in many of the motor dysfunctions. In a chronic study, cats fed tuna contaminated with methylmercury, showed degenerative changes in the cerebellum and the cortex (Chang *et al*., 1974). Neonatal monkeys exposed to methylmercuric chloride at 0.5 mgHg/kg/day for 28–29 days exhibited stumbling, and falling, blindness, crying, tember tomtrums, and coma. Histopathological analysis revealed diffuse degeneration in the cerebral cortex, cerebellum basal ganglia, thalamus, amygdala, and lateral geniculate nuclei (Willes *et al*., 1978).

Rats acutely intoxicated with methylmercury (19.9 mg Hg/kg, oral gavage) showed signs of lethargy and ataxia, which was not accompanied by histopathological changes. Symptoms disappeared within 2–3 h. Administration of a single dose of methylmercuric chloride (0.8 mg Hg/kg) produced blood–brain barrier dysfunction in rats (Chang and Hartman, 1972) similar to that described for inorganic mercury.

Following inhalation exposure to metallic mercury vapors, the CNS has been found to be the most sensitive organ in guinea pigs, rats, and mice. With increasing concentrations of mercury, damage to CNS becomes irreversible. Rabbits appear to be less sensitive.

In rabbits given 5.5 mgHg/kg as methylmercuric acetate for 1–4 days, widespread neuronal degenerative changes in cervical ganglia cells, cerebellum, and cerebral cortex have been observed without accompanying behavioral changes (Jacobs *et al*., 1977). In similar studies, mice exposed to 1.9 or 9.5 mgHg/kg/day as methylmercury in the drinking water for 28 weeks exhibited degeneration of Purkinje cells and loss of granular cells in the cerebellum (MacDonald and Harbison, 1977). At higher doses, hind limb paralysis was observed. Neuronal degeneration and microgliosis were observed in the corpus striatum, cerebral cortex, thalamus, and hypothalamus, accompanied by hind leg weakness, in mice given with 1 or 4 mgHg/kg/day as methylmercuric chloride by gavage for 60 days (Berthoud *et al*., 1976).

Neurotoxic signs observed in rats exposed to methylmercury (4 mg Hg/kg/day for 8 days) include muscle spasms, gait disturbances, flailing, and hind limb crossing (Inouye and Murakami, 1975; Fuyuta *et al.*, 1978; Magos *et al.*, 1980, 1985). Histopathological examination of the nervous system of affected rats has shown degeneration of cerebellar granule cells and dorsal root ganglia (Magos *et al.*, 1980, 1985) and degenerative changes in peripheral nerves (Fehling *et al.*, 1975; Miyakawa *et al.*, 1976).

## Renal system

Mercury, in all forms, has been shown to cause renal toxicity (structural and functional damage) in humans and animal species that are tested. Renal toxicity has been observed in rats (Fisher 344) and mice ($B6C3F_1$) following acute, intermediate, and chronic exposures to mercuric chloride (Dieter *et al.*, 1992; NTP, 1993). In a 14-day study, male and female rats were exposed by gavage to 0.93–14.8 mgHg/kg/day as mercuric chloride for 5 days a week. There was a significant increase in the absolute and relative kidney weights of males beginning at the 1.9 mgHg/kg/day dose level. An increased incidence of tubular necrosis was observed in rats exposed to at least 3.7 mgHg/kg/day. Severity was dose dependent. In chronic studies, mercuric chloride produced a variety of pathological changes in kidneys (Carmignani *et al.*, 1992; Hultman and Enestrom, 1992; NTP, 1993). Degenerative effects have been found in the kidneys of animals exposed to moderate-to-high levels of metallic mercury vapors following acute or subacute exposures (Ashe *et al.*, 1953). Effects ranging from marked cellular degeneration to tissue destruction and widespread necrosis were observed in rabbits exposed to mercury vapor at a concentration of 28.8 $mg/m^3$ for 2–3 h. In rats, slight degenerative changes (i.e. dense deposits in tubule cells and lysosomal inclusions) in the renal tubular epithelium were evident following exposure to 3 $mg/m^3$ mercury vapor for 3 h/day, 5 days a week, for 12–42 weeks (Kishi *et al.*, 1978). Low-level, long-term exposure to mercury (0.1 $mg/m^3$) has not been found toxic to kidneys of rats, rabbits, and dogs (Asche *et al.*, 1953). Exposure to organic mercury via inhalation is extremely rare.

## Cardiovascular system

Mercury has been shown to have adverse effects on cardiovascular system. A decrease in heart rate was observed in male rats given two gavage doses of 2 mgHg/kg as methylmercuric chloride (Arito and Takahashi, 1991). An increase in systolic blood pressure was observed in male rats after daily oral gavage doses of 0.4 mgHg/kg/day as methylmercuric chloride for 3–4 weeks (Wakita, 1987). This effect began approximately 60 days after initiation of exposure and persisted for at least 9 months.

## GI tract

Ingestion of mercuric chloride is highly irritating to the tissues of GI tract. Inflammation and necrosis of the glandular stomach were observed in mice that were given oral doses of 59 mg/kg as mercuric chloride 5 days a week for 2 weeks (NTP, 1993). In a 2-year gavage study, an increased incidence of forestomach hyperplasia was observed in male rats exposed to 1.9 or 3.7 mgHg/kg/day as mercuric chloride compared to the control group. Mice showed ulceration of the glandular stomach compared to the control group. It showed ulceration of the glandular stomach after 2 years of dietary exposure to methylmercuric chloride at 0.69 mg Hg/kg/day (Mitsumori *et al.*, 1990). In experimental studies, methylmercury has been found very strain- and sex-specific in mice. A single oral dose of methylmercuric chloride at 16 mgHg/kg resulted in the death of 4 of 6 male mice (C57BL/6N Jc1 strain) but no death was noted in females (Yasutake *et al.*, 1991). Mortality in female mice (4 of 6) was noted at 40 mg Hg/ kg dose. In a chronic study, 26 weeks of dietary exposure to methylmercuric chloride resulted in increased mortality in both male and female mice (ICR strain) at 3.1 mgHg/kg/day (Mitsumori *et al.*, 1981).

## Hematopoietic system

In general, acute mercury toxicity does not produce any characteristic hematological changes. In a chronic study conducted in rats, phenylmercuric acetate given in water at a dose of 4.2 mgHg/kg/day caused decreases in hemoglobin, hematocrit, and RBC counts (Solecki *et al.*, 1991). The anemia observed in this study may have been secondary to blood loss associated with the ulcerative lesions in the large intestine. However, methylmercuric chloride at low dose (0.1 mgHg/kg/day for 2 years) given in diet for 2 years caused no changes in hematological parameters (Verschuuren *et al.*, 1976).

## Other effects

In laboratory animals, mercury has been found to have potential for inducing genotoxicity (Ghosh *et al.*, 1991), carcinotoxicity (Solecki *et al.*, 1991; NTP, 1993), reproductive and developmental toxicity (Fuyuta *et al.*, 1979), and immunotoxicity (Thuvander *et al.*, 1996).

## Diagnosis

Presently there are reliable and accurate ways to measure mercury levels in the body. These tests involve mercury analysis in blood, urine, milk, hair, and liver and kidney. Levels found in blood, urine, and hair may be used together to predict possible health effects that may be

caused by the different forms of the mercury. Mercury levels in the blood provide more useful information after recent exposures than after long-term exposures. Mercury in urine is determined to test for exposure to metallic or inorganic mercury, while whole blood or hair is used to determine exposure to methylmercury. Kidney is an ideal specimen for mercury analysis from dead animals.

## TREATMENT

Activated charcoal (1–3 g/kg body weight, p.o.) is very effective in reducing further absorption of mercury. Specific treatment of mercury poisoning rests with the use of chelators along with protein solutions to bind and neutralize mercury compounds. The use of a particular chelator is dependent on the type of mercury exposure. Among several chelators, dimercaprol (British anti-Lewisite (BAL), 3 mg/kg, i.m.) has been found to be the most effective against mercury poisoning. However, chelation releases mercury from soft tissues which can be redistributed to the brain. Oral administration of sodium thiosulfate (1 g/kg) can assist in eliminating mercury. Animal studies suggest that antioxidants (particularly vitamin E) may be useful for decreasing the toxicity of mercury. Improved chelation and drug therapies for treating acute and chronic mercury poisonings are greatly needed.

## CONCLUSION

Toxicity by mercury depends on the form of mercury, dose, duration, and route of exposure. Organic mercury tends to bioaccumulate in the higher food chain, and as result, the maximum concentrations are found in the meat of fish, marine mammals, and fish-eating birds and wildlife. Methylmercury is the most toxic among the mercury species because of its volatility and its ability to pass through biological membranes such as the blood–brain barrier and the placental barrier. Nervous system and kidneys are the two major target organs. Not all forms of mercury cross the blood–brain barrier (e.g. inorganic mercury), but in all forms it accumulate in the kidney and thereby causes damage to this organ. Chelation therapy appears to be the best treatment.

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## REFERENCES


Agency for Toxic Substances and Disease Registry (ATSDR) (1999) *Toxicological Profile for Mercury*. US Department of Health and Human Services. Atlanta, GA.

Al-Saleem T (1976) Levels of mercury and pathologic changes in patients with organomercury poisoning. *Bull World Health Org* **53**(Suppl.): 99–104.

Arito H, Takahashi M (1991) Effect of methylmercury on sleep patterns in the rat. In *Advances in Mercury Toxicology*, Suzuki T, Imura N, Clarkson TW (eds). Plenum Press, New York, pp. 381–94.

Ashe W, Largent E, Dutra F, *et al.* (1953) Behaviour of mercury in the animal organism following inhalation. *Arch Ind Hyg Occup Med* **17**: 19–43.

Bakir F, Damluji SF, Amin-Zaki L, *et al.* (1973) Methylmercury poisoning in Iraq. *Science* **181**: 230–41.

Berthoud HR, Garman RH, Weiss B (1976) Food intake, body weight, and brain histopathology in mice following chronic methylmercury treatment. *Toxicol Appl Pharmacol* **36**: 19–30.

Bressa G, Cima L, Costa P (1988) Bioaccumulation of Hg in the mushroom *Pleurotus ostreatus. Ecotoxicol Environ Safety* **16**: 85–9.

Carmignani M, Boscolo P, Artese L, *et al.* (1992) Renal mechanism in the cardiovascular effects of chronic exposure to inorganic mercury in rats. *Br J Ind Med* **49**(4): 226–32.

Chang L, Hartman HA (1972) Ultrastructural studies of the nervous system after mercury intoxication. *Acta Neuropathol (Berlin)* **20**: 122–38.

Chang LW, Yamaguchi S, Dudley JAW (1974) Neurological changes in cats following long-term diet of mercury contaminated tuna. *Acta Neuropathol (Berlin)* **27**: 171–6.

Cheung MK, Verity MA (1985) Experimental methylmercury neurotoxicity: locus of mercurial inhibition of brain protein synthesis *in vivo* and *in vitro*. *J Neurochem* **44**: 1799–808.

Choi CM, Lapham LW, Amin-Zaki L, *et al.* (1978) Abnormal neuronal migration, deranged cerebral cortical organization and diffuse white matter astrocytosis of human fetal brain: a major effect of methylmercury poisoning *in utero. J Neuropathol Exp Neurol* **37**: 719–32.

Clarkson TW (1987) Metal toxicity in the central nervous system. *Environ Health Perspect* **75**: 59–64.

Dieter MP, Boorman GA, Jameson CW, *et al.* (1992) Development of renal toxicity in F344 rats gavaged with mercuric chloride for 2 weeks, or 2, 4, 6, 15, and 24 months. *J Toxicol Environ Health* **24**: 319–40.

Fehling C, Abdulla M, Brun A, *et al.* (1975) Methylmercury poisoning in the rat: a combined neurological, chemical, and histopathological study. *Toxicol Appl Pharmacol* **33**: 27–37.

Fuyuta M, Fujimoto T, Hirata S (1978) Embryotoxic effects of methylmercuric chloride administered to mice and rats during organogenesis. *Teratology* **18**: 353–66.

Fuyuta M, Fujimoto T, Kiyofuji E (1979) Teratogenic effects of a single oral administration methylmercuric chloride in mice. *Acta Anat* **104**: 356–62.

Ghosh AK, Sen S, Sharma A, *et al.* (1991) Effect of chlorophyllin on mercuric chloride-induced clastogenicity in mice. *Food Chem Toxicol* **29**: 777–9.

Hansen JC, Danscher G (1995) Quantitative and qualitative distribution of mercury in organs from arctic sledgedogs: an atomic absorption spectrophotometric and histochemical study of tissue samples from natural long-termed high dietary organic mercury-exposed dogs for Thule, Greenland. *Toxicol Appl Pharmacol* **77**: 189–95.

Hultman P, Enestrom S (1992) Dose–response studies in murine mercury-induced autoimmunity and immune-complex disease. *Toxicol Appl Pharmacol* **113**: 199–208.

Inouye M, Murakami U (1975) Teratogenic effects of orally administered methylmercuric chloride in rats and mice. *Congenit Abnormal* **15**: 1–9.

Jacobs JM, Carmichael N, Cavanagh JB (1977) Ultrastructural changes in the nervous system of rabbits poisoned with methylmercury. *Toxicol Appl Pharmacol* **39**: 249–61.

Kishi R, Hashimoto K, Shimizu S, *et al.* (1978) Behavioral changes and mercury concentrations in tissues of rats exposed to mercury vapor. *Toxicol Appl Pharmacol* **46**: 555–66.

Lecavalier PR, Chu I, Villeneuve D, *et al.* (1994) Combined effects of mercury and hexachlorobenzene in rat. *J Environ Sci Health* **29**: 951–61.

MacDonald JS, Harbison RD (1977) Methylmercury-induced encephalopathy in mice. *Toxicol Appl Pharmacol* **39**: 195–205.

Magos L, Peristianis GC, Clarkson TW, *et al.* (1980) The effect of lactation on methylmercury intoxication. *Arch Toxicol* **45**: 143–8.

Magos L, Brown AW, Sparrow S, *et al.* (1985) The comparative toxicology of ethyl and methylmercury. *Arch Toxicol* **57**: 260–7.

Matsumoto H, Koya G, Takeuchi T (1965) Fetal Minamata disease–a neuropathological study of two cases of intrauterine intoxication by a methylmercury compound. *J Neuropathol Exp Neurol* **24**: 563–74.

Mitsumori K, Maita K, Saito T, *et al.* (1981) Carcinogenicity of methylmercury chloride in ICR mice: preliminary note on renal carcinogenesis. *Cancer Lett* **12**: 305–10.

Mitsumori K, Hirano M, Ueda H, *et al.* (1990) Chronic toxicity and carcinogenicity of methylmercury chloride in B6C3F1 mice. *Fundam Appl Toxicol* **14**: 179–90.

Miyakawa T, Murayama E, Sumiyoshi S, *et al.* (1976) Late changes in human sural nerves in Minamata disease and in nerves of rats with experimental organic mercury poisoning. *Acta Neuropathol (Berlin)* **35**: 131–8.

NTP (1993) *Toxicology and Carcinogenesis Studies of Mercuric Chloride (CAS No. 7487-94-7) in F344/N Rats and B6C3F1 Mice* (*Gavage Studies*). National Toxicology Program, US Department of Health and Human Service, National Institutes of Health, Research Triangle Park, NC. NTP TR 408. NIH Publication No. 91-3139.

Puls R (1994) *Mineral Levels in Animal Health: Diagnostic Data*, 2nd edn. Sherpa International, Clearbrook, BC, Canada, pp, 184–91.

Sager PR, Doherty RA, Olmsted JB (1983) Interaction of methylmercury with microtubules in cultured cells and *in vitro. Exp Cell Res* **146**: 127–37.

Schuh, *et al.* (1988) Concurrent mercuric blister and dimethyl sulfoxide (DMSO) application as a cause of mercury toxicity in two horses. *Equine Vet J* **20**: 68–71.

Solecki R, Hothorn L, Holzweissig M, *et al.* (1991) Computerized analysis of pathological findings in longterm trials with phenylmercuric acetate in rats. *Arch Toxicol* **14**(Suppl.): 100–3.

Thuvander A, Sundberg J, Oskarsson A (1996) Immunomodulating effects after perinatal exposure to methylmercury in mice. *Toxicology* **114:** 163–75.

Tsubaki T, Krukuyama K (1977) *Minamata Disease.* Elsevier Scientific Publishing Company, Amsterdam.

Tsubaki T, Takahashi H (1986) *Recent Advances in Minamata Disease Studies.* Kodansha, Tokyo, Japan.

Vahter M, Mottet NK, Friberg L, *et al.* (1994) Speciation of mercury in the primate blood and brain following long-term exposure to methylmercury. *Toxicol Appl Pharmacol* **124**: 221–9.

Verschuuren HG, Kroes R, Den Tonkelaar EM, *et al.* (1976) Toxicity of methylmercury chloride in rats. III. Long-term toxicity study. *Toxicology* **6**: 107–23.

Wakita Y (1987) Hypertension induced by methylmercury in rats. *Toxicol Appl Pharmacol* **89**: 144–7.

Willes RF, Truelove JF, Nera EA (1978) Neurotoxic response of infant monkeys to methylmercury. *Toxicology* **9**: 125–35.

Yasutake A, Hirayama K, Inouye M, *et al.* (1991) Sex differences of nephrotoxicity by methylmercury in mice. In *Nephrotoxicity: Mechanisms Early Diagnosis and Therapeutic Management, Proceedings of the 4th International Symposium on Nephrotoxicity*, Guilford, England, UK, 1989. Marcel Dekker Inc., New York, NY, pp. 389–396.

CHAPTER

# 33

# Molybdenum

*Jeffery O. Hall*

## INTRODUCTION

Molybdenum (Mo) is an essential nutrient in plants and animals. Thorough reviews on Mo have been published (Dick, 1956; Underwood, 1977; Ward, 1978; Friberg and Lener, 1986; Mills and Davis, 1987; Rajagopalan, 1988; Nielsen, 1996; Johnson, 1997; NRC, 2006). In plants and microbes, reduction of nitrate to nitrite and nitrogen fixation requires Mo (Williams and daSilva, 2002). Higher animals require Mo for oxygen transfer reactions of aldehyde oxidase, sulfite oxidase, and xanthine oxidase, where Mo is bound to a pterin nucleus (Johnson *et al*., 1980). Although dietary clinical deficiencies have not been reported under natural conditions (Mills and Davis, 1987), deficiency has been produced in animals fed purified Mo deficient diets (Mills and Bremner, 1980; Anke *et al*., 1985). Functional Mo deficiency has been caused by genetic disorders in humans (Reiss, 2000) and competitive replacement of tungsten for Mo in enzymes (Nell *et al*., 1980). And, iatrogenic Mo deficiency, resulting in aberrant sulfur-containing amino acid metabolism, has been reported following prolonged total parenteral nutrition (Abumrad *et al*., 1981).

Mo toxicity is intricately tied to interactions with copper and sulfur. Predominant manifestations of Mo poisoning are associated with secondary copper deficiency, but not all clinical symptoms are alleviated by copper supplementation. The copper–sulfur–Mo interactions are complex and vary greatly in degree of severity among species.

## BACKGROUND

Mo is a transition metal within group VI of the periodic table. It has an atomic number of 42, an atomic weight of 95.95, and has 7 different naturally occurring atomic masses from 92 to 100 (Rosman and Taylor, 1998). Mo can occur in a variety of oxidation states that range from (-II) to (VI) (IMOA, 2006), but valence states IV, V, and VI are the most common in biological systems (Johnson, 1997). Mo is utilized in the production of oxidation catalysts, pigments, corrosion-resistant steel, smoke suppressants, lubricants, fertilizers, and metal alloys. Although uniformly found in nature, the United States has the greatest producible Mo reserves.

Mo is commonly found in low concentrations in most dietary constituents (Rajagopalan, 1988), but excess intake can occur from plants grown on soils naturally high in Mo or from areas contaminated by mining or smelting operations. Daily dietary requirements for all species are such that requirements are met, even with low intake.

## PHARMACOKINETICS/ TOXICOKINETICS

### Absorption

Mo absorption differs between monogastrics and ruminants. In monogastrics, Mo absorption occurs from the stomach throughout the intestinal tract (Bell *et al*., 1964; Miller *et al*., 1972; Nielsen, 1996;). In contrast, ruminant absorption occurs in the intestinal tract, as an extensive delay in peak blood concentration would indicate that rumen absorption does not occur. This mucosal absorption is via an active carrier-mediated process that is also utilized by sulfate (Mason and Cardin, 1977). Absorption is quite efficient, being from 40% to 90% (Friberg and Lener, 1986; Turnlund *et al*., 1995). But, Mo absorption does not appear to be regulated at the point of mucosal

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absorption, as increasing Mo concentrations presented to the mucosa results in concomitant increased absorption (Miller *et al*., 1972; Turnlund *et al*., 1995; ).

Dietary constituents can limit Mo absorption. Dietary sulfate present at the point of absorption can competitively inhibit Mo uptake (Mason and Cardin, 1977). Furthermore, in the presence of sulfur/sulfates, the reductive rumen metabolism results in di-, tri-, and tetra-thiomolybdates, which can then bind copper and form a non-absorbable complex (Dick, 1956; Price *et al*., 1987; Gooneratne *et al*., 1989).

### Distribution

Mo is widely distributed in tissues, but has highest concentrations in the liver, kidney, and bone (Schroeder *et al*., 1970; Friberg and Lener, 1986). In light of the essential nature of Mo, it is somewhat unusual that very little tissue retention/reserve is maintained. Post-absorptive circulation occurs by transport bound to the red blood cell proteins or as free ionic molybdate (Allway *et al*., 1968; Versieck *et al*., 1981). But, absorbed or systemically produced thiomolybdates can bind copper and result in circulating copper–thiomolybdate complexes which are not biologically available for tissue utilization.

### Elimination

Mo is eliminated from the body fairly rapidly, with little, and only short-term, tissue retention. Although urinary is the primary route of elimination, biliary elimination also occurs (Friberg and Lener, 1986; Vyskocil and Viau, 1999; NRC, 2006) and likely is the primary route of elimination in ruminants (Grace and Suttle, 1979; Pott *et al*., 1999). Urinary elimination is concentration dependent, resulting in relatively rapid elimination even with very large exposures. In lactating animals, Mo is excreted in the milk with content being dependent on the concentration being ingested (Archibald, 1951; Anke *et al*., 1985). Thus, exposure can be approximated by analysis of urine or milk for Mo content across time and extrapolating back to the time of exposure (Lesperance *et al*., 1985). Just as sulfate can inhibit the absorption of Mo, it can also compete for reabsorption sites in the renal tubules and enhance the rate of elimination (Friberg and Lener, 1986).

## MECHANISM OF ACTION

The mechanism by which Mo is active in biologic systems is through it redox activity in functional molybdo-enzymes (Mills and Davis, 1987). The readily changeable oxidation states of Mo lend it to functional utilization in these types of reactions.

The primary mechanisms by which Mo is toxic are directly tied to its interactions with sulfur and copper. These interactions result in functional or overt copper deficiency. But, these interactions differ significantly among species, with ruminants being much more susceptible than monogastrics. The reducing environment of the rumen converts sulfate or sulfur from sulfur-containing amino acids to sulfide, which then forms mono-, di-, tri-, and tetra-thiomolybdates (Price *et al*., 1987; Spears, 2003). Thiomolybdates binding of copper in the digestive tract prevents absorption of ingested copper, while systemic binding renders it non-bioavailable for tissue utilization (Gooneratne *et al*., 1989; Suttle, 1991). These copper–thiomolybdate complexes also result in enhanced copper excretion (Howell and Gooneratne, 1987). Price *et al*. (1987) found that the ruminal binding was predominantly via tri- and tetra-thiomolybdates, while systemic effects were predominantly via di- and tri-thiomolybdates. In practical means, the thiomolybdates serve as effective chelators of copper and deplete functional body stores.

Most of the clinical syndromes of Mo poisoning can be tied to deficiencies in copper-containing enzyme systems (NRC, 2006). Although most clinical effects of Mo poisoning are reversed by supplementation of copper, Mo may have some direct toxic effects. It is possible that permanent tissue damage, caused by severe copper depletion, results in non-response to copper supplementation in clinically affected animals. The exact mechanisms of non-copper responsive toxic effects of Mo are poorly defined or investigated, but high Mo concentrations can inhibit the *in vivo* activity of succinic acid oxidase, sulfide oxidase, glutaminase, cholinesterase, and cytochrome oxidase (Venugopal and Luckey, 1978).

## TOXICITY

Both acute and chronic toxicity of Mo varies greatly among species. Monogastric animals are much less sensitive than ruminants, due to the rumen metabolism of sulfur and formation of thiomolybdates. The relative tolerance to Mo has been ranked: horses > pigs > rats > rabbits > guinea pigs > sheep > cattle (Venugopal and Luckey, 1978), but more recent literature suggests horses may be more sensitive (Ladefoged and Sturup, 1995). In total, the toxicity of Mo needs to be evaluated with consideration of dietary sulfur/sulfates and copper. This is with knowledge that sulfates competitively inhibit uptake from the intestinal tract, but in ruminants results in the formation of thiomolybdates which will enhance the toxic effects on copper status.

Natural toxic effects of Mo are primarily via ingestion, but toxicity has been demonstrated by both inhalation and injection in laboratory rodents. Intraperitoneal $LD_{50}$ of Mo in rats ranges from 99 to 228 mg/kg body wt. (Venugopal and Luckey, 1978), with similar lethal doses in mice and guinea pigs. Chronic inflammatory lesions and hyaline degeneration within the respiratory tree was induced by Mo trioxide exposure of 10–100 mg/m$^3$ 6 h per day, 5 days a week (Chan *et al.*, 1998). Increased incidence of respiratory adenomas was also seen in the rats and mice.

Concentrations of Mo required to produce acute poisoning orally differ significantly among species. For cats, rabbits, and guinea pigs, the oral $LD_{100}$ for Mo is 1310, 1020, and 1200 kg/kg body wt., while the oral $LD_{50}$ for rats is 125–370 mg/kg body wt. (Venugopal and Luckey, 1978). Little acute toxicity data are available for domestic animals, but cattle have been acutely poisoned with feed containing 7400 mg Mo/kg diet (group average intake of 31 mg Mo/kg body wt./day) and Mo was acutely lethal in sheep at 132–137 mg Mo/kg body wt./day for 2–3 days (Swan *et al.*, 1998).

Clinical signs and pathologic lesions in acutely poisoned animals differ from those seen with more chronic poisonings. Acutely poisoned cattle and sheep developed feed withdrawal, lethargy, weakness, hind limb ataxia that progressed to the front limbs, and recumbency (Swan *et al.*, 1998). The cattle also had profuse salivation, ocular discharge, and mucoid feces. Hydropic hepatocellular degeneration/necrosis and hydropic degeneration/necrosis of the proximal and distal renal tubules was observed in both cattle and sheep.

Relative chronic toxicity of Mo is just as species dependent as acute poisoning. Lethal doses of Mo administered chronically to laboratory animals (rats, mice, guinea pigs, and rabbits) range from 60 to 333 mg Mo/kg body wt./day, while cattle are poisoned with as little as 3 mg Mo/kg body wt./day (NRC, 2006). Literature reports of toxic dietary Mo content range from 10 to 1200 ppm for rats, 300 to 8000 ppm for poultry, 200 to 4000 ppm for rabbits, 1000 to 8000 ppm for Guinea pigs, 2.5 to 20 ppm for sheep, and 2 to 400 ppm for cattle (Pitt, 1976). Ruminants commonly succumb to molybdenosis when Mo intake is greater than 20 ppm in the diet. However, due to the intrinsic nature of the Mo–copper–sulfur interactions, chronic Mo poisoning in ruminants can be divided into three classes: dietary Mo greater than 20 ppm, low Cu:Mo ratio ($\leq$2:1), and high dietary sulfur with normal copper and Mo (Ward, 1978). The desired Cu:Mo ratio in ruminants is between 6:1 and 10:1 (Thompson *et al.*, 1991). Thus, Mo toxicosis can occur at much low concentrations in copper deficient animals than those on diets adequate in copper.

Most clinical signs of chronic Mo poisoning are associated with induced copper deficiency. Commonly, the first recognized clinical sign of chronic Mo poisoning is severe diarrhea (Dick, 1956; Pitt, 1976; Underwood, 1977; Ward, 1978; Friberg and Lener, 1986; Mills and Davis, 1987; Rajagopalan, 1988; Nielsen, 1996; Johnson, 1997; Coppock and Dziwenka, 2004; NRC, 2006). "Teart" is used to refer to soil or plants that contain unusually high amounts of Mo, thus the term teart scours is commonly used to describe the diarrhea associated with excessive Mo intake. Although the exact mechanism is not well defined, copper supplementation alleviates this clinical sign. Other common clinical signs of chronic Mo poisoning include poor body weight gain, weight loss, anemia, decreased milk production, achromotrichia, alopecia, limb deformities, bone fractures, periostosis, lameness, lack of libido, and ataxia. Abortions have been reported in horses.

Pathologic alterations of chronic Mo poisoning are not specifically diagnostic. These lesions are secondary to induced copper deficiency and include emaciation, periostosis, and epiphyseal plate growth abnormalities. Because of the nature of chronic Mo poisoning, primary copper deficiency must always be ruled out when these lesions are identified.

## TREATMENT

The two primary mechanisms of treating Mo toxicosis involve removal from the source of high Mo and copper supplementation, but administration of sulfate to monogastrics will enhance elimination rates. With rapid clearance from the body, removal from the high Mo source will quickly remove excess Mo from the body. However, permanent damage may be present that results in sustained animal deaths for weeks to months post-exposure (Swan *et al.*, 1998). Supplementation of copper in Mo poisoned animals must be done with care, especially in sheep, to prevent excessive copper accumulation and subsequent copper toxicosis.

## CONCLUDING REMARKS AND/OR FUTURE DIRECTION

The known mechanisms of Mo poisoning are a complex interaction with sulfur and copper that differ significantly across animal species. But, some clinical effects of Mo poisoning have not been related to absolute or functional copper deficits. These clinical effects of Mo poisoning need further investigation to delineate the mechanism by which Mo is producing adverse animal health.

# REFERENCES


Abumrad NN, Schneider AJ, Steel D, Rogers LS (1981) Amino acid intolerance during prolonged total parenteral nutrition reversed by molybdate therapy. *Am J Clin Nutr* **34**: 2551–9.

Allway WH, Kubota J, Losee F, Roth M (1968) Selenium, molybdenum, and vanadium in human blood. *Arch Environ Health* **16**: 342–9.

Anke M, Groppel B, Grun M (1985) Essentiality, toxicity, requirement and supply of molybdenum in human and animals. In *International Symposium on Trace Elements in Man and Animals*, vol. 5, Mills CT, Brenner I, Chesters (eds). Commonwealth Agriculture Bureaux, Farnham Royal, UK.

Archibald JG (1951) Molybdenum in cows milk. *J Dairy Sci* **34**: 1026–9.

Bell MD, Diggs GB, *et al.* (1964) Comparison of $Mo^{99}$ metabolism in swine and cattle as affected by stable molybdate. *J Nutr* **84**: 367–72.

Chan PC, Herbert RA, Roycroft JH, *et al.* (1998) Lung tumor induction by inhalation exposure to molybdenum trioxide in rats and mice. *Toxicol Sci* **45**: 58–65.

Coppock R, Dziwenka MM (2004) Molybdenum. In *Clinical Veterinary Toxicology*, Plumlee KH (ed.). Mosby, St. Louis, MO, pp. 211–14.

Dick AT (1956) Molybdenum in animal nutrition. *Soil Sci* **81**: 229–58.

Friberg L, Lener J (1986) Molybdenum. In *Handbook on the Toxicology of Metals*, 2nd edn, Friberg L, Nordberg GF, Vouk V (eds). Elsevier Science, New York, p. 446.

Gooneratne SR, Buckley WT, Christensen DA (1989) Review of copper deficiency and metabolism in ruminants. *Can J Anim Sci* **69**: 819–45.

Grace ND, Suttle NF (1979) Some effects of sulfur intake on molybdenum metabolism in sheep. *Br J Nutr* **41**: 125–36.

Howell JM, Gooneratne SR (1987) The pathology of copper toxicity in animals. In *Copper in Animals and Man*, Howell JM, Gawthorne JM (eds). CRC Press, Boca Raton, FL, pp. 53–78.

IMOA (International Molybdenum Association) (2006) Available at http://www.imoa.info.

Johnson JL (1997) Molybdenum. In *Handbook of Nutritionally Essential Mineral Elements*, O'Dell BL, Sunde RA (eds). Marcel Dekker, New York, pp. 413–38.

Johnson JL, Hainline BE, Rajagopalan KV (1980) Characterization of the molybdenum cofactor of sulfite oxidase, xanthine oxidase, and nitrate reductase. *J Biol Chem* **255**: 1783–6.

Ladefoged O, Sturup S (1995) Copper deficiency in cattle, sheep, and horses caused by excess molybdenum. *Vet Hum Tocicol* **37**: 63.

Lesperance AL, Bohman VR, Oldfield JE (1985) Interactions of molybdenum, sulfate, and alfalfa in the bovine. *J Anim Sci* **60**: 791–802.

Mason J, Cardin CJ (1977) The competition of molybdate and sulfate ions for a transport system in the ovine small intestine. *Res Vet Sci* **22**: 313–15.

Miller JK, Moss BR, Bell MC, Sneed NN (1972) Comparison of $Mo^{99}$ metabolism in young cattle and swine. *J Anim Sci* **34**: 846–50.

Mills CF, Bremner I (1980) Nutritional aspects of molybdenum in animals and man. In *Molybdenum and Molybdenum Containing Enzymes*, Coughlan MP (ed.). Pergamon, Oxford, pp. 517–42.

Mills CF, Davis GK (1987) Molybdenum. In *Trace Elements in Human and Animal Nutrition*, 5th edn, Mertz W (ed.). Academic Press, New York, pp. 429–65.

National Research Council (NRC) (2006) Molybdenum. In *Mineral Tolerance of Animals*, 2nd edn. National Academies Press, Washington, DC, pp. 262–75.

Nell JA, Annison EF, Balnave D (1980) The influence of tungsten on the molybdenum status of poultry. *Br Poult Sci* **21**: 193–202.

Nielsen FH (1996) Other trace elements. In *Present Knowledge in Nutrition*, 7th edn, Ziegler EE, Filer LJ (eds). International Life Science Institute Press, Washington, DC, pp. 353–77.

Pitt MA (1976) Molybdenum toxicity: interactions between copper, molybdenum, and sulfate. *Agent Action* **6**: 758–69.

Price J, Will AM, Paschaleris G, Chesters JK (1987) Identification of thiomolybdates in digesta and plasma from sheep after administration of $Mo^{99}$-labeled compounds into the rumen. *Br J Nutr* **58**: 127–38.

Pott EB, Henry PR, Zanetti MA, *et al.* (1999) Effects of high dietary molybdenum concentration and duration of feeding time on molybdenum and copper metabolism in sheep. *Anim Feed Sci Technol* **79**: 93.

Rajagopalan KV (1988) Molybdenum: an essential trace element in human nutrition. *Ann Rev Nutr* **8**: 401–27.

Reiss J (2000) Genetics of molybdenum cofactor deficiency. *Hum Genet* **106**: 157–63.

Rosman KJR, Taylor PDP (1998) Isotopic composition of the elements 1997. *Pure Appl Chem* **70**: 217–35.

Schroeder HA, Balassa JJ, Tipton IH (1970) Essential trace metals in man: molybdenum. *J Chron Dis* **23**: 481–99.

Spears JW (2003) Trace mineral bioavailability in ruminants. *J Nutr* **133**: 1506S–9S.

Suttle NF (1991) The interactions between copper, molybdenum, and sulfur in ruminant nutrition. *Annu Rev Nutr* **11**: 121–40.

Swan DA, Creeper JH, White CL, *et al.* (1998) Molybdenum poisoning in feedlot cattle. *Aust Vet J* **76**: 345–9.

Thompson LJ, Hall JO, Meerdink GL (1991) Toxic effects of trace element excess. *Vet Clin N Am-Food Anim Pract* **7**: 277–306.

Turnlund JR, Keyes WR, Peiffer GL, Chiang G (1995) Molybdenum absorption, excretion, and retention studied with stable isotopes in young men during depletion and repletion. *Am J Clin Nutr* **61**: 1102–9.

Underwood EJ (1977) Molybdenum. In *Trace Elements in Human and Animal Nutrition*, 4th edn, Underwood EJ (ed.). Academic Press, New York, pp. 429–65.

Versieck J, Hoste J, Vanballengerghe L, *et al*. (1981) Serum molybdenum in diseases of the liver and biliary system. *J Lab Clin Med* **97**: 535–44.

Venugopal B, Luckey TD (1978) Molybdenum. In *Metal Toxicity in Mammals 2: Chemical Toxicity of Metals and Metalloids*. Plenum Press, New York, pp. 253–7.

Vyskocil A, Viau C (1999) Assessment of molybdenum toxicity in humans. *J Appl Toxicol* **19**: 185–92.

Ward GM (1978) Molybdenum toxicity and hypocuprosis in ruminants:a review. *J Anim Sci* **46**: 1078–85.

Williams RJP, daSilva JJRF (2002) The involvement of molybdenum in life. *Biochem Biophys Res Comm* **292**: 293–9.

C H A P T E R

# 34

# Selenium

*Jeffery O. Hall*

## INTRODUCTION

Selenium is an essential nutrient that has a relatively narrow window between ingested amounts that result in deficiencies and those that cause toxicoses. Historically, occurrences of livestock disease that mimic clinical presentation of chronic selenium poisoning were recorded in the 13th century (Martin, 1973). Marco Polo wrote of such cases in western China in 1295. In 1560, Father Simon Pedro described human cases of presumably chronic selenosis in Columbia (Benavides and Mojica, 1965). The first documented record of selenium poisoning in livestock was reported in 1860 by US Army surgeon (Martin, 1973). T.W. Madison described a fatal disease of horses that grazed near Fort Randall, South Dakota. It also has been speculated that the horse illness that slowed General Custer's Cavalry relief may have been due to selenium, but chronic selenosis generally takes weeks to develop.

Selenium deficiency had been historically linked to a variety of clinical effects. Since 1949, vitamin E, cysteine and a "factor 3" were known to protect rats from fatal liver necrosis (Schwarz and Foltz, 1957). When rats were fed torula, a brewer's yeast, they developed liver necrosis that could be avoided by use of baker's yeast (*Saccharomyces sp.*). It was postulated that an essential nutrient, "factor 3", was deficient in torula. After much research, the active, preventative element present in "factor 3" was identified as selenium. Several metabolic diseases of previously unknown origin were later found to relate to selenium deficiency, including "white muscle disease" (WMD) in calves and lambs (Muth *et al*., 1958; Godwin and Fraser, 1966), hepatosis dietetica in pigs (Eggert *et al*., 1957), exudative diathesis in poultry (Patterson *et al*., 1957), and pancreatic degeneration in poultry (Thompson and Scott, 1969).

Since its first discovery as an essential nutrient, selenium has been found to act in numerous body systems. In 1973, Se was identified as an essential component of glutathione peroxidase (GSH) enzyme (Flohe *et al*., 1973; Rotruck *et al*., 1973). Selenium was shown to be essential in humans when added dietary selenium prevented a cardiomyopathy known as "Keshan disease" (Chen, 1986). Selenium supplementation may also be protective against certain types of cancer (Combs, 1997), cardiovascular disease (Duthie *et al*., 1989), and viral infections (Schrauzer, 1994; Levander, 2000). Because of the essential nature of selenium, poisoning cases from both natural plant accumulations of selenium and nutritional overdoses are encountered.

The identified essential functions of selenium are still increasing. The most notable is GSH, where selenocysteine is a required component of the enzyme system (Brown and Arthur, 2001). Reduced GSH is the primary physiologic first defense against free radical damage to tissues, helping to maintain functional membrane integrity. Several subclasses of GSH are now recognized (Cohen and Avissar, 1994; Sunde, 1994). To date over 30 selenoproteins have been identified, many of which have vital enzymatic functions (Tiwary, 2004). Thioredoxin reductase I, II, and III (Brown and Arthur, 2001), 5′ triiodothyronine deiodinase (Arthur and Beckett, 1994), and "selenoprotein" are also selenium dependent. In addition, selenium plays several roles in normal immune function, reproductive function, hepatic biotransformation reactions, neurotransmitter turnover, and anti-carcinogenic functions.

## BACKGROUND

Selenium is a member of the non-metallic elements within group VIa of the periodic table. It has an atomic number of 34, an atomic weight of 78.96, and has six different naturally occurring stable isotopic masses from 74 to 82

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(Rosman and Taylor, 1997). Selenium has four natural oxidation states: −2 (selenides), 0 (elemental), +4 (selenites), and +6 (selenates) (Barceloux, 1999). Selenium was first identified in 1817 by Jons Jakob Berzelius, a Swedish chemist, who investigated worker illnesses in a sulfuric acid plant at Gripsholm, Sweden (Fredga, 1972). He named this element after "Selene", the green moon Goddess.

Many areas within the Northern Great Plains of the United States, such as the Dakotas, Wyoming, Montana, Nebraska, and Kansas, have high soil selenium content (4–5 ppm Selenium or more), resulting in high plant uptake and subsequent Se toxicosis in herbivores (Rosenfeld and Beath, 1964). High soil selenium also occurs in alkaline soils of some localities in Algeria, Argentina, Australia, Bulgaria, Canada, China , Columbia, Ireland, Israel, Mexico, Morocco, New Zealand, South Africa, the former Soviet Union, Spain, and Venezuela (NRC, 1983). However, total soil selenium is not the best indicator of potential selenium poisonings, as Hawaii and Puerto Rico have areas of high soil selenium that is not available to the plants due to the acidic soil types, which result in lowered water soluble, bioavailable selenium for plant uptake (Lakin, 1961).

Inorganic forms of selenium are the primary form in soil. Only the water soluble forms are readily available for plant uptake, with the greatest absorption being in the form of selenate via the sulfate transporter. Elemental selenium and precipitated metal-selenides are not bioavailable for plant uptake. Some "indicator plants" or "obligatory selenium accumulator plants" can accumulate several thousand ppm selenium and are often found in selenium rich areas, since they require high selenium for growth (Rosenfeld and Beath, 1964). These plants include genera such as *Astragalus* (milk vetch), *Xylorhiza, Machaeranthera* (woody aster), *Haplopappus* (golden weed) – formerly known as *Oonopsis* and *Stanleya* (prince's plume). Selenium content as high as 14,990 ppm have been reported for a sample of *Astragalus racemosus* (Beath, 1937). Although these indicator plants have poor palatability, during times of limited forage, they are eaten. Secondary or facultative accumulating plants can survive with high selenium content, but do not require it for growth. These plants are often more palatable than the indicator plants and include *Aster*, *Atriplex* (salt bush), *Castilleja* (paintbrush), *Gutierrezia* (snakeweed), *Grindelia* (gumweeds), *Sideranthus* (ironweed), *Eurotia* (winter fat), *Mentzelia, Machaeranthera*, and *Gyria sp.,* as well as some crop plants such as western wheat grass, barley, wheat, alfalfa, onions, and Swiss chard (Beath *et al.*, 1935; Williams and Byers, 1936).

Most of the selenium in plants and other biological matrices is in an organic form, but small amounts of inorganic selenate and selenite can also be present. The vast majority of plant selenium, especially in non-indicator plants, is in the form of selenomethionine, but selenocysteine and a variety of other seleno-amino acid derivatives can also be found (Peterson and Butler, 1962; Olson *et al.*, 1970; Whanger, 2002). In contrast, the majority of selenium in indicator plants, such as *Astragalus*, is a water soluble Se-methyl-selenocysteine (Shrift, 1973), but can also have selenocystathionine (Lewis, 1976). Garlic was found to contain significant selenomethionine, as well as glutamyl-Se-methyl-selenocysteine and possibly $\gamma$-glutamyl-selenomethionine (Kotrebai *et al.*, 1999). The non-protein associated selenium compounds may be a protective mechanism of the plants to prevent excessive replacement of methionine in plant proteins resulting in misfolding and altered protein properties (Peterson and Butler, 1962). Some microbial populations, as well as plants, can reduce selenium to volatile chemical forms (Shrift, 1973).

## PHARMACOKINETICS/ TOXICOKINETICS

### Absorption

The majority of ingested selenium compounds are absorbed from the duodenum, with lesser amounts in the jejunum and ilium (Wright and Bell, 1966; Whanger *et al.*, 1976). Little to no absorption reportedly occurs from the stomach and rumen. However, one report suggests that minimal absorption of selenomethionine occurs through the rumen wall and into the blood (Hidiroglou and Jenkins, 1973).

The chemical form of selenium greatly impacts the overall absorption. Selenite absorption is via passive diffusion through the brush border membranes (Vendeland *et al.*, 1992, 1994). In contrast, selenate has little affinity for the brush border membranes. Selenate is absorbed via a sodium co-transport system that is also utilized by sulfate (Wolffram *et al.*, 1988). Selenium in the form of seleno-amino acids, selenomethionine, and selenocysteine are absorbed through active amino acid transport mechanisms and are more bioavailable than selenite or selenate (McConnell and Cho, 1967; Ammerman and Miller, 1974; Vendeland *et al.*, 1994). The selenium status did not affect overall absorption, indicating that absorption was not under homeostatic regulation.

In monogastrics, the relative selenium absorption is greater than in ruminants, ranging from 45% to 95% (Thomson and Stewart, 1974; Furchner *et al.*, 1975; Bopp *et al.*, 1982). And organic forms of selenium are better absorbed (Robinson *et al.*, 1978). In ruminants, the relative absorption ranges from 29% to 50% (Wright and Bell, 1966; Suttle and Jones, 1989). The decreased absorption in ruminants is due to microbial reduction of selenium forms in the rumen to selenides and elemental selenium which are not bioavailable (Cousins and Cairney, 1961; Whanger *et al.*, 1968; Peter *et al.*, 1982). This reduction in

bioavailability is generally exacerbated by high carbohydrate diets, but can be altered by differing rumen microbial populations (Hudman and Glenn, 1984; Koenig *et al.*, 1997). Some rumen microbes more efficiently reduce selenium, while others effectively incorporate it into selenium containing amino acids. The incorporation of selenium into microbial proteins, as well as systemic absorption, can be competitively inhibited by natural methionine and cysteine (Serra *et al.*, 1996).

## Distribution

Tissue distribution is dependent on the chemical form of selenium absorbed. Selenium is generally utilized for synthesis of selenoproteins, incorporated into tissue proteins, or eliminated. Selenomethionine can be non-specifically incorporated into tissue proteins in place of methionine (Awadeh *et al.*, 1998), but selenocysteine is not (Burk *et al.*, 2001), with highest incorporation occurring in tissues with high rates of protein synthesis (Hansson and Jacobsson, 1966). The non-specific incorporation of selenomethionine effectively serves as a pool of selenium reserve with a long biological half-life (Schroeder and Mitchener, 1972). Highest total selenium content is typically found in the kidney and liver, with lesser amounts in all other tissues (Muth *et al.*, 1967; Levander, 1987; Echevarria *et al.*, 1988; Davidson and Kennedy, 1993). Both specific and non-specific selenium incorporation into proteins was greater in selenium deficient animals.

Time to peak tissue concentrations is tissue dependent. Peak selenium content of blood, liver, muscle, kidney, spleen, and lung was reached within 24 h after an injection of $^{75}$Se as selenite (Muth *et al.*, 1967). In contrast, brain, thymus, and reproductive organs do not reach maximal content until much later (Brown and Burk, 1973; McConnell *et al.*, 1979; Smith *et al.*, 1979; Behne *et al.*, 1988).

Selenium is efficiently transferred across the placenta into feti during gestation. The overall maternal selenium content is positively correlated with fetal and newborn selenium status (McConnell and Roth, 1964). Newborns get some selenium from milk, with much higher content in colostrums than in milk later in lactation.

## Metabolism

Selenite is metabolized in red blood cells to hydrogen selenide (Gasiewicz and Smith, 1978). Sequential methylation reactions result in the formation of monomethylselenide, dimethylselenide, and trimethylselenide (Kajander *et al.*, 1991; Itoh and Suzuki, 1997). These reactions utilize *S*-adenosylmethionine for methyl groups which are transferred by methyltransferases (Kajander *et al.*, 1991). These sequential reactions can deplete available *S*-adenosylmethionine, which would limit the degree of methylation. In rats given selenomethionine, trimethylselenide occurred in the urine more rapidly than in rats given sodium selenite or selenocysteine, indicating that selenomethionine may be converted to methylselenol which is easily further methylated.

Selenomethionine is metabolized by demethylation to selenocysteine. This set of pathways is similar to the metabolism of methionine. The selenocysteine is then metabolized by selenocysteine-β-lyase in the liver and kidney to alanine and selenide (Soda *et al.*, 1987).

## Elimination

Selenium is primarily excreted in the urine and feces, but the form and extent of elimination by different routes are dose and species dependent. In monogastric animals, urinary elimination predominates, irrespective of the route of exposure (Leng *et al.*, 2000), with less than 10% recovered in feces (Burk *et al.*, 1972). Some literature suggests that urinary eliminated selenium is predominantly metabolites of selenium, with trimethylselenide predominating at higher doses (McConnell and Roth, 1966; Palmer *et al.*, 1969; Zeisel *et al.*, 1987; Itoh and Suzuki, 1997), but monomethylselenide is more abundant at lower doses. Human elimination is tri-exponential for selenite and selenomethionine (Alexander *et al.*, 1987). The terminal elimination phase was 8–20 and 230 days for selenite and selenomethionine, respectively. Overall selenium retention and maintenance of adequate seleno-enzymes are for a much longer time period in animals supplemented with selenomethionine than selenite. Elimination rate is dose dependent, with half-lives of 19.5 and 1.2 days with selenite of 0.1 and 1.0 ppm in the diet, respectively (Burk *et al.*, 1972). Due to non-specific protein incorporation of selenomethionine, urinary and fecal recovery after dosing was less than 30% of that for selenite or selenate (Thomson, 1998).

The literature suggests that the predominant selenium elimination in ruminants is fecal when ingested, but urinary with parenteral administration or in non-ruminating young animals. This is actually an error in terminology, as the fecal loss of selenium is primarily in the forms of elemental selenium and precipitated selenides from ruminal reduction (Langlands *et al.*, 1986). Thus, this selenium is just non-absorbed material and not truly being eliminated from the central compartment. However, a small amount of metabolized selenium excesses is excreted in the bile (Cousins and Cairney, 1961). The metabolic metabolites eliminated in the urine follow a similar pattern to that seen with monogastrics. Urinary elimination is predominant with parenteral administrations and in non-ruminating young animals (NRC, 1983).

Renal selenium elimination is dependent of glomerular filtration and degree of re-absorption. Increasing renal fluid absorption did not increase the selenium content in urine, indicating a tubular re-absorptive process (Oster and Prellwitz, 1990). Thus, dehydration or renal insufficiency would decrease rates of elimination. Excretion and renal clearance rates correlate with creatinine, indicating glomerular filtration is the mechanism of elimination.

Some selenium is eliminated via respired air, but the relative importance of this route is dose dependent. At normal intake, only about 10% or less is eliminated from the respiratory tract (Burk *et al.*, 1972), but as dose increases the percent eliminated in respired air increases (Jacobsson, 1966; McConnell and Roth, 1966). Dimethylselenide and dimethyldiselenide are the predominant forms eliminated in respired air at toxic doses. Dimethylselenide predominates when mice were dosed with selenite or selenocysteine, while dimethyldiselenide is most abundant when rats were dosed with selenomethionine. Respiratory elimination is primarily when renal elimination thresholds are maximized, which results in most respiratory elimination occurring in a short-time period soon after exposure to toxic doses (McConnell and Roth, 1966; Tiwary *et al.*, 2005).

## MECHANISM OF ACTION

Although much research has been conducted with regard to selenium poisoning, the exact mechanism of the toxic effects in the body are still not clear. With acute poisoning, one theory is the depletion of intermediate substrates, such as glutathione and *S*-adenosylmethionine, which disturbs their respective enzyme activities (Vernie *et al.*, 1978). Another potentially interactive theory is the production of free radicals by reaction of selenium with thiols, causing subsequent oxidative tissue damage (Hoffman, 2002; Kaur *et al.*, 2003; Balogh *et al.*, 2004). A third theory is the incorporation of selenium compounds in place of sulfur, such as in proteins, in which it disrupts normal cellular functions (Raisbeck, 2000). This is an especially likely mechanism for the hair and hoof lesions of chronic selenium poisoning, with the loss of disulfide bridges which provides structural integrity to these tissues. This would also apply to inhibition of DNA methylation by *S*-adenosylmethionine or indirect inhibition by increased *S*-adenosyl homocysteine content (Hoffman, 1977). And, it is possible that each of these proposed mechanisms is valid with respect to specific chemical forms of selenium.

## TOXICITY

Selenium poisoning cases generally fall into three types of exposure history. The first is from ingestion of selenium in plants that have accumulated it from naturally seleniferous soils. The second is from accidental overdoses by injection or errors in feed mixing. And the third is from environmental contamination, which often results in exposure from plant accumulation. With each of these types of poisonings, one may see acute, subacute, or chronic selenium poisoning, depending upon the daily exposure rate. However, one must understand that an animal's age plays a role in susceptibility to selenium poisoning, as young animals are less tolerant than adults (Raisbeck, 2000).

Acute oral selenium poisoning occurs with sudden exposure ranging from 2.2 mg/kg (Rosenfeld and Beath, 1964) to greater than 20 mg/kg body wt. (Miller and Williams, 1940; Mahan and Moxon, 1984) across species. The relative acute toxicity of selenium containing compounds is dependent on their solubility, with poorly soluble selenides and elemental selenium being much less toxic than soluble selenates, selenites, and organic selenium (NRC, 2006). Minimum lethal dose for rabbits, rats, dogs, and cats is 1.5–3 mg/kg body wt. (NRC, 1983). The $LD_{50}$ for oral selenite has been estimated to be 1.9–8.3 mg/kg body wt. in ruminants (Grace, 1994), but other references suggest it to be 9–20 mg/kg body wt. (Puls, 1994). In poultry, the acute oral $LD_{50}$ of selenium is 33 mg/kg body wt. Injectable selenium is more acutely toxic than oral, with intramuscular $LD_{50}$ of 0.5 mg/kg in lambs (Caravaggi *et al.*, 1970). Subcutaneous $LD_{50}$ of selenium is 1 mg/kg in lambs and 1.9 mg/kg in adult cattle (Grace, 1994).

Clinical manifestation of acute selenium poisoning begins as early as 8–10 h, but can be delayed for up to 36 h (Franke and Moxon, 1936; NRC, 1983; Raisbeck, 2000; Tiwary *et al.*, 2006). Early in the clinical syndrome, one can smell the garlicky smell of dimethylselenide on the breath. Clinical signs that follow include by respiratory distress, restlessness or lethargy, head down, droopy ears, anorexia, gaunt appearance, salivation, watery diarrhea, fever, sweating, tachycardia, teeth grinding, tilted gait, tetanic spasms, and/or death. Clinical signs tent to progress quickly after first observed. Gross and histologic lesions include systemic congestion, pulmonary edema, and petechial hemorrhages in and on the myocardium.

"Blind staggers" has historically associated with subacute to chronic selenium. However, this association was due to its occurrence in known seleniferous areas. The areas with seleniferous soils also tend to have highly alkaline soils with high potential for excessive sulfur exposure. It has been stated that blind staggers cannot be reproduced with pure selenium compounds alone and likely involves other factors, such as alkaloid poisoning, starvation, or polioencephalomalasia (O'Toole and Raisbeck, 1995). However, one can still find references that tie it to selenium (Underwood and Suttle, 1999; NRC, 2006).

Chronic selenosis, often referred to as "alkali disease", is the results of long-term ingestion of seleniferous forages (NRC, 1983; Raisbeck, 2000 NRC, 2006;). High selenium intake is generally for greater than 30 days and, due to

plant selenium content, is usually associated with facultative accumulators, not indicator plants, although chronic selenosis can also be reproduced by long-term feeding of high inorganic selenium (Kaur *et al*., 2003). Calves were chronically poisoned with selenite at 0.25 mg/kg body wt. daily for 16 weeks. In a similar study in yearlings, selenium as selenite at 0.8 mg/kg/day and as selenomethionine at 0.28 mg/kg/day resulted in alkali disease (O'Toole and Raisbeck, 1995). However, other studies did not produce alkali disease with selenium doses as high as 11.9 mg/kg of diet in feeders or 118 mg/kg body wt. daily for 128 days in dairy cows (Ellis *et al*., 1997 Lawler *et al*., 2004;). Differences in susceptibility to chronic selenium poisoning may be a product of historical exposure and variability in rumen microbial population. As stated previously, certain microbes can reduce selenium to non-bioavailable forms, resulting in decreased systemic absorption. Pigs develop chronic selenosis with exposure to selenium as low as 8 mg/kg of diet (Goehring *et al*., 1984; Mahan and Magee, 1991; Stowe and Herdt, 1992). And horses exposed to 20 mg Se/kg DM for 3 weeks developed lesions (Stowe and Herdt, 1992).

Clinical signs of chronic selenosis include depression, weakness, emaciation, anemia, hair loss, anorexia, diarrhea, weight loss, lameness, and death (Rosenfeld and Beath, 1964; O'Toole and Raisbeck, 1995; Underwood and Suttle, 1999 Raisbeck, 2000;). Hoof wall abnormalities are frequently identified in cattle, horses, and pigs and include swelling of the coronary band, hoof deformities, and/or separation and sloughing of the hoof wall. Hair loss from the base of the tail and switch in cattle, horses, and mules is sometimes referred to as "Bobtail disease". Interestingly, sheep do not develop the alopecia or hoof lesions that are seen in cattle, but they have decreased wool growth rates. In pigs, goats, and horses, there may be a general alopecia (Franke, 1934). Pigs also develop neurologic signs of paralysis (Goehring *et al*., 1984).

Pathologic lesions of chronic selenium poisoning are generally related to hoof lesions and to the effects of starvation (Raisbeck, 2000). Lesions of nephritis, hepatic cirrhosis, and myocardial necrosis can be expected. In pigs, bilateral malacia of the gray matter in the spinal cord can be seen.

Reproductive abnormalities are seen in several species when excessive selenium is ingested. Field reports indicate that reproductive performance can be reduced without the other typical signs of alkali disease at 5–10 ppm Se in diet (Olson *et al*., 1970), but direct experimental evidence is lacking (Raisbeck, 2000). Decreased conception rate and an increased fetal resorption rate in cattle, sheep, and horses were observed when they were fed natural diets containing 20–50 mg Se/kg diet (Harr and Muth, 1972). In addition, it must be noted that Se accumulates in the fetus at the expense of the dam (Puls, 1994). Thus, higher accumulation of selenium in the fetus may result in abortions, stillbirths, or weak/lethargic calves. However, at least some of the adverse effects on reproduction caused by excess selenium in ruminants are caused by interference with absorption and retention of copper that results in copper deficiency.

Selenosis in poultry has major effects in reproduction. Poor hatchability, embryonic deformities, and embryonic death are common sequelle to selenium poisoning (Latshaw *et al*., 2004).

## TREATMENT

The most effective treatment is to prevent excessive exposure. Although the maximal tolerable level for selenium was once set at 2 mg/kg of diet per day for all species, this has now been changed to 5 mg/kg of diet per day for ruminants (NRC, 2006). It is stated that this new tolerance for ruminants is appropriate for horses as well. Swines have a maximum tolerance level of 4 mg/kg of diet, for poultry it is set at 3 mg/kg of diet, and for fish it is set at 2 mg/kg of diet.

As there is no specific mechanism of chelation and removal of selenium in animals, the primary treatment protocol is of supportive care with both acute and chronic selenium poisoning. With chronic poisoning, it is important to understand the long-term commitment necessary to allow an animal with hoof lesions time to re-grow the hoof wall once exposure has been stopped. Especially with organic selenium's incorporation into body proteins, the time necessary to just decrease the body load of selenium, once excessive exposure has stopped, is quite long.

## CONCLUDING REMARKS AND FUTURE DIRECTIONS

Selenium deficiency and toxicity are problems that veterinarians, nutritionists, and animal owners need to understand. The small difference between deficiency and toxicity in terms of dietary intake makes the risk of accidental selenium poisoning high. Many areas of the world have seleniferous soils which can cause poisoning via ingestion of natural forages grown on them. As there is no specific treatment for selenium toxicosis, it is critical that appropriate education be utilized in order to minimize the risks to the livestock industry.

Future research needs to address the mechanisms of physiologic damage caused by both acute and chronic selenium poisoning with differing chemical forms of selenium. With an understanding of the cellular mechanisms of selenium poisoning, more specific means of treatment might be developed. In addition, a clearer understanding of thresholds for chronic selenium poisoning is needed.

With the knowledge that certain ruminal microbes can reduce various forms of selenium to non-absorbable forms, preventative use of these microbes in seleniferous areas to prevent systemic accumulation and poisonings should be investigated.

## REFERENCES


Alexander J, Hogberg J, Thomassen Y, Aaseth J (1987) Selenium. In *Handbook on Toxicity of Inorganic Compounds*, Seiler HG (ed.). Marcel Dekker, New York, p. 585.

Ammerman CB, Miller SM (1974) Selenium in ruminant nutrition: a review. *J Dairy Sci* **58**: 1561–76.

Arthur JR, Beckett GJ (1994) New metabolic roles for selenium. *Proc Nutr Soc* **53**: 615–24.

Awadeh FT, Rahman A, Kincaid RL, Finley JW (1998) Effect of selenium supplements on the distribution of selenium among serum proteins in cattle. *J Dairy Sci* **81**: 1089–94.

Balogh K, Weber M, Erdelyi M, Mezes M (2004) Effects of excess selenium supplementation on the glutathione redox system in broiler chickens. *Acta Vet Hung* **52**: 403–11.

Barceloux DG (1999) Selenium. *Clin Toxicol* **37**: 145–72.

Beath OA (1937) The occurrence of selenium and seleniferous vegetation in Wyoming. II. Seleniferous vegetation. *Wyo Agric Exp Sta Bull* **221**: 29–64. Laramie, WY.

Beath OA, Eppson HF, Gilbert CS (1935) Selenium and other toxic minerals in soils and vegetation. *Wyo Agric Exp Sta Bull* **206**: 1–55. Laramie, WY.

Behne D, Hillmert H, Scheid S, Gessner H, Elger W (1988) Evidence for specific target tissues and new biologically important SelP. *Biochim Biophys Acta* **966**: 12–21.

Benavides ST, Mojica FS (1965) *Selenosis*, 2nd edn. Instituto Geografico Augustin Codazzi, Bogota, Columbia.

Bopp BA, Sonders RC, Kesterson JW (1982) Metabolic fate of selected selenium compounds in laboratory animals and man. *Drug Metab Rev* **13**: 271–318.

Brown KM, Arthur JR (2001) Selenium, selenoproteins and human health: a review. *Public Health Nutr* **4**: 593–9.

Brown DG, Burk RF (1973) Selenium retention in tissues and sperm of rats fed a torula yeast diet. *J Nutr* **103**: 102–8.

Burk RF, Brown DG, Seely RJ, Scaief III CC (1972) Influence of dietary and injected selenium on whole-body retention, route of excretion, and tissue retention of $^{75}SeO_3^{2-}$ in the rat. *J Nutr* **102**: 1049–55.

Burk RF, Hill KE, Motley AK (2001) Plasma selenium in specific and non-specific forms. *Biofactor* **14**: 107–14.

Caravaggi C, Clark FL, Jackson ARB (1970) Acute selenium toxicity in lambs following intramuscular injection of sodium selenite. *Res Vet Sci* **11**: 146–9.

Chen X (1986) Selenium and cardiomyopathy (Keshan disease). *Acta Pharmacol Toxicol* **59**: 325–30.

Cohen HJ, Avissar N (1994) Extracellular glutathione peroxidase: a distinct selenoprotein. In *Selenium in Biology and Human Health*, Burk RF (ed.). Springer-Verlag, New York, pp. 79–92.

Combs GF (1997) Selenium and cancer prevention. In *Antioxidants and Disease Prevention*, Garewal HA (ed.). CRC Press, New York, pp. 97–113.

Cousins FB, Cairney IM (1961) Some aspects of selenium metabolism in sheep. *Aust J Agric Res* **12**: 927–33.

Davidson WB, Kennedy DG (1993) Synthesis of [$^{75}$Se] selenoproteins is greater in selenium-deficient sheep. *J Nutr* **123**: 689–94.

Duthie GG, Wahle KWJ, James WPJ (1989) Oxidants, antioxidants and cardiovascular disease. *Nutr Res Rev* **2**: 51–62.

Echevarria M, Henry PR, Ammerman CB, Rao PV (1988) Effects of time and dietary selenium concentration as sodium selenite on tissue selenium uptake by sheep. *J Anim Sci* **66**: 2299–305.

Eggert RO, Patterson E, Akers WJ, Stokstad ELR (1957) The role of vitamin E and selenium in the nutrition of the pig. *J Anim Sci* **16**: 1037–45.

Ellis RG, Herdt TH, Stowe HD (1997) Physical, hematologic, biochemical, and immunological effects of supranutritional supplementation with dietary selenium in Holstein cows. *Am J Vet Res* **58**: 760–4.

Flohe L, Gunzler WA, Shock HH (1973) Glutathione peroxidase: a selenoenzyme. *FEBS Lett* **32**: 132–4.

Franke KW (1934) A new toxicant occurring naturally in certain samples of plant foodstuffs. I. Results obtained in preliminary feeding trials. *J Nutr* **8**: 597–608.

Franke KW, Moxon AL (1936) A comparison of the minimum fatal doses of selenium, tellurium, arsenic, and vanadium. *J Pharm Exptl Therap* **58**: 454–9.

Fredga A (1972) Organic selenium chemistry. *Ann NY Acad Sci* **192**: 1–9.

Furchner JE, London JE, Wilson JS (1975) Comparative metabolism of radionuclides in mammals – IX. Retention of $^{75}$Se in the mouse, rat, monkey and dog. *Health Phys* **29**: 641–8.

Gasiewicz TA, Smith JC (1978) The metabolism of selenite by intact rat erythrocytes *in vitro*. *Chem Biol Interact* **21**: 299–313.

Godwin KO, Fraser FJ (1966) Abnormal electrocardiograms, blood pressure changes, and some aspects of the histopathology of selenium deficiency in lambs. *Quart J Exp Physiol* **51**: 94–102.

Goehring TB, Palmer IS, Olson OE, Libal GW, Wahlstorm RC (1984) Effects of seleniferous grains and inorganic selenium on tissue and blood composition of and growth performance of rats and swine. *J Anim Sci* **59**: 725–32.

Grace ND, Selenium (1994). *Managing Trace Element Deficiencies*, Grace ND (ed.). New Zealand Pastoral Agricultural Research Institute, Simon Print, Palmerston North, New Zealand.

Hansson E, Jacobsson SO (1966) Uptake of [$^{75}$Se] selenomethionine in the tissues of the mouse studied by whole-body autoradiography. *Biochim Biophys Acta* **115**: 285–93.

Harr JR, Muth OH (1972) Selenium poisoning in domestic animals and its relationship to man. *Clin Toxicol* **5**: 175–86.

Hidiroglou M, Jenkins KJ (1973) Absorption of $^{75}$Se-selenomethionine from the rumen of sheep. *Can J Anim Sci* **53**: 345–7.

Hoffman DJ (2002) Role of selenium toxicity and oxidative stress in aquatic birds. *Aquat Toxicol* **57**: 11–26.

Hoffman JL (1977) Selenite toxicity, depletion of liver *S*-adenosylmethionine, and inactivation of methionine adenosyltransferase. *Arch Biochem Biophys* **179**: 136–40.

Hudman JF, Glenn AR (1984) Selenite uptake and incorporation by selenomonas ruminantium. *Arch Microbiol* **140**: 252–6.

Itoh M, Suzuki KT (1997) Effects of dose on the methylation of selenium to monomethylselenol and trimethyl selenonium ion in rats. *Arch Toxicol* **71**: 461–6.

Jacobsson SO (1966) Excretion of a single dose of selenium in sheep. *Acta Vet Scand* **7**: 226–39.

Kajander EO, Harvima RJ, Elonranta TO, Martikainen H, Kantola M, Karenlampi SO, Akerman K (1991) Metabolism, cellular actions, and cytotoxicity of selenomethionine in cultured cells. *Biol Trace Elem Res* **28**: 57–68.

Kaur R, Sharma S, Rampal S (2003). Effects of subchronic selenium toxicosis on lipid peroxidation, glutathione redox cycle, and antioxidant enzymes in calves. *Vet Hum Toxicol* **45**: 190–2.

Koenig KM, Rode LM, Cohen RDH, Buckley WT (1997) Effect of diet and chemical form of selenium in sheep. *J Anim Sci* **75**: 817–27.

Kotrebai M, Birringer, Tyson JF, Block E, Uden PC (1999) Identification of the principal selenium compounds in selenium-enriched natural

sample extracts by ion-pair liquid chromatography with inductively coupled plasma and electrospray ionization-mass spectrometric detection. *Anal Commun* **36**: 249–52.

Lakin HW (1961) Geochemistry of selenium in relation to agriculture. In *Selenium in Agriculture. US Department of Agriculture, Agric Handb* 2001, US Government Printing Office, Washington DC.

Langlands JP, Bowles JE, Donald GE, Smith AJ (1986) Selenium excretion in sheep. *Aust J Agric Res* **37**: 201–9.

Latshaw DJ, Morishita TY, Sarver CF, Thilsted J (2004) Selenium toxicity in breeding ring-necked pheasants (*Phasianus colchicus*). *Avian Dis* **48**: 935–9.

Lawler TL, Taylor JB, Finley JW, Canton JS (2004) Effects of supranutritional and organically bound selenium on performance, carcass characteristics, and selenium distribution in finishing beef steers. *J Anim Sci* **82**: 1488–93.

Leng L, Boldizarova K, Faix S, Kovac G (2000) The urinary excretion of selenium in sheep treated with a vasopressin analogue. *Vet Res* **31**: 499–505.

Levander OA (1987) Selenium. In *Trace Elements in Human and Animal Nutrition*, 5th edn, vol. 2, Mertz W (ed.). Academic Press, New York, pp. 209–79.

Levander OA (2000) The selenium-coxsackievirus connection: Chronicle of collaboration. *J Nutr* **130**: 485S–8S.

Lewis BG (1976) Selenium in biological systems, and pathways for its volatilization in higher plants. In *Environmental Biogeochemistry*, Nriagu JO (ed.). Ann Arbor Science, Ann Arbor, MI, pp. 389–409.

Mahan DC, Magee PL (1991) Efficacy of dietary sodium selenite and calcium selenite provided in the diet at approved, marginally toxic, and toxic levels to growing swine. *J Anim Sci* **69**: 722–5.

Mahan DC, Moxon AL (1984) Effect of inorganic selenium supplementation on selenosis in post-weaning swine. *J Anim Sci* **58**: 216–21.

Martin JL (1973) Selenium compounds in nature and medicine. In *Organic Selenium Compounds: Their Chemistry and Biology*, Klayman DL, Gunther WHH (eds). John Wiley & Sons, New York, pp. 663–91.

McConnell KP, Cho GJ (1967) Active transport of *L*-selenomethionine in the intestine. *Am J Physiol* **213**: 50–6.

McConnell KP, Roth DM (1964) Passage of selenium across the placenta and also into the milk of the dog. *J Nutr* **84**: 40–4.

McConnell KP, Roth DM (1966) Respiratory excretion of selenium. *Proc Soc Exp Biol Med* **123**: 19–21.

McConnell KP, Burton RM, Kute T, Higgins PJ (1979) Selenoproteins from rat testis cytosol. *Biochim Biophys Acta* **588**: 13–19.

Miller WT, Williams KT (1940) Minimum lethal dose of selenium as sodium selenite for horses, mules, cattle and swine. *J Agric Res* **60**: 53–73.

Muth OH, Oldfield JE, Remmert LF, Schubert JR (1958) Effects of selenium and vit. E on white muscle disease. *Science* **128**: 1090–7.

Muth OH, Pendell HW, Watson CR, Oldfield JE, Weswig PH (1967) Uptake and retention of parenterally administered $^{75}$Se in ewes on different selenium regimens. *Am J Vet Res* **28**: 397–406.

National Research Council (NRC) (1983) *Selenium in Nutrition* revised edition. Subcommittee on Selenium, Committee on Animal Nutrition, Washington, DC.

National Research Council (NRC) (2006) Selenium. In *Mineral Tolerance of Animals*, 2nd edn. National Academies Press, Washington, DC, pp. 321–47.

Olson OE, Novacek EJ, Whitehead EI, Palmer IS (1970) Investigations on selenium in wheat. *Phytochem* **9**: 181–90.

Oster O, Prellwitz W (1990) The renal excretion of selenium. *Biol Trace Elem Res* **24**: 19–46.

O'Toole D, Raisbeck MF (1995) Pathology of experimentally induced chronic selenosis ("alkali disease") in yearling cattle. *J Vet Diagn Invest* **7**: 64–73.

Palmer IS, Fischer DD, Halverson AW, Olson OE (1969) Identification of a major selenium excretory product in rat urine. *Biochim Biophys Acta* **177**: 336–42.

Patterson EL, Milstrey R, Stokstad ELR (1957) Effect of selenium in preventing exudative diathesis in chicks. *Proc Soc Exp Biol Med* **95**: 17–20.

Peter DW, Whanger PD, Lindsay JP, Buscall DJ (1982) Excretion of selenium, zinc and copper by sheep receiving continuous intraruminal infusions of selenite or selenomethionine. *Proc Nutr Soc* **7**: 78.

Peterson PJ, Butler GW (1962) The uptake and assimilation of selenite by higher plants. *Australian J Biol Sci* **15**: 26–46.

Puls R (1994) *Mineral Levels in Animal Health,* 2nd edn, Diagnostic data. Sherpa International, British Columbia, Canada.

Raisbeck MF (2000) Selenosis. Veterinary clinics of North America: Food animal practice. **16**(3): 465–80.

Robinson MF, Rea RM, Friend GM, Stewart RDR, Scow PC, Thomson CD (1978) On supplementing the selenium intake of New Zealanders. 2. Prolonged metabolic experiments with daily supplements of selenomethionine, selenite and fish. *Br J Nutr* **39**: 89–95.

Rosenfeld I, Beath OA (1964) *Selenium: Geobotany, Biochemistry, Toxicity, and Nutrition*. Academic Press, New York.

Rosman KJR, Taylor PDP (1997) Isotopic composition of the elements 1997. *Pure Appl Chem* **70**: 217–35.

Rotruck JT, Pope AL, Ganther HE, Swanson AB, Hafeman DL, Hoekstra WG (1973) Selenium: biochemical role as a component of glutathione peroxidase. *Science* **179**: 588–90.

Schrauzer GN (1994) Selenium in the maintenance and therapy of HIV-infected patients. *Chem Biol Interact* **91**: 199–205.

Schroeder HA, Mitchener M (1972) Selenium and tellurium in rats: effect on growth, survival, and tumors. *J Nutr* **101**: 1531–40.

Schwarz K, Foltz CM (1957) Selenium as an integral part of Factor 3 against dietary necrotic liver degeneration. *J Am Chem Soc* **78**: 3292.

Serra AB, Serra SD, Fujihara T (1996) Influence of dietary protein on the fractionation of selenium in the rumen of sheep. *Biol Trace Elem Res* **9**: 557–62.

Shrift A (1973) Selenium compounds in nature and medicine. In *Organic Selenium Compounds: Their Chemistry and Biology*, Klayman DL, Gunther WH (eds). Wiley-Interscience, New York, pp. 763–814.

Smith DG, Senger PL, McCutchan JF, Landa CA (1979) Selenium and glutathione peroxidase distribution in bovine semen and selenium-75 retention by the tissues of the reproductive tract in the bull. *Biol Reprod* **20**: 377.

Soda K, Esaki N, Nakamura T, Tanaka H (1987) Selenocysteine β -lyase: an enzymological aspect of mammalian selenocysteine metabolism. In *Selenium in Biology and Medicine. Part A. Proceedings of the Third International Symposium on Selenium in Biology and Medicine.* May 27–June 1, 1984 at Beijing, China. AVI Book Pub by Van Nostrand Reinhold Co., New York, pp. 160–71.

Stowe HD, Herdt TH (1992) Clinical assessment of selenium status of livestock. *J Anim Sci* **70**: 3928–33.

Sunde RA (1994) In *Selenium in Biology and Human Health*, Burk RF (ed.). Springer-Verlag, New York, pp. 45–78.

Suttle NF, Jones DG (1989) Recent developments in trace element metabolism and function: trace elements, disease resistance and immune responsiveness in ruminants. *J Nutr* **119**: 1055–61.

Thomson CD (1998) Selenium speciation in human body fluids. *Analyst* **123**: 827–31.

Thompson JM, Scott ML (1969) Role of selenium in the nutrition of the chick. *J Nutr* **97**: 335–42.

Thomson CD, Stewart RDH (1974) The metabolism of [$^{75}$Se] in young women. *Br J Nutr* **32**: 47–57.

Tiwary AK (2004) Differences between inorganic and organic selenium toxicosis in sheep. Masters Thesis, Utah State University, Logan, UT.

Tiwary AK, Panter KE, Stegelmeier BL, James LF, Hall JO (2005) Evaluation of respiratory elimination kinetics of selenium after oral administration in sheep. *Am J Vet Res* **66**: 1–7.

Tiwary AK, Stegelmei BL, Panter KE, James LF, Hall JO (2006) Comparative toxicosis of sodium selenite and selenomethionine in lambs. *J Vet Diag Invest* **18**: 60–9.

Underwood EJ, Suttle NF (1999) *The Mineral Nutrition of Livestock*, 3rd edn. Wallingford, Oxon, UK.

Vendeland SC, Butler JA, Whanger PD (1992) Intestinal absorption of selenite, selenate and selenomethionine in the rat. *J Nutr Biochem* **3**: 359–65.

Vendeland SC, Deagen JT, Butler JA, Whanger PD (1994) Uptake of selenite, selenomethionine and selenate by brush border membrane vesicles isolated from rat small intestine. *Biometals* **7**: 305–12.

Vernie LN, Ginjarr HB, Wilders IT, Bont WS (1978) Amino acid incorporation in a cell-free systemderived from rat liver studied with the aid of selenodiglutathione. *Biochem Biophys Acta* **518**: 507–17.

Whanger PD (2002) Selenocompounds in plants and animals and their biological significance. *J Am Coll Nutr* **21**: 223–32.

Whanger PD, Pedersen ND, Hatfield, Weswig PH (1976) Absorption of selenite and selenomethionine from ligated digestive tract segments in rats. *Proc Soc Exp Biol Med* **153**: 295.

Whanger PD, Weswig PH, Muth OH (1968) Metabolism of $^{75}$Se-selenite and $^{75}$Se-selenomethionine by rumen microorganisms. *Fed Proc* **27**: 418.

Williams KT, Byers HG (1936) Se compounds in soils. *Ind Eng Chem* **28**: 912.

Wolffram S, Grenacher B, Scharrer E (1988) Transport of selenate and sulphate across the intestinal brush-border membrane of pig jejunum by two common mechanisms. *Q J Exp Physiol* **73**: 103–11.

Wright PL, Bell MC (1966) Comparative metabolism of selenium and tellurium in sheep and swine. *Am J Physiol* **211**: 6–10.

Zeisel SH, Ellis AL, Sun XF, Pomfret EA, Ting BTG, Janghorbani M (1987) Dose–response relations in urinary excretion of trimethylselenonium ion in the rat. *J Nutr* **117**: 1609–14.

CHAPTER

# 35

# Sodium chloride (salt)

*Larry J. Thompson*

## INTRODUCTION

Sodium chloride is often referred to as table salt, common salt or just simply as salt. Salt is an essential nutrient and can give an attractive taste to foods and feeds. It has been stated that salt is the only mineral compound for which animals can truly develop a craving.

Salt is a necessary nutrient for the health of animals and many nutrition texts divide it into separate requirements for sodium (Na) and chloride (Cl). Daily requirements for salt will increase due to lactation, exertion and increases in ambient temperatures. These same conditions will also require an increase in water intake, which must always be considered in any discussion concerning salt intake.

An excess of salt intake can lead to the condition known by various names including salt poisoning, hypernatremia, sodium ion toxicosis and water deprivation-sodium ion intoxication. The last name in this list is the most descriptive, giving both the result (sodium ion intoxication) and the most common predisposing condition (water deprivation).

## BACKGROUND

Sodium is the main cation and chloride is the main anion in the regulation of osmotic balance in the extracellular fluid (ECF) of the body. Serum sodium concentration and serum osmolarity are normally maintained under precise control by homeostatic mechanisms involving thirst, antidiuretic hormone and renal reabsorption of filtered sodium. Normal reference ranges for serum sodium in adult animals (given in mmol/l) include porcine 135–150, bovine 132–152, canine 141–152, feline 147–156 and equine 132–146 (Kaneko *et al*., 1997). For sodium concentration measurement, mmol/l = mEq/l and can be used interchangeably. Hyponatremia is the result of the loss of excess sodium compared to loss of water and can be the result of a large number of disease processes and conditions. For a more complete explanation on the recognition and correction of hyponatremia the reader is directed to other references (Angelos and Van Metre, 1999).

Salt is normally present in animal diets at 0.5–1%. Production animals are often given free access to salt blocks or mineral mixes as supplements to the diet. Additional sources of salt may include high-saline ground water, brine or seawater. The use of whey as a feed or to produce wet mash can add dramatically to sodium intake. High concentrations of salt in the diet (up to 13%) have been used to limit feed intake of cattle. In general, animals can tolerate high concentrations of salt in the feed if they have free access to fresh water. Salt-deprived animals or animals not acclimated to high-salt feeds can overconsume these feeds making the animal prone to hypernatremia. Improperly formulated or improperly mixed feed can be an additional source of excess salt. Companion animal exposures to excess salt have included the use of salt as an emetic (this practice is no longer recommended) and the consumption of various salt-containing objects including rock salt and dough-salt mixtures. Hypernatremia has also been reported in animals treated with improperly mixed oral electrolyte solutions and remedies for diarrhea.

Excess ingested salt can be irritating to mucosal surfaces and can result in anorexia, vomiting or diarrhea. Ingested dietary salt is approximately 90% absorbed across the gastrointestinal tract. Ingestion of excess sodium on an acute basis can result in hypernatremia with this condition being variously termed direct sodium ion toxicosis, acute sodium ion toxicosis or acute hypernatremia. Clinical signs develop within 1–2 days. The more common form of hypernatremia develops due to restricted water intake and is termed indirect sodium ion toxicosis, chronic

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sodium ion toxicosis or chronic hypernatremia. Clinical signs will develop over a period of 4–7 days but the early changes may be missed or ignored. There are several common reasons for restricted water intake including frozen water sources, unpalatable water sources, mechanical failure, overcrowding or naïve animals and owner neglect.

## PHARMACOKINETICS/MECHANISM OF ACTION

An increase in sodium intake leads to a rise in sodium concentration in the serum and a rapid distribution throughout the body. Osmolarity of the ECF is monitored by osmoreceptors in the hypothalamus and the body reacts to increases by stimulating thirst for increased water intake. Additionally, the release of antidiuretic hormone from the posterior pituitary will cause increased water retention by the kidneys. These responses should function to quickly restore normal osmolarity but may only be effective if the osmolar changes are gradual and sufficient water is available to the animal. As the sodium ion concentration of the serum increases, water will move out of the interstitium and intracellular fluid into the ECF along the osmotic gradient. Sodium will passively diffuse across the blood–brain barrier increasing the sodium concentration of the cerebral spinal fluid above the normal range (135–150 mmol/l). During this developing hypernatremia, the cells of the brain will also increase their intracellular osmolarity to prevent excess water loss to the ECF, which would cause cell shrinkage. If the hypernatremia develops too quickly and this protective mechanism fails, significant cell shrinkage occurs and the entire brain shrinks and pulls away from the calvarium resulting in the disruption of the blood supply to the brain. This can result in subarachnoid, subdural or intravascular hemorrhages (Hardy, 1989). In severe cellular dehydration, the result can be seizure-like activity and death. If the increase in sodium concentration of the brain cells continues, there will be an inhibition of glycolysis and a decrease in the energy available in the cell. While sodium will passively diffuse into the brain, it is an energy-requiring active process that transports sodium out. Thus the brain response to a rapid decrease in serum sodium is delayed and the developing osmotic gradient will cause water to move into the brain causing swelling, cerebral edema and the development of clinical signs.

Changes in cellular osmolarity will occur in both acute and chronic hypernatremia situations, but changes to osmolarity on a chronic basis will involve the accumulation of more osmotically active organic compounds, termed idiogenic osmoles. These include taurine, myoinositol, glycerophosphoryl-choline, glutamate, glutamine, betaine and phosphocreatine. Maximum concentrations of idiogenic osmoles occur within 48–72 h and can account for 60% of the change in cellular osmolarity. Once the hypernatremia situation is corrected, the idiogenic osmoles will take 48–72 h to decrease back to normal levels. As in the acute situation, a rapid decrease in serum sodium will develop an osmotic gradient causing water to move into the brain with resulting cerebral edema and the development of clinical signs.

## TOXICITY

In all situations involving salt intake, the intake of water will have great impact and must also be considered. The acute toxic dose of sodium chloride is approximately 2.2 g/kg in swine, equine and bovine species with the ovine toxic dose approximately 6 g/kg (Osweiler *et al*., 1985). Swine appear to be most sensitive domestic animal and involve the greatest number of clinical reports. Both swine and poultry can be severely affected when water intake is severely restricted or with high-salt diets and only moderate water restriction. Increased water requirements will increase the susceptibility of lactating cows and sows to salt poisoning, making them more sensitive to sudden restrictions in water. The acute toxic dose of sodium chloride in dogs is given as 4 g/kg, but clinical signs have been reported for lesser ingestions (Barr *et al*., 2004) and an ingestion greater than this was reported with only mild clinical signs. Horses appear to be rarely affected with classic salt poisoning but can develop it with conditions of increased salt intake and sudden water restriction. Horses are, however, subject to dehydration and electrolyte abnormalities especially under conditions of exercise and high ambient temperatures (Cohen *et al*., 1993).

Clinical signs have best been described in swine and include loss of appetite, thirst, restlessness, pruritus and constipation. These early clinical signs can progress over several days to aimless wandering, head pressing, circling or pivoting around a limb. The animal may display seizure-like activity and assume a dog-sitting position, draw its head back in a jerking motion and fall over on its side (Osweiler *et al*., 1985; Niles, 2004). Terminally, the animal will be in lateral recumbency with paddling and opisthotonus. Cattle with acute excess salt intake may develop gastroenteritis, weakness, dehydration, tremors and ataxia. The cattle may appear to be blind and develop seizure-like activity or partial paralysis including knuckling over at the fetlocks. Terminally, cattle can also be in lateral recumbency with paddling and opisthotonus. Cattle can die within 24 h following the appearance of severe clinical signs. Recovered animals may drag the rear

feet or knuckle over at the rear fetlock without exhibiting pain (Osweiler *et al.*, 1985). Poultry and other birds may exhibit clinical signs of depression, weakness, dyspnea and sudden death. Excess salt intake in the dog will result in vomiting within several hours of ingestion. The clinical signs can progress to diarrhea, muscle tremors and seizure-like activity. Increased severity of clinical signs in the dog have been seen when serum sodium levels have been above 180 mEq/l (Barr *et al.*, 2004).

Post mortem examination of salt-poisoned animals may include some degree of gastric irritation, including ulceration and hemorrhages. The content of the gastrointestinal tract may be abnormally dry. Histopathologic lesions may be limited to the brain and include cerebral edema and inflammation of the meninges. In swine, the appearance of eosinophilic perivascular cuffing is seen if the animal dies early in the syndrome with the lesion not found after 48 h. Brain sodium concentrations above 2000 ppm are considered diagnostic in cattle and swine. Upper normal brain sodium concentrations are 1600 ppm for cattle and 1800 ppm for swine, both on a wet weight basis. There is a paucity of data on normal brain sodium concentrations in other common domestic species but normal ranges should be similar. Serum sodium concentrations taken from the live animal will be significantly above the normal ranges listed previously. Post mortem analysis of aqueous humor, vitreous humor or cerebral spinal fluid will show a significant increase over values from normal animals (Osweiler *et al.*, 1995). Optimally, the values obtained should be compared to normal values for that species generated by the same laboratory.

## TREATMENT

Prior to the onset of clinical signs, the acute ingestion of salt can best be treated by allowing the animal full access to water and closely observing the animal for several hours. Emetics may be used in the dog if known ingestions occur and the animal is not yet showing clinical signs. However, most cases are discovered long after the excess salt ingestion or the water deprivation has occurred and the affected animals are showing obvious clinical signs. The overriding concept of treatment is to slowly return the animal to normal water and electrolyte balance over a 2–3-day period of time. Quickly lowering the serum sodium concentration will increase the osmotic gradient between the serum and the brain with water following the gradient into the brain increasing the likelihood of severe cerebral edema. The prognosis for an animal hypernatremic from salt ingestion/water deprivation with significant clinical signs on either an acute or chronic basis should be guarded at best.

On a herd basis with large animals, water intake should be limited to 0.5% of body weight at hourly intervals until normal hydration is accomplished. Monitoring serum sodium concentration is the first step in treatment on an individual animal basis. This information can be used to correct the free water deficit (*FWD*) in the animal, based on the following formula:

$$FWD\ (\text{l}) = 0.6 \times \text{bodyweight (kg)} \times [(\text{measured serum Na}/\text{normal serum Na}) - 1]$$

Not more than 50% of the *FWD* should be replaced in the first 24 h with the remaining deficit replaced in the following 24–48 h. Serum sodium levels should be lowered at a rate of 0.5–1.0 mEq/l/hour, with the slower rate recommended for cases of chronic hypernatremia (Schaer, 2000). In acute hypernatremia without clinical dehydration, the use of 5% dextrose solution in combination with a loop diuretic has been suggested at 3.7 ml/kg/h to decrease serum sodium at 1 mEq/l/hour (Barr *et al.*, 2004). Diuretics such as furosemide can be used to prevent the development of pulmonary edema during fluid therapy. The use of slightly hypertonic intravenous fluids has been recommended to reduce the likelihood of cerebral edema developing. Intravenous fluids should be made to approximate the serum sodium concentration of the animal, or the clinician may start with a solution containing 170 mEq/l of sodium and decrease this concentration as clinical signs improve (Angelos and Van Metre, 1999; Niles, 2004). If brain edema is suspected, the use of mannitol, dexamethasone or dimethyl sulfoxide may aid in control.

## CONCLUDING REMARKS

While the term "salt poisoning" may not be the most accurate way to describe the above syndrome, it is certainly in common usage. A similar and confusing term is "water intoxication" which has been used to describe the situation of excess water intake or infusion over a short period of time which can dramatically decrease the serum sodium concentration and make the serum hypoosmolar. Water intoxication has been used to describe the exacerbation of cerebral edema when the correction of hypernatremia occurs too quickly. Water intoxication has also been used to describe the brain swelling and seizure-like activity which occurs when a normal animal drinks excessive amounts of water over a short period of time. In addition to the possible neurological effects, hemolysis has also been described (Middleton *et al.*, 1997). In the above situations, acute and dramatic osmotic changes are the cause.

## REFERENCES

Angelos SM, Van Metre DC (1999) Treatment of sodium balance disorders. In *Veterinary Clinics of North America: Food Animal Practice*, vol. 15, Roussel Jr A, Constable PD (eds). W.B. Saunders Co., Philadelphia, PA, pp. 587–607.

Barr JM, Khan SA, McCullough SM, Volmer PA (2004) Hypernatremia secondary to homemade play dough ingestion in dogs: a review of 14 cases from 1998 to 2001. *J Vet Emerg Crit Care* **14**: 196–202.

Cohen ND, Roussel AJ, Lumsden JH, Cohen AC, Grift E, Lewis C (1993) Alterations of fluid and electrolyte balance in thoroughbred racehorses following strenuous exercise during training. *Can J Vet Res* **57**: 9–13.

Hardy RM (1989) Hypernatremia. In *Veterinary Clinics of North America: Small Animal Practice*, vol. 19, Schaer M (ed.). W.B. Saunder Co., Philadelphia, PA, pp. 231–40.

Kaneko J, Harvey J, Bruss M (1997) Appendices VII and VIII. In *Clinical Biochemistry of Domestic Animals*, 5th edn, Kaneko J, Harvey J, Bruss M (eds). Academic Press, San Diego, CA, p. 894.

Middleton JR, Katz L, Angelos JA, Tyler JW (1997) Hemolysis associated with water administration using nipple bottle for human infants in juvenile pygmy goats. *J Vet Intern Med* **11**: 382–4.

Niles G (2004) Sodium. In *Clinical Veterinary Toxicology*, Plumlee KH (ed.). Mosby, St. Louis, MO, pp. 218–21.

Osweiler GD, Carson TL, Buck WB, Van Gelder GA (1985) Water deprivation-sodium salt. In *Clinical and Diagnostic Veterinary Toxicology*, 3rd edn. Kendall/Hunt Publishing Co., Dubuque, IA, pp. 167–70.

Osweiler GD, Carr TF, Sanderson TP, Carson TL, Kinker JA (1995) Water deprivation-sodium ion intoxication in cattle. *J Vet Diagn Invest* **7**: 583–5.

Schaer M (2000) Hyperkalemia and hypernatremia. In *Textbook of Veterinary Internal Medicine*, 5th edn, Ettinger SJ, Feldman BF (eds). W.B. Saunders Co., Philadelphia, PA, pp. 227–32.

C H A P T E R

# 36

# Sulfur

*Jeffery O. Hall*

## INTRODUCTION

Sulfur is a necessary dietary component that can be toxic at excessive concentrations. Animal bodies are about 0.15% sulfur by weight (NRC, 1989, 2006). Sulfur is incorporated into many essential molecules, including biotin, chondroitin sulfate, cartilage mucopolysaccharides, co-enzyme A, fibrinogen, glutathione, heparin, lipoic acid, mucins, and thiamine (NRC, 1989, 1998, 2006). In addition to these biologically active compounds, sulfur is an intricate component of sulfur containing amino acids, such as methionine, cysteine, cystine, homocysteine, and taurine. With the exception of thiamine and biotin, all sulfur containing compounds in the body can be synthesized from methionine (NRC, 1996). Thus, thiamine, biotin, and methionine are essential nutrients in the diet of monogastric animals, but ruminant microbes can synthesize these compounds from inorganic sulfate in the diet (Block *et al.*, 1951). Species differences are such that cats cannot synthesize taurine from methionine, making it an essential nutrient in their diets. Recommended daily dietary intakes of sulfur are 0.15%, 0.14–0.26%, 0.15–0.2%, and 0.2–0.25% of the diet for horses, sheep, beef cattle, and dairy cattle, respectively (NRC, 1985, 1988, 1989, 1996).

Ruminants tend to be more sensitive to the toxic effects of dietary sulfur/sulfate due to efficient microbial conversion to bioactive species in the rumen. But both dietary and water sources of sulfur/sulfate have similar toxic potential and must be factored into the total daily intake in order to establish potential risk.

In addition to dietary sulfur, other sulfur containing compounds can be toxic. Sulfur dioxide gas from industrial waste gas, as well as hydrogen sulfide gas from manure pits, natural gas production, and crude oil production, can be toxic to livestock. The toxic effects of these gaseous forms of sulfur are better summarized separately from the dietary toxicoses.

Plants can accumulate high sulfur concentrations. High sulfate water can cause a dual increase in total daily sulfur intake by way of the water and ingested proximal vegetation. Plant sulfur concentrations have been shown to increase with increasing sulfate in the soil (Reddy *et al.*, 1981; Hardt *et al.*, 1991; Leustek and Saito, 1999). In soil matrixes, sulfate can be actively reduced and precipitated; however, this only sequesters the sulfur until environmental change allows the re-oxidation of the sulfur back to sulfate. During drought conditions, precipitated sulfur is exposed to oxygen and re-oxidized. The resultant sulfate is then bioavailable for plant uptake.

## BACKGROUND

Sulfur is a non-metal within group VIA of the periodic table. This group is sometimes referred to as the chalcogenides or ore-formers, since many metal ores are sulfide or sulfate salts. Sulfur has an atomic number of 16, an atomic weight of 32.07, and has four different naturally occurring atomic masses from 32 to 36 (Rosman and Taylor, 1998). It can occur in four different oxidation states: −2 (sulfide), 0 (elemental sulfur), +4 (sulfite), and +6 (sulfate). All valence states, except elemental sulfur, are found in biologic molecules. Sulfur is utilized in the production of sulfuric acid, fertilizers, pigments, dyes, drugs, explosives, rubber, insecticides, and detergents, as well as many inorganic salts and esters. Although uniformly found in nature, industrialized countries are the largest users of sulfur materials.

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## PHARMACOKINETICS/ TOXICOKINETICS

When evaluating the absorption of sulfur, the chemical form must be considered. The intestinal mucosal absorption of sulfate is via an active carrier mediated process that is also utilized by molybdate (Mason and Cardin, 1977). Active intestinal absorption of sulfate has been shown in sheep, rats, dogs, rabbits, and hamsters (Bird and Moir, 1971). Similarly, the sulfur containing amino acids and other sulfur containing compounds are absorbed via specific transport mechanisms across the intestinal mucosa (NRC, 2006). These specific transport processes are specific for the individual compounds. Rumen microbes convert a percentage of dietary sulfur containing compounds to sulfide, which can then be incorporated into microbial sulfur containing amino acids, thiamine, biotin, other microbial sulfur metabolites, or absorbed as sulfide. In addition to gastrointestinal absorption of sulfides, hydrogen sulfide can be absorbed across the respiratory epithelium. Large amounts of sulfide, as hydrogen sulfide, produced in the rumen can be eructated, inhaled, and absorbed (Dougherty *et al*., 1965). And, the inhaled sulfide is important in the toxicosis, as sheep that had trachea blocked to prevent eructation and inhalation of sulfide did not succumb while those without tracheal blockage were poisoned (NRC, 2006).

Sulfur is widely distributed in the body. All tissues in the body have significant sulfur components, with the body being made up of approximately 0.15% sulfur (NRC, 2006). Absorbed sulfides and thiomolybdates, the primary toxic sulfur metabolites, are well distributed in the body. This is evidenced by the facts that thiomolybdates can deplete tissue stores of copper and sulfides can cross the blood–brain barrier causing neurological effects.

Sulfur containing amino acids and sulfate are extensively metabolized in order to produce biologically utilized sulfur compounds. In comparison, absorbed sulfide is efficiently metabolized in the liver to sulfate, with a high first pass clearance (NRC, 2006). Inhaled sulfide would not be subject to this rapid hepatic removal, resulting in increased circulating concentrations that can result in neurologic effects.

Sulfur containing compounds are eliminated by both renal and biliary routes. Just as molybdate can compete for the intestinal absorption sites for sulfate, it can also compete for re-absorption sites in the renal tubules (Friberg and Lener, 1986). The relative quantities of sulfur elimination from renal and biliary routes can differ depending on the form ingested. In sheep, Bird (1972) found the greatest percent elimination of sulfate was via urine, while taurine was predominantly eliminated in the bile. Intestinally absorbed sulfide efficiently undergoes hepatic metabolism to sulfate, which is eliminated in the urine (NRC, 2006).

## MECHANISM OF ACTION

Acute oral poisoning with elemental sulfur results in formation of hydrogen sulfide, as well as many other potential metabolites. The gastric and respiratory effects are postulated to be due to coagulative effects of rumen produced sulfurous acids and the irritating effects of hydrogen sulfide, respectively (Julian and Harrison, 1975; Kandylis, 1984; Gunn *et al*., 1987). However, the exact mechanisms are not well delineated. Inhaled sulfide at high concentrations may act in a similar mechanism to high concentrations of hydrogen sulfide gas, causing acute respiratory paralysis.

The mechanism of sub-acute sulfur poisoning is much better researched. This condition is correlated with the reduction of the sulfate or other forms of sulfur to sulfide in the rumen (Gould *et al*., 1991, 1997; Loneragan *et al*., 1998). The current literature suggests that inhibition of cytochrome C oxidase, which is essential for cellular respiration, is the primary mechanism (Smith *et al*., 1977; Beauchamp *et al*., 1984). But, cerebral vasospasms and regional ischemia could also account for the localization of the lesions (Siesjo, 1984; McAllister, 1991). Although once thought to be associated with a true thiamine deficiency from either inhibition of rumen microbial production or cleavage of thiamine (Edwin and Jackman, 1982), it has been shown that systemic thiamine concentrations are within the normal range for most animals (Olkowski *et al*., 1992; Gould, 2000). Slight decline in the blood thiamine concentration can also being seen in some animals (Olkowski *et al*., 1991). And, thiamine supplementation in the presence of high sulfate/sulfur-associated polioencephalomalacia (PEM) alleviates the clinical disease (Olkowski *et al*., 1992). This would indicate that the sulfide or some other sulfur metabolite is either competitively inhibiting the cellular uptake/utilization of thiamine or therapeutic doses of thiamine diminish the effects of sulfide on the cytochrome C oxidase enzyme.

The sub-acute to chronic, indirect effects of excessive sulfur are seen in ruminants, due to the efficient conversion of sulfur compounds to sulfide. The sulfide can form insoluble salts with copper and zinc (Suttle, 1974), but it can also form thiomolybdate complexes which bind copper making it non-bioavailable (Suttle, 1991) (see Molybdenum-mechanism of action). Systemic copper decreases, associated with increased sulfur/sulfate, have been reported in sheep (Moshtaghi-Nia *et al*., 1989; Van Niekerk and Van Niekerk, 1989a, b) and cattle (Wittenberg and Boila, 1988). High forage and water sulfur have also been associated with selenium deficiency (Ivancic and Weiss, 2001). Decreased serum and wool selenium has been reported with increasing dietary sulfate (White and Somers, 1977; White, 1980). In addition, increased soil sulfur inhibits the plant uptake of selenium, thereby increasing the potential for inducing a

selenium deficiency in ingesting herbivores (Newman and Schreiber, 1985), which may be an important mechanism in grazing animals.

## TOXICITY

Toxicity of sulfur can be divided into three main categories that are likely to be encountered. The first is acute oral poisoning. The second is sub-acute to chronic direct toxicosis. And the third is sub-acute to chronic indirect toxicosis, as a secondary interference with other essential minerals that result in mineral deficiencies.

Reports of acute oral sulfur poisoning are scarce in the literature. In a group of Holstein heifers, sulfur ingested at 0.85–3.8 g/kg body weight resulted in high morbidity and moderate mortality (Gunn *et al.*, 1987), while 20 heifers given 250 g sulfur in grain had high mortality (Julian and Harrison, 1975). Ewes fed a barley–sulfur mix that provided approximately 40–45 g sulfur/ewe were poisoned (White, 1964). Five horses administered 300 g sulfur succumbed to sulfur poisoning (Ales, 1907).

Clinical and pathologic manifestations of acute oral sulfur poisoning are similar across species (White, 1964; Julian and Harrison, 1975; Gunn *et al.*, 1987). Abdominal pain, colic, rumen stasis, fetid diarrhea, dehydration, metabolic acidosis, tachypnea, recumbency, and hydrogen sulfide smell are expected clinical signs. Irritation, edema, and hemorrhage of the gastrointestinal tract and respiratory tract should be expected. In addition, renal tubular necrosis can be seen.

Monogastric animals are much less susceptible to the sub-acute direct and indirect toxic effects of excessive sulfur intake than ruminants. Pigs can tolerate 1000 mg/l sulfur in the drinking water with only a mild cathartic effect (Paterson *et al.*, 1979) and 0.42% in the diet for several months without adverse effects (Dale *et al.*, 1973). Similarly, chicks had decreased growth rates at 1.2% dietary sulfur (Leach *et al.*, 1960). And, chickens had decreased egg production, decreased feed intake, and deaths at 4000 mg/l sulfate in their drinking water (Adams *et al.*, 1975). Since the indirect toxic effects of excessive sulfur are related to rumen conversion to sulfide, these effects are not observed in monogastric animals.

In contrast, for sheep, beef cattle, and dairy cattle the maximum tolerable content of sulfur in the total diet is 0.4% (NRC, 1985, 1988, 1996), while concentrations slightly below this tolerable content can occasionally result in toxic effects (Gould *et al.*, 1991). Since dietary toxicity is not mutually exclusive, as sulfur and sulfate ion can have the same clinical effects, total doses of sulfur/sulfate from both water and dietary material must be taken into account when evaluating potential toxicity (Suttle, 1974). For ruminants, the typical clinical presentation is one of ataxia, weakness, blindness, recumbency, seizures, and death.

Sub-acute ingestion of toxic doses of sulfate/sulfur has been associated with PEM, a necrotizing lesion of the brain (Beke and Hironaka, 1991; Gould *et al.*, 1991; Olkowski *et al.*, 1992; Hamlen *et al.*, 1993; McAllister *et al.*, 1997). Gross and histologic lesions are primarily in the brain, but ruminal changes can be observed. Gross pathologic lesions include a darkening of the rumen contents from precipitated sulfide salts, swelling of the cerebral hemispheres, softening of the cerebral hemispheres, and yellow discoloration of the cortical gray matter. Histological lesions include necrosis of the cortical gray matter and occasional areas of necrosis in the thalamus or midbrain. The clinical condition can be an additive effect of the total sulfur in the diet and sulfate in the drinking water (Beke and Hironaka, 1991). PEM has been associated with high sulfur/sulfate ingestion in cattle (Beke and Hironaka, 1991; Gould *et al.*, 1991; Hamlen *et al.*, 1993), pigs (Dow *et al.*, 1963), and sheep (Olkowski *et al.*, 1992). However, sodium ion poisoning in the pigs was likely the primary causative factor, as the exposure was to sodium sulfate.

The peak rumen production of hydrogen sulfide can be somewhat delayed from the time of initiating high sulfur intake. Peak rumen gas cap sulfide occurred at 1–3 weeks after placing cattle on a high sulfur diet (Gould *et al.*, 1997). But, continued exposure resulted in a gradual decline in the sulfide content. This would indicate an adaptation of the rumen microbes to favor direct utilization of the sulfide or diminished rates of production.

Sub-acute to chronic sulfur-induced mineral deficiencies can result in severe health problems. Copper deficiency can cause poor growth, weakness, poor immune function, poor reproductive function, and death. In addition, sulfur-induced copper deficiency may play a role in PEM (Gooneratne *et al.*, 1989). Severe copper deficiency also causes myelin degeneration (enzootic ataxia) in lambs, deer, and other ruminants (Cordy, 1971; Faye *et al.*, 1991; Audige *et al.*, 1995). Sulfate-induced selenium deficiency can cause poor growth, weakness, poor immune function, poor reproductive function, damage to the cardiac or skeletal muscles, and death.

## TREATMENT

Treatment for acute sulfur poisoning is predominantly supportive in nature, with removal of the causative material, as well as administration of fluids and electrolytes. However, the human literature points to successful treatment of acute hydrogen sulfide poisoning by induction of methemoglobinemia with nitrite to allow for the formation of sulfmethemoglobin, similar to therapeutic protocols for

treatment for cyanide poisoning (Stine *et al*., 1976; Peters, 1981). This type of therapy may also be beneficial in the treatment of sub-acute direct sulfur poisoning. Since sulfides act on and split disulfide bridged, the use of oxidized glutathione or other simple disulfide compounds could be protective or antidotal (Smith and Abbanat, 1966). The use of thiamine in the treatment of PEM has been suggested to be beneficial, even though there is no overt thiamine deficiency. The primary treatment of indirect mineral deficiencies resultant from high sulfur intake would also include supplementation of copper and potentially selenium.

In addition to direct treatment, management can play a role in the prevention of sulfur poisoning. Testing of water and dietary materials will identify high sulfur prior to utilization. With this information, management strategies can be utilized that would incorporate the sulfur at gradually increasing content in an attempt to allow for microbial adaptation.

## CONCLUDING REMARKS AND/OR FUTURE DIRECTION

Although much is known about the different clinical disease conditions that excessive sulfur can cause, treatment remains only somewhat effective. Thus, it is important to evaluate the use of nitrite or disulfide containing compounds in the treatment of domestic animals.

## REFERENCES


Adams AW, Cunningham FE, Munger LL (1975) Some effects on layers of sodium sulfate and magnesium sulfate in their drinking water. *Poult Sci* **54**: 707–14.

Ales (1907) Case of poisoning by sulfur in the horse. *Vet J* **63**: 524.

Audige L, Wilson PR, Morris RS, Davidson GW (1995) Osteochondrosis, skeletal abnormalities and enzootic ataxia associated with copper deficiency in a farmed red deer (*Cervus elaphus*) herd. *N Zeal Vet J* **43**(2): 70–6.

Beauchamp Jr RO, Bus JS, Popp JA, Boreiko CJ, Andjelkovich DA (1984) A critical review of the literature on hydrogen sulfide toxicity. *Crit Rev Toxicol* **13**: 25–97.

Beke GJ, Hironaka R (1991) Toxicity to beef cattle of sulfur in saline well water: a case study. *Sci Total Environ* **101**(3): 281–90.

Bird PR (1972) Sulfur metabolism and excretion studies in ruminants: VII. Secretion of sulfur and nitrogen in sheep pancreatic and bile fluids. *Aust J Biol Sci* **25**: 817–34.

Bird PR, Moir RJ (1971) Sulfur metabolism and excretion studies in ruminants. I. The absorption of sulfate in sheep after intraruminal or intraduodenal infusions of sodium sulfate. *Aust J Biol Sci* **24**: 1319–28.

Block RJ, Stekol JA, Loosli JK (1951) Synthesis of sulfur amino acids from inorganic sulfate by ruminants. II. Synthesis of cystine and methionine from sodium sulfate by the goat and by the microorganisms of the rumen of the ewe. *Arch Biochem* **33**: 353–63.

Cordy DR (1971) Enzootic ataxia in California lambs. *J Am Vet Med Assoc* **158**(11): 1940–2.

Dale SE, Ewan RC, Speer VC, Zimmerman DR (1973) Copper, molybdenum, and sulfate interaction in young swine. *J Anim Sci* **37**: 913–17.

Dougherty RW, Mullenax CH, Allison MJ (1965) Physiological phenomena associated with eructation in ruminants. In *Physiology of Digestion in the Ruminant*, Dougherty RW (ed.). Buttersworth, Washington, DC, p. 159.

Dow C, Lawson GHK, Todd JR (1963) Sodium sulfate toxicity in pigs. *Vet Rec* **75**: 1052–5.

Edwin EE, Jackman R (1982) Ruminant thiamine requirement in retrospect. *Vet Res Commun* **5**: 237–50.

Faye B, Grillet C, Tessema A, Kamil M (1991) Copper deficiency in ruminants in the Rift Valley of East Africa. *Trop Anim Health Prod* **23**(3): 172–80.

Friberg L, Lener J (1986) Molybdenum. In *Handbook on the Toxicology of Metals*, 2nd edn, Friberg L, Nordberg GF, Vouk VB (eds). Elsevier/North-Holland Biomedical Press, New York, pp. 446–61.

Gooneratne SR, Olkowski AA, Klemmer RG, Kessler GA, Christensen DA (1989) High sulfur related thiamine deficiency in cattle: a field study. *Can Vet J* **30**: 139–46.

Gould DH (2000) Update on sulfur-related polioencephalomalacia. *Vet Clin North Am Food Anim Pract* **16**: 481–96.

Gould DH, McAllister MM, Savage JC, Hamar DW (1991) High sulfide concentrations in rumen fluid associated with nutritionally induced polioencephalomalasia. *Am J Vet Res* **52**: 1164–9.

Gould DH, Cummings BA, Hamar DW (1997) *In vivo* indicators of pathologic ruminal sulfide production in steers with diet-induced polioencephalomalacia. *J Vet Diagn Invest* **9**: 72–6.

Gunn MF, Baird JD, Wilke JSN (1987) Accidental sulfur poisoning in a group if Holstein heifers. *Can Vet J* **28**: 188–92.

Hamlen H, Clark E, Janzen E (1993) Polioencephalomalasia in cattle consuming water with elevated sodium sulfate levels: a herd investigation. *Can Vet J* **34**: 153–8.

Hardt PF, Ocumpaugh WR, Greene LW (1991) Forage mineral concentration, animal performance, and mineral status of heifers grazing cereal pastures fertilized with sulfur. *J Anim Sci* **69**: 2310–20.

Ivancic Jr J, Weiss WP (2001) Effects of dietary sulfur and selenium concentrations on selenium balance of lactating Holstein cows. *J Dairy Sci* **84**: 225–32.

Julian RJ, Harrison KB (1975) Sulfur poisoning in cattle. *Can Vet J* **16**: 28–9.

Kandylis K (1984) Toxicology of sulfur in ruminants: review. *J Dairy Sci* **67**: 2179–87.

Leach RM, Ziegler TR, Norris LC (1960) The effects of dietary sulfate in the growth rate of chicks fed purified a diet. *Poult Sci* **39**: 1577–82.

Leustek T, Saito K (1999) Sulfate transport and assimilation in plants. *Plant Physiol* **120**: 637–44.

Loneragan GH, Gould DH, Callan RJ, Sigurdson CJ, Hamar DW (1998) Association of excess sulfur intake and an increase in hydrogen sulfide concentrations in the ruminal gas cap of recently weaned beef calves with polioencephalomalacia. *J Am Vet Med Assoc* 213: 1599–604.

Mason J, Cardin CJ (1977) The competition of molybdate and sulphate ions for a transport system in the ovine small intestine. *Res Vet Sci* **22**(3): 313–15.

McAllister MM (1991) Sulfur toxicosis and polioencephalomalasia in ruminants. PhD Dissertation, Colorado State University, Fort Collins, CO.

McAllister MM, Gould DH, Raisbeck MF, Cummings BA, Longeragan GH (1997) Evaluation of ruminal sulfide concentrations

and seasonal outbreaks of polioencephalomalacia in beef cattle in a feedlot. *J Am Vet Med Assoc* **211**(10): 1275–9.

Moshtaghi-nia SA, Devlin TJ, Phillips GD (1989) Influence of dietary copper, molybdenum, and sulfur on copper metabolism of sheep. *Can J Anim Sci* **69**: 187–94.

Newman JR, Schreiber RK (1985) Effects of acidic deposition and other energy emissions on wildlife: a compendium. *Vet Hum Toxicol* **27**: 394–401.

NRC (1985) *Nutrient Requirements of Sheep*, 6th revised edition. National Academic Press, Washington, DC, p. 15.

NRC (1988) *Nutrient Requirements of Dairy Cattle*, 6th revised edition. National Academic Press, Washington, DC, pp. 28–9.

NRC (1989) *Nutrient Requirements of Horses*, 6th revised edition. National Academic Press, Washington, DC, pp. 28–9.

NRC (1996) *Nutrient Requirements of Beef Cattle*, 7th revised edition. National Academic Press, Washington, DC, pp. 60–1.

NRC (1998) *Nutrient Requirements of Swine*, 10th revised edition. National Academic Press, Washington, DC, pp. 60–1.

NRC (2006) Molybdenum. In *Mineral Tolerance of Animals*, 2nd edn. National Academies Press, Washington DC, pp. 262–75.

Olkowski AA, Rousseaux CG, Christensen DA (1991) Association of sulfate-water and blood thiamine concentration in beef cattle: field studies. *Can J Anim Sci* **71**: 825–32.

Olkowski AA, Gooneratne SR, Rousseaux CG, Christensen DA (1992) Role of thiamine in sulfur induced polioencephalomalacia in sheep. *Res Vet Sci* **52**: 78–85.

Paterson DW, Wahlstrom RC, Libal GW, Olson OE (1979) Effects of sulfate water on swine reproduction and young pig performance. *J Anim Sci* **49**: 664–7.

Peters JW (1981) Hydrogen sulfide poisoning in a hospital setting. *J Am Med Assoc* **246**: 1588–9.

Reddy GD, Alston AM, Tiller KG (1981) Effects of fertilizer on concentrations of copper, molybdenum, and sulfur in subterranean clover (*Trifilium subterraneum*). *Aust J Exp Anim Husb* **21**: 491–7.

Rosman KJR, Taylor PDP (1998) Isotopic composition of the elements 1997. *Pure Appl Chem* **70**: 217–35.

Siesjo BK (1984) Cerebral circulation and metabolism. *J Neurosurg* **60**: 883–908.

Smith RP, Abbanat RA (1966) Protective effects of oxidized glutathione in acute sulfide poisoning. *Toxicol Appl Pharmacol* **9**: 209–17.

Smith L, Kruszyana H, Smith RP (1977) The effects of methemoglobin on the inhibition of cytochrome *c* oxidase by cyanide, sulfide, and azide. *Biochem Pharmacol* **26**: 2247–50.

Stine RJ, Slosberg B, Beacham BE (1976) Hydrogen sulfide intoxication: a case report and discussion of treatment. *Ann Intern Med* **85**: 756–8.

Suttle NF (1974) Effects of organic and inorganic sulfur on the availability of dietary copper to sheep. *Br J Nutr* **32**: 559–68.

Suttle NF (1991) The interactions between copper, molybdenum, and sulfur in ruminant nutrition. *Annu Rev Nutr* **11**: 121–40.

Van Niekerk FE, Van Niekerk CH (1989a) Effects of high levels of dietary molybdenum and sulfate on SA Mutton Merino sheep. I. mineral status and haematologic parameters. *S Afr Tydskr Veek* **19**: 107–13.

Van Niekerk FE, Van Niekerk CH (1989b) Effects of high levels of dietary molybdenum and sulfate on SA Mutton Merino sheep. II. Certain aspects of the oestrous cycle and pregnancy. *S Afr Tydskr Veek* **19**: 114–20.

White CL (1980) Sulfur–selenium studies in sheep. Effect of dietary sulfur deficiency on selenium and sulfur metabolism in sheep fed varying levels of selenomethionine. *Aust J Biol Sci* **33**: 699–707.

White CL, Somers M (1977) Sulfur–selenium studies in sheep. The effect of varying dietary sulfate and selenomethionine on sulfur, nitrogen, and selenium metabolism in sheep. *Aust J Biol Sci* **30**: 47–56.

White JB (1964) Sulfur poisoning in ewes. *Vet Rec* **76**: 278–9.

Wittenberg KM, Boila RJ (1988) Supplementary copper for growing cattle consuming diets high in molybdenum or molybdenum plus sulfur. *Can J Anim Sci* **68**: 1143–54.

CHAPTER

# 37

# Zinc

*Tam Garland*

## INTRODUCTION

Zinc (Zn) is a transitional metal in group XII on the periodic chart and is the fourth most commonly used metal today. It is a moderately reactive metal with a common valence state of +2. It is also an essential element in mammals and birds and is a component of approximately 200 metaloenzymes. Although zinc has an essential role in nutrition and consequences of nutritional deficiency, this chapter will focus on its toxicity.

## BACKGROUND

As the fourth most commonly used metal it is expected to be found in a variety of places with a multitude of uses. Zinc is an economical metal to use and is relatively non-toxic. Table 37.1 details some of those uses. Since zinc has nutritional qualities, it is not uncommon to find it added as a supplement to feed. Problems with toxicity generally occur when the levels are 1000 parts per million (ppm) or greater.

**TABLE 37.1 Uses and purposes of zinc**

| Uses | Purposes |
|---|---|
| Galvanized steel | Prevent corrosion |
| Parkerize steel | Prevent rust and corrosion |
| Used in numerous metal alloys | Brass, nickelled silver, typewriter metal, various soldering formulas, German silver |
| Primary metal | American Pennies, nuts and bolts |
| Die casting | Automotive industry |
| Zinc oxide | Paints, sun protectants, rubber activator, diaper rash ointments |
| Wall tiles | Germicidal properties |
| Zinc chloride | Wood preservative and deodorant |
| Zinc methyl ($Zn(CH_3)_2$) | number of organic syntheses |
| Zinc stearate | a lubricative plastic additive |

## PHARMACOKINETICS/ TOXICOKINETICS

Ingested zinc is primarily absorbed from the duodenum and the intestine by a carrier-mediated mechanism. Approximately 25–50% of ingested zinc is absorbed. However, absorption of zinc in influenced by many factors, including whether or not food is in the stomach. Plant phytates can bind zinc and in an alkaline pH environment can form insoluble complexes. Similarly zinc absorption is decreased in the presence of phosphates and calcium in the diet. However, the presence of some peptides, amino acids and the presence of ethylenediamine tetra acetic acid disodium (EDTA) may cause an increase in absorption. Generally, the stomach acid provides for rapid release of zinc from ingested metallic objects.

Once zinc has been absorbed it is bound to plasma albumin and to macroglobulins, and transported to the liver. Zinc is extracted by the liver and returned to the bloodstream for distribution to the liver, pancreas, kidney, and spleen which all rapidly accumulate the zinc. These tissues, as well as muscle and prostate, are induced to synthesize methallothionein in the cells.

Under normal dietary conditions, excess zinc is excreted through the feces. Nevertheless, excretion of zinc, especially in toxic situations, is limited. Excretion occurs through the bile and feces but may also occur through urine (Abdel-Mageed and Oehme, 1990), and saliva.

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## MECHANISM OF ACTION

The mechanism of action producing clinical signs is not well defined or understood. The characteristic clinical signs are more easily recognized. The most recognized abnormality observed is severe intravascular hemolysis and gastroenteritis. This may be a result of excess zinc interfering with copper and iron storage and utilization, resulting in a suppression of hematopoiesis.

Diets high in zinc interfere with hepatic copper storage and may compete with calcium for intestinal absorption.

## TOXICITY

The different forms of zinc have different toxicities. The zinc salts have a median lethal dose ($LD_{50}$) of approximately 100 mg/kg body weight. Zinc oxides are less toxic. Zinc oxides are frequently found in ointments, such as for preventing sunburn or treating diaper rash. Dogs often ingest it when it is applied topically to them or to someone they can lick it off. Dogs also ingested the ointment by chewing on the tube container. It has been estimated that the toxic dose is approximately 108 g of zinc in the dog (Breitschwerdt *et al.*, 1986).

The ingestion of pennies, which are 96% zinc (Latimer *et al.*, 1989), by dogs has resulted in the development of a subacute zinc toxicosis. The pennies remain in the acidic environment of the stomach and slowly release metallic zinc. It is unclear how many pennies are required to produce a toxicosis. The pennies can be vomited up or passed in the feces prior to presentation at the veterinary clinic.

Other forms of zinc containing hardware such as transport kennel bolts or machine nuts and bolts have caused problems in animals. Analysis of these objects has shown them to be about 97% zinc (Breitschwerdt *et al.*, 1986; Torrance and Fulton, 1987). They remain in the stomach producing a subclinical toxicosis or are vomited or passed out prior to the animal's presentation.

Other sources of toxicity have been galvanized wire used in cages in aviaries (Reece *et al.*, 1986) and for housing ferrets (Straube *et al.*, 1980).

An additional component of zinc toxicosis is acute renal failure observed in some dogs. (Breitschwerdt *et al.*, 1986). The clinical findings include hypercreatinemia, azotemia, hyperphosphatemia, and granular casts in the urine (Breitschwerdt *et al.*, 1986; Torrance and Fulton, 1987; Latimer *et al.*, 1989). Other signs of acute intoxication are signs of pancreatitis and acute arthritis. Also, non-viable new-borns may be observed in zinc intoxication.

Foals are susceptible to zinc intoxication. It appears to be a chronic zinc toxicosis. Initially, the foals have non-painful joint enlargement lasting 7–21 days. They are reluctant to rise and have stiff gait and increased joint fluid (Gunson *et al.*, 1982).

Other livestock have early signs of lethargy and anorexia followed by diarrhea. Later, they display a decreased rate of gain or decreased milk production. As the toxic condition progress, the animals have anemia and icterus. Other signs include exophthalmia, polydipsia, polyphagia, and seizures.

Generally most species display some degree of hemolytic anemia, often with an erythrocytic regenerative response. Additionally, there is renal damage with hematuria, urinary casts, and proteinuria. Diagnostically, there may be a radiodense area in the gastrointestinal tract indicating the presence of zinc or some other metal such as lead. Postmortem lesions include renal tubular necrosis, hepatocyte necrosis and gastroenteritis.

## TREATMENT

As with many metal intoxications, removal of the source of the toxin from the animal, followed by supportive therapy are critical to the patient's recovery. This procedure with zinc will result in dramatic drops in serum and tissue levels within a relatively short time. Removal of the source will allow the normal excretory pathways to work appropriately.

Particularly when treating foals it is important to evaluate the copper status of the animal. Copper and zinc seem to work in tandem and adding copper to copper deficient animals may help decrease the overriding effects of zinc.

Supportive care is critically important as the gastroenteritis and anemia must be addressed. The vomiting, in species capable of vomiting, has resulted in usually a severe gastroenteritis. Vomiting may be controlled with metoclopramide (0.2–0.4 mg/kg) administered every 6 h intramuscularly, subcutaneously or per os. Blood transfusions may be in order to address the anemia. Additionally, fluid therapy with a balanced solution such as Lactated Ringers solution should be considered. Good supportive care includes continuous monitoring of various blood parameters such as red blood cells, platelets, packed cell volume, and serum chemistries including liver enzymes, serum urea nitrogen, and electrolytes. Treatment efforts must be directed at correcting pancreatic, renal and/or liver dysfunctions that are so closely associated with zinc intoxication.

Chelation therapy is an option in the patient's care. Chelation therapy is effective but not without risk and so must be evaluated with regard to the patient's condition. Patient conditions requiring evaluation include the hydration status, the degree of dysfunction of the excretory organs and the severity of the serum zinc concentration.

Another important factor is whether the offending object could be removed from the patient. The most commonly suggested chelator is calcium disodium EDTA. Calcium disodium EDTA is most commonly used to treat lead intoxication and the dose for zinc intoxication has thus been extrapolated (100 mg/kg, divided into four doses per day administered subcutaneously, diluted in 5% dextrose in water to decrease local irritation). Especially with chelation therapy, daily monitoring of the patient is essential to determine the length of therapy.

## CONCLUSIONS

Evidence of pancreatitis, liver and kidney dysfunction must be considered along with gastroenteritis and a hemolytic event when considering the differentials. The clinical sign of acute gastroenteritis is common to viral and bacterial diseases, parasitic diseases and various neoplasms. Gastroenteritis and a hemolytic event must be differentiated from other metal intoxications such as copper poisoning. Additional considerations include acetaminophen or onion intoxication, mustard poisoning, immune mediated diseases, and certain snake bites.

As zinc is a very economically useful metal it is likely to be found in more products that animals are exposed to in various fashions. It would be beneficial to have a larger array of safe chelation products. Unfortunately, the importance of chelation therapy outweighs the available markets for the product. Therefore, it is unlikely research dollars will be directed to finding new chelation therapies.

## REFERENCES

Abdel-Mageed AB, Oehme FW (1990) A review of the biochemical roles, toxicity and interactions of zinc, copper and iron. 1. Zinc. *Vet Hum Toxicol* **32**(1): 34–9.

Breitschwerdt EB, Armstrong PJ, Robinette CL, Dillman RC, Karl ML, Lowry EC (1986) Three cases of acute zinc toxicosis in dogs. *Vet Hum Toxicol* **28**: 109.

Gunson DE, Kowalczyk DF, Shoop CR, Ramberg Jr CF (1982) Environmental zinc and cadmium pollution associated with generalized osteochondrosis, osteoporosis, and nephrocalcinosis in horses. *J Am Vet Med Assoc* **180**(3): 295–9.

Latimer KS, Jain AV, Inglesby HB, Clarkson WD, Johnson GB (1989) Zinc-induced hemolytic anemia caused by ingestion of pennies by a pup. *J Am Vet Med Assoc* **195**(1): 77–80.

Reece RL, Dickson DB, Burrowes PJ (1986) Zinc toxicity (new wire disease) in aviary birds. *Aust Vet J* **63**: 199.

Straube EF, Schuster NH, Sinclair AJ (1980) Zinc toxicity in the ferret. *J Comp Pathol* **90**: 355.

Torrance AG, Fulton RB (1987) Zinc-induced hemolytic anemia in a dog. *J Am Vet Med Assoc* **191**: 443.

CHAPTER

# 38

# Chromium, iodine and phosphorus

*Larry J. Thompson*

## CHROMIUM

Chromium is a metallic element that can exist in six valence states, with the trivalent chromium form most commonly found in nature as ferrochromite ores. Both trivalent and hexavalent (+6) are widely used in various industrial and manufacturing processes. Chromium is an essential trace element and functions in a number of metabolic processes including glucose, lipid and amino acid metabolism. Hexavalent chromium is considered to be more toxic than the trivalent form which may be a direct result of its increased systemic availability.

While hexavalent chromium is considered to be a human carcinogen, acute chromium toxicosis in animals is probably of minimum concern in all but the most unusual circumstances. Both hexavalent chromium and dichromate are easily converted to trivalent chromium in mammalian systems but the burning of chromium-treated lumber does not produce enough heat to convert the dichromate to the trivalent chromium form. Oil field contamination with hexavalent chromium has been associated with cases of cattle death (Thompson *et al.*, 1991) and a solution of strong, oxidizing chromium was responsible for dairy cattle deaths when the solution dripped on cattle and was absorbed dermally (Talcott *et al.*, 2005). Acute chromium toxicosis is associated with severe congestion and inflammation of the digestive tract, kidney damage and liver damage. Chronic toxicosis has been associated with gastroenteritis and dermatitis.

## IODINE

Iodine is a non-metallic element of the halogen group that occurs as a purple-black crystalline solid but has several common other forms including iodide ($I^{-1}$) and iodate ($IO^{-3}$). Iodine is widely distributed in nature in both organic and inorganic forms but only in low concentrations. Iodine is essential for the normal synthesis of thyroid hormones and a deficiency of iodine can result in thyroid enlargement or goiter. Deficiencies may occur from eating feeds grown on iodine-deficient soils or from the presence of goitrogenic substances (NRC, 2005a). High dietary iodine for a prolonged period of time can reduce the iodine uptake by the thyroid, causing the clinical syndrome of iodine deficiency.

Common dietary sources of iodine include iodized salt, ethylenediamine dihydroiodide (EDDI) and calcium iodate. Iodized salt can contain 0.01% iodine (100 ppm) and has not been associated with excess iodine exposure. EDDI has been used to prevent and treat foot rot in cattle, although its efficacy is unclear (Morgan, 2004). Toxic effects of iodine excess have been reported in cattle consuming iodine-containing feed additives (Thompson *et al.*, 1991). Clinical signs include decreased feed intake, decreased milk production, rapid breathing, nasal and ocular discharge, dry hair coat and non-responsive hock lesions. Affected cattle were on high intakes of iodine (68–600 mg/head/day and above) for several weeks before obvious clinical signs appeared. Young calves exhibited chronic coughing and profuse nasal discharge at 100–200 ppm dietary iodine. Removal of the excess iodine from the diet and supportive care usually results in rapid return to normal.

## PHOSPHORUS

Most phosphorus in nature exists in combination with oxygen in the form of phosphates, primarily in igneous

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and sedimentary rocks. Inorganic phosphates are commonly used as chemical fertilizers, food and feed supplements and have many industrial uses. Phosphorus is abundant in the animal body primarily as a structural component of crystalline hydroxyapatite in bone and teeth but also as required components of phospholipids, nucleic acids, nucleotides and enzyme cofactors. Phosphate ions also function in acid–base balance and other essential body functions. Phosphorus is an essential macroelement in nutrition and is an important consideration in the formulation of animal diets. The largest dietary source of phosphate will be in the form of inorganic phosphate supplements and other dietary sources may include plant-origin feeds as well as bone, meat, poultry and fish meals. Normal phosphorus nutrition and metabolism requires adequate calcium in the diet with an appropriate calcium-to-phosphorus ratio (Ca:P). While adverse effects of excess phosphorus are rare, they can occur with either excess dietary phosphates or deficient dietary calcium. If the Ca:P ratio is balanced, usually no wider than 2:1, animals can tolerate a wide range of dietary phosphorus levels (NRC, 2005b).

Excess phosphorus in the diet of ruminants, especially sheep, can result in the formation of urinary calculi in the kidney or bladder. This formation of stones can obstruct or completely block urine flow, especially in males, resulting in the bladder filling with urine and eventually rupturing into the abdominal cavity causing death. The problem can be prevented by correctly balancing calcium and phosphorus in the diet. Excess phosphorus in the diet of horses has resulted in nutritional secondary hyperparathyroidism, a condition usually associated with a high grain diet without appropriate calcium supplementation. The high dietary phosphate will depress the intestinal absorption of calcium with a decrease in plasma calcium and an increase in plasma phosphate levels. Low plasma calcium will stimulate the secretion of parathyroid hormone which will increase bone mineral resorption activity. The skeletal bones will lose calcium and the demineralized bone will be replaced by fibrous connective tissue with the facial bones often becoming enlarged (Joyce *et al*., 1971), leading to the common term of big head disease in horses. It is also known as bran disease, since feeding high dietary levels of bran, which is high in phosphate and low in calcium, has historically been a cause of the disease. In all animals, optimum animal performance will be closely associated with optimum dietary calcium and phosphorus balance.

Phosphorus, in the form of white or yellow phosphorus, has historically been used as a rodenticide but is uncommon today. Initial clinical signs following ingestion would include gastroenteritis with vomiting and diarrhea. If the animal survived several days it would often develop a secondary phase of severe liver damage with renal insult also occurring.

## REFERENCES

Joyce JR, Pierce KR, Romane WM, Baker JM (1971) Clinical study of nutritional secondary hyperparathyroidism in horses. *J Am Vet Med Assoc* **158**: 2033.

Morgan SE (2004) Iodine. In *Clinical Veterinary Toxicology*, Plumlee KH (ed.). Mosby, St. Louis, MO, pp. 200–2.

National Research Council (NRC) (2005a) Iodine. In *Mineral Tolerance of Animals*, 2nd revised edition. The National Academies Press, Washington, DC, pp. 182–98.

National Research Council (NRC) (2005b) Phosphorus. In *Mineral Tolerance of Animals*, 2nd revised edition. The National Academies Press, Washington, DC, pp. 290–9.

Talcott PA, Haldorson GJ, Sathre P (2005) Chromium poisoning in a group of dairy cows. *Proceedings of the AAVLD 48th Annual Conference*, Hershey, PA, p. 45.

Thompson LJ, Hall JO, Meerdink GL (1991) Toxic effects of trace element excess. *Vet Clin North Am Food Anim Pract* **7**: 277–306.

# Part 6

# Insecticides and Molluscicides

CHAPTER

# 39

# Organophosphates and carbamates

*Ramesh C. Gupta*

## INTRODUCTION

Organophosphates (OPs) and carbamates (CMs) are commonly used as pesticides in agriculture, industry, and around the home/garden throughout the world. In addition, these chemicals are used as parasiticides in veterinary medicine. Both types of chemicals produce their toxicity by virtue of inhibition of acetylcholinesterase (AChE) enzyme, which terminates the action of the neurotransmitter acetylcholine (ACh) at the synapses in nervous tissue and at the neuromuscular junctions. These chemicals are referred to as "anticholinesterases". Some of the OPs with strong AChE inhibiting potential are also used as nerve agents or nerve gases.

Many compounds of both classes are extremely toxic and lack species selectivity, and therefore their inadvertent/accidental use continues to pose a threat to the environment, human and animal health, wildlife, and aquatic systems. Small animals often encounter poisoning with these insecticides by malicious activity, while livestock by ingesting freshly sprayed crop or contaminated feed. Although these compounds are neurotoxicants, they produce a variety of cholinergic and non-cholinergic effects. Latest evidence suggests that while cholinergic mechanisms play a critical role in the initial stage of toxicity, neuronal damage/death appears to occur through non-cholinergic mechanisms. OPs and CMs are discussed here together because they produce similar toxic effects in poisoned animals. This chapter covers various aspects of toxicity of OP and CM compounds and therapeutic measures in animals. For more details, readers are referred to Gupta (2006).

## BACKGROUND

The first OP compound, tetraethyl pyrophosphate, was synthesized in 1854 by Philipe de Clermont. In 1932, Lange and Kruger described the synthesis of dimethyl and diethyl phosphorofluoridate. Based on the chemistry of these compounds, Gerhard Schrader (a chemist at the I.G. Farbenindustrie) led the exploration of OP class of compounds that could be used as insecticides. One of the earliest OP insecticides synthesized by Schrader was parathion, which is still used worldwide. Prior to World War II (WWII), the German Ministry of Defense developed highly toxic OP compounds of G series (tabun, sarin, and soman) and diisopropyl phosphorofluoridate. In the 1950s, OP compounds with super toxicity, such as VX and VR, were synthesized in the United Kingdom and Soviet Union. After WWII, thousands of OPs have been synthesized in the search for compounds with species selectivity, i.e. more toxic to insects and less toxic to mammals. Malathion is an example. This compound has been used for about half-a-century as the most popular insecticide. Today, more than 100 OPs are in use for a variety of purposes, such as protection of crops, grains, gardens, homes, and public health.

The first CM compound, physostigmine (eserine alkaloid), was isolated from calabar beans (ordeal poison) of a perennial plant *Physostigma venenosum* in the mid-1860s. The compound was used to treat glaucoma. About 50 years later, an aromatic ester of carbamic acid, neostigmine, was synthesized and used in the treatment of myasthenia gravis. Most of the CMs (esters of carbamic acid) that are used as pesticides were synthesized in the 1960s and 1970s. Carbaryl was the first CM compound used as an insecticide.

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To mimic the structure of acetylcholine (ACh), aldicarb was synthesized which has a toxicity greater than any other compounds of this class. Like OPs, thousands of CMs have been synthesized, but less than two dozen compounds have been used practically. Today, CMs are preferred for pesticide use over OPs because some OPs have been found to be extremely toxic, whereas others cause delayed neuropathy in animals as well as in humans. In essence, both OPs and CMs have broad applications in agriculture and veterinary medicine and as a result of their indiscriminate use acute poisonings often result in animals, birds, fish, and wildlife.

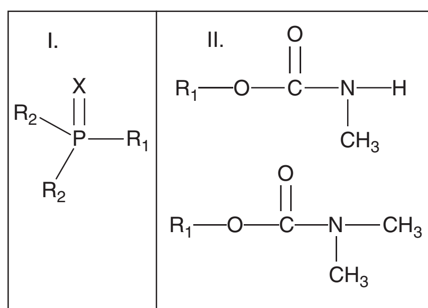


**FIGURE 39.1** General structure for organophosphorus (I) and CM (II) insecticides (adapted from Timchalk, 2006).

## TYPES OF OPs AND CMs

Basic structures of organophosphorus and CM compounds are shown in Figure 39.1. There are at least 13 types of OPs (see Table 39.1). Despite differences in chemical structures, all OPs share one thing in common: they all have a pentavalent phosphorus atom and a characteristic phosphoryl bond (P = O) or thiophosphoryl bond (P = S). Essentially, OPs are esters of phosphoric acid with varying combinations of oxygen, carbon, sulfur, and/or nitrogen attached. Of course, the chemistry of these compounds is much more complex. The OPs that are derivatives of phosphoric or phosphonic acid possess anticholinesterase activity, unlike those that are derivatives of phosphinic acid. Usually, OP compounds have two alkyl substituents and an additional substituents group (leaving group, which is more labile to hydrolysis than the alkyl group (Marrs, 1993). Basically, some OPs (such as dichlorvos, monocrotophos, and trichlorfon) are direct AChE inhibitors, while those of phosphorothioates type (such as bromophos, diazinon, fenthion, and parathion) possess minimal or no anticholinesterase (anti-AChE) activity and require desulfuration to the analogous oxon before acquiring anti-AChE activity. Also, OPs which are used as defoliants (*S*,*S*,*S*-tributyl phosphorotrithioate and *S*,*S*,*S*-tributyl phosphorotrithioite) and

**TABLE 39.1** Types of OPs

| Type of OP | Chemical structure | Example |
|---|---|---|
| Phosphates | RO—P(=O)(—OR)—OR | Chlorfenvinphos<br>Dichlorvos<br>Monocrotophos |
| Phosphonates | RO—P(=O)(—OR)—R | Trichlorfon |
| Phosphinates | R—P(=O)(—OR)—R | Gluphosinate |
| Phosphorothioates (S=) | RO—P(=S)(—OR)—OR | Bromophos<br>Diazinon<br>Fenthion<br>Parathion<br>Pirimiphos-methyl |
| Phosphonothioates (S=) | RO—P(=S)(—OR)—R | EPN<br>Leptophos |

(*Continued*)

TABLE 39.1 (Continued)

| Type of OP | Chemical structure | Example |
|---|---|---|
| Phosphorothioates (S-substituted) | RS—P(=O)(—OR)—OR | Demeton-*S*-methyl<br>echothiophate |
| Phosphonothioates (S-substituted) | RS—P(=O)(—R)—OR | VX |
| Phosphorodithioates | RS—P(=O)(—SR)—OR or RS—P(=S)(—OR)—OR | Azinphos-ethyl<br>Azinphos-methyl<br>Dimethoate<br>Disulfoton<br>Malathion<br>Methidathion |
| Phosphorotrithioates | RS—P(=O)(—SR)—SR | DEF (tribufos) |
| Phosphoramidates | RO—P(=O)(—OR)—N(R)R | Fenamiphos |
| Phosphoramidothioates | RO—P(=S)(—OR)—N(R)R or RS—P(=O)(—OR)—N(R)R | Methamidophos<br>Isofenphos |
| Phosphorofluoridates | RO—P(=O)(—OR)—F | Diisopropyl<br>phosphorofluoridate (DFP) |
| Phosphonofluoridates | RO—P(=O)(—R)—F | Cyclosarin<br>Sarin<br>Soman |

*Note*: Adapted from Marrs (1993).

herbicides (glyphosate and gluphosinate) are of very low mammalian toxicity.

Chemical structures of some of the commonly used OP pesticides are shown in Figure 39.2.

## OP PESTICIDES

The majority of OP compounds are used as pesticides, and a brief chemical description for commonly used compounds is given in Table 39.2.

## OP NERVE GASES/AGENTS

OP nerve agents include tabun (GA), sarin (GB), soman (GD), cyclosarin (GF), VX, and VR. These compounds are highly toxic and pose continuous threats for the lives of

TABLE 39.2 Brief chemical description of commonly used OP pesticides

| Chemical | Chemical name | Molecular weight | Oral $LD_{50}$ in rat (mg/kg) | Dermal $LD_{50}$ in rabbit (mg/kg) |
|---|---|---|---|---|
| Acephate | *O,S*-dimethyl acetylamidothiophosphate | 183.17 | 866 | >2000 |
| Azinphos-ethyl | *O,O*-diethyl *S*-[(4-oxo-1,2,3-benzotriazin-3(4*H*)-yl)methyl] dithiophosphate | 345.38 | 13 | 250 |
| Azinphos-methyl | *O,O*-dimethyl *S*-[(4-oxo-1,2,3-benzotriazin-3(4*H*)-yl)methyl] dithiophosphate | 317.32 | 5 | 220 |
| Bromophos | *O*-(4-bromo-2,5-dichlorophenyl) *O,O*-dimethyl thiophosphate | 366.00 | 1600 | 2188 |
| Cadusaphos | *S,S*-di-*sec*-butyl *O*-ethyl dithiophosphate | 270.40 | 391 | 143 |
| Carbophenothion | *S*-{[(4-chlorophenyl)thio] methyl} *O,O*-diethyl dithiophosphate | 342.87 | 6 | 22 |
| Chlorethoxyphos | *O,O*-diethyl *O*-(1,2,2,2-tetrachloroethyl) thiophosphate | 336.00 | 1.8 | 12.5 |
| Chlorfenvinphos | 2-chloro-1-(2,4-dichloro-phenyl)vinyl diethyl phosphate | 359.57 | 12 | 3200 |
| Chlorpyrifos | *O,O*-diethyl *O*-(3,5,6-trichloropyridin-2-yl) thiophosphate | 350.59 | 135 | 2000 |
| Chlorpyrifos-methyl | *O,O*-dimethyl *O*-(3,5,6-trichloropyridin-2-yl) thiophosphate | 322.53 | 941 | 2000 |
| Coumaphos | *O*-(3-chloro-4-methyl-2-oxo-2*H*-chromen-7-yl) *O,O*-diethyl thiophosphate | 362.77 | 13 | – |
| Crotoxyphos | 1-phenylethyl (2*E*)-3-[(dimethoxyphosphoryl)oxy]but-2-enoate | 314.27 | 125 | 385 |
| Cyanophos | *O*-(4-cyanophenyl) *O,O*-dimethyl thiophosphate | 243.22 | 610 | 800 |
| Demeton-O | *O,O*-diethyl *O*-[2-(ethylthio)ethyl] thiophosphate | 258.34 | 2.5 | 8 |
| Diazinon | *O,O*-diethyl *O*-(2-isopropyl-6-methylpyrimidin-4-yl) thiophosphate | 304.35 | 300 | 379 |
| Dichlorvos | 2,2-dichlorovinyl dimethyl phosphate | 220.98 | 25 | 59 |
| Dicrotophos | (1*E*)-3-(dimethylamino)-1-methyl-3-oxoprop-1-en-1-yl dimethyl phosphate | 237.19 | 22 | 223 |
| Dimethoate | *O,O*-dimethyl *S*-[2-(methylamino)-2-oxoethyl] dithiophosphate | 229.26 | 250 | 400 |
| Disulfoton | *O,O*-diethyl *S*-[2-(ethylthio)ethyl] dithiophosphate | 274.40 | 2 | 6 |
| Ethion | *O,O,O′,O′* -tetraethyl *S,S′* -methylene bis(dithiophosphate) | 384.48 | 27 | 915 |
| Famphur | *O*-{4-[(dimethylamino)sulfonyl]phenyl} *O,O*-dimethyl thiophosphate | 325.34 | 35 | 2730 |
| Fenamiphos | ethyl 3-methyl-4-(methylthio)phenyl isopropylamidophosphate | 303.36 | 15.3 | – |
| Fenitrothion | *O,O*-dimethyl *O*-(3-methyl-4-nitrophenyl) thiophosphate | 277.23 | 250 | 1300 |
| Fenthion | *O,O*-dimethyl *O*-[3-methyl-4-(methylthio)phenyl] thiophosphate | 278.33 | 255 | 330 |
| Fonofos | *O*-ethyl *S*-phenyl ethylphosphonodithioate | 246.33 | 8 | 25 |
| Glyphosate | *N*-(phosphonomethyl)-glycine | 169.07 | 4300 | >5000 |
| Gluphosinate ammonium | 2-amino-4-[hydroxy(methyl)phosphoryl]butanoic acid ammoniate | 198.16 | 2000 | >4000 |
| Glyphosine | *N,N*-bis(phosphonomethyl)glycine | 263.08 | 3925 | >5010 |
| Isazophos | *O*-(5-chloro-1-isopropyl-1*H*-1,2,4-triazol-3-yl) *O,O*-diethyl thiophosphate | 313.75 | 40 | >3100 |
| Isofenphos | isopropyl 2-{[ethoxy(isopropylamino)phosphorothioyl] oxy}benzoate | 345.40 | 32 | 162 |
| Malathion | diethyl 2-[(dimethoxyphos-phorothioyl)thio] succinate | 330.36 | 885 | 4000 |
| Methamidophos | *O,S*-dimethyl amidothiophosphate | 141.13 | 13 | 110 |
| Methidathion | *S*-[(5-methoxy-2-oxo-1,3,4-thiadiazol-3(2*H*)-yl)methyl] *O, O*-dimethyl dithiophosphate | 302.33 | 25 | 200 |
| Methyl parathion | *O,O*-dimethyl *O*-(4-nitrophenyl) thiophosphate | 263.21 | 9 | 63 |
| Mevinphos | methyl (2*E*)-3-[(dimethoxyphos-phoryl)oxy]but-2-enoate | 224.15 | 3 | 16 |
| Monocrotophos | dimethyl (1*E*)-1-methyl-3-(methylamino)-3-oxoprop-1-en-1-yl phosphate | 223.16 | 8 | 354 |
| Omethoate | *O,O*-dimethyl *S*-[2-(methylamino)-2-oxoethyl] thiophosphate | 213.19 | 50 | 1400 |
| Paraoxon | diethyl 4-nitrophenyl phosphate | 275.19 | 1.8 | – |
| Parathion | *O,O*-diethyl *O*-(4-nitrophenyl) thiophosphate | 291.26 | 3 | 6.8 |
| Phenthoate | ethyl [(dimethoxyphosphorothioyl)thio] (phenyl)acetate | 320.36 | 200 | 4000 |
| Phorate | *O,O*-diethyl *S*-[(ethylthio)methyl] dithiophosphate | 260.38 | 1.6 | 2.5 |
| Phosmet | *S*-[(1,3-dioxo-1,3-dihydro-2*H*-iso-indol-2-yl)methyl] *O, O*-dimethyl dithiophosphate | 317.32 | 147 | 3160 |
| Phosphamidon | (1*Z*)-2-chloro-3-(diethylamino)-1-methyl-3-oxoprop-1-en-1-yl dimethyl phosphate | 299.69 | 15 | 125 |
| Phoxim | phenylglyoxylonitrile oxime, *O, O*-diethyl phosphorthioate | 289.30 | 1845 | 1126 |

(*Continued*)

**TABLE 39.2 (Continued)**

| Chemical | Chemical name | Molecular Weight | Oral $LD_{50}$ in rat (mg/kg) | Dermal $LD_{50}$ in rabbit (mg/kg) |
|---|---|---|---|---|
| Profenofos | *O*-(4-bromo-2-chlorophenyl) *O*-ethyl *S*-propyl thiophosphate | 373.63 | 400 | 472 |
| Propetamphos | isopropyl (2*E*)-3-{[(ethylamino)(methoxy)phosphorothioyl] oxy}but-2-enoate | 281.31 | 82 | 2300 |
| Quinalphos | *O,O*-diethyl *O*-quinoxalin-2-yl thiophosphate | 298.30 | 65 | 340 |
| Ronnel | *O,O*-dimethyl *O*-(2,4,5-trichlorophenyl) thiophosphate | 321.55 | 1250 | 2000 |
| Sulfotepp | *O,O,O,O*-tetraethyl dithiodiphosphate | 322.32 | 5 | – |
| Sulprofos | *O*-ethyl *O*-[4-(methylthio)phenyl] *S*-propyl dithiophosphate | 322.45 | 107 | 820 |
| Terbufos | *S*-[(*tert*-butylthio)methyl] *O,O*-diethyl dithiophosphate | 288.43 | 1.6 | 1 |
| Triazophos | *O,O*-diethyl *O*-(1-phenyl-1*H*-1,2,4-triazol-3-yl) thiophosphate | 313.32 | 83 | 280 |
| Trichlorfon | dimethyl (2,2,2-trichloro-1-hydroxyethyl)phosphonate | 257.44 | 630 | >2100 |

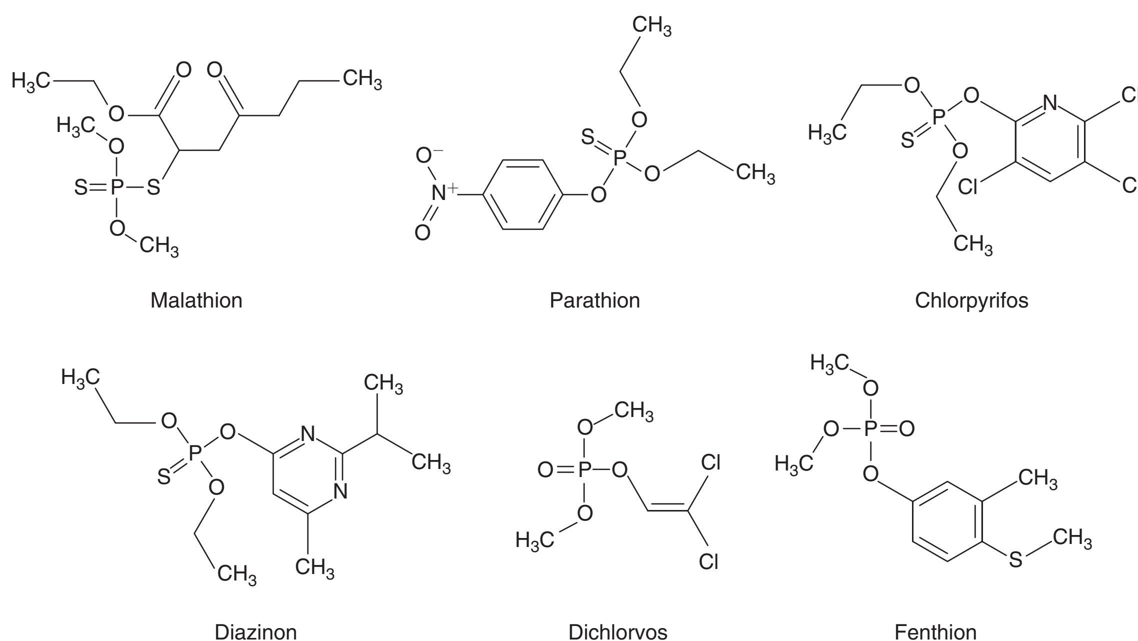


**FIGURE 39.2** Chemical structures of commonly used OP pesticides.

humans as well as animals, because they can be used as chemical weapons of mass destruction. So far these agents have been used by dictators and terrorists. These compounds produce toxicity by directly inhibiting AChE, and are much more potent than OP pesticides, as they cause lethality to animals in the submilligram range. Their chemical structures are shown in Figure 39.3. For details of toxicity of these compounds, refer to Watson *et al.* (2006).

## CARBAMATES

The CM compounds are esters of carbamic acid. Unlike OPs, CM compounds are not structurally complex. Currently, CMs are used as pesticides in agricultural crops and gardens and in veterinary medicine as parasiticides.

## CM PESTICIDES

Currently, the volume of CMs used exceeds OPs because they are comparatively safer than OPs. Some of the commonly used *N*-methyl CM insecticides are briefly described in Table 39.3. For other CMs, readers are referred to Gupta (2006).

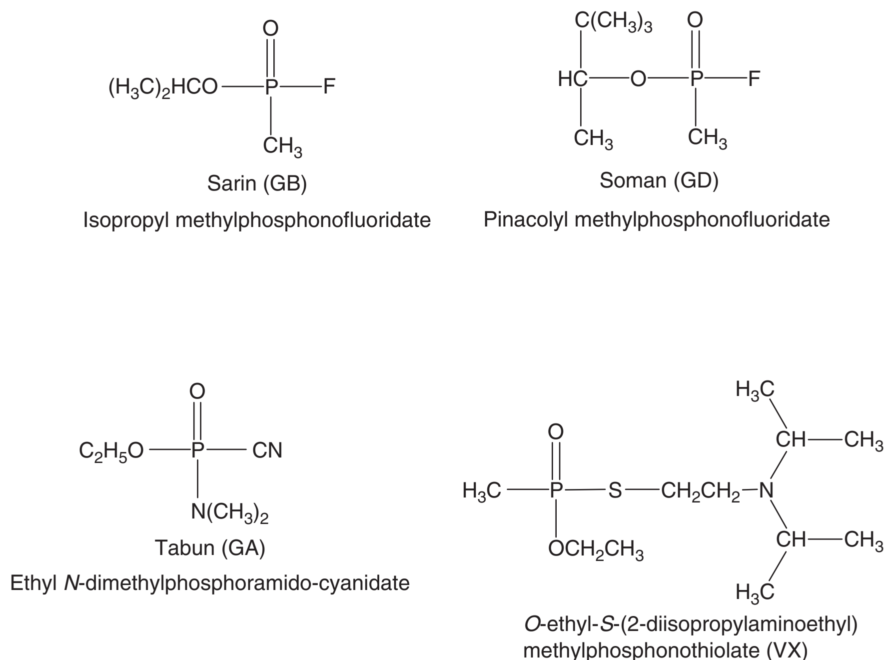


**FIGURE 39.3** Chemical structures of OP nerve agents.

**TABLE 39.3 Brief chemical description of commonly used CM pesticides**

| Chemical | Chemical name | Molecular weight | Oral $LD_{50}$ in rat (mg/kg) | Dermal $LD_{50}$ in rabbit (mg/kg) |
|---|---|---|---|---|
| Aldicarb | (1*E*)-2-methyl-2-(methylthio)propanal *O*-[(methyl-amino) carbonyl] oxime | 190.26 | 0.9 | 5 |
| Aminocarb | 4-(dimethylamino)-3-methylphenyl methylcarbamate | 208.26 | 30 | 275 |
| Bendiocarb | 2,2-dimethyl-1,3-benzodioxol-4-yl methylcarbamate | 223.23 | 34 | 566 |
| Benfuracarb | 2,3-dihydro-2,2-dimethyl-7-benzofuranyl *N*-[n[2-(ethylcarbonyl) ethyl]-*N*-isopropyl sulfenamoyl]-*N*-methylcarbamate | 410.53 | 138 | >2000 |
| BPMC | 2-*sec*-butylphenyl *N*-methylcarbamate | 422.87 | 340 | 4200 |
| Carbaryl | 1-naphthyl methylcarbamate | 201.22 | 307 | 2000 |
| Carbofuran | 2,2-dimethyl-2,3-dihydro-1-benzofuran-7-yl methylcarbamate | 221.25 | 8 | 2550 |
| Carbosulfan | 2,3-dihydro-2,2-dimethyl-7-benzofuranyl-[(di-butylamino)thio] methyl carbamate | 380.55 | 209 | >2000 |
| Croneton | 2-[(ethylthio)methyl]phenyl methylcarbamate | 225.31 | 200 | 1000 |
| Fenoxycarb | ethyl [2-(4-phenoxyphenoxy)ethyl] carbamate | 301.34 | 10,000 | 2000 |
| Isoprocarb | 2-isopropylphenyl methylcarbamate | 193.24 | 450 | – |
| Methiocarb | 3,5-dimethyl-4-(methylthio)phenyl methylcarbamate | 225.31 | 15 | 2000 |
| Methomyl | methyl (1*E*)-*N*-{[(methylamino) carbonyl]oxy} ethanimidothioate | 162.21 | 17 | 5000 |
| Metolcarb | 3-methylphenyl methylcarbamate | 165 | 268 | – |
| Mexacarbate | 4-(dimethylamino)-3,5-dimethylphenyl methylcarbamate | 222.28 | 15 | 5000 |
| Oxamyl | methyl 2-(dimethyl-amino)-*N*-{[(methyl-amino) carbonyl] oxy}-2-oxoethan-imidothioate | 219.26 | 5 | 710 |
| Pirimicarb | 2-(dimethylamino)-5,6-dimethyl-pyrimidin-4-yl dimethylcarbamate | 238.29 | 147 | >500 |
| Promecarb | 3-isopropyl-5-methylphenyl methylcarbamate | 207.27 | 61 | >1000 |
| Propoxur | 2-isopropoxyphenyl methylcarbamate | 209.24 | 95 | >1000 |
| Trimethacarb | 3,4,5-trimethylphenyl methylcarbamate | 193.24 | 125 | >2000 |
| XMC | 3,5-dimethylphenyl methylcarbamate | 179.22 | 542 | – |
| Xylylcarb | 3,4-dimethylphenyl methylcarbamate | 179.22 | 384 | – |

## PHARMACOKINETICS OF OPs AND CMs

Pharmacokinetics deals with the processes of absorption, distribution, metabolism, and excretion (ADME). The ADME of OP and CM insecticides have been studied in animals (Tomokuni *et al.*, 1985; Moody and Franklin, 1987; Gupta, 1994; Wu *et al.*, 1996; Tos-Luty *et al.*, 2001; Timchalk *et al.*, 2002, 2004; Poet *et al.*, 2004). These insecticides gain entry into the body mainly through oral, dermal, or inhalation exposure. Ingestion encounters with contaminated feed/food with pesticides residue, while dermal exposure is more relevant when these insecticides are used as ectoparasiticides in the form of dust, dip, or oily solution. Inhalation of airborne insecticides occurs during or soon after aerial spray, particularly due to chemical drift. Once the insecticide reaches a portal of entry, it is available for absorption. It is established that following absorption, these insecticides are well distributed in tissue throughout the body. OP insecticides may follow either activation or detoxification, or both. Activation implies that the metabolite is more toxic than the parent compound, e.g. the conversion of malathion to malaoxon. This process is often referred to as "lethal synthesis". On the other hand, detoxification implies that the metabolite is less toxic than the parent compound, e.g. the conversion of malathion to malathion monoacid and malathion diacid. Unlike OPs, CMs are metabolized to less toxic or non-toxic metabolites, though some of the metabolites of CMs are quite toxic. For example, the two major metabolites of carbofuran (3-hydroxycarbofuran and 3-ketocarbofuran) have a significant impact on overall toxicity of carbofuran. A bulk of the metabolic activation and detoxification reactions occur within the liver (Sultatos *et al.*, 1984a, b; Sultatos, 1988). Finally, due to extensive metabolism in the body, only few metabolites are excreted in the urine that can be used as biomarkers of insecticides exposure (Figure 39.4).

Residues of some OPs and CMs can also be detected in the feces, saliva, and milk.

## MECHANISM OF ACTION

OP and CM insecticides share a common mode of insecticidal and toxicological action associated with their ability to inhibit the enzyme AChE within nerve tissue and at the neuromuscular junctions. Both types of insecticides have a high affinity for binding to and inhibiting the enzyme AChE, an enzyme specifically responsible for the destruction of the neurotransmitter ACh. Since the cholinergic system is widely distributed within both the central and peripheral nervous systems, chemicals that inhibit AChE are known to produce a broad range of well-characterized symptoms of anticholinesterases (Savolainen, 2001; Timchalk, 2006). A graphic representation for the comparison of the AChE inhibition dynamics for the interaction of ACh, carbaryl (CM), or chlorpyrifos-oxon (OP) with AChE is shown in Figure 39.5 (Timchalk, 2006).

The cholinesterases (ChE) are serine hydrolases that catalyze the breakdown of ACh through an acyl-transfer, where water is the acceptor molecule to which the substrate acyl moiety is transferred. A serine oxygen of the active site gorge in ChEs carries out a nucleophilic attack on the electrophilic carbon of the carbonyl group of ACh, resulting in an acetylated enzyme intermediate and the release of choline. Deacetylation occurs when an attacking water molecule (hydroxyl ion) acts as a more effective nucleophile, thereby releasing acetate (Sultatos, 2006). The molecular interactions between OPs and AChE (see Figure 39.6) have been studied in much more detail than

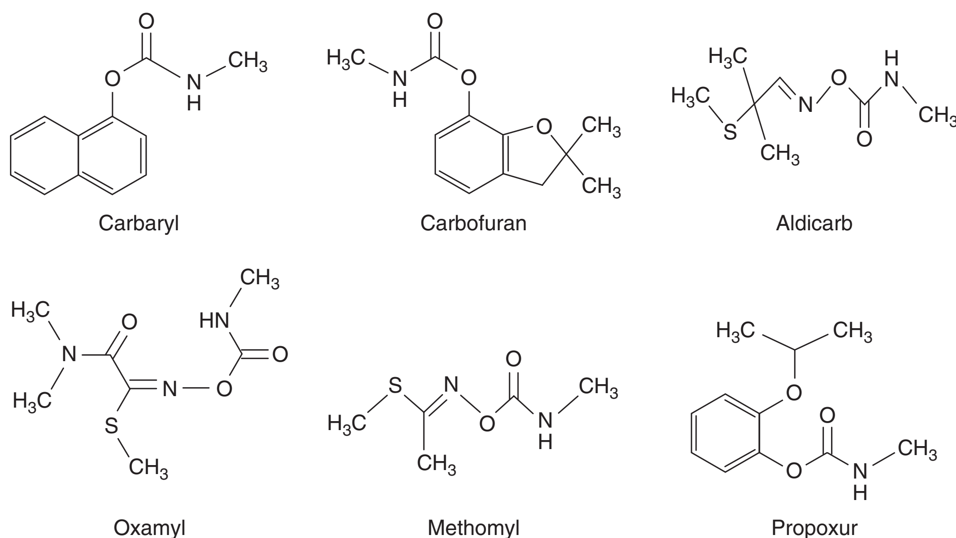


**FIGURE 39.4** Chemical structures of commonly used CM pesticides.

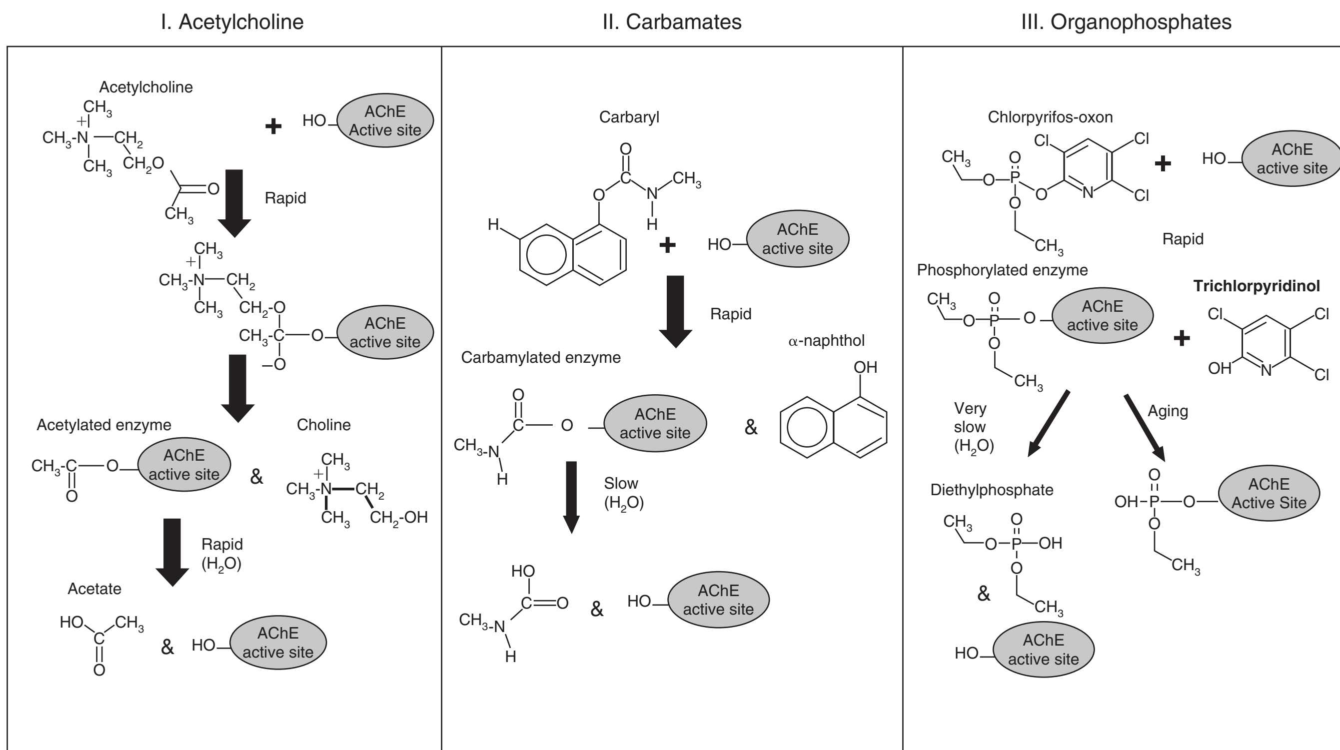


**FIGURE 39.5** Interaction of acetylcholine (I), the CM carbaryl (II), and the OP chlorpyrifos-oxon (III) with the active site of AChE. The general rate of bound AChE hydrolysis is ACh>carbaryl>chlorpyrifos-oxon (Timchalk, 2006).

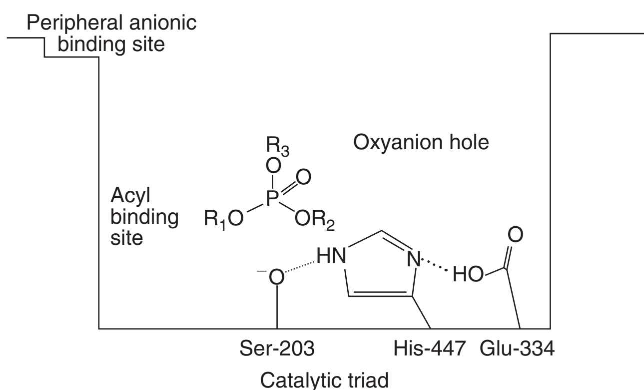


**FIGURE 39.6** Schematic drawing of the active site gorge of AChE, with the entry of an OP molecule. $R_1$ and $R_2$ on the OP are usually identical alkyl chains, whereas $R_3$ is the leaving group. The catalytic triad consists of Ser203, His447, and Glu334. The acyl binding site is likely important in positioning the inhibitor for the nucleophilic attack from Ser203 (Ordentlich *et al*., 1996), whereas the oxyanion hole may polarize the P=O bond, thereby facilitating the nucleophilic attack (Ordentlich *et al*., 1998). Binding of ligand to the peripheral anionic site can lead to inhibition or activation. Additionally, the peripheral anionic site plays an important role in the stereoselectivity of AChE toward methylphosphonates (Ordentlich *et al*., 2004). Adapted from Sultatos (2006).

between CMs and AChE. The rates of hydrolysis and reactivation of AChE following carbamylation and phosphorylation of the active site appear to be drastically slower than for the hydrolysis of the acetylated enzyme. The turnover time for ACh is of the order of ~150 μs, whereas the carbamylated enzyme *t*½ for hydrolysis is substantially slower (~15–30 min). The phosphorylated enzyme is highly stable (*t*½ ~days), and further dealkylation of the phosphorylation group produces an "aged" AChE that is irreversibly inhibited (Sogorb and Villanova, 2002; Taylor, 2006; Timchalk, 2006). In general, OPs and CMs are considered as irreversible and reversible AChE inhibitors, respectively. Details of ChEs, interaction of OPs and CMs with ChEs, and reactivation/regeneration of ChEs are described elsewhere (Radic and Taylor, 2006; Sultatos, 2006; Timchalk, 2006).

# TOXICITY

Most animal poisoning cases in the field are acute in nature. Onset of clinical signs usually occurs within 15 min to 1 h, which is soon followed by the signs of maximal severity, although these timings tend to vary depending upon the OP compound and its dose, and species. For example, onset of clinical signs is delayed with chlorpyrifos (Dursban) and dimethoate (Rogor). Clinical signs observed in poisoned animals can be divided into local and systemic effects. The local effects involve the eyes and the lungs, owing to their exposure to vapors or droplets of the insecticides. These effects, however, are of significance in the case of animals only when exposure is via spraying.

**TABLE 39.4 Normal acetylcholinesterase (AChE) activity in brain cortex of different species**

| Species | AChE (μmol/g/h) |
|---|---|
| Cattle | 160 |
| Swine | 163 |
| Sheep | 170 |
| Horse | 124 |
| Chicken | 1098 |
| Dog | 200 |
| Rat | 255 |

The systemic effects are primarily on the brain, skeletal muscles, lungs, heart, and other organs.

The clinical signs can also be classified as muscarinic, nicotinic, and central. Muscarinic receptor-associated effects are manifested by vomiting, abdominal and chest pain, salivation, lacrimation, urination, diarrhea (SLUD), miosis (pinpoint pupils), tracheobronchial secretion, lung edema, and cyanosis. The nicotinic receptor-associated effects are produced on autonomic ganglia and skeletal muscles, and the affected animals show twitching of muscles, tremors, followed by convulsions, and seizures. This condition may lead to paralysis. The central effects include apprehension, stimulation, followed by depression. The affected animals may also show restlessness, ataxia, stiffness of the neck, and coma. Death occurs due to respiratory failure and cardiac arrest. It is important to mention that all poisoned animals may not show all the clinical signs (as described above) with every OP or CM compounds. In other words, there is a great variation in symptomatology among the individual cases of poisoning. Surviving animals usually recover within 3–6 h with CMs and within 24 h with OPs.

Poisoning cases of OP or CM are usually diagnosed by determining the level of inhibition of AChE activity in blood from a live animal and brain from a dead animal. Inhibition of AChE activity >70% is considered positive case of poisoning. It should be noted that great species variability exists in normal values of AChE activity (Table 39.4). Also, analyzing the cortex of the brain and not the striatum for AChE analysis is preferred, since more than 6-fold variability exists (Gupta, 2004). Therefore, interpretation should be made with great caution. Residue analysis of an insecticide and/or its metabolite(s), followed by confirmation with GC/MS or LC/MS, seems an ideal approach for diagnosis.

## TREATMENT OF ACUTE POISONING

Before instituting antidotal therapy, monogastric animals, such as dog, should be given gastric lavage. Animals of any species can be given activated charcoal to stop further absorption of insecticides. Animals should be washed thoroughly with water if they are exposed to insecticides dermally. Intravenous (IV) fluid therapy is always beneficial.

In the case of OP poisoning, antidotal treatment requires the combined use of atropine sulfate and pyridine-2-aldoxime methochloride (2-PAM). Atropine sulfate acts by blocking the muscarinic receptors from ACh. In ruminants, one-fourth of the total recommended dose (0.5 mg/kg) can be given as a slow IV injection, and the remainder through intramuscular (IM) or subcutaneous (SC) injection (Gupta, 1984). The total dose of atropine sulfate for an average size horse is about 65 mg, and for a dog is about 2 mg. Atropine sulfate treatment can be repeated at an interval of every hour until all hyper-secretory signs have subsided. 2-PAM reactivates the AChE inhibited by OPs. The recommended therapeutic dose of 2-PAM is 20 mg/kg, IV. The injection of 2-PAM can be repeated once after 1 h at half of its initial dose. Care should be taken that only a freshly prepared solution of 2-PAM be used. It needs to be emphasized that the combined therapy of atropine sulfate and 2-PAM is superior to any other treatment till today in the case of OP poisoning. Although many other oximes have been tested against many OPs, none has been proven to be better than 2-PAM. Furthermore, depressant drugs, such as morphine and barbiturates, are contraindicated, since they aggravate the condition. Diazepam without atropine sulfate also accentuates the toxicity of OPs.

Unlike with OP poisoning, 2-PAM and other oximes are ineffective in CM poisoning cases. In fact, in the case of some CMs, such as carbaryl and carbofuran, 2-PAM therapy accentuates the toxicity. Some anticonvulsant drugs, such as barbiturates and diazepam, also aggravate the toxicity of CMs. Therefore, atropine sulfate, with doses as described for OPs, is the only preferred antidote. When the animals are exposed to very higher doses of CMs, atropine sulfate does not appear to be a life saving antidote.

## OP-INDUCED DELAYED POLYNEUROPATHY

OP compounds that produce delayed neurotoxic effects are esters of phosphorus-containing acids. Over 35 years ago, tri-*o*-cresyl phosphate (TOCP) was known to produce delayed neurotoxic effects in man and chicken, characterized by ataxia and weakness of the limbs, developing 10–14 days after exposure (Johnson, 1969). This syndrome was called OP-induced delayed neuropathy (OPIDN). In recent literature, the syndrome has been renamed as OP-induced delayed polyneuropathy (OPIDP). OPIDP is characterized by distal degeneration of long- and large-diameter motor and sensory axons of both peripheral nerves

and spinal cord. Among all animal species hen appears to be the most sensitive and therefore used as an animal model. TOCP and certain other compounds have minimal or no anti-AChE property, however they cause phosphorylation and aging (dealkylation) of a protein in neurons called neuropathy target esterase (NTE), and subsequently lead to OPIDP. Studies on the sensitivity of the target enzymes of a variety of OPs showed that the comparative inhibitory power of OPs against hen AChE and NTE *in vitro* correlates with their comparative effects *in vivo* (i.e. delayed neuropathy or death). The relationship between the degree of NTE inhibition and the severity of OPIDP changes according to the compound involved. For example, certain compounds cause OPIDP with a minimum of 70% NTE inhibition, while others require almost complete inhibition to cause OPIDP. However, the cascade of events from NTE inhibition/aging to impairment of retrograde axonal transport and axonal degeneration is yet to be explained (Moretto and Lotti, 2006). Today, many compounds, such as DFP, *N,N′* -diisopropyl phosphorodiamidic fluoride (mipafox), tetraethyl pyrophosphate (TEPP), paraoxon, parathion, *o*-cresyl saligenin phosphate, and haloxon, are known to produce this syndrome. For the details of OPIDP syndrome, refer to Moretto and Lotti (2006). Treatment of this syndrome is symptomatic.

## OP-INDUCED INTERMEDIATE SYNDROME

OP insecticide-induced intermediate syndrome (IMS) was reported for the first time in human patients in Sri Lanka in 1987 (Senanayake and Karalliedde 1987). Thereafter this syndrome has been diagnosed in OP-poisoned patients in South Africa (1989), Turkey (1990), Belgium (1992), the United States (1992), Venezuela (1998), France (2000), and elsewhere. To date, OPs that are known to cause IMS include bromophos, chlorpyrifos, diazinon, dicrotophos, dimethoate, disulfoton, fenthion, malathion, merphos, methamidophos, methyl parathion, monocrotophos, omethoate, parathion, phosmet, and trichlorfon. IMS is usually observed in individuals who have ingested a massive dose of an OP insecticide either accidentally or in a suicide attempt. IMS is clearly a separate clinical entity from acute toxicity and delayed neuropathy. Clinically, IMS is characterized by acute paralysis and weakness in the areas of several cranial motor nerves, neck flexors, facial, extraocular, palatal, nuchal, proximal limb, and respiratory muscles 24–96 h after poisoning. Generalized weakness, depressed deep tendon reflexes, ptosis, and diplopia are also evident. These symptoms may last for several days or weeks depending on the OP involved. A similar syndrome has also been observed in dogs and cats poisoned maliciously or accidentally with massive doses of certain OPs. It should be noted that despite severe AChE inhibition, muscle fasciculations and muscarinic receptor-associated hyper-secretory activities are absent.

Although the exact mechanism involved in pathogenesis of IMS is unclear, studies suggest that decrease of AChE and nicotinic ACh receptor mRNA expression occurs after oral poisoning with disulfoton in rats. Based on electromyographic (EMG) findings from OP-poisoned patients and experimental studies on laboratory animals, it has been found that the defect in IMS is at the neuromuscular endplate and postsynaptic level, but the effects of neural and central components in producing muscular weakness have not been ruled out. It seems clear that some OPs are greatly distributed to muscles and have higher affinity for nicotinic ACh receptors. Currently, very little is known about the type of damage at the motor endplate or about risk factors contributing to its development. There is no specific treatment, and therapy relies upon atropine sulfate and 2-PAM. The administration of atropine sulfate and 2-PAM should be continued for a long period, even if efficacy of these drugs on the development of IMS appears to be limited. For further details about IMS, readers are referred to Gupta (2005) and De Bleecker (2006).

## TOLERANCE DEVELOPMENT

Tolerance development to the toxicity of OPs was noted more than 50 years ago. Following prolonged exposure to an OP, the physiological effects often diminish more than expected from the degree of AChE inhibition or repeated additions of OP give lower responses with time. Tolerance to AChE inhibiting OPs (such as DFP, disulfoton, methyl parathion, and others) has been observed using different forms of administration and in different species, such as mice, rats, guinea pig, and man (Fonnum and Sterri, 1981; Gupta, 2004; Gupta and Dettbarn, 1986; Gupta *et al.*, 1986; Fonnum and Sterri, 2006).

Tolerance to OP toxicity can develop in several ways. Most often, it occurs due to receptor changes either in the number of receptors or by decreased affinity of the receptor molecule. However, it can also occur due to the presence of other proteins that can bind or inactivate the inhibitor and thereby make it less readily available. Some of the examples for binding to the OPs are carboxylesterases (CarbEs), butyrylcholinesterases (BuChEs), or other binding proteins such as albumin. In addition, tolerance can be achieved through more rapid metabolism of the OP compounds by OP-hydrolyzing enzymes such as paraoxonases (PONs) and somanases (Fonnum and Sterri, 2006).

ACh receptors (both mAChRs and nAChRs) are involved in the development of tolerance to OP toxicity. Treatment

with a cholinergic agonist for a prolonged time leads to a decrease in the muscarinic ACh receptors (mAChRs). This is common for G protein-linked receptors. In some studies, OPs have been found to cause decrease in the numbers of mAChRs in the brain, while in others both the number of mAChRs and the affinity to the ligand in ileum and striatum. Significant reductions in nAChRs numbers ($B_{max}$), without change in affinity ($K_D$), have been found in the brain of tolerant rats treated with disulfoton (Costa and Murphy, 1983) in skeletal muscle of rats treated with DFP (Gupta and Dettbarn, 1986; Gupta *et al*., 1986). In tolerant rats, significant recovery of CarbEs and BuChEs has also been found. In essence, tolerance development following subchronic or chronic treatment with AChE inhibiting OPs occurs through multiple mechanisms.

## CONCLUSIONS AND FUTURE DIRECTIONS

OPs and CMs constitute a large number of chemicals that are used in agriculture primarily as insecticides and in veterinary medicine as parasiticides. These chemicals exert a broad range of toxic effects, varying from mild effects as salivation and tremors to as serious as convulsions, seizures, paralysis, and death. Basically, OPs and CMs are neurotoxicants, but directly or indirectly several vital organs are affected, as these chemicals produce a variety of toxicological effects on the central nervous system, peripheral nervous system, cardiovascular, pulmonary, ocular, neurobehavioral, immunological, reproductive, placental, cutaneous, and other body systems. In addition, these insecticides cause neurodegeneration, oxidative stress, endocrine disruption, and many other complications. In general, OPs produce more serious and lingering health effects than CMs. For example, some of the complex syndromes like OPIDP and IMS have devastating effects, and have yet to be thoroughly characterized mechanistically and need to be well defined. It is expected that newer compounds of both OP and CM classes will be developed with greater selective toxicity. Also, newer antidotes need to be developed that can be effective in patients with OPIDP or IMS, or against CMs.

## REFERENCES

Costa LG, Murphy SD (1983) [$^3$H]-nicotine binding in rat brain: alteration after chronic acetylcholinesterase inhibition. *J Pharmacol Exp Ther* **226**: 392–7.

De Bleecker J (2006) Intermediate syndrome in organophosphate poisoning. In *Toxicology of Organophosphate and Carbamate Compounds*, Gupta RC (ed.). Academic Press/Elsevier, Amsterdam, pp. 371–80.

Fonnum F, Sterri SH (1981) Factors modifying the toxicity of organophosphorus compounds including soman and sarin. *Fund Appl Toxicol* **1**: 143–7.

Fonnum F, Sterri SH (2006) Tolerance development to toxicity of cholinesterase inhibitors. In *Toxicology of Organophosphate and Carbamate Compounds*, Gupta RC (ed.). Academic Press/Elsevier, Amsterdam, pp. 257–67.

Gupta RC (1984) Acute malathion toxicosis and related enzymatic alterations in *Bubalus bubalis*: antidotal treatment with atropine, 2-PAM, and diazepam. *J Toxicol Environ Health* **14**: 291–303.

Gupta RC (1994) Carbofuran toxicity. *J Toxicol Environ Health* **42**: 383–418.

Gupta RC (2004) Brain regional heterogeneity and toxicological mechanisms of organophosphates and carbamates. *Toxicol Mechan Meth* **14**: 103–43.

Gupta RC (2005) Organophosphate poisoning, intermediate syndrome. In *Encyclopedia of Toxicology*, 2nd edn, Wexler P (ed.). Elsevier, San Diego, CA, pp. 306–8.

Gupta RC (2006) Classification and uses of organophosphates and carbamates. In *Toxicology of Organophosphate and Carbamate Compounds*, Gupta RC (ed.). Academic Press/Elsevier, Amsterdam, pp. 5–24.

Gupta RC, Dettbarn W-D (1986) Role of uptake of [$^{14}$C]valine into protein in the development of tolerance to diisopropyl phosphorofluoridate (DFP) toxicity. *Toxicol Appl Pharmacol* **84**: 551–60.

Gupta RC, Patterson GT, Dettbarn W-D (1986) Mechanisms of toxicity and tolerance to diisopropyl phosphorofluoridate at the neuromuscular junction of the rat. *Toxicol Appl Pharmacol* **84**: 541–50.

Johnson MK (1969) Delayed neurotoxic action of some organophosphorus compounds. *Br Med Bull* **25**: 231–5.

Marrs TC (1993) Organophosphate poisoning. *Pharmacol Ther* **58**: 51–66.

Moody RP, Franklin CA (1987) Percutaneous absorption of the insecticide fenitrothion and aminocarb in rats and monkeys. *J Toxicol Environ Health* **20**(1–2): 209–18.

Moretto A, Lotti M (2006) Peripheral nervous system effects and delayed neuropathy. In *Toxicology of Organophosphate and Carbamate Compounds*, Gupta RC (ed.). Academic Press/Elsevier, Amsterdam, pp. 361–70.

Ordentlich A, Barak D, Kronman C, Ariel N, Segall Y, Velan N, Shafferman A (1996) The architecture of human acetylcholinesterase active center probed by interactions with selected organophosphate inhibitors. *J Biol Chem* **271**: 11953–62.

Ordentlich A, Barak D, Kronman C, Ariel N, Segall Y, Velan N, Shafferman A (1998) Functional characteristics of the oxyanion hole in human acetylcholinesterase. *J Biol Chem* **273**: 19509–17.

Ordentlich A, Barak D, Sod-Moriah G, Kaplan D, Mizrahi D, Segall Y, Kronman C, Karton Y, Lazar A, Marcus D, Velan B, Shafferman A (2004) Stereoselectivity toward VX is determined by interactions with residues of the acyl pocket as well as of the peripheral anionic site of AChE. *Biochemistry* **43**: 11255–65.

Poet TS, Kousba AA, Dennison S, Timchalk C (2004) Physiologically based pharmacokinetic/pharmacodynamic model for the organophosphate pesticide diazinon. *Neurotoxicology* **25**(6): 1013–30.

Radic Z, Taylor P (2006) Structure and function of cholinesterases. In *Toxicology of Organophosphate and Carbamate Compounds*, Gupta RC (ed.). Academic Press/Elsevier, Amsterdam, pp. 161–86.

Savolainen K (2001) Understanding the toxic actions of organophosphates. In *Handbook of Pesticide Toxicology*, 2nd edn, Krieger R (ed.). Academic Press, San Diego, CA, pp. 1013–42.

Senanayake N, Karalliedde L (1987) Neurotoxic effects of organophosphorus insecticides. An intermediate syndrome. *N Engl J Med* **316**: 761–3.

Sogorb MA, Villanova E (2002) Enzymes involved in the detoxification of organophosphorus, carbamate and pyrethroid insecticides through hydrolysis. *Toxicol Lett* **128**: 215–28.

Sultatos LG (1988) Factors affecting the hepatic biotransformation of the phosphorothioates pesticide chlorpyrifos. *Toxicology* **51**: 191–200.

Sultatos LG (2006). Interactions of organophosphorus and carbamate compounds with cholinesterases. In *Toxicology of Organophosphate and Carbamate Compounds*, Gupta RC (ed.). Academic Press/Elsevier, Amsterdam, pp. 209–18.

Sultatos LG, Basker KM, Shao M, Murphy SD (1984a) The interaction of the phosphorothioate insecticides chlorpyrifos and parathion and their oxygen analogues with bovine serum albumin. *Mol Pharmacol* **26**(1): 99–104.

Sultatos LG, Shao M, Murphy SD (1984b) The role of hepatic biotransformation in mediating the acute toxicity of the phosphorothionate insecticide chlorpyrifos. *Toxicol Appl Pharmacol* **73**: 60–8.

Taylor P (2006) Anticholinesterase agents. In *Goodman and Gilmam's The Pharmacological Basis of Therapeutics*, Brunton LL, Lazo JS, Parker KL (eds). McGraw-Hill, New York, pp. 201–16.

Timchalk C (2006) Physiologically based pharmacokinetic modeling of organophosphorus and carbamate pesticides. In *Toxicology of Organophosphate and Carbamate Compounds*, Gupta RC (ed.). Academic Press/Elsevier, Amsterdam, pp. 103–25.

Timchalk C, Nolan RJ, Mendrala AL, Dittenber DA, Brzak KA, Mattsson A (2002) A physiologically based pharmacokinetic and pharmacodynamic (PBPK/PD) model for the organophosphate insecticide chlorpyrifos in rats and humans. *Toxicol Sci* **66**: 34–53.

Timchalk C, Poet TS, Kousba AA, Campbell JA, Lin Y (2004) Noninvasive biomonitoring approaches to determine dosimetry and risk following acute chemical exposure: analysis of lead or organophosphate insecticide in saliva. *J Toxicol Environ Health A* **67**: 635–50.

Tomokuni K, Gasegawa T, Hirai Y, Koga N (1985) The tissue distribution of diazinon and the inhibition of blood cholinesterase activities in rats and mice receiving a single intraperitoneal dose of diazinon. *Toxicology* **37**: 91–8.

Tos-Luty S, Tokarska-Rodak M, Latuszysnki J, Prezebirowska D (2001) Dermal absorption and distribution of $^{14}$C-carbaryl in Wistar rats. *Ann Agric Environ Med* **8**(1): 47–50.

Watson A, Bakshi K, Opresko D, Young R, Houschild V, King J (2006) Cholinesterase inhibitors as chemical warfare agents: community preparedness guidelines. In *Toxicology of Organophosphate and Carbamate Compounds*, Gupta RC (ed.). Academic Press/Elsevier, Amsterdam, pp. 47–68.

Wu HX, Evereux-Gros C, Descotes J (1996) Diazinon toxicokinetics, tissue distribution and anticholinesterase activity in the rat. *Biomed Environ Sci* **9**: 359–69.

C H A P T E R

# 40

# Organochlorines

*Steve Ensley*

## INTRODUCTION

Chlorinated compounds, cyclodienes such as aldrin and dieldrin, used as insecticides, became available for use in the 1940's. Many methods have been used and developed to control or eliminate insects and other plant and animal pests that have threatened man and his food supply. There were major changes in the development and use of insecticides in the 1930s when the synthetic organic chemical industry began developing compounds to control insects. Dichlorodiphenyltrichloroethane (DDT) became available during World War II (WWII) and was used extensively as an insecticide worldwide. One of the reasons that organochlorines were effective insecticides was their ability to persist in the environment (Leonard *et al.*, 1999; Hites *et al.*, 2004; Hoekstra *et al.*, 2005). Because of that persistence, most have been eliminated from use today. Lindane (gamma-hexachlorocyclohexane) and endosulfan are the most biodegradable organochlorines and are still used today.

The diphenyl aliphatic organochlorines such as DDT, affect the peripheral nerves and brain by slowing sodium ($Na^+$) influx and inhibiting potassium ($K^+$) outflow. This results in excess intracellular $K^+$ in the neuron, which partially depolarizes the cell. In the 1980s, the mechanism of toxicity for the cyclodiene organochlorine insecticides was determined. These compounds were found to be non-competitive antagonists acting on the chloride ion channel of the gamma-aminobutyric acid A (GABA) receptor.

## BACKGROUND

Even though DDT was first synthesized by Othmar Zeidler in 1874, it was another 40 years before this compound was used as an insecticide. Paul Mueller, a Swiss chemist, rediscovered DDT in 1939 while investigating insecticides for use against clothes moths and carpet beetles. Mueller won the Noble Prize in 1948 for this work. This pleasant smelling, greasy white powder (DDT) has had an influence on human ecology perhaps unmatched by any other chemical discovery including gunpowder, sulfanilamide, penicillin, and plutonium (Metcalf, 1973).

The insecticidal properties of technical hexachlorocyclohexane (t-HCH; commonly known as benzene hexachloride) and the first cyclodiene insecticides (e.g., aldrin, dieldrin, chlordane) were discovered as a result of the commercial interest in new uses for chlorine and hydrocarbons such as cyclopentadiene and benzene. The first use of the chlorinated hydrocarbons was for dielectrics and as fire retardants. The use of these compounds as insecticides occurred when benzene was added to liquid chlorine in the field and it was noted that the product killed insects.

As with many insecticides there are many unintentional secondary effects for every insecticide (Uzoukwu and Sleight, 1972; Furie and Trubowitz, 1976; Hathway, 1977). The following is a brief discussion about the organochlorines and some of the intermediates used in their production. Hexachlorocyclopentadiene, a raw material used in manufacturing chemicals, was known to be stable and was found to react easily with cyclopentadiene in a Diels–Alder reaction, which leads to the production of chlordane. It was discovered later to react with norbornadiene (a bicyclic hydrocarbon) to produce aldrin. Allylic chlorination of chlordane produces heptachlor. The intermediate, hexachloronorbornadiene (HCNB), reacts with cyclopentadiene to produce isodrin and after epoxidation, dieldrin and endrin are produced. The t-HCH can be used to produce the gamma isomer, lindane. One of the problems with the production of lindane is the inefficiency of the process; for every ton of lindane produced; 8–10 tons of the inactive alpha and beta isomers are formed. Because of the widespread worldwide use of t-HCH, the environment

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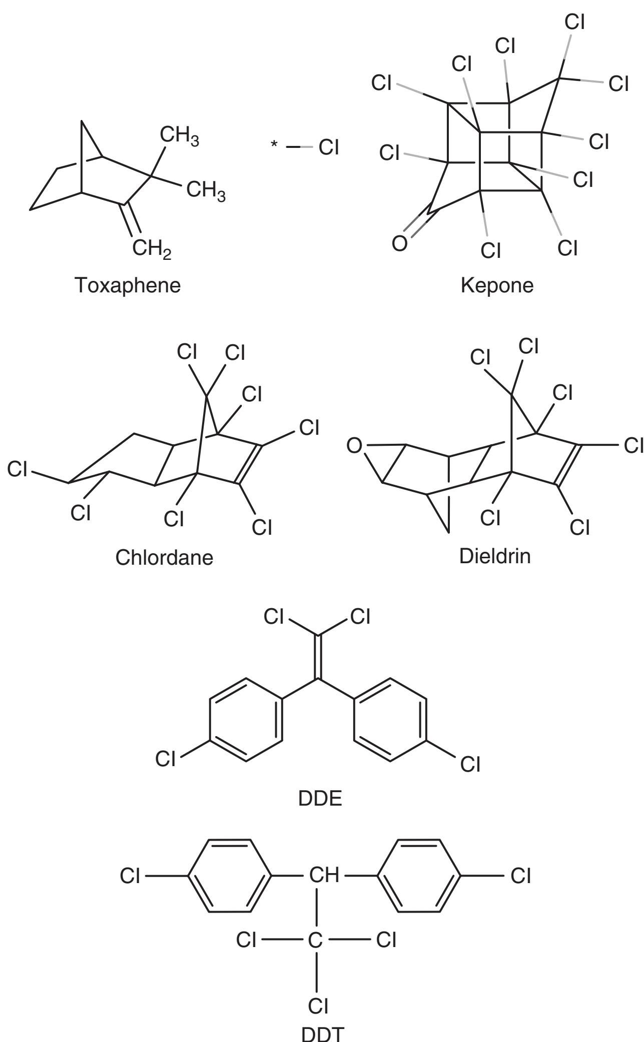


**FIGURE 40.1** Structures of common organochlorines.

has become contaminated with the inactive alpha- and beta-HCH isomers.

Overcoming the problem of insect resistance to the organochlorines has also been associated with toxicity. A major mechanism of insect resistance to DDT was found to be enzymatic dechlorination of DDT to dichlorodiphenyldichloroethylene (DDE) (Bonner and Yarbrough, 1988). While working to overcome resistance to DDT, it was discovered that certain non-toxic DDT analogs and other compounds suppressed resistance when co-applied with DDT. Toxicity to parent compounds as well as congeners has been associated with use of the organochlorines.

The beginning of the science of toxicology can be traced to the problems associated with use of DDT and the subsequent impact on man and the environment. Rachel Carson's book "Silent Spring" brought the problems associated with the use of DDT to national attention in 1962.

The structures of various organochlorine insecticides are shown in Figure 40.1.

## PHARMACOKINETICS/ TOXICOKINETICS

Organochlorine insecticides can be absorbed orally and topically, with absorption being rapid due to the lipid solubility of these compounds (Buck *et al*., 1976; Marth *et al*., 1989). Organochlorine insecticides are not highly volatile, so inhalation is not a normal route of exposure (Jaeger *et al*., 1973). Distribution is to the liver, kidney, brain, and adipose tissue (Buck and Van Note, 1968; Buck, 1970; Booth and McDowell, 1975). Acute toxicity caused is of concern, but bioaccumulation from chronic exposures is equally important (Starr and Clifford, 1972). Chlorinated hydrocarbons are highly lipid soluble and persist in the environment; as a result bioaccumulation occurs in the food chain from the environment to animals and humans (Mount *et al*., 1980; Oehme, 1991; Safe and Krishnan, 1995; Watanabe *et al*., 1999; Backer *et al*., 2001; Smith and Gangolli, 2002; Harris *et al*., 2005 ).

As with all xenobiotics, the toxicity of the organochlorines is related to the absorption, distribution, metabolism and elimination (Jaeger *et al*., 1975; Beasley *et al*., 1994). The diphenyl aliphatics, such as DDT, are dechlorinated by mixed function oxidases (MFOs). Aryl hydrocarbons, like paradichlorobenzene, undergo glucuronidation and sulfation. The cyclodiene insecticides, such as endrin are rapidly converted to epoxides by MFOs. Methoxychlor is rapidly eliminated compared to DDT by dechlorination and oxidation. The intermediates of organochlorine insecticide production may be more toxic than the parent compound.

The major excretory route of organochlorines is from bile into the digestive tract, and as a result enterohepatic recycling can occur. Metabolites are also lipophilic, will move into adipose tissue and are released slowly from lipid depot storage (Sell *et al*., 1977). The half-life of some diphenyl aliphatics, such as DDT and the cyclodienes may range from days to years (Council for Agricultural Science and Technology, 1974). Elimination can sometimes be explained by a two compartment model, where the first phase is rapid elimination and the second is prolonged.

## MECHANISM OF ACTION

There are at least two different mechanisms of action for organochlorine insecticides (Shankland, 1982; Narahashi, 1987; Osweiler, 1996). DDT-type organochlorine (dichlorodiphenylethanes) insecticides affect the peripheral nerves and brain by slowing sodium ($Na^+$) influx and inhibiting potassium ($K^+$) efflux. That results in excess intracellular $K^+$ in the neuron, which partially depolarizes the cell.

The threshold for another action potential is decreased, resulting in premature depolarization of the neuron.

The aryl hydrocarbons and cyclodienes, in addition to decreasing action potentials, may inhibit the post-synaptic binding of GABA (Bloomquist and Soderlund, 1985; Lummis *et al*., 1990; French-Constant, 1993; Hahn, 1998; Carr *et al*., 1999). The cyclodiene organochlorine insecticides act by competitive inhibition of the binding of GABA at its receptor, causing stimulation of the neuron, as described below (Joy, 1976, 1982; Gandolfi *et al*., 1984).

GABA is a neurotransmitter in the mammalian and insect central nervous system and the inhibitory neurotransmitter for insects at the neuromuscular junction. $GABA_A$ receptors, present in mammalian and insect synapse, are ligand gated chloride ion channels. In mammals, $GABA_B$ receptors are coupled to calcium and potassium channels and the action of GABA is mediated by G-proteins. $GABA_B$ receptors are not important in insect physiology. When GABA is released in the synapse it diffuses to the pre-synaptic terminal of another nerve, where it binds to a $GABA_A$ receptor. This causes chloride ions to enter the synapse resulting in hyperpolarization of the terminal and inhibition of release of other neurotransmitters. Because of this inhibition, post-synaptic stimulation of other nerves by other transmitters (e.g. acetylcholine) is reduced. When GABA is inhibited, there is no synaptic down-regulation and other neurotransmitters can be released in excess. The inhibitory mechanism of GABA explains the cholinergic effects (over stimulation by acetylcholine) of dieldrin and lindane on some species.

## TOXICITY

Cats are the most sensitive species to organochlorine insecticides, with the $LD_{50}$ for endrin in cats of 3–6 mg/kg. The cyclodiene organochlorine insecticides cause more seizure activity and have a lower $LD_{50}$ than the DDT-type insecticides in most species. The oral $LD_{50}$ for DDT in rats is 113–2500 mg/kg and the IV $LD_{50}$ is 47 mg/kg. In humans, toxic signs from oral exposure to organochlorines can be observed at 10 mg/kg. Shown below is a Table 40.1 with the acute toxicity to rats and the housefly of other chlorinated insecticides.

A description of the $GABA_A$ receptor in the human brain is presented in Figures 40.2 and 40.3 to clearly describe the chloride ion channel. The $GABA_A$ receptor of the human brain consists of four or five 50–60 kDa glycoprotein subunits, each of which contains four ($M_1$–$M_4$) hydrophobic domains. The five $M_2$ domains are arranged to form a 5.6 Å diameter ion channel.

In addition to the effects on the nervous system, DDT metabolites can inhibit the output of the adrenal gland by selective necrosis of the zona fasciculata and the zona reticularis.

Chronic exposure to DDT has been documented to cause eggshell thinning and reduced fertility in wild birds.

**TABLE 40.1 Toxicity data for some organochlorines**

| Compound | Rat acute oral $LD_{50}$ (mg/kg) | Rabbit dermal $LD_{50}$ (mg/kg) |
|---|---|---|
| Lindane | 76–190 | 500 |
| Aldrin | 39–60 | 65 |
| Dieldrin | 40 | 65 |
| Endrin | 3 | 12 |
| Chlordane | 283–1,100 | 580 |
| Endosulfan | 18–76 | 74 |
| Mirex | 235–3000 | 800 |
| Kepone | 95–125 | 345 |
| Toxophene | 40–127 | 600 |

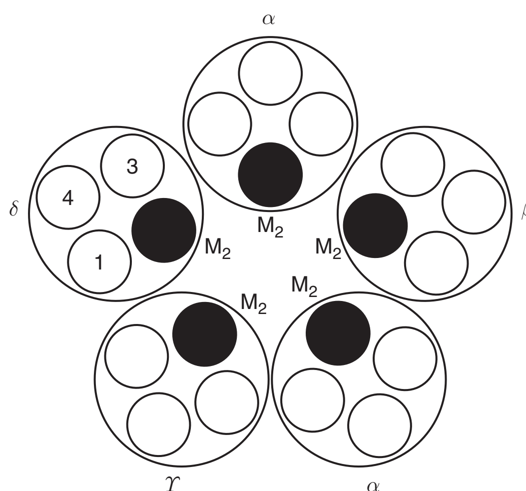


**FIGURE 40.2** Illustration of the $GABA_A$ receptor of the mammalian brain. The $M_2$ segments form the chloride ion channel (McDonald and Olsen, 1994).

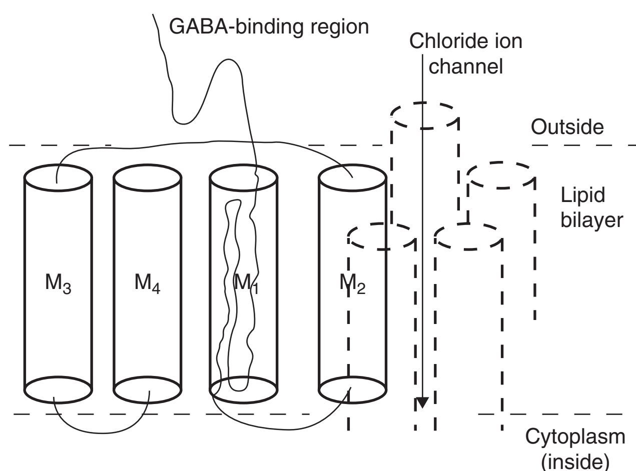


**FIGURE 40.3** Illustration of the $GABA_A$ receptor of the mammalian brain (McDonald and Olsen, 1994).

## TREATMENT

No specific antidotes for organochlorine insecticides are available (Osweiler, 1996). Detoxification is the most essential component of therapy for organochlorine toxicity. If dermal exposure has occurred, the animal should be thoroughly washed with a detergent and water to remove the insecticide so absorption ceases. The hair of heavily contaminated long-haired animals should be clipped. Personnel treating animals should exercise caution and prevent themselves from becoming contaminated with the insecticide by wearing gloves, aprons, or raincoats.

For oral exposure to organochlorines, activated charcoal (1–2 g/kg) should be administered orally. An alternative but less effective treatment is mineral oil. The insecticide can dissolve in mineral oil, which decreases its absorption systemically. The approximate oral dose of mineral oil is 2–6 ml in cats, 5–15 ml in dogs, and 1–3 l in large animals. Charcoal or non-absorbable oils are most effective when given within 4 h of ingestion of the pesticide (Aslani, 1996).

General supportive care includes the use of antiseizure medications such as diazepam, phenobarbital, or pentobarbital. Animals should to be placed in a warm and comfortable area to minimize trauma when they are seizuring. Animals recovering from organochlorine insecticide exposure may have to be monitored long term because organochlorines can persist in the body for months or years. The source of the exposure must be identified and removed to stop exposure. One decontamination strategy is to reduce feed intake so that the animal loses body fat, thereby reducing organochlorine residues in adipose tissue. Lactating animals rapidly eliminate organochlorine residues because the residues are excreted in milk. An additional treatment option in large animals is to feed activated charcoal (500–1000 g/day) to reduce enterohepatic recycling.

## CONCLUDING REMARKS AND FUTURE DIRECTIONS

The use of organochlorine insecticides is decreasing in the United States but they are still used worldwide. In the United States, with the ability of these compounds to persist in the environment and bioaccumulate, the organochlorine insecticides will continue to be an important toxicants for many years (Custer *et al.*, 2005).

## REFERENCES


Aslani MR (1996) Endosulfan toxicosis in calves. *Vet Hum Toxicol* **38**: 364.

Backer LC, Grindem CB, *et al.* (2001) Pet dogs as sentinels for environmental contamination. *Sci Total Environ* **274**: 161–9.

Beasley VR, Dorman DC, Fikes FD, Diana SG (1994) *A Systems Approach to Veterinary Toxicology*. University of Illinois, Champagne, IL.

Bloomquist JR, Soderlund DM (1985) Neurotoxic insecticides inhibit GABA-dependent chloride uptake by mouse brain vesicles. *Biochem Biophys Res Commun* **133**: 37–43.

Bonner JC, Yarbrough JD (1988) Vertebrate cyclodiene insecticide resistance: role of gamma-aminobutyric acid and diazepam binding sites. *Arch Toxicol* **62**: 311–5.

Booth NH, McDowell JR (1975) Toxicity of hexachlorobenzene and associated residues in edible animal tissues. *J Am Vet Med Assoc* **166**: 591–5.

Buck WB (1970) Lead and organic pesticide poisonings in cattle. *J Am Vet Med Assoc* **156**: 1468–72.

Buck WB, Van Note, W (1968) Aldrin poisoning resulting in dieldrin residues in meat and milk. *J Am Vet Med Assoc* **153**: 1472–5.

Buck WB, Osweiler GD, VanGelder GA (1976) *Clinical and Diagnostic Veterinary Toxicology*, 2nd edn. Kendall/Hunt Publishing, Dubuque, IA.

Carr RL, Couch TA, *et al.* (1999) The interaction of chlorinated alicyclic insecticides with brain GABA(A) receptors in channel catfish (*Ictalurus punctatus*). *J Toxicol Environ Health A* **56**: 543–53.

Council for Agricultural Science and Technology (1974) Aldrin and dieldrin in agriculture. Report No. 34.

Custer CM, Custer TW, *et al.* (2005) Exposure and effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in tree swallows (*Tachycineta bicolor*) nesting along the Woonasquatucket River, Rhode Island, USA. *Environ Toxicol Chem* **24**: 93–109.

French-Constant RH (1993) Cloning of a putative $GABA_A$ receptor from cyclodiene-resistant *Drosophila*: a case study in the use of insecticide-resistant mutants to isolate neuroreceptors. *Exs* **63**: 210–23.

Furie B, Trubowitz S (1976) Insecticides and blood dyscrasias. *J Am Med Assoc* **235**: 1720–2.

Gandolfi O, Cheney DL, *et al.* (1984) On the neurotoxicity of chlordecone: a role for gamma-aminobutyric acid and serotonin. *Brain Res* **303**: 117–23.

Hahn ME (1998) The aryl hydrocarbon receptor: a comparative perspective. *Comp Biochem Physiol C Pharmacol Toxicol Endocrinol* **121**: 23–53.

Harris ML, Wilson LK, *et al.* (2005) An assessment of PCBs and OC pesticides in eggs of double-crested (*Phalacrocorax auritus*) and Pelagic (*P. pelagicus*) cormorants from the west coast of Canada, 1970 to 2002. *Ecotoxicology* **14**: 607–25.

Hathway DE (1977) Comparative mammalian metabolism of vinyl chloride and vinylidene chloride in relation to oncogenic potential. *Environ Health Perspect* **21**: 55–9.

Hites RA, Foran JA, *et al.* (2004) Global assessment of organic contaminants in farmed salmon. *Science* **303**: 226–9.

Hoekstra PF, O'Hara TM, *et al.* (2005) Concentrations of persistent organochlorine contaminants in bowhead whale tissues and other biota from northern Alaska: implications for human exposure from a subsistence diet. *Environ Res* **98**: 329–40.

Jaeger RJ, Conolly RB, *et al.* (1973) Diurnal variation of hepatic glutathione concentration and its correlation with 1,1-dichloroethylene inhalation toxicity in rats. *Res Commun Chem Pathol Pharmacol* **6**: 465–71.

Jaeger RJ, Conolly RB, *et al.* (1975) Biochemical toxicology of unsaturated halogenated monomers. *Environ Health Perspect* **11**: 121–8.

Joy R (1976) The alteration by dieldrin of corticol excitability conditioned by sensory stimuli. *Toxicol Appl Pharm* **38**: 357–68.

Joy RM (1982) Mode of action of lindane, dieldrin and related insecticides in the central nervous system. *Neurobehav Toxicol Teratol* **4**: 813–23.

Leonard AW, Hyne RV, *et al.* (1999) Effect of endosulfan runoff from cotton fields on macroinvertebrates in the Namoi river. *Ecotoxicol Environ Saf* **42**: 125–34.

Lummis SC, Buckingham SD, *et al.* (1990) Blocking actions of heptachlor at an insect central nervous system GABA receptor. *Proc R Soc Lond B Biol Sci* **240**: 97–106.

Marth E, Stunzner D, *et al.* (1989) Toxicokinetics of chlorinated hydrocarbons. *J Hyg Epidemiol Microbiol Immunol* **33**(4 Suppl.): 514–20.

McDonald RL, Olsen RW (1994) $GABA_a$-receptor channels. *Ann Rev Neurosci* **17**: 569–602.

Metcalf, RL (1973) A century of DDT. *J Agric Food Chem*. Jul–Aug **21**(4): 511–9.

Mount ME, Traffas V, Milleret RJ, Oehme FW (1980) An unusual occurrence of toxaphene poisoning in swine. *J Am Vet Med Assoc* **177**: 445–7.

Narahashi T (1987) Nerve membrane ion channels as the target site of environmental toxicants. *Environ Health Perspect* **71**: 25–9.

Oehme M (1991) Dispersion and transport paths of toxic persistent organochlorines to the Arctic-levels and consequences. *Sci Total Environ* **106**(1–2): 43–53.

Osweiler GD (1996) *Toxicology*. Williams and Wilkins, Philadelphia, PA.

Safe S, Krishnan V (1995) Chlorinated hydrocarbons: estrogens and antiestrogens. *Toxicol Lett* **82–3**: 731–6.

Sell JL, Davison KL, Bristol DW (1977) Depletion of dieldrin from Turkeys. *Poult Sci* **56**: 2045–51.

Shankland DL (1982) Neurotoxic action of chlorinated hydrocarbon insecticides. *Neurobehav Toxicol Teratol* **4**: 805–11.

Smith AG, Gangolli SD (2002) Organochlorine chemicals in seafood: occurrence and health concerns. *Food Chem Toxicol* **40**: 767–79.

Starr HG, Clifford NJ (1972) Acute lindane intoxication. *Arch Environ Health* **25**: 374–5.

Uzoukwu M, Sleight SD (1972) Effects of dieldrin in pregnant sows. *J Am Vet Med Assoc* **160**: 1641–3.

Watanabe M, Tanabe S, *et al.* (1999) Contamination levels and specific accumulation of persistent organochlorines in Caspian seal (*Phoca caspica*) from the Caspian sea, Russia. *Arch Environ Contam Toxicol* **37**: 396–407.

CHAPTER

# 41

# Pyrethrins and pyrethroids

*Steve Ensley*

## INTRODUCTION

Pyrethrins are the insecticidal compounds obtained from the flowers of the plant *Tanacetum cinerariaefolium*, also called *Chrysanthemum cinerariaefolium* or *Pyrethrum cinerariaefolium*. Pyrethrum denotes extracts from the flowers that contain the active pyrethrin compounds (Proudfoot, 2005). The use of pyrethrum in insecticide preparations dates back to Persia, about 400 BC Pyrethroids are synthetic analogs of pyrethrins. Because of stability problems with the natural pyrethrins, these insecticides were replaced by the more stable organophosphate and organochlorine insecticides developed after World War II (Valentine, 1990). As a result of the toxicity and environmental contamination associated with the organophosphate and organochlorine insecticides, interest in the use of pyrethrins and pyrethroids re-emerged in the 1970s. Pyrethrin and pyrethroid insecticides are effective against a variety of insect pests on companion animals and livestock, and are used on farms, in the home and garden and have many public health applications because of the safety associated with these compounds.

## BACKGROUND

There are six compounds that comprise the natural pyrethrins: pyrethrin I and II, jasmolin I and II and cinerin I and II. Synthetic pyrethroids have been developed because the natural pyrethrins breakdown quickly when exposed to air, light, and heat. The synthetic pyrethroids can be classified as first and second generation. First generation pyrethroids are esters of chrysanthemic acid and an alcohol, having a furan ring and terminal side chain moieties. Second generation pyrethrins have 3-phenoxybenzyl alcohols derivatives in the alcohol moiety, and have had some of the terminal side chain moieties replaced with a dichlorovinyl or dibromovinyl substitute and aromatic rings. Addition of the alpha-cyano group to the 3-phenoxybenyl alcohol group in the second generation pyrethroids (type II) has increased the insecticidal potency.

Pyrethrins cause hyperexcitability with very little cytotoxicity. The molecular targets of the pyrethrins and pyrethroids are similar in mammals and insects and include voltage gated sodium, chloride, and calcium channels, gamma-aminobutyric acid (GABA)-gated chloride channels, nicotinic receptors, membrane depolarization, and intercellular gap junctions (Forshaw and Ray, 1990; Song and Narahashi, 1996a). Mammals are less susceptible to pyrethrin and pyrethroid toxicoses than insects primarily because they have a faster metabolic clearance, higher body temperatures, and a lower affinity for the pyrethrins/pyrethroids (Song and Narahashi, 1996b) (Figure 41.1).

## PHARMACOKINETICS/ TOXICOKINETICS

Determination of the toxicity of pyrethroids *in vivo* is difficult because they have low water solubility, easily partition into lipids, and will bind to plastics and glass. The reported toxicity of this class of insecticides has high variability.

Most pyrethrin and pyrethroid products are applied dermally in animals, but because of grooming, there can be oral and inhalation exposures too. Less than 2% of topically applied pyrethrin and pyrethroids insecticides are

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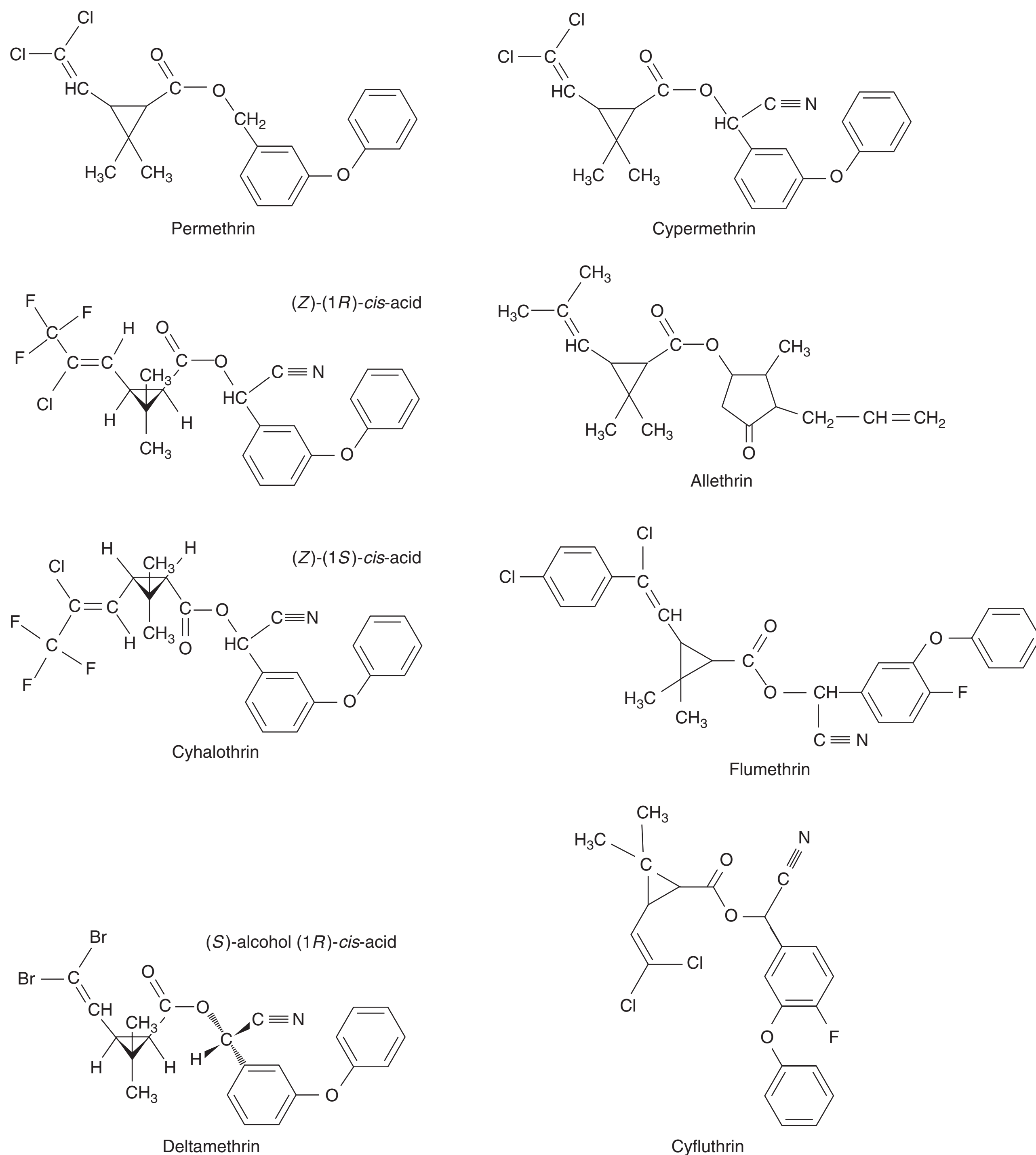


**FIGURE 41.1** Structures of selected pyrethrins and pyrethroids.

absorbed dermally (Wollen *et al.*, 1992). One study confirmed that absorption of cypermethrin across human skin is minimal and peak excretion rates after dermal application were not observed until 12–36 h after dosing. Pyrethrins may be sequestered in the skin and slowly released into the systemic circulation (He *et al.*, 1989). Oral or inhalation exposure results in faster systemic exposure (Anadon *et al.*, 1996). Approximately 40–60% of an orally ingested dose is absorbed. When cypermethrin was administered orally to six adult male volunteers, oral absorption ranged from 27% to 57% of the administered dose and peak excretion rates were measured in the urine between 8 and 24 h after dosing. When adult human males were exposed to cyfluthrin at 160 $\mu g/m^3$, 93% of the metabolites

were excreted within the first 24 h with peak excretion rates ranging from 0.5 to 3 h.

Pyrethroids are lipophilic and will distribute to tissues with high lipid content such as fat, and nervous tissue in addition to liver, kidney, and milk.

Pyrethroids and pyrethrins are rapidly hydrolyzed in the gastrointestinal tract. Once absorbed these compounds are metabolized by mixed function oxidases and esterases. Metabolism of the pyrethroids results in water-soluble metabolites. Metabolism includes hydrolysis of the central ester bond, oxidation at several sites, and conjugation with glycine, sulfate, glucuronide, or glucosides. Cleavage of the ester bond results in substantial reduction in toxicity. The presence of the alpha-cyano group, as in type II pyrethroids, will decrease the rate of hydrolysis of the ester bond. Cleavage of the alpha-cyano group results in rapid conversion of the cyano group to thiocyanate. Pyrethroids are eliminated by first order kinetics and most of the dose is eliminated in the first 12–24 h after absorption. The primary routes of excretion are urinary and fecal, as a mix of parent compound and metabolites.

## MECHANISM OF ACTION

Pyrethroids primarily affect the sodium channel of cells, but chloride and calcium channels are also affected. Pyrethrins and pyrethroids slow the opening and closing of the sodium channels, resulting in excitation of the cell (Marban *et al.*, 1989; Conley and Brammar, 1999). The increase of sodium in the sodium channels results in a cell that is in a stable, hyperexcitable state. The duration of the sodium action potential is much longer for type II pyrethroids than for type I. Type I pyrethroids result in primarily repetitive charges and in type II pyrethroid toxicity cell membrane depolarization is the main mechanism of action. Paresthesia results from the direct action of pyrethroids on sensory nerve endings, causing repetitive firing of these fibers. Less than 1% of sodium channels must be modified by pyrethroids to produce neurological signs. High concentrations of type II pyrethroids may also act on GABA-gated chloride channels (Bloomquist *et al.*, 1986).

Pyrethrins also affect the voltage-dependant chloride channels. These channels are found in the brain, nerve, muscle, and salivary gland and control cell excitability. There are many different functional types of chloride channels in contrast to sodium channels. Most pyrethroid-sensitive channels belong to the Maxi chloride channel class. Maxi channels are activated by depolarization, have high conductance, are calcium independent, and are activated by protein kinase C phosphorylation. Pyrethroids cause a decrease in the Maxi chloride channel current, which increases excitability of the cell just as the action of pyrethroids on the sodium channel.

The decreased sensitivity of mammals to this class of compounds compared to insects is due to several factors. Pyrethroids bind more strongly with the sodium channel at low temperatures than at high temperatures. Insects ambient temperature is approximately 25°C compared to mammals at 37°C. Mammalian sodium channels are at least 1000 times less sensitive to pyrethroids than insect sodium channels. Mammalian sodium channels recover much more quickly from depolarization than do insect sodium channels. Mammals are much more likely to detoxify pyrethroids before they reach their target site than are insects.

Pyrethroids cause a phenomenon in insects called "knock down" (Narahashi, 1985). Knock down is caused by inhibiting the cell but not causing a lethal effect. Knock down is caused by the ability of the sodium channels to retain many of the normal functions, such as selectivity for sodium ions and conductance after exposure to pyrethroids. After exposure to moderate doses of pyrethroids, cells function in a new state of hyperexcitability. If the level of sodium in the ion channel does not exceed the ability of the sodium pump to remove it, the cell continues to function normally. High concentrations of pyrethroids or hyperactivity beyond what the cell can tolerate, will cause depolarization and conduction block. The pyrethroids that hold the sodium channel open the longest will cause the greatest amount of depolarization.

There is marked stereospecificity of the action of pyrethroids on the sodium channel; some isomers are more toxic than others (Soderland, 1985). The *cis* isomers are usually more toxic than the *trans* isomers. As an example, the 1*R* and 1*S cis* isomers bind competitively to one site, and the 1*R* and 1*S trans* isomers bind non-competitively to another (Narahashi, 1986). In mammals the 1*R* isomers are active and the 1*S* isomers inactive, making the 1*S* isomers non-toxic. Deltamethrin has been produced using stereospecificity to produce a high degree of selective toxicity. This is the reason that the toxicity of different batches of pyrethroids can vary. The rat oral $LD_{50}$ of commercial permethrin can vary from 430 to 8900 mg/kg, with toxicity depending on the amount of *cis* isomer present in the batch.

## TOXICITY

Dermal exposure to pyrethroids is most common (Osweiler, 1996). In humans, the bioavailability of pyrethroids applied dermally is approximately 1%. Absorption after oral exposure in humans is 36%, mostly from the stomach. Once absorbed, the pyrethroids are rapidly distributed due to their lipophilicity. Systemic distribution produces effects

that can be difficult to control and may be confused with poisoning by other pesticides, such as organophosphates which also cause increased salivation and hyperexcitability. Many pyrethroid formulations also contain solvents which can also cause toxicity. Cats are very sensitive to pyrethroid exposure (Meyer, 1999).

The half-life of pyrethroids in general in plasma is in hours, while oral exposure can be equally short. Cyfluthrin has a plasma half-life of 19–86 min. Intravenous $LD_{50}$'s for pyrethroids range from 0.5 to 250 mg/kg. The major neurotoxicity observed in adults with pyrethroids toxicity is acute toxicity with no chronic or cumulative toxicity being observed. The excitatory motor signs are generated at the spinal level.

Fish are highly sensitive to pyrethrin and pyrethroid products, and contamination of lakes, streams, ponds, or any aquatic habitat should be avoided (Bradbury and Coats, 1986, 1989; Ansari and Kumar, 1988). Household exposure of fish can occur when the premise is sprayed or fogged with insecticides and especially when the aquarium aerator is left on. The tank and aerator should be covered during use of insecticides and the home should be well ventilated before uncovering and starting the pump.

Most avian species are thought to be tolerant of pyrethrin and pyrethroid products but carriers or propellants in spray formulations may be hazardous (Bradbury and Coats, 1982). There is very little literature about pyrethrin or pyrethroid toxicity of exotic avian species, reptiles, or lagomorphs.

Tables 41.1 and 41.2 refer to oral toxicity of some types I and II pyrethroids.

In dogs, cats, and large animals the clinical signs are similar for both type I and II compounds. Clinical signs include salivation, vomiting, hyperexcitability, tremors, seizures, dyspnea, weakness, prostration, and death (Murphy, 1996). In rats with type I toxicity there is an increased response to stimulation, muscle tremors, excitement, and paralysis (Beasley *et al.*, 1994). These clinical signs can also be compatible with strychnine toxicities. Type II over exposure will cause increased salivation, weakness, and choreoathetosis.

**TABLE 41.1** Toxicity of selected type I pyrethroids

| Type I compounds | Oral $LD_{50}$ (mg/kg body weight) in rat |
|---|---|
| Pyrethrin I | 900 |
| Allethrin | 680 |
| Tetramethrin | 4640 |
| Resmethrin | 100 |
| Permethrin | 2000 |

**TABLE 41.2** Toxicity of selected type II pyrethroids

| Type II compounds | Oral $LD_{50}$ (mg/kg body weight) in rat |
|---|---|
| Cypermethrin | 500 |
| Deltamethrin | 31 |
| Fenvalerate | 450 |
| Fluvalinate | 1000 |

## TREATMENT

There is no specific antidote for pyrethroid toxicity, animals should be treated symptomatically. The main treatment for dermal exposure is to wash the animal with a mild detergent and water. Do not use any shampoos that contain additional insecticides as that could increase exposure to insecticides. Large and small animals should be treated the same way. The pyrethroids bound to the skin cannot be removed by washing with soap and water, but dermal paresthesia can be reduced by applying corn oil to the site(s) of application. For oral exposure, emetics or gastric lavage can be used to empty the stomach, if within 1–2 h of ingestion. Activated charcoal and a saline or sorbitol cathartic will reduce oral absorption and increase elimination.

Supportive therapy using diazepam or barbiturates to control hyperexcitability or seizures can be used. Phenothiazine tranquilizers should not be used because they can lower the threshold for seizures. Atropine can be used to control excess salivation or gastrointestinal hypermotility.

The prognosis for pyrethroid toxicity is usually good because of the low toxicity.

## CONCLUDING REMARKS AND FUTURE DIRECTIONS

Pyrethroid insecticides, being of plant origin, are attractive to people that prefer to use organic insecticides on their companion animals or livestock, or who are engaged in organic food production. Pyrethrin insecticides, while toxic to selected species, have a wider margin of safety than organophosphate or organochlorine insecticides. Biomarkers of exposure to pyrethroids are being investigated. Urine is the matrix that is being most heavily investigated to determine which metabolites can be used to identify exposure to the parent compound. Advances in analytical methods to detect low concentrations of the pyrethroid metabolites will allow improved assessment of exposure in the future.

## REFERENCES

Anadon A, Martinez Larranage MR, Fernandez Cruz ML, Diaz MJ, Fernandez MC, Martinez MA (1996) Toxicokinetics of deltamethrin

and its 4′-HO-metabolite in the rat. *Toxicol Appl Pharmacol* **141**: 8–16.

Ansari BA, Kumar K (1988) Cypermethrin toxicity: effect on the carbohydrate metabolism of the Indian catfish, *Heteropneustes fossilis*. *Sci Total Environ* **72**: 161–6.

Beasley VR, Dorman DC, Fikes FD, Diana SG (1994) *A Systems Approach to Veterinary Toxicology*. University of Illinois, Champagne, IL.

Bloomquist JR, Adams PM, Soderlund DM (1986) Inhibition of gamma-aminobutyric acid-stimulated chloride flux in mouse brain vesicles by polychloroalkane and pyrethroid insecticides. *Neurotoxicology* **7**: 11–20.

Bradbury SP, Coats JR (1982) Toxicity of fenvalerate to bobwhite quail (*Colinus virginianus*) including brain and liver residues associated with mortality. *J Toxicol Environ Health* **10**: 307–19.

Bradbury SP, Coats JR (1986) Toxicokinetics of fenvalerate in rainbow trout (*Salmo gairdneri*). *Environ Toxicol Chem* **5**: 567–76.

Bradbury SP, Coats JR (1989) Comparative toxicology of the pyrethroid insecticides. *Rev Environ Contam Toxicol* **108**: 133–77. US Environmental Research Laboratory Edition. Springer-Verlag, New York.

Conley EC, Brammar WJ (1999) *The Ion Channel Facts Book*. Academic Press, San Diego, CA.

Forshaw PJ, Ray DE (1990) A novel action of deltamethrin on membrane resistance in mammalian skeletal-muscle and non-myelinated nerve-fibers. *Neuropharmacology* **29**: 75–81.

He FS, Wang SG, Liu LH, Chen SY, Zhang ZW, Sun JX (1989) Clinical manifestations and diagnosis of acute pyrethroid poisoning. *Arch Toxicol* **63**: 54–8.

Maraban E, Yamagishi T, Tomaselli GF (1989) Structure and function of voltage-gated sodium channels. *J Physiol* **508**: 647–57.

Meyer KE (1999) Toxicosis in cats erroneously treated with 45 to 65% permethrin products. *J Am Vet Med Assoc* **215**: 198–203.

Murphy M (1996) *A Field Guide to Common Animal Poisons*. State University Press, Ames, IA.

Narahashi T (1985) Nerve membrane ionic channels as the primary target of pyrethroids. *Neurotoxicology* **6**: 3–22.

Narahashi T (1986) Mechanisms of action of pyrethroids on sodium and calcium channel gating. In *Neuropharmacology of Pesticide Action*, Ford GG, Lunt GG, Reay RC, Usherwood, PNR (eds). Ellis Horwood, Chichester, pp. 36–40.

Osweiler GD (1996) *Toxicology*. Williams and Wilkins, Philadelphia, PA.

Proudfoot AT (2005) Poisoning due to pyrethrins. *Toxicol Rev* **24**: 107–13.

Soderland DM (1985) Pyrethroid–receptor interactions: stereospecific binding and effects on sodium channels in mouse brain preparations. *Neurotoxicology* **6**: 35–46.

Song JH, Narahashi T (1996a) Differential effects of the pyrethroid tetramethrin on tetrodotoxin-sensitive and tetrodotoxin-resistant single sodium channels. *Brain Res* **712**: 258–64.

Song JH, Narahashi T (1996b) Modulation of sodium channels of rat cerebellar Purkinje neurons by the pyrethroid tetramethrin. *J Pharmacol Exp Ther* **277**: 445–53.

Valentine WM (1990) Pyrethrin and pyrethroid insecticides. *Vet Clin North Am: Small Anim Pract* **20**: 375–82.

Wollen BH, Marsh JR, Laird WJD, Lesser JE (1992) The metabolism of cypermethrin in man – differences in urinary metobolite profiles following oral and dermal administration. *Xenobiotica* **22**: 983–91.

C H A P T E R

# 42

# Rotenone

*Ramesh C. Gupta*

## INTRODUCTION

Rotenone is one of the oldest botanical insecticides, which has been used for centuries and is still used worldwide. Rotenone is the trivial name of the main insecticidal component of certain plant of "*Derris*", "*Lonchocarpus*", and "*Tephrosia*" species. Rotenone has also acaricidal properties. It has molecular formula of $C_{23}H_{22}O_6$ and molecular weight 394.42. Its chemical structure is shown in Figure 42.1. It is present in the form of colorless crystals, but readily oxidized by light, and becomes yellow, orange, and then deep red. Rotenone and its formulatory products have other names, such as Barbasco, Chem-Fish, Cubé, Cuberol, Derris, FishTox, Haiari, Nicouline, PrenFish, Prentox, Rotacide, SprenFish, Tubatoxin, and Tox-R. It is also marketed as Control Garden Dust, Chem-Mite, Cibe Extract, Curex Flea Dust, Derrin, and Green Cross Warble Powder.

Rotenone is formulated along with other pesticides such as carbaryl, pyrethrins, piperonyl butoxide, lindane, and others in pesticide products to control insects, mites, ticks, lice, spiders, and undesirable fish. In general, rotenone is used in home gardens for insect control, for lice and ticks on pets, and fish eradications as part of water body management. In veterinary medicine, rotenone is used in the powder form to control parasitic mites on chickens and other fowl, and for lice and ticks on dogs, cats, and horses. According to a survey conducted by Environmental protection Agency (EPA) in 1990, rotenone was found to be one of the pesticides most commonly used in and around the home. Rotenone has been found toxic to humans, animals, and fish.

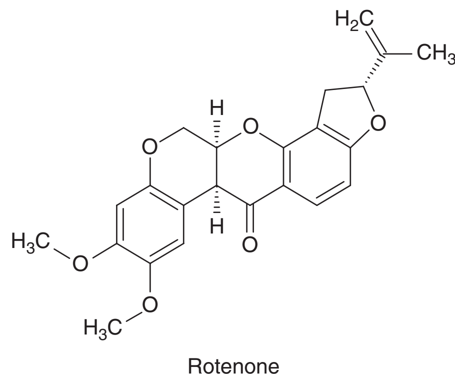


**FIGURE 42.1** Chemical structure of rotenone.

## BACKGROUND

Rotenone and rotenoids have been used as crop insecticides since 1848, when they were applied to plants to control leaf eating caterpillars (Ware, 1994). These compounds have been used for centuries (since 1649) in South America to paralyze fish, causing them to surface. Rotenone and rotenoids are extracted mainly from the roots, but also from the seeds and leaves of certain plants grown in Malaya, East Indies, and Central and South America. Presently, Peru is the major source of the root of the plant, which may be ground as a dust or extracted to provide concentrates. Other rotenone-related compounds such as deguelin, tephrosin, and toxicarol have been isolated from various parts of rotenone-containing plants, such as legume shrubs. Rotenone has been a registered pesticide in the United States under Federal Insecticide Fungicide Rodenticide Act (FIFRA) since 1947. It is a selective and non-specific insecticide with some acaricidal properties. Its formulations include crystalline preparations (about 95%), emulsified solutions (about 50%), and dust (0.75%). Rotenone dust is used for insects, lice, and ticks on animals; and beetles and aphids on vegetables, fruits, berries, and flowers. Rotenone emulsions are used for eliminating unwanted fish in the management of bodies of water.

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It is a very safe compound when properly used, but in higher doses it is toxic to humans, animals, and fish. Rotenone has been involved in suicidal attempts, in which acute congestive heart failure was the characteristic feature at autopsy.

Selective toxicity of rotenone in insects and fish versus mammals can be explained based on metabolism of this compound. Rotenone converts to highly toxic metabolites in large quantities in insects and fish, while it converts to non-toxic metabolites in mammals.

## PHARMACOKINETICS/ TOXICOKINETICS

Absorption of rotenone from the gastrointestinal (GI) tract is low and incomplete. In animals, rotenone has been found to be hundreds of times more toxic by IV route than by oral route. Fats and oils increase the absorption of rotenone from the GI tract. Rotenone is metabolized in the liver by nicotinamide adenine dinucleotide phosphate (NADP)-linked hepatic microsomal enzymes. Several metabolites have been identified as rotenoids, such as rotenolone I and II, hydroxyl and dihydroxyrotenones, etc. (Hayes, 1982; Gosselin *et al.*, 1984). It has been reported from the studies conducted on rats and mice that approximately 20% of a dose is excreted in urine within 24 h of oral administration (Hayes, 1982). Unabsorbed rotenone from the GI tract excretes in feces.

## MECHANISM OF ACTION

In insects, rotenone is both contact and systemic insecticide. It is used as a broad-spectrum insecticide that works by inhibiting the transfer of electrons from Fe–S centers in Complex I to ubiquonone in electron transport chain. This prevents NADH from being converted into usable cellular energy, i.e. adenosine triphosphate (ATP).

In mammals and fish, rotenone inhibits the oxidation of reduced nicotinamide adenine dinucleotide (NADH) to nicotinamide adenine dinucleotide (NAD), thereby blocking the oxidation by NAD of substrates such as glutamate, $\alpha$-ketoglutarate, and pyruvate. Rotenone is a strong inhibitor of mitochondrial electron transport. It inhibits the mitochondrial respiratory chain between diphosphopyridine nucleotide and flavine. Following chronic exposure of rotenone, fatty acid synthesis is altered in mitochondria, resulting in fatty changes in the liver (Hayes, 1982; Gosselin *et al.*, 1984). In *in vitro* studies, Hayes (1982) demonstrated that the aerobic oxidation of pyruvic acid was completely inhibited by rotenone in the isolated rat liver mitochondria. In a recent study, Nianyu *et al.* (2003) showed that rotenone can cause cell death via apoptosis by excess production of mitochondrial reactive oxygen species. In addition, rotenone causes a definite anesthetic effect when it comes in contact with nerve axons (Hayes, 1982).

## TOXICITY

Rotenone is a naturally occurring chemical with insecticidal, acaricidal, and fish-killing properties. Rotenone is toxic to humans as well as animals. WHO classifies rotenone as a moderately hazardous Class II pesticide.

In mammals, the acute toxicity of rotenone is moderate and widely varies between and within species (Ellenhorn and Barceloux, 1988). The oral $LD_{50}$value of rotenone in rats is approximately 60–135 mg/kg body weight, while in mice the value is approximately 350 mg/kg. $LD_{50}$ intraperitoneal (IP) in mice is 2.8 mg/kg. Rotenone is more toxic to female rats than the males. Rotenone is less toxic in mice and hamsters than in rats. The pig seems to be especially sensitive. In rabbits, the $LD_{50}$ values for rotenone following intravenous (IV), oral, and dermal routes are 0.35–0.65, 1.5, and 100–200 mg /kg, respectively.

Following oral ingestion, rotenone induces mild irritation to vomition because of irritant effect on mucus membranes (Gosselin *et al.*, 1984). Inhalation of dusts of rotenone is known to cause severe pulmonary irritation. In the rats and dogs, experimental inhalation of rotenone dust produced onset of signs earlier than following oral ingestion (Hayes, 1982). Toxicity is greater if the particles are of smaller size, because these particles can enter deep regions of the lungs.

In general, depression and convulsions are the common clinical signs with acute toxicity of rotenone. Following oral ingestion, clinical signs of toxicosis may include pharyngitis, nausea, vomiting, gastric pain, clonic convulsions, muscle tremors, lethargy, incontinence, and respiratory stimulation followed by depression (Hayes, 1982; Gosselin *et al.*, 1984). In animals, severe signs of hypoglycemia and liver failure are evident. Rotenone causes alterations in arterial blood gases and acid–base balance. It induces hypoxemia and hypercapnia due to respiratory depression and seizures. In addition, rotenone can impair myocardial contractile force.

Following inhalation exposure, rotenone causes severe pulmonary irritation and asphyxia. Studies suggest that following parenteral administration, rotenone can induce vomiting, incoordination, muscle tremors, clonic convulsions, and respiratory failure. Terminal symptoms of rotenone poisoning are convulsions and cardio-respiratory failure (Gosselin *et al.*, 1984; Ellenhorn and Barceloux, 1988). Most often, cardiovascular effects

include tachycardia, hypotension, and impaired myocardial contractility.

Rotenone is a neurotoxicant. In the rat model, IV infusion of rotenone (2–3 mg/kg/day) is known to produce Parkinson's-like pathology.

In a chronic study, dogs receiving rotenone (10 mg/kg/day) for 6 months showed weight loss and hematological changes. Chronic exposure to rotenone may produce fatty changes in the liver (Windholz, 1983). A no observed adverse effect level (NOAEL) of 0.4 mg/kg/day has been determined in rats and dogs.

Studies suggest that rotenone has no potential to induce teratogenicity or mutagenicity (Waters *et al.*, 1982; Moriya *et al.*, 1983) or endocrine disruption. Its potential to cause carcinogenicity is controversial. Rotenone may produce tumors only in vitamin-deficient animals (Gosalvez, 1983).

Rotenone is highly toxic to fish, while posing low risk to wildlife if properly used.

### Diagnosis

Cases of rotenone poisoning in animals are rare. Diagnosis can be based on circumstantial evidence and detection of rotenone residue in blood, urine, feces, and vomitus. It is expected that rotenone residue is more likely to be present in the liver. Rotenone residue can be determined using high pressure liquid chromatography (HPLC) coupled with fluorescence detector.

## TREATMENT

There is no specific antidote available for rotenone or rotenoids. Treatment relies upon symptomatic and supportive measures. Wash contaminated skin to decontaminate rotenone residue. Avoid emesis if convulsions are present or the ingested product has petroleum distillate. Perform gastric lavage if a large amount of rotenone is ingested. Control seizures and agitation with diazepam and correct hypoglycemia with glucose (5%, IV).

## REFERENCES

Ellenhorn MJ, Barceloux DG (1988) *Medical Toxicology. Diagnosis and Treatment of Human Poisoning.* Elsevier, New York.

Gosalvez M (1983) Carcinogenesis with the insecticide rotenone. *Life Sci* **32**: 809–16.

Gosselin RE, Smith RP, Hodge HC (1984) *Clinical Toxicology of Commercial Products*, 5th edn. Williams & Wilkins, Baltimore, MD/London, pp. 366–7.

Hayes Jr WJ (1982) *Pesticides Studies in Man.* Williams & Wilkins, Baltimore, MD/London, pp. 81–6.

Moriya M, Ohta T, Watanabe K, *et al.* (1983) Further mutagenicity studies on pesticides in bacterial reversion assay system. *Mutation Res* **116**: 185–216.

Nianyu L, Ragheb K, Lawler G, *et al.* (2003) Mitochondrial complex I inhibitor rotenone induces apoptosis through enhancing mitochondrial reactive oxygen species production. *J Biol Chem* **278**: 8516–25.

Ware GW (1994) Rotenone. In *The Pesticide Book*, 4th edn. Thompson Publications, Fresno, CA, p. 58.

Waters MD, Sandhu SS, Simon VF, *et al.* (1982) Study of pesticide genotoxicity. *Basic Life Sci* **21**: 275–326.

Windholz M (1983) *The Merck Index: An Encyclopedia of Chemicals, Drugs, and Biologicals.* 10th edn. Merck and Co., Inc., Rahway, NJ.

CHAPTER

# 43

# Fipronil

*Ramesh C. Gupta*

## INTRODUCTION

Fipronil is a member of a new class of insecticides called phenylpyrazoles. Chemically, it is a (5-amino-1-[2,6-dichloro-4-(trifluoromethyl)phenyl]-4-[(trifluoromethyl)sulfinyl]-1*H*-pyrazole). Chemical structure of fipronil is shown in Figure 43.1. Fipronil is an active ingredient of one of the popular ectoparasiticide products, Frontline™. There are currently two forms of Frontline preparation commercially available (the spray and the spot on application) for dogs and cats. The product is meant to kill fleas and all stages of brown dog ticks, American dog ticks, lone star ticks, which may carry Lyme disease, and mites (Cutler, 1998; Hutchinson *et al*., 1998; Anonymous, 2000). Fipronil is also formulated as insect bait for roaches, ants, and termites; sprays for pets; and as a granular turf control and mole crickets. Presently, Fipronil is widely used for soil treatment and seed coating.

Currently, fipronil-based products with various trade names (Chipco®, Choice, ICON 6.2FS™, and Over n' Out™, TeckPac) have gained the popularity worldwide for pest management, including residential insect control, rice and cotton production, and turf-grass management. The Environmental Protection Agency (EPA) has determined fipronil to be safe for use on dogs and cats, with no harm to humans who handle these dogs and cats (EPA, 1997). Poisoning cases from accidental or misuse of fipronil occur in animals. This chapter describes the toxicity of fipronil in various species of animals and birds.

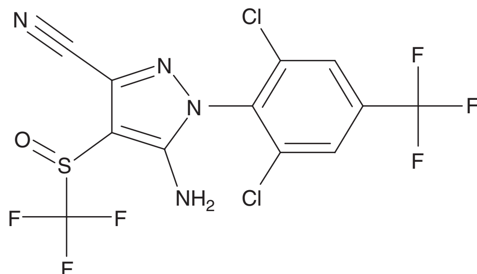


**FIGURE 43.1** Chemical structure of fipronil.

## BACKGROUND

Fipronil was discovered for the first time by Rhone-Poulenc Agro in 1987. It represents the second generation of insecticides that acts through a different mechanism compared to other conventional insecticides. In 1996, it was registered as a pesticide in the United States (Bobe *et al*., 1998). It can be formulated as roach or ant baits, flea and tick sprays for pets, and in granular turf products to control mole crickets (Kidd and James, 1991; EPA, 1996). In addition, fipronil is used for soil treatment and on crops to protect from insects.

Most of the time poisoning cases of fipronil occur in dogs and cats due to accidental ingestion or licking the product Frontline. Frontline preparation for dogs contains 132 mg fipronil in a volume of 1.3 ml tube that is meant for a topical application. In humans, poisoning is mainly due to accidental or suicidal attempt. In a recent report, the Paris Poison Center, France, recorded 81 human cases of fipronil exposure from 1994 to 1999. Out of these 81 cases, 57 involved veterinary ectoparasiticides and 7 used domestic insecticide preparations (Gasmi *et al*., 2001).

## PHARMACOKINETICS/ TOXICOKINETICS

Fipronil in Frontline preparation (132 mg in a 1.34 ml liquid) is placed between the dog's shoulder blades at the

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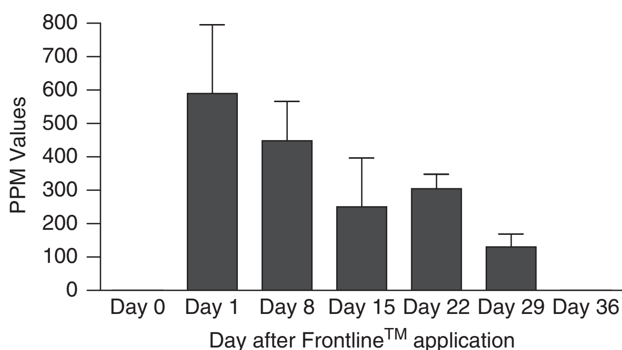


**FIGURE 43.2** Transferable residue of fipronil from the dog coat after a single application of Frontline spot-on.

nape of the neck. After application, fipronil spreads and sequesters in the lipids of the skin and hair follicles, and continues to be released onto the skin and coat, resulting in long-lasting activity against fleas and ticks. Residue of fipronil lasts on dog's hair coat for about a month. The maximum concentration of fipronil on the canine hair coat is found 24 h after a single application of Frontline top spot (Jennings *et al.*, 2002). With a descending concentration trend fipronil residue can be detected on dog's hair coat for a period of 30 days (Figure 43.2). Although fipronil binds to the lipids of the skin cells and hair follicles, the transferable residue can be detected up to a month after application. Studies have revealed that in rats, fipronil is excreted mainly in the feces (45–75%) and little in the urine (5–25%).

Fipronil is degraded by sunlight to produce a variety of metabolites, one of which is fipronil desulfinyl. This metabolite is extremely stable, bioaccumulates in the fatty tissues, and is more toxic than the parent compound in insects (EPA, 1998). Information on other metabolites of fipronil in the living system and nonliving system can be found elsewhere (Feung and Yenne, 1997: Aajoud *et al.*, 2003). This photoproduct exerts high neurotoxicity by blocking the $\gamma$-aminobutyric acid (GABA)-regulated chloride channels. Fortunately, this metabolite is not formed in mammals. However, it does have high-affinity toward the insect's GABA system, thereby contributing to fipronil's selective toxicity toward insects.

## MECHANISM OF ACTION

In mammalian system, the mechanism of fipronil is quite different from other classes of insecticides, and it is better understood in insects than in mammals. Fipronil sulfone is a major metabolite of fipronil in mammals and insects. In insects, fipronil or its metabolite noncompetitively inhibits GABA-induced ion influx by targeting the GABA-regulated chloride channels (Cole *et al.*, 1993). Consequently, fipronil binding blocks the inhibitory action of GABA, leading to hyperexcitation and, in appropriate concentrations, death (Bobe *et al.*, 1998). Fipronil exhibits >500-fold selective toxicity to insects over mammals, primarily because of affinity differences in receptor binding between insect and mammalian receptors (Cole *et al.*, 1993; Grant *et al.*, 1998; Hainzl *et al.*, 1998; Kamijima and Casida, 2000; Ratra *et al.*, 2001; Zhao *et al.*, 2005). In essence, fipronil binds more tightly to $GABA_A$ receptors in insects than in mammals. It is important to note that since fipronil sulfone is rapidly formed *in vivo*, the toxicological effects are likely due to the sulfone metabolite (Zhao *et al.*, 2005).

## TOXICITY

Fipronil produces toxicity in insects and mammals by the same mechanism, however due to selective action, the toxicity is much more severe in insects than in mammals. Fipronil exerts neurotoxicity by blocking the GABA-regulated chloride channels of neurons.

There are numerous reports regarding the effects of fipronil in small animals, birds, and fish (http://www.cdpr.ca.gov/docs/empm/pubs/fatememo/fipronil.pdf). Adequate acute toxicity data is available from small animals. The oral $LD_{50}$ value for rats and mice is reported to be 97 and 95 mg/kg body weight, respectively. It is slightly toxic to nontoxic via the dermal route, with a reported dermal $LD_{50}$ of greater than 2000 mg/kg in rats (EPA, 1996). In rabbits also, dermal $LD_{50}$ is 2000 mg/kg (WHO, 1998-1999). In general, dermal absorption of fipronil is less than 1% after 24 h and therefore dermal toxicity is considered low. Fipronil has moderate inhalation toxicity with an acute $LC_{50}$ of 0.682 mg/l in rats (EPA, 1996). On an acute and subchronic level fipronil is practically nontoxic to waterfowl with an acute oral $LC_{50}$ of >2000 g/kg and a 5-day dietary $LC_{50}$ of >5000 mg/kg for mallard ducks. The oral $LC_{50}$ for Bobwhite quail is 11.3 mg/kg, and the $LC_{50}$ for 5-day dietary is 49 mg/kg (EPA, 1996). The sulfone metabolite is more toxic than the parent compound to certain bird species. This metabolite has shown a very high toxicity in game birds and moderate toxicity in waterfowl (EPA, 1996; Bobe *et al.*, 1997). Fipronil is highly toxic to rainbow trout and very highly toxic to bluegill sunfish with an $LC_{50}$ of 0.246 and 0.083 ppm, respectively. WHO classifies fipronil as a Class II moderately hazardous pesticide.

Poisoning cases of fipronil occur in dogs and cats due to accidental ingestion/licking of Frontline product. There is some indication that dogs are more severely affected than cats. Application of Frontline top spot on dogs and cats can cause skin irritation and/or hair loss at the site of application. Common clinical signs of fipronil toxicosis

are of CNS hyperexcitability, including tremors, convulsions, seizures, and death (Grant *et al*., 1990; Hainzl *et al*., 1998; Kamijima and Casida, 2000). In rabbits, fipronil produces serious adverse reactions, suggesting that this species is unusually sensitive to fipronil. Young rabbits are especially more sensitive than adults. Frontline has been considered to be unsuitable for rabbits.

Diagnosis of a fipronil poisoning can be based on circumstantial evidence, clinical manifestations, and chemical confirmation. Fipronil residue can be detected in the blood, tissue, or hair using GC/MS. Transferable residue of fipronil can be detected on a dog hair coat up to 4 weeks after a single spot on application (Figure 43.2).

## TREATMENT

There is no specific treatment for the toxicity of fipronil, and therefore treatment relies upon symptomatic and supportive measures. If a dog or cat shows adverse reaction to topical application of Frontline, decontamination by washing at the site of application is advisable.

## CONCLUSIONS

Fipronil elicits neurotoxicity in mammals by inhibition of $GABA_A$-gated chloride channels, producing hyperexcitability of CNS. Overdosage due to accidental ingestion often leads to serious toxicosis. There is no specific antidote for the toxicity of fipronil.

## REFERENCES

Aajoud A, Ravanel P, Tissut M (2003) Fipronil metabolism and dissipation in a simplified aquatic ecosystem. *J Agric Food Chem* **51**: 1347–52.

Anonymous (2000) Fipronil. *Pestic News* **48**: 20.

Bobe A, Coste CM, Cooper J (1997) Factors influencing the adsorption of fipronil on soils. *J Agric Food Chem* **45**: 4861–965.

Bobe A, Meallier P, Copper J, Coste CM (1998) Kinetics and mechanisms of abiotic degration of fipronil. *J Agric Food Chem* **46**: 2834–39.

Cole LM, Nicholson R, Casida JE (1993) Action of phenylpyrazole insecticides at the GABA-gated chloride channel. *Pestic Biochem Physiol* **46**: 47–54.

Cutler SL (1998) Ectopic psoroptes cuniculi infestation in a pet rabbit. *J Small Aim Pract* **39**: 86–7.

Environmental Protection Agency (1996) New Pesticide Fact Sheet. PB96-181516. EPA 737-F-96-005. US EPA Office of Prevention, Pesticides and Toxic Substances, May 1996.

Environmental Protection Agency (1997) Federal Register. Rules and Regulations. 62970–79.

Environmental Protection Agency (1998) Office of Prevention, Pesticides and Toxic Substances. Washington, DC, 90.

Feung CS, Yenne SP (1997) Fipronil: aerobic aquatic metabolism. Rhone-Poulenc Agricultural Limited. Data Package ID No. 169043. DPR Document No. 52062–180.

Gasmi A, Chataigner D, Garnier R, *et al.* (2001) Toxicity of fipronil-containing insecticides. Report of 81 cases from the Paris Poison Center. *Vet Hum Toxicol* **43**: 247.

Grant DB, Bloomquist JR, Ayad HH, *et al*. (1990) A comparison of mammalian and insect: GABA receptor chloride channels. *Pest Sci* **30**: 355–356.

Grant DB, Chalmers A, Wolff M, Hoffman H, Bushey D (1998) Fipronil: action at the GABA receptor. *Rev Toxicol* **2**: 147–56.

Hainzl D, Cole LM, Casida JE (1998) Mechanism for selective toxicity of fipronil insecticide and its sulfone metabolite and desulfinyl photoproduct. *Chem Res Toxicol* **11**: 1529–35.

Hutchinson MJ, Jacobs DE, Fox MT, *et al.* (1998) Evaluation of flea control strategies using fipronil on cats in a controlled simulates home environment. *Vet Rec* **142**: 356–7.

Jennings KA, Canerdy TD, Keller RJ, Atich BH, Doss RB, Gupta RC (2002) Human exposure to fipronil from dogs treated with frontline. *Vet Hum Toxicol*, **44**: 301–3.

Kamijima M, Casida JE (2000) Regional modification of [$^3$H]ethynylbicyclo-orthobenzoate binding in mouse brain $GABA_A$ receptor by endosulfan, fipronil, and avermectin $B_{1a}$. *Toxicol Appl Pharmacol* **160**: 188–94.

Kidd H, James D (1991) *The Agrochemicals Handbook*, 3rd edn. Royal Society of Chemistry Information Services, Cambridge, UK.

Ratra G, Kamita SG, Casida JE (2001) Role of human $GABA_A$ receptor fl3 subunit in insecticide toxicity. *Toxicol Appl Pharmacol* **172**: 233–40.

WHO (1998-1999) Classification of pesticides by hazard. International Program on Chemical Safety. WHO/IPCS/98.21.

Zhao X, Yeh JZ, Salgado VL, Narahashi T (2005) Sulfone metabolite of fipronil blocks GABA- and glutamate-activated chloride channels in mammalian and insect neurons. *Toxicol Sci* **84**: 401–2.

CHAPTER

# 44

# Imidacloprid

*Steve Ensley*

## INTRODUCTION

Imidacloprid is relatively new compound with many uses. Imidacloprid is a neonicotinoid compound that is used as an insecticide for dermal application on animals, for termite and grub control and as an insecticide for crop protection. The neonicotinoids act on nicotinic receptors in insects and vertebrates. To reduce toxicity to mammals and increase toxicity to insects, neonicotinic compounds have been selected that are highly specific for subtypes of nicotinic receptors that occur in insects. The neonicotinoids do not readily pass the blood–brain barrier, further reducing the potential for mammalian toxicity (Yamamoto *et al.*, 1995). When administered orally, imidacloprid is rapidly absorbed, metabolized primarily in the liver and excreted primarily in urine. Imidacloprid does not accumulate in the body, and it is neither carcinogenic, mutagenic, teratogenic nor a reproductive toxicant. Imidacloprid has a high margin of safety due to the high insecticidal specificity and low mammalian toxicity (Nagata *et al.*, 1999).

## BACKGROUND

The neonicotinoids were developed in the late 1970s by chemists at Shell Chemical Company doing research with the heterocyclic nitromethylenes for use as insecticides (Soloway *et al.*, 1978; Schroeder and Flattum, 1984). The neonicotinoids and chloronicotinyls are a separate class of compounds from the nicotinoids (Tomizawa and Yamamoto, 1993). Chemists at Nihon Bayer Agrochem discovered imidacloprid in 1984 when the 3-pyridylmethyl group was added to the nitromethylene heterocyclic parent molecule. This addition to nitromethylene greatly increased the insecticidal activity and reduced mammalian toxicity. After imidacloprid was discovered several other analogs with the 6-chloro-3-pyridylmethyl moiety have been developed, such as acetamiprid, nitenpyram and thiacloprid (Takahashi *et al.*, 1992; Minamida *et al.*, 1993; Yamada *et al.*, 1999).

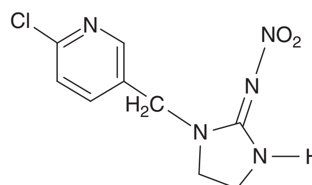


**FIGURE 44.1** Structure of imidacloprid.

## PHARMACOKINETICS/ TOXICOKINETICS

There are two routes of imidacloprid metabolism in mammals. The first route of metabolism involves oxidative cleavage of imidacloprid to imidazolidine and 6-chloronicotinic acid. The imidazolidine moiety is excreted in the urine. The 6-chloronicotinic acid is further degraded by glutathione conjugation to a derivative of mercapturic acid then to methyl mercaptonicotinic acid. The mercaptonicotinic acid is then conjugated with glycine to form a hippuric acid conjugate that is excreted. A second route of metabolism involves hydroxylation of the imidazolidine ring followed by elimination of water and formation of an unsaturated metabolite. Specific information on the toxicokinetics of the rat can be found in Thyssen and Machemer (1999) (Figure 44.1).

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The following studies were conducted by Bayer CropScience (Sheets, 2001). Acute oral toxicity of imidacloprid in rats has been demonstrated at doses above 300 mg/kg with 100% mortality at 500 mg/kg. At doses in rats above 300 mg/kg, clinical signs were observed with 15 min of dosing and recovery was observed within 8–24 h. A subchronic 13-week oral dosing study of imidacloprid in rats also demonstrated toxicity at a dose of 300 mg/kg in male rats. At a dose of 300 mg/kg in male rats, hypertrophy of hepatocytes and sporadic cell necrosis was observed in the liver. This mild liver damage was not observed after a 4-week recovery period. Serum alkaline phosphatase and alanine aminotransferase were elevated in male and female rats treated at 300 mg/kg and above. In dogs, a 13-week oral dose of imidacloprid at 15 mg/kg and above produced a tremor that increased with dose. A 52-week study in dogs at oral doses up to 72 mg/kg/day of imidacloprid did not produce tremors. The no observed effective level (NOEL) for this chronic oral exposure dog study was 15 mg/kg. Carcinogenicity was not observed in rats dosed orally at doses up to 103 mg/kg for 2 years. These studies would indicate that imidacloprid is non-mutagenic, non-embryotoxic and non-teratogenic.

The most common adverse effects observed with dosing of neonicotinoids at low levels (40 mg/kg) in rodents is decreased activity. At higher doses (150 mg/kg) tremors, impaired pupillary function (dilated or pin point pupils), incoordinated gait, and hypothermia is observed. At lethal doses (307 mg/kg), deaths are observed within 4 hours of dosing. If death did not occur, recovery was observed within 8 to 24 hours of dosing.

## MECHANISM OF ACTION

Absorption and distribution of imidacloprid in rats occurs within 1 h following oral administration. In rats more than 90% of a dose of imidacloprid is eliminated within 24 h. Approximately 80% of the dose is excreted by the urine with the remainder eliminated in the feces. Imidacloprid is not distributed to the central nervous system, fatty tissues or bone. This indicates that the blood–brain barrier allows little distribution to the central nervous system for imidacloprid in particular and the neonicotinoids in general.

The neonicotinoids act on post-synaptic nicotinic receptors (Buckingham *et al.*, 1997). These receptors are located entirely in the central nervous system of insects. Imidacloprid acts on at least three different subtypes of nicotinic receptors in the cockroach. Neonicotinoids cause a bi-phasic response: an initial increase in the frequency of spontaneous discharge followed by a complete block to nerve propagation. Insecticidal activity is increased by adding synergists that inhibit oxidative degradation (Liu M-Y, Casida IE, 1993).

Mammalian tissue also contains multiple subtypes of nicotinic receptors. The various subtypes are formed from different combinations of nine $\alpha$ , four $\beta$ , $\gamma$ , $\delta$ and $\varepsilon$ subunits (Tomizawa *et al.*, 1999). Nicotinic receptors in mammals are located in the autonomic ganglia, skeletal muscle, spinal cord and in different regions of the brain. Neonicotinoids have much lower activity in vertebrates compared to insects due to the different binding properties of the various receptor subtypes (Yamamoto *et al.*, 1998). Acute toxicity of the neonicotinoids in mammals is related to the potency at the $\alpha_7$ nicotinic receptor subtype with the activity at the $\alpha_4$, $\beta_2$, $\alpha_3$ and $\alpha_1$ receptors having a decreasing effect on toxicity. Toxicity in mammals involves complex interactions at multiple receptor sites with some of the receptor types even having a combination of agonist and antagonist effects on the synapse.

## TREATMENT

There is no specific antidote to treatment of overdoses of imidacloprid. Treatment is symptomatic. If the exposure is dermal, washing the affected animal with soap and water is indicated. If the exposure is oral, emetics, adsorbents or cathartics could be used depending on what clinical signs are being observed. Absorption and elimination of imidacloprid is rapid so immediate supportive care should allow for rapid recovery.

## CONCLUDING REMARKS AND FUTURE DIRECTIONS

The neonicotinoids have a wide safety margin in mammals and the insecticidal activity can be targeted so this class of compounds will have wide future use as an insecticide.

## REFERENCES

Buckingham SD, Lapied B, LeCorronc H, Grolleau F, Sattelle DB (1997) Imidacloprid actions on insect neuronal acetylcholine receptors. *J Exp Biol* **200**: 2685–92.

Liu M-Y, Casida JE (1993) High affinity binding of [$^3$H]imidacloprid in the insect acetylcholine receptor. *Pestic Biochem Physiol* **46**: 40–6.

Minamida I, Iwanaga K, Tabuchi T, Aoki I, Fusaka T, Ishizuka H, Okauchi T (1993) Synthesis and insecticidal activity of acyclic nitroethene compounds containing a heteroarylmethylamino group. *J Pestic Sci* **18**: 41.

Nagata K, Aoyama E, Ikeda T, Shono T (1999) Effects of nitenpyram on the neuronal nicotinic acetylcholine receptor-channel in rat phaeochromocytoma PC12 cells. *J Pestic Sci* **24**: 143–8.

Schroeder ME, Flattum RF (1984) The mode of action and neurotoxic properties of the nitromethylene heterocycle insecticides. *Pest Biochem Physiol* **22**: 148–60.

Sheets LP (2001) The neonicotinoid insecticides. In *Handbook of Neurotoxicology*, vol. 1. E. Massaro (ed.). Humana Press, Totowa, New Jersey, pp. 79–87.

Soloway SB, Henry AC, Kollmeyer WD, Padgett WM, Powell JE, Roman SA, Tieman CH, Corey RA, Horne CA (1978) Nitromethylene insecticides. *Adv Pestic Sci* **4**: 206–17.

Takahashi H, Mitsui J, Takakusa N, Matsuda M, Yoneda H, Suzuki J, Ishimitsu K, Kishmoto T (1992) Ni-25, a new type of systemic and broad spectrum insecticide. In *Brighton Crop Protection Conferences B Pest and Diseases*, vol. 1, Market Scope Europe Ltd, London, England, pp. 89–96.

Thyssen J, Machemer L (1999) Imidacloprid: toxicology and metabolism. In *Nicotinoid Insecticides and the Nicotinic Acetylcholine Receptor*, Yamamoto I, Casida, JE (eds). Springer-Verlag, Tokyo, pp. 213–22.

Tomizawa M, Yamamoto I (1993) Structure–activity relationships of nicotinoids and Imidacloprid analogs. *J Pestic Sci* **18**: 91–8.

Tomizawa M, Latli B, Casida, JE (1999) Structure and function of insect nicotinic acetylcholine receptors studied with nicotinoids insecticide affinity probes. In *Nicotinoid Insecticides and the Nicotinic Acetylcholine Receptor*, Yamamoto I, Casida JE (eds). Springer-Verlag, Tokyo, pp. 271–92.

Yamada T, Takashi H, Hatano R (1999) A novel insecticide, acetamiprid. In *Nicotinoid Insecticides and the Nicotinic Acetylcholine Receptor*, Yamamoto I, Casida JE (eds). Springer-Verlag, Tokyo, pp. 149–76.

Yamamoto I, Yabuta G, Tomizawa M, Saito T, Miyamoto T, Kagabu S (1995) Molecular mechanism for selective toxicity of nicotinoids and neonicotinoids. *J Pestic Sci* **20**: 33–40.

Yamamoto I, Tomizawa M, Saito T, Miyamoto T, Walcott EC, Sumikawa, K (1998) Structural factors contributing to insecticidal and selective actions of neonicotinoids. *Arch Insect Biochem Physiol* **37**: 24–32.

CHAPTER

# 45

# Ivermectin and selamectin

*Ramesh C. Gupta*

## INTRODUCTION

### Ivermectin

Ivermectin is a semi-synthetic macrocyclic lactone which was first obtained from *Streptomyces avermitilis.* Ivermectin is a mixture of homologues, i.e. $B_{1a}$ and $B_{1b}$, and their structural formulas are shown in Figure 45.1. It was introduced to the market in 1981, as a potent antiparasitic animal health drug. It has been found to be effective against virtually all external and internal parasites thus far tested, except trematodes and cestodes. The drug is approved at very low dosage for the control of parasites in many animal species (cattle, sheep, swine, horse, cat, and dog), but not approved in lactating cows, sheep, and goats. In agriculture, ivermectin is used for its miticidal, insecticidal, and acaricidal activities.

Although ivermectin poisoning has been reported in many animal species due to inadvertent or misuse of the product, major concern of ivermectin toxicity is in collie dogs (Paul *et al*., 1987), which are very sensitive to this drug. In addition, an Old English sheepdog (Houston *et al*., 1987), Murray Grey Cattle (Seaman *et al*., 1987), and in general, young animals are more sensitive to ivermectin toxicity.

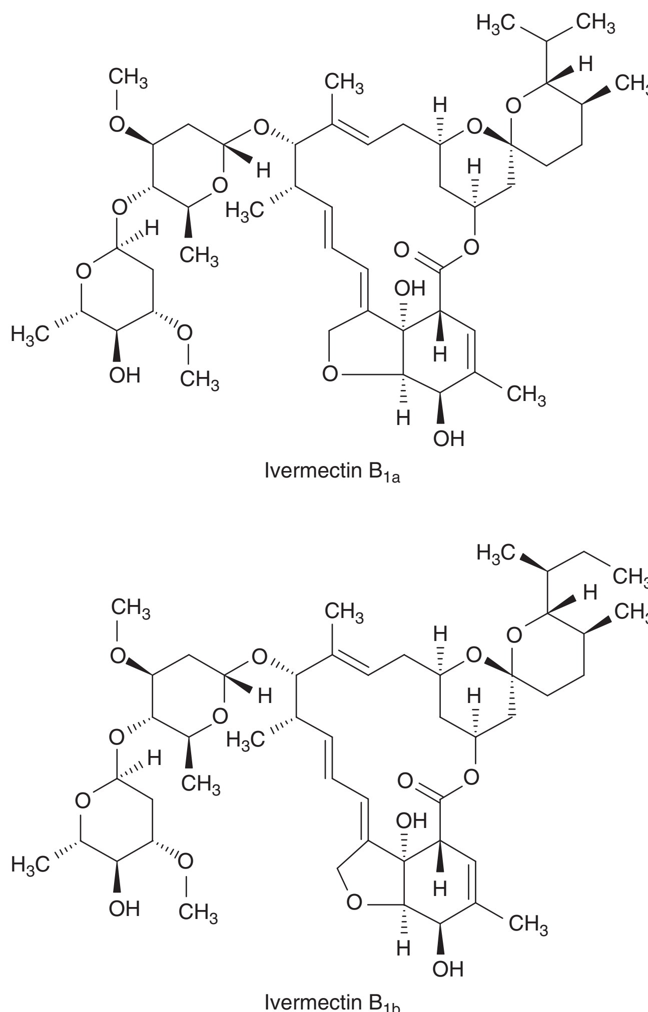


**FIGURE 45.1** Chemical structure of ivermectin $B_{1a}$ and $B_{1b}$.

### Selamectin

Selamectin is a novel semi-synthetic avermectin (Figure 45.2), which is marketed as Revolution. Revolution is a topical antiparasitic preparation recommended for use in dogs and cats 6 weeks of age and older. Selamectin is used to kill fleas (*Ctenocephalides felis*) and ear mites (*Otodectis cynotis*) in dogs and cats. It is also indicated for the treatment and

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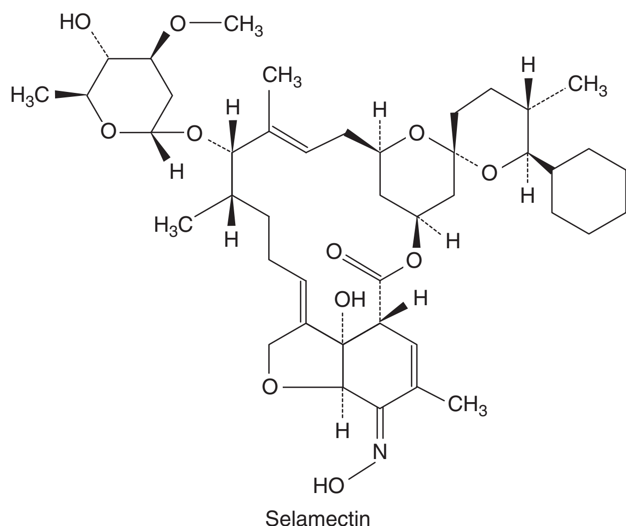


**FIGURE 45.2** Chemical structure of selamectin.

control of sarcoptic mange (*Sarcoptes scabiei*) and for the control of ticks (*Dermacentor variabilis*) infestations in puppies, and the treatment of hookworm (*Ancylostoma tubaeforme*) and roundworm (*Toxocara* cati) infections in kittens. Selamectin is also used to prevent heartworm disease caused by *Dirofilaria immitis*. It is recommended in the dogs and cats at a dose of 6 mg/kg body weight.

This chapter describes toxicity of ivermectin and selamectin in animals.

## BACKGROUND

### Ivermectin

Ivermectin is a mixture of homologues, i.e. not less than 80% of 22,23-dihydroavermectin $B_{1a}$ and not more than 20% of 22,23-dihydroavermectin $B_{1b}$. The mixture is commercialized as abamectin. Ivermectin is a drug of choice because of its broad spectrum nematicidal, insecticidal, and acaricidal properties, and has been approved for prophylactic use against heartworm (*Dirofilaria immitis*) in dogs.

Ivermectin is recommended at very low dosage. It is effective against a wide range of helminths in sheep and cattle in single oral or parenteral dose of 0.1 mg/kg body weight (Egerton *et al*., 1979). It is recommended in dogs, cats, sheep, goats, swine, feedlot beef cattle, horse, and reindeer. The tolerances for beef cattle, reindeer, swine, and sheep are 15, 15, 20, and 30 ppb ivermectin $B_{1a}$ (Code of Federal Regulation, 1990). Ivermectin is not approved for lactating animals.

In the case of beagle dogs the dose required for prevention of heartworm disease is 6 μg/kg body weight, while the $LD_{50}$ of this drug in the same breed is 80,000 μg/kg body weight (Pullium *et al*., 1985). However, certain dogs, primarily rough-coated collies, exhibit signs of toxicity when given ivermectin at doses in the range of 100–2500 μg/kg body weight (Easby, 1984; Houston, 1987). Although this dosage level exceeds the recommended dose for dogs, it has been administered inadvertently by improper use of formulations which were meant for cattle, sheep, horses, and swine. Poisoning in several animal species has been reported due to overdosage of ivermectin. Young animals in general are more sensitive than adults because their blood–brain barrier (BBB) is more permeable to ivermectin (Sanford *et al*., 1988).

### Selamectin

Topical application of Revolution (6 mg selamectin/kg body weight) has been shown to have a broad range of efficacy against many external and internal parasites of dogs and cats (Jacobs, 2000; Dryden *et al*., 2001). Following topical administration of Revolution to a single site on the skin, selamectin is absorbed by the animal through the skin, enters the blood stream and the gastrointestinal (GI) tract, and is ingested by the external and internal parasites as they feed on the treated host. Selamectin has been shown to have a direct parasiticidal effect following ingestion (Bishop *et al*., 2000; Pfizer, 2001). Selamectin is a very safe drug, but the poisoning can occur due to overdosage or accidental exposure.

## PHARMACOKINETICS/ TOXICOKINETICS

### Ivermectin

The pharmacokinetics of ivermectin differs according to the animal species, formulation, and route of administration. In general, the pharmacokinetic properties of these compounds are characterized by their large volume of distribution, slow elimination from the body, and linear relationship to dose, as well as differences between species and variation within individuals of the same species. Bioavailability and pharmacokinetic studies, based upon plasma analysis, revealed that ivermectin was slowly eliminated with a terminal half-life in the range of 5–7 days (Lo *et al*., 1985; Prichard *et al*., 1985). Tissue distribution of ivermectin residues in cattle and sheep has been studied (Tway *et al*., 1981) and it was shown that the major simple component in the edible tissue of the animals was the unaltered drug. In this regard, the liver is the tissue in which the drug is most persistent, as the residue was detected over a period of 28 days post-administration. Due to high lipid solubility,

ivermectin also deposits in adipose tissue, which act as a reservoir that contributes to the long persistence of this drug in the body. The half-life of ivermectin is 4.03 d in goat, 3.68 d in sheep, and 3.66 d in horse following a subcutaneous dose (Prichard *et al.*, 1985; Alvinerie *et al.*, 1993).

Pharmacokinetics of ivermectin in a foal has been found to be similar to that reported in sheep (Prichard *et al.*, 1985 Godber *et al.*, 1995; ). In pigs, ivermectin is distributed to all tissues and body fluids (GI tract contents, GI tract wall and mucus, lungs, skin, earwax, feces) following a subcutaneously injection at 300 μg/kg (Scott and McKellar (1991). Due to high lipid solubility, ivermectin also concentrates in the skin and earwax. The mean elimination half-life of ivermectin was 41.5 h, and the residue was detectable in the plasma for 6–10 days.

Ivermectin residues can be detected in the milk up to 3–4 weeks post-administration (Alvinerie *et al.*, 1987, 1993). These authors determined the pharmacokinetics and mammary excretion of ivermectin in goats following a single subcutaneous (SC) injection (0.2 mg/kg). Kinetic analysis of plasma and milk, using one-compartment model, revealed that the maximum plasma concentration of 6.12 ng/ml occurred at 2.85 d. Ivermectin was detected in the milk as early as 0.3 d, and for at least 25 d. Comparison of the milk and plasma data shows the parallel disposition of the drug in milk and plasma with a milk–plasma concentration ratio of 1.08±0.23. Ivermectin is highly lipid soluble, and it has been found that its concentrations in the milk of cows (Alvinerie *et al.*, 1987; Bogan and McKellar, 1988), ewes (Marriner *et al.*, 1987) and goats (Alvinerie *et al.*, 1993) were similar to the plasma concentrations. Furthermore, following oral or pour-on administration, the excretion pattern of ivermectin is similar (Scott *et al.*, 1990). Ivermectin in the milk of goats can excrete 0.31±0.21 % of the administered dose (Alvinerie *et al.*, 1993), 4.0% of the dose in sheep (Marriner *et al.*, 1987), and 5.46% in cows (Toutain *et al.*, 1988). This large difference appears to be related to differences in the volume of milk produced in the various species and to fat content of the milk (Alvinerie *et al.*, 1993).

Regardless of the method of application (oral, subcutaneous, topical), ivermectin is excreted in bile (Chiu *et al.*, 1990), with high concentrations in feces of the treated animals, where it is associated with reductions in number of dung breeding insects. For further details of pharmacokinetics of ivermectin in animals and humans, readers are referred to Fink and Porras (1989).

## Selamectin

The pharmacokinetics of selamectin has been evaluated in dogs and cats following intravenous (IV) (0.5, 0.1, and 0.2 mg/kg), topical (24 mg/kg), and oral (24 mg/kg) administration (Sarasola *et al.*, 2002). After IV administration of selamectin, the mean maximum plasma concentrations and area under the concentration (AUC)–time curve were linearly related to the dose, and mean systemic clearance ($Cl_b$) and steady state volume of distribution ($Vd_{ss}$) were independent of dose. Plasma concentrations decline polyexponentially in cats and biphasically in dogs with mean terminal phase half-life ($t_{1/2}$) of approximately 69 h in cats and 14 h in dogs. After topical administration, the mean $C_{max}$ in cats was 5513 ± 2173 ng/ml reached at a time ($T_{max}$) of 15 ± 12 h post administration; in dogs, $C_{max}$ was 86.5 ± 34.0 ng/ml at $T_{max}$ of 72 ± 48 h. Bioavailability was 74% in cats and 4.4% in dogs. There is no evidence of sex differences in the pharmacokinetics of selamectin in dogs or cats.

In dogs and cats, selamectin is rapidly absorbed from the skin into the bloodstream, where it kills heartworms. Selamectin excretes into the intestinal tract where it kills intestinal parasites. Finally, selamectin is selectively distributed from the bloodstream into the sebaceous glands of the skin, forming reservoirs that provide persistent efficacy against fleas, ear mites, and sarcoptic mites. Active concentrations of selamectin are found in the plasma for at least 30 days. It is excreted mostly in the feces and a small unmetabolized amount in the urine. Selamectin eliminates more slowly in cats than in dogs following IV administration, as reflected in longer $t_{1/2}$ (69 h versus 14 h) and slower body clearance (0.47 versus 1.18 ml/min/kg). These findings indicate that selamectin persists longer in cats than in dogs, possibly because of differences in metabolism and/or another elimination process between the species.

In dogs, following a single topical application of Revolution, transferable residue of selamectin persists up to a period of 30 days (Gupta *et al.*, 2005) (Figure 45.3). Blood analysis at the 24 h interval revealed no residue of selamectin, but at 72 h the concentration was 10.26±1.06 ng/ml. By day 7, no selamectin residue was

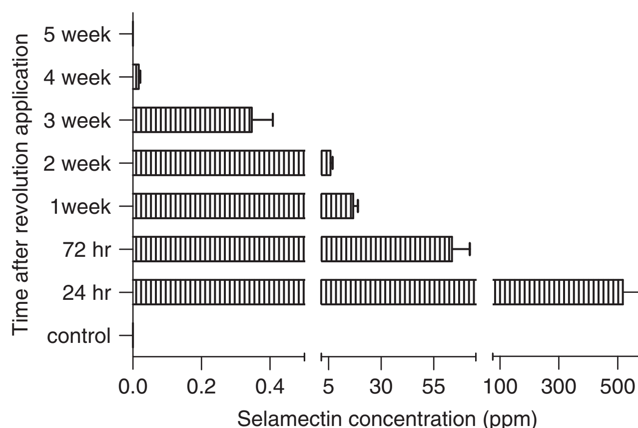


**FIGURE 45.3** Transferable selamectin residue in gloves (ppm) following a single topical application of Revolution (240 mg selamectin per dog).

present. The findings suggested that due to rapid elimination of selamectin from circulation, by day 7, all traces of selamectin in the blood were no longer present.

# MECHANISM OF ACTION

## Ivermectin

Ivermectin is effective against all external and internal parasites, except trematodes and cestodes. This is because of the fact that the drug acts as a $\gamma$-aminobutyric acid (GABA) agonist (Pong and Wang, 1982), and trematodes and cestodes apparently lack a GABA system.

In mammals, ivermectin exerts toxicity by blocking the post-synaptic transmission of nerve impulses by potentiating the release and binding of GABA, and thus blocking GABA-mediated transmission (Campbell and Benz, 1984; Bennett, 1986; Barragry, 1987). In mammals, GABA system is found only in the central nervous system (CNS). Although ivermectin does not readily cross the BBB, there are several reports of ivermectin-induced CNS toxicosis in collie dogs (Campbell and Benz, 1984; Pullman *et al.*, 1985; Bennett, 1986; Barragry, 1987; Paul *et al.*, 1987), and in other domestic animals (Houston, 1987; Houston *et al.*, 1987). Some adverse effects seen in dogs, horses, and cattle given ivermectin may be due to GABA-mediated cholinergic effects (Basudde, 1989). It has been observed that serum pseudocholinesterase levels are significantly increased following SC or IV injection of ivermectin.

## Selamectin

Selamectin binds to glutamate gated chloride channels in the parasite's nervous system, causing them to remain open. This causes chloride ions to continuously flow into the nerve cell, changing the charge of the cell membrane. The continuous flow of chloride ions blocks neurotransmission, and transmission of stimuli to muscles is prevented. Selamectin binding is irreversible causing prolonged channel opening and permanent hyperpolarization. Selamectin has no such effect in the mammalian nervous system, and therefore, it is much safer than common insecticides and parasiticides.

# TOXICITY

## Ivermectin

Ivermectin is a mixture of homologues, in which $B_{1a}$ and $B_{1b}$ components differ from each other by a single methylene group. $B_{1a}$ is the principal component of the mixture (>80%). $B_{1a}$ and $B_{1b}$ have very similar biological and toxicological properties and can, for all practical purposes, be considered equipotent (Campbell, 1989).

The clinical signs and symptoms of ivermectin toxicosis in collie dogs have been reported in several studies (Pullium *et al.*, 1985; Paul *et al.*, 1987; Tranquilli *et al.*, 1987). Ivermectin is a neurotoxicant, as it exerts its toxic effects on the CNS of these sensitive dogs. Further support for CNS involvement was the finding that the ivermectin concentrations in the brain were higher in dogs displaying symptoms of ivermectin toxicosis than in non-sensitive dogs (Pullium *et al.*, 1985). These findings not only suggest the involvement of CNS, but also suggest a difference in the BBB permeability to ivermectin in sensitive versus non-sensitive dogs. However, no differences in the binding characteristics of ivermectin in plasma (albumin and the high density lipoprotein) from ivermectin sensitive and non-sensitive Collies were found (Rohrer and Evans, 1990). The difference between sensitive and non-sensitive dogs lies within the anatomy or physiology of BBB itself or in the presence of high affinity binding sites within the brain rather than difference in the bioavailability of the drug (Kitagawa *et al.*, 1988; Sasaki *et al.*, 1988; Tranquilli *et al.*, 1989). A collie dog after ingesting ivermectin at about 200 μg/kg (in an equine anthelmintics paste) exhibited signs of dehydration, bradycardia, respiratory depression, cyanosis, dilated pupils, and a diminished gag reflex (Heit *et al.*, 1989). Toxic signs may also include vomiting, ataxia, tremors, hypersalivation, coma, and death. Toxic reactions in cats receiving ivermectin (500 μg/kg, sc) showed signs such as vocalization, ataxia, tremors, sternal recumbency, coma, and death. Ivermectin should not be given to kittens, as it can cross the BBB in young animals. The kittens also showed signs of mild diarrhea, posterior ataxia, and mild miosis (Song, 1991). In contrast, in mammals in which ivermectin does not readily cross the BBB, the drug has a wide margin of safety and at least 10 times the therapeutic dose is required to produce toxic effects in most normal mammals (Campbell *et al.*, 1983; Bennett, 1986).

Ivermectin given IV or SC to 8-month-old jersey bull calves at 600 μg/kg caused depression, ataxia, diarrhea, dyspnea, tachycardia, and miosis. Signs were less severe after SC than were after IV injection. Some of these signs were associated with GABA-mediated cholinergic effects. Calves receiving 8.0 mg/kg developed ataxia and recumbency within 24 h after ivermectin exposure. Calves developed depression, increased respiratory rates, muscular fasciculations, mydriasis, and extensor rigidity of the limbs. In cattle, maximum tolerated dose of $B_{1a}$ is about 1.0 mg/kg. In sheep, with higher dose of ivermectin, the toxicity is characterized by depression and incoordination.

Horses exposed to higher doses of ivermectin (6–10 times greater than therapeutic dose) showed ataxia,

depression, and vision impairment within 24 h. With a dose of 60 times the recommended dose (12 mg/kg) horses showed depression, mydriasis, ataxia, depressed respiratory rate, and drooping lower lip (Leaning, 1983).

### Selamectin

In acute cases, signs of toxicosis may include hair loss at the site of application, vomiting, diarrhea with or without blood, anorexia, lethargy, salivation, tachypnea, pruritis, urticaria, arythema, ataxia, and fever. In rare instances, seizures followed by death occur with overt acute overdose. In chronic cases, in dogs and cats, seven monthly treatments of 60 mg/kg (10 times the recommended dose) produced no adverse reactions when given to 6-week-old kittens or puppies. An exposure of 18 mg/kg (3 times the recommended dose) produced no effect on reproduction in females or males. Three monthly doses of 30 mg/kg produced no adverse effects in ivermectin sensitive collies. There have also been rare reports of muscle spasms, seizures, ataxia, and other neurological signs. In a recent study, dogs treated with a single topical application of Revolution (6 mg/kg body weight) showed no signs of any poisoning (Gupta *et al*., 2005).

### Diagnosis

Diagnosis of ivermectin/selamectin can be based on history of exposure to a product containing ivermectin or selamectin, clinical signs, and residue analysis in the body tissue or fluids. These compounds are analyzed using high performance liquid chromatograph coupled with UV detector fluorescence detector, or photo diode array detector (Maynard and Maynard, 1989; Rabel *et al*., 1993; Payne *et al*., 1995; Reising *et al*., 1998; Anastaseo *et al*., 2002; Gupta *et al*., 2005). GI content, liver, fat, and feces are usually the specimens analyzed for ivermectin residue.

## TREATMENT

There is no specific antidote for ivermectin or selamectin toxicity. If poisoning occurs by oral ingestion, the initial therapy should focus on ivermectin removal by the use of oral activated charcoal and saline cathartics (Roder and Stair, 1998). With symptomatic and supportive therapies, even severely affected animals can recover from toxicosis, although recovery is slow. In severely intoxicated cases, use of physostigmine has been found to be beneficial, but physostigmine should not be considered as an antidote.

## CONCLUSIONS

Acute toxicity is more often encountered with ivermectin than selamectin. Poisoning occurs in dogs (especially collies) and cats due to inadvertent or misuse of the product meant for another species. In general, young animals are affected with a greater frequency than the adults. Treatment relies upon symptomatic and supportive therapies.

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## REFERENCES

Alvinerie M, Sutra JF, Galtier P, Toutain PL (1987) Determination of invermectin in milk by high performance liquid chromatography. *Ann Rech Vet* **18**: 269–74.

Alvinerie M, Sutra JF, Galtier P (1993) Ivermectin in goat plasma and milk after subcutaneous injection. *Ann Rech Vet* **24**: 417–21.

Anastaseo A, Esposito M, Amorena M, *et al.* (2002) Residue study of ivermectin in plasma, milk, and mozzarella cheese following subcutaneous administration to buffalo (*Bubalus bubalis*). *J Agric Food Chem* **50**: 5244–5.

Barragry TB (1987) A review of the pharmacology and clinical uses of ivermectin. *Can Vet J* **28**: 512–17.

Basudde CDK (1989) Clinical signs and biochemical changes in calves caused by injection of ivermectin. *Vet Quart* **11**: 29–32.

Bennett DG (1986) Clinical pharmacology of ivermectin. *J Am Vet Med Assoc* **189**: 100–4.

Bishop BF, Bruce CI, Evans NA, *et al.* (2000) Selamectin: a novel broad spectrum endoctocide for dogs and cats. *Vet Parasitol* **91**: 163–76.

Bogan JA, McKellar QA (1988) The pharmacokinetics of ivermectin in sheep and cattle. *J Vet Pharmacol Ther* **11**: 260–68.

Campbell WC (1989) *Ivermectin and Abamectin*. Springer-Verlag, New York.

Campbell WC, Benz GW (1984) Ivermectin: a review of efficacy and safety. *J Vet Pharmacol Ther* **7**: 1–16.

Campbell WC, Fisher MH, Stapley EO, *et al*. (1983) Avermectin: a potent new antiparasitic agent. *Science* **222**: 823.

Chiu S-HL, Sestokas E, Taub R, *et al.* (1990) Metabolic disposition of ivermectin in swine. *J Agric Food Chem* **38**: 2079–85.

Code of Federal Regulations (1990) Title 21, Sec. 556.344. US Government Printing Office, Washington, DC.

Dryden MW, Atkins CE, Evans NA, *et al.* (2001) Insight: new perceptions for veterinary innovators. (Sym.). Pfizer, pp. 7–55.

Egerton JR, Ostlind DA, Blair LS, *et al.* (1979) Avermectins, new family of potent anthelmintic agents: efficacy of the $B_{1a}$ component. *Antimicrob Agents Chemother* **15**: 372–8.

Easby SM (1984) Ivermectin in the dog. *Vet Rec* **115**: 45.

Fink DW, Porras AG (1989) Pharmacokinetics of ivermectin in animals and humans. In *Ivermectin and Abamectin*, Campbbell WC (ed.). Springer-Verlag, New York, pp. 113–30.

Godber LM, Derksen FJ, Williams JF, Mahmoud B (1995) Ivermectin toxicosis in a neonatal foal. *Aust Vet J* **72**: 191–2.

Gupta R, Masthay MB, Canerdy TD, Acosta TM, Provost RJ, Britton D, Atich BN, Keller RJ (2005) Human exposure to selamectin from dogs treated with Revolution™. Methodological consideration for selamectin isolation. *Toxicol Mechan Methods* **15**: 317–21.

Heit JE, Tranquili WJ, Paul AJ, *et al.* (1989) Clinical management of ivermectin toxicosis in a collie dog. Compan Anim Pract **19**: 3–7.

Houston DM (1987) Ivermectin toxicity in small animals. *Can Vet J* **28**: 18.

Houston DM, Parent J, Matushek KJ (1987) Ivermectin toxicosis in a dog. *J Am Vet Med Assoc* **191**: 78–80.

Jacobs DE (2000) Selamectin – a novel endoctocide for dogs and cats. *Vet Parasitol* **91**: 161–2.

Kitagawa H, Sasaki Y, Ishihara K, Ishizake K (1988) Plasma milbemycin D concentrations in collies, shelties, and Japanese mongrel dogs. *Jpn J Vet Sci* **50**: 1184–91.

Leaning WHD (1983) The efficacy and safety evaluation of ivermectin as a parenteral and oral antiparasitic agent in horses. *Proc Am Assoc Equine Pract* **29**: 319.

Lo PKA, Fink DW, Williams JB, Blodinger J (1985) Pharmacokinetic studies of ivermectin: effects of formulation. *Vet Res Commun* **9**: 251–68.

Marriner SC, Mc Kinnon I, Bogan JA (1987) The pharmacokinetics of ivermectin after oral and subcutaneous administration to sheep and horses. *J Vet Pharmacol Ther* **10**: 175–9.

Maynard MS, Maynard HD (1989) HPLC assay for avermectin $B_{1a}$ and its two photoisomers using a photo diode array detector. *Bull Environ Contam Toxicol* **43**: 499–504.

Paul AJ, Tranquilli WJ, Seward RL, *et al.* (1987) Clinical observations in collies given ivermectin orally. *Am J Vet Res* **48**: 684–5.

Payne LD, Hicks MB, Wehner TA (1995) Determination of abamectin and/or ivermectin in cattle feces at low parts per billion levels using HPLC with fluorescence detection. *J Agric Food Chem* **43**: 1233–5.

Pfizer (2001) Revolution: mechanism of action. http://www.revolutionvet.com/action3.htm (accessed on October 28, 2002)

Pong SS, Wang CC (1982) Ivermectin $B_1$ modulation of gamma-aminobutyric acid receptor in rat brain membranes. *J Neurochem* **38**: 375–9.

Prichard RK, Steel JW, Lacey E, Hennessy DR (1985) Pharmacokinetics of ivermectin in sheep following intravenous, intra-abomasal or intra-ruminal administration. *J Vet Pharmacol Ther* **8**: 88–94.

Pullium JD, Seward RL, Henry RT, Steinberg SA (1985) Investigating ivermectin toxicity in collies. *Vet Med* **80**: 33–40.

Pullman JD, Seward RC, Henry RD, Steinberg SA (1985) Investigating ivermectin toxicity in Collies. *Vet Med* **80**: 33–40.

Rabel SR, Stobaugh, Heining R, Bostick JM (1993) Improvements in detection sensitivity for the determination of ivermectin in plasma using chromatographic techniques and laser-induced fluorescence detection with automated derivatization. *J Chromatogr* **617**: 79–86.

Reising KP, Migdal N, Benedetto D (1998) Solid-phase extraction cleanup for ivermectin in liver tissue. *AOAC Intl* **81**: 484–7.

Roder JD, Stair EL (1998) An overview of ivermectin toxicosis. *Vet Hum Toxicol* **40**: 369–70.

Rohrer SP, Evans DV (1990) Binding characteristics of ivermectin in plasma from collie dogs. *Vet Res Commun* **14**: 157–65.

Sanford SE, Rehmtulla AJ, Josephson GKA (1988) Ivermectin overdose and toxicosis in neonatal pigs. *Can Vet J* **29**: 735–6.

Sarasola P, Jernigan AD, Walker DK, *et al.* (2002) Pharmacokinetics of selamectin following intravenous, oral, and topical administration in cats and dogs. *J Vet Pharmacol Ther* **25**: 265–72.

Sasaki Y, Kitagawa H, Ishihara K, Ishizake K (1988) Milbemycin D concentrations in tissues after administration in collies, shelties, and Japanese mongrel dogs. *Jpn J Vet Sci* **50**: 1177–83.

Scott EW, Kinabo LD, McKellar QA (1990) Pharmacokinetics of ivermectin after oral or percutaneous administration to adult milking goats. *J Vet Pharmacol Ther* **13**: 432–5.

Scott EW, McKellar OA (1991) Pharmacokinetics and pharmacodynamics of ivermectin administered subcutaneously to pigs. Proceedings of the 5th Congress. *Eur Assoc Vet Pharm Tox* 383–4.

Seaman TJ, Eagleson JS, Carrigan MJ, Web RF (1987) *Aust Vet J* **64**: 284.

Song MD (1991) Using ivermectin to treat feline dermatoses caused by external parasites. *Vet Med* **86**: 498–502.

Toutain PL, Chapman M, Galtier P, Alvinerie M (1988) Kinetic and insecticidal properties of ivermectin residues in the milk of dairy cows. *J Vet Pharmacol Ther* **11**: 288–91.

Tway PC, Woods JS, Downing GV (1981) Determination of ivermectin in cattle and sheep tissue using high performance liquid chromatography with fluorescence detection. *J Agric Food Chem* **29**: 1059–63.

Tranquilli WJ, Paul AJ, Seward RL, *et al.* (1987) Response to physostigmine administration on collie dogs exhibiting ivermectin toxicosis. *J Vet Pharmacol Ther* **10**: 96–100.

Tranquilli WJ, Paul AJ, Seward RL (1989) Ivermectin plasma concentrations in collies sensitive to ivermectin-induced toxicosis. *Am J Vet Res* **50**: 769–70.

CHAPTER

# 46

# Amitraz

*Ramesh C. Gupta*

## INTRODUCTION

Amitraz is a triazapentadiene compound, which is a member of the formamidine pesticide family. It has a chemical formula of $C_{19}H_{23}N_3$ and molecular weight 293.33. Its chemical structure is shown in Figure 46.1. Amitraz is commonly used in agriculture and horticulture as an insecticide and acaricide to control red spider mite, leaf miners, scale insects, aphids, and other infestations. Amitraz is used to control ticks, mites, lice, and many other pests on dogs, sheep, cattle, pigs, and horses. Amitraz is available in the form of a wetable powder or a pour-on powder, emulsifiable liquid, and spray. It is also used as a synergist and formulated with other insecticides. Amitraz is sold with various products names, including Aazdieno, Acadrex, Acarac, Amitik, Amitraze, Baam, Bumetran, Ectodex, Edrizan, Kenaz, Mitac, Maitac, Metaban, Preventic, Tickoff, Taktic, Triatix, Ovasyn, Ovidrex, and many others. Amitraz poisoning is frequently encountered in dogs and cats (Grossman *et al.*, 1993; Hugnet *et al.*, 1996). In children the poisoning occurs due to widespread use of amitraz veterinary products (Ertekin *et al.*, 2002). This chapter describes the toxicity of amitraz in animals with a special focus on dogs.

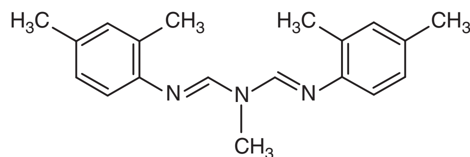


**FIGURE 46.1** Chemical structure of amitraz.

## BACKGROUND

Amitraz is a broad spectrum insecticide and acaricide used in veterinary medicine and in agriculture and horticulture throughout the world since 1974. Amitraz has rapid action on the control of animal ectoparasites, such as mites, ticks, kids, etc., and it persists on hair and wool long enough to control all stages of the parasites. Amitraz was a restricted use pesticide in 1985 because some studies showed it caused cancer in mice. But re-evaluation of the evidence has led to the current classification of amitraz as an unrestricted or general use pesticide. In veterinary medicine, the most common use of amitraz is in tick collars, which contains 9% amitraz as an active ingredient. A collar for a large size dog contains 2.4 g of amitraz (Hugnet *et al.*, 1996). Amitraz is available as a 19.9% topical solution for dogs. All tickicide dips for cattle and sheep contain 0.025% amitraz as the active ingredient. In France, tick collars contain 8–9% amitraz, and external lotions contain 5–12.5%. The amitraz containing product Metaban, which is a liquid concentrate and labeled for veterinary use, is commonly used for demodectic mange (*Demodex canis*) in dogs (Farmer and Seawright, 1980). This product is not recommended for cats and horses (Auer *et al.*, 1984; Gunaratnam *et al.*, 1993). Amitraz is used to control ectoparasites on cattle, sheep, goats, and pigs. There are several reports that have described side effects and toxic effects of amitraz in animals (Turnbull, 1983; Auer *et al.*, 1984; Hsu *et al.*, 1984; Grossman *et al.*, 1993). In the case of dogs, most of the time poisoning is associated with accidental ingestion of the collar, resulting in severe and sometimes fatal poisoning (Grossman *et al.*, 1993; Hugnet *et al.*, 1996). Amitraz should not be used in diabetic animals as it affects the levels of glucose and insulin.

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## PHARMACOKINETICS/ TOXICOKINETICS

Dogs receiving amitraz (100 mg/kg, PO) in a gelatin capsule revealed the pharmacokinetic parameters as follows: area under the curve ($AUC_{0-48\,h}$ = 265.3 ± 12.3 mg/h/ml), elimination $t_{1/2}$ (23.4 ± 2.3 h), time to reach peak plasma concentration ($t_{max}$ = 5.0 ± 0.7 h), and mean peak plasma concentration ($C_{max}$ = 20.7 ± 2.3 mg/l) (Hugnet *et al.*, 1996). From these data it is clear that amitraz has a long elimination half-life and a significant amount is absorbed. Although some amount of amitraz degrades into two metabolites in the stomach, a substantial amount of amitraz is absorbed which is accountable for most of the observed signs. There is a strong association between plasma amitraz concentration and clinical/hematological modifications (Hugnet *et al.*, 1996). In ponies and sheep, amitraz has a brief half-life after intravenous (IV) administration because it is hydrolyzed in blood by formaminidases (Pass and Mogg, 1995).

Hugnet *et al*. (1996) observed that clinical signs of toxicosis usually appeared around 1 h after ingestion, with plasma concentration around 5 mg/l, and lasted until the concentration of amitraz decreased. This information can be used to monitor accidental ingestion of amitraz by dogs (Hugnet *et al.*, 1996).

The amitraz gets hydrolyzed into two metabolites such as 2,4-dimethylaniline and *N*-(2,4-dimethylphenyl)-*N'*-methylformamidine. The former metabolite is a relatively weak methemoglobin-former in dogs and man. These metabolites are further metabolized to 2,4-demethylaniline and ultimately to 4-amino-3-methylbenzoic acid, which is the principal metabolite found in the urine and liver (Aronson *et al.*, 1988; Jones, 1990).

## MECHANISM OF ACTION

Amitraz kills mites and ticks by interfering with their nervous system. The tick's sharp barbed mouth parts become paralyzed and cannot pierce the skin, thereby inhibiting the tick from feeding on dogs. Attached ticks detach.

Amitraz adversely affects the nervous system of animals if exposed to higher doses. Amitraz stimulates $\alpha_2$-adrenoceptor resulting in impairment of consciousness, respiratory depression, convulsions, bradycardia, hypotension, hypothermia, and hyperglycemia. Amitraz has been shown to produce side effects and toxicity in several animal species by targeting $\alpha_2$-adrenergic receptors. It produced effects similar to that produced by a pure $\alpha_2$-adrenergic agonist drugs such as clonidine (Costa *et al.*, 1988; Jorens *et al.*, 1997). As a pharmacologically active compound, amitraz in higher doses is responsible for neurotoxic and preconvulsant effects. In addition to $\alpha_2$-adrenergic agonist, amitraz is a potent inhibitor of monoamine oxidase (MAO) enzyme (Aziz and Knowles, 1973; Moser and Macphail, 1988).

It is suggested that the central $\alpha_2$-agonist activity of amitraz is responsible for central nervous system (CNS) depression (Cullen and Reyoldson, 1990). Other toxic effects associated with $\alpha_2$-agonist are hypothermia and increased heart rate.

Amitraz acts centrally to influence blood pressure and heart rate by $\alpha_2$-adrenoceptor agonism which causes a reduction in peripheral sympathetic tone (Kobinger, 1978; Cullen and Reyoldson, 1990). In the peripheral vasculature both $\alpha_1$ and $\alpha_2$ adrenoceptors are involved in contributing to a vasopressor action of amitraz which results in hypotension.

## TOXICITY

Acute toxicity data of amitraz is available for laboratory animals. Oral $LD_{50}$ is 650 mg/kg in rats and 1600 mg/kg in mice. Values of dermal $LD_{50}$ in rabbit and rat are >200 and 1600 mg/kg, respectively. The inhalation $LC_{50}$ (6 h) of amitraz for rats is 65 mg/l of air. The oral $LD_{50}$ in birds (bobwhite) is estimated at 788 mg/kg, and dietary $LD_{50}$ in mallard is 7000 mg/kg and in Japanese quail is 1800 mg/kg. $LC_{50}$ in rainbow trout is 0.74 ppm and in bluegill is 0.5 ppm and in harlequin fish is 3.2–4.2 ppm. The Environmental Protection Agency (EPA) classifies amitraz as a Class III- slightly toxic pesticide.

Signs of acute amitraz poisoning in male and female rats treated with 440 and 365 mg/kg, respectively, include coolness to touch, reduced spontaneous activity, episodes of increased induced activity such as aggression in response to handling, and signs of debilitation (Hayes and Laws, 1991). Due to its widespread use, acute amitraz poisoning is often encountered in dogs and cats. Poisoning may occur by oral, inhalation, or dermal exposure, but poisoning is most often by oral route. Onset of clinical signs is noted within 30 min to 2 h after ingestion. Clinical signs of poisoning include gastrointestinal (GI) disturbance, nausea, and vomiting (in species capable of vomition), CNS and respiratory depression, bradycardia, hypotension, and hypothermia. Biochemical changes include hyperglycemia and elevation of liver enzyme (transaminases) activity. When amitraz is formulated in xylene and propylene oxide, signs such as depression, ataxia, stupor, and coma are likely attributed to the xylene and propylene oxide (Jones, 1990). Generally, signs and symptoms of amitraz toxicity subside within 24 h; while in some cases it may take 7–10 days.

In dogs and cats, common side effects of the medication include anorexia, sedation, and a dry skin and hair coat.

Serious side effects include low blood pressure, hypothermia, hyperglycemia, mydriasis, bradycardia, slow intestinal rate, incoordination, ataxia, vasoconstriction, vomiting, diarrhea, seizures, and in rare instances death. Clinical signs in humans are similar to those described in animals, especially dogs. In brief, clinical signs include CNS depression, hypothermia, bradycardia, hypotension, hyperglycemia, vomiting, and respiratory failure (Aydin *et al.*, 1997; Kennel *et al.*, 1996; Ulukaya *et al.*, 2001). Death occurs due to cardiac and respiratory failure.

Evidence from animal studies suggests that amitraz is neurotoxicant (Moser and Macphail, 1988). Amitraz has been shown to produce many behavioral and physiological changes in rats. Amitraz at a dose greater than 100 mg/kg caused inhibition of MOA within 2 h of dosing and lasted up to 7 days. Amitraz appears to be more selective for type MAO-B when given *in vivo*, although MAO-A was also inhibited at doses ≥300 mg/kg. However, no selectivity was indicated by the $IC_{50}$ values determined *in vitro* ($IC_{50}$ = 31 and 28 μM for MAO-A and MAO-B, respectively). The findings revealed that the MAO inhibition is probably not due to amitraz-induced alterations in motor activity. Amitraz produced only negligible inhibition of acetylcholinesterase at very high doses. In a dose-dependent manner, amitraz produced depressed arousal and rearing activity, hypothermia, body- weight loss; and autonomic changes including ptosis, chromodacryorrhea resulting in facial crustiness, loss of the pupil reflex, and decreased defecation in rats (Moser, 1991).

Animals exposed to amitraz may show signs of CNS depression or CNS stimulation, depending on the dose level and to some extent the species involved. Generally, high doses have a CNS depressive effect with reduced spontaneous activity, bradycardia, respiratory depression, and hypothermia. Death results from respiratory failure. At low doses CNS stimulation may occur, as manifested by hyperactivity to external stimuli such as handling, and considerably increased food consumption (Pfister and Vim, 1977).

Studies suggest that amitraz exerts endocrine disrupting activity. In dogs, following a topical application, amitraz has been shown to increase plasma glucose and decrease insulin release when dogs were dipped at twice the recommended concentration (Hsu and Schaffer, 1988).

Most of the time, poisoned animals survive and show complete recovery from all signs and symptoms in about 7–10 days, even when exposed to higher doses (Bonsall and Turnbull, 1983).

In chronic 2-year feeding trials, rats receiving 50 mg/kg/day in their diet or dogs receiving 0.25 mg/ kg/day of amitraz did not show any ill effects. Male and female rats receiving amitraz at 200 mg/kg/day for 10 weeks showed decreased fertility. Female mice treated with amitraz orally (50 mg/kg/day) for 5 days and then mated showed a slight increase in loss of fetuses and decrease in the number of living offspring. When male mice were given the same dose and same duration and then mated, the resulting embryos were significantly less likely to grow in the mother's uterus. Rabbits who received 25 mg/kg/day of amitraz from days 6 to 18 of pregnancy had fewer and smaller litters (Meister, 1994).

Studies in laboratory animals have shown that amitraz has no potential for mutagenic activity and does not cause damage to DNA (Hayes and Laws, 1991). Amitraz can cause tumors in female mice but not in male mice or male or female rats. Amitraz also has potential for teratogenic activity. In rats treated with 12 mg/kg/day of amitraz from days 8 to 20 of pregnancy, the offspring were heavier but had less bone development than the offspring of untreated rats (Hayes and Laws, 1991). EPA has established the teratogenic no observed effect level (NOEL) in rats at 12 mg/kg/day (Walker and Keith, 1992). The teratogenic NOEL of rabbits is 25 mg/kg/day. Amitraz and its metabolite 2,4-dimethylaniline have been shown to induce teratogenic effects in frog embryos (Osano *et al.*, 2002). In addition, amitraz has potential for endocrine disruption.

Most of the time, poisoning in animals (especially dogs and cats) with amitraz is acute. Diagnosis is based on history of exposure, clinical signs of toxicity associated with stimulation of $\alpha_2$-adrenoceptor, and residue analysis. Due to a long half-life, residue of amitraz and its major metabolites can be detected in the plasma of a poisoned animal using gas chromatography (GC) coupled with nitrogen–phosphorus detection (Ameno *et al.*, 1991) or high-performance thin-layer chromatographic (HPTLC) coupled with UV detection (Hugnet *et al.*, 1996).

## TREATMENT

Studies from animals suggest that the $\alpha_2$-adrenoceptor antagonists (yohimbine and atipamezole) can reverse amitraz-induced toxicity. In dogs, yohimbine has been found to be very effective. Bradycardia and hypotension respond to yohimbine administration (Hsu and Schaffer, 1988). Severely poisoned dogs with amitraz have been successfully treated with low doses (50 μg/kg, IM) of the potent $\alpha_2$-receptor antagonist atipamezole (Hugnet *et al.*, 1996). Doses of atipamezole can be repeated after 3–4 h, if necessary. Atipamezole can control all the clinical effects of amitraz within 20 min. In most cases, symptomatic and supportive therapies are of great value. Remove the tick collar or any other source of amitraz from pets. In the case of dermal exposure, bathing is helpful. For oral ingestion, induction of vomiting and gastric lavage is recommended. Administration of activated charcoal with a saline cathartic is also beneficial. Animals should be kept in a room

with warm temperature, as amitraz causes hypothermia. Continuous monitoring of cardiovascular, respiratory, and CNSs, and of glucose and insulin levels is advised. With good care and aggressive treatment, even severe cases can be recovered.

## CONCLUSIONS

Amitraz is widely used as an insecticide and acaricide in veterinary medicine. Dogs and cats are intoxicated due to accidental ingestion of amitraz-containing tick collars and other products. Major toxic signs are associated with $\alpha_2$-adrenergic receptor agonism, such as CNS depression, hypotension, bradycardia, and hypothermia. Increased blood glucose and decreased insulin secretion are characteristics of amitraz poisoning and are also due to $\alpha_2$-receptor stimulation. With timely administration of a $\alpha_2$-adrenoceptor blocker (yohimbine or atipamezole), and conventional therapeutic measures, even seriously intoxicated cases can recover.

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## REFERENCES

Ameno K, Fuke C, Ameno S, *et al.* (1991) A rapid and sensitive quantitation of amitraz in plasma by gas chromatography with nitrogen–phosphorus detection and its application for pharmacokinetics. *J Anal Toxicol* **15**: 116–18.

Aronson CE, Powers TE, Davis LE (1988) *Veterinary Pharmacological Biology*, 6th edn. Veterinary Medicine Publishing Co., Lenexa, KS, pp. 905–6.

Auer DE, Seawright AA, Pollitt CC, Williams G (1984) Illness in horses following spraying with amitraz. *Aust Vet J* **61**: 257–9.

Aydin K, Kurtoglu S, Poyrazoglu MH, Uzum K, Ustunbas HB, Hallay IK (1997) Amitraz poisoning in children: clinical and laboratory findings of eight cases. *Hum Exp Toxicol* **16**: 680–2.

Aziz SA, Knowles CO (1973) Inhibition of monoamine oxidase by the pesticide chlordimeform and related compounds. *Nature* **242**: 417–18.

Bonsall JL, Turnbull GJ (1983) Extrapolation from safety data to management of poisoning with reference to amitraz and xylene. *Hum Toxicol* **2**: 587–92.

Costa LG, Olibet G, Murphy SD (1988) Alpha-2 adrenoceptors as a target for formamidine pesticides. *In vitro* and *in vivo* studies in mice. *Toxicol Appl Pharmacol* **93**: 319–28.

Cullen LK, Reynoldson JA (1990) Central and peripheral alpha-adrenoceptor actions of amitraz in the dog. *J Vet Pharmacol Ther* **13**: 86–92.

Ertekin V, Alp H, Selimoglu M, Karacan M (2002) Amitraz poisoning in children: retrospective analysis of 21 cases. *J Intl Med Res* **30**: 203–5.

Farmer H, Seawright AA (1980) The use of amitraz (*N'*-2,4 (dimethylphenyl)-*N*-[(2,4-dimethylphenyl) imino)-methyl]-*N*-methylmethanimidamide) in demodecosis in dogs. *Aust Vet J* **56**: 537–41.

Grossman MR, Garvey MS, Murphy MJ (1993) Amitraz toxicosis associated with ingestion of an acaricide collar in a dog. *J Am Vet Med Assoc* **203**: 55–7.

Gunaratnam P, Wilkinson GT, Seawright AA (1993) A study of amitraz toxicity in cats. *Aust Vet J* **60**: 278–89.

Hayes Jr WJ, Laws Jr ER (1991) *Handbook of Pesticide Toxicology*, vol. 1. Academic Press, Inc., New York.

Hsu WH, Schaffer DO (1988) Effects of topical application of amitraz on plasma glucose and insulin concentrations in dogs. *Am J Vet Res* **49**: 130–1.

Hsu WH, Lu ZX, Hembrough FB (1984) Effects of amitraz on heart rate and aortic blood pressure in conscious dogs; influence of atropine, prazosin, talazoline, and yohimbine. *Toxicol Appl Pharmacol* **84**: 418–22.

Hugnet C, Buronfusse F, Pineau X, Cadore J-L, Lorgue G, Berney PJ (1996) Toxicity and kinetics of amitraz in dogs. *Am J Vet Res* **57**: 1506–10.

Jones RD (1990) Xylene/amitraz: a pharmacologic review and profile. *Vet Hum Toxicol* **32**: 446–8.

Jorens PG, Zandijk E, Belmans L, Schepens PA, Bossaert LL (1997) *Hum Exp Toxicol* **16**: 600–1.

Kennel O, Prince C, Garnier R (1996) Four cases of amitraz poisoning in humans. *Vet Hum Toxicol* **38**: 28–30.

Kobinger W (1978) Central alpha-adrenergic system as targets for hypersensitive drugs. *Rev Phys Biochem Pharm* **81**: 39–100.

Meister RT (1994) *Farm Chemicals Handbook*. Meister Publishing Co., Willoughby, OH.

Moser VC (1991) Investigations of amitraz neurotoxicity in rats. IV. Assessment of toxicity syndrome using a functional observational battery. *Toxicol Sci* **17**: 7–16.

Moser VC, Macphail RC (1988) Investigations of amitraz neurotoxicity in rats. Effects on motor activity and inhibition of monoamine oxidase. *Toxicol Sci* **12**: 12–22.

Osano O, Oladimeji AA, Kraak MHS, Admiral W (2002) Teratogenic effects of amitraz, 2,4-dimethylaniline, and paraquat on developing frog (*Xenopus*) embryos. *Arch Environ Contam Toxicol* **43**: 22–49.

Pass MA, Mogg TD (1995) Pharmacokinetics and metabolism of amitraz in ponies and sheep. *J Vet Pharmacol Ther* **18**: 210–15.

Pfister WR, Vim GKW (1977) Formamidine induced feeding and behavioral alteration in the rat. *Fed Proc* **36**: 352–3.

Turnbull GJ (1983) Animal studies on the treatment of poisoning by amitraz (a formamidine pesticide) and xylene. *Hum Toxicol* **2**: 579–86.

Ulukaya S, Demirag K, Moral AR (2001) Acute amitraz intoxication in human. *Intens Care Med* **27**: 930–3.

Walker MM, Keith LH (1992) *EPA Fact Sheet Database*. Lewis Publishers, Ann Arbor, MI.

C H A P T E R

47

# Metaldehyde

*Ramesh C. Gupta*

## INTRODUCTION

Metaldehyde is a cyclic polymer of acetaldehyde (Figure 47.1), which is found in the form of white or colorless crystalline solid or powder. It is used primarily as a molluscicide to control slugs and snails in a very wide range of gardens and croplands, worldwide. Secondary use of this pesticide is for the control of fish, leeches, and frogs. Metaldehyde is commonly used in many pesticide products along with other compounds, such as carbamates, organophosphates, and arsenates. In addition, metaldehyde is a main ingredient of solid fuel used for making camp fires (fire starter; camp fuel for portable stove) or in small heaters, and is also marketed as a color flame tablet for party goods (ENGELFIRE). Metaldehyde has been involved in suicide attempts. Oral ingestion of metaldehyde in the forms of molluscicide and fuel tablets is the most common route of exposure. In recent years, there has been a widespread concern about the increasing number of poisoning incidents that have occurred in pets, birds, and wild animals. Poisoning cases have also been encountered in large animals. This chapter describes the toxicity of metaldehyde and its metabolites in mammalian and avian species.

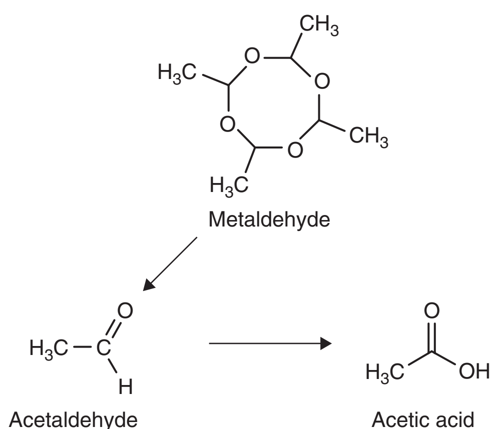


FIGURE 47.1 Chemical structures of metaldehyde and its two major metabolites (acetaldehyde and acetic acid).

## BACKGROUND

Metaldehyde is a molluscicide used in a variety of vegetables and crops in the field, gardens, and greenhouses. It is applied in the form of liquid, granules, sprays, dusts, or pelleted/grain bait to kill slugs, snails, and other garden pests. Usually, the commercial baits contain 4% or less metaldehyde as active ingredient. Some of the granule baits can have up to 5–10% metaldehyde. In Europe, the bait can have up to 50% metaldehyde. Baits formulated in pellets resemble dog food and are flavored with bran or molasses to attract snails. Unfortunately, the flavor attracts dogs and other pets as well. Acute poisoning is common in pets, birds, domestic, and wild animals. A very small amount of metaldehyde is required to cause poisoning or death. The major target organs for metaldehyde toxicity include central nervous system (CNS), liver, kidney, and lung. Animals that ingest metaldehyde may exhibit a variety of toxicological signs including vomiting, tachycardia, ataxia, tremors, seizures, and death (Dobler, 2003). Upon dermal contact, metaldehyde may cause irritation. Currently, there is an increase in the number of metaldehyde poisoning cases attributed to accidental/inadvertent or malicious use of this chemical in baits.

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## TOXICOKINETICS

Metaldehyde and its metabolites are readily absorbed from the gastrointestinal (GI) tract. Metaldehyde can also be absorbed from the lungs and skin. Following oral ingestion, maximal concentrations in the circulation and tissues can be attained within 1–3 h. Metaldehyde secretes back into the GI tract because it gets trapped in enterohepatic circulation (Sax, 1984; Dreisbach, 1987; Knowles, 1991). Residues of metaldehyde have been detected in the brain, blood, and liver of mice (NLM, 1995; Puschner, 2001).

Metaldehyde undergoes hydrolysis reaction at acidic pH in the stomach forming acetaldehyde as a major degradation product. Acetaldehyde is then oxidized to acetic acid (Figure 47.1). Metaldehyde, but not acetaldehyde, was found in plasma and urine of dogs given a single oral dose of 600 mg/kg body weight (Booze and Oehme, 1986). Similar findings were reported in a human case (Moody and Inglis, 1992). Metaldehyde can be excreted in urine and feces. Urinary excretion is found to be less than 1% of the oral dose (600 mg/kg, p.o.) administered in dogs (Booze and Oehme, 1986). Its elimination half-life is reported to be 27 h (Olson, 1999).

## MECHANISM OF ACTION

Pesticidal action of metaldehyde in mollusks and snails is due to contact, making them torpid and increasing the secretion of mucus leading to fatal dehydration (RSC, 1987).

In mammalian and avian species, the toxicity of metaldehyde is characterized by CNS signs, metabolic acidosis, and respiratory alkalosis; and the exact mechanisms involved are yet to be elucidated. The proposed mechanism of action is that the acetaldehyde, which is formed from metaldehyde at low pH in the stomach, is responsible for the toxic effects observed with metaldehyde exposure (Knowles, 1991). Other toxic products are probably also formed. Metaldehyde and its metabolite acetaldehyde can cross the blood–brain barrier, as evidenced by their neurotoxic effects in animals (NLM, 1995). Acetaldehyde acts as a releasing factor for 5-hydroxytryptamine (5-HT) and norepinephrine. CNS signs of metaldehyde toxicosis may be due to decreased brain concentrations of γ-aminobutyric acid (GABA), norepinephrine, and 5-HT, and increased monoamine oxidase (MAO) activity (Dobler, 2003). Acetaldehyde competitively inhibits biogenic amine oxidation, which in turn decreases 5-hydroxyindoleacetic acid, a metabolite of 5-HT, by competitively inhibiting 5-HT oxidation. Acetaldehyde also increases MAO activity and decreases central 5-HT levels (Booze and Oehme, 1985). The old theory that the toxicity of metaldehyde is attributable to its metabolite acetaldehyde is questionable, because acetaldehyde was not present in the plasma and urine of dogs or serum of rats that were given metaldehyde (Shintani *et al.*, 1999; Puschner, 2001). Metaldehyde also affects electrolyte and acid–base balances, which can cause metabolic acidosis that is often associated with CNS depression and hyperpnea (Puschner, 2001).

## TOXICITY

Metaldehyde is toxic to all animals. Secondary poisoning is also not uncommon. It is highly toxic by inhalation, moderately toxic by ingestion, and slightly toxic by dermal absorption. Ingestion is the most common route of poisoning. WHO classifies metaldehyde as a moderately hazardous pesticide; and Environmental Protection Agency (EPA) classifies it as a slightly toxic chemical (Toxicity Class II or III). This chemical has a label of "Restricted Use Pesticide" because of its potential short- and long-term effects on wildlife.

The oral $LD_{50}$ of metaldehyde is 100 mg/kg body weight in dogs, 60 mg/kg in horses, 207 mg/kg in cats (Beasley, 1999; Plumlee, 2001), 227–690 mg/kg in rats, 200 mg/kg in mice, 175–700 mg/kg in guinea pigs, and 290–1250 mg/kg in rabbits (Knowles, 1991; NLM, 1995). The toxic dose of metaldehyde in dogs and cats is reported to be 45 mg/pound of body weight. $LC_{50}$ values of metaldehyde in rainbow trout and bluegill are 62 and 10 ppm, respectively.

Metaldehyde is primarily a neurotoxicant, as it produces CNS depression, convulsions, and violent muscular contractions in several mammalian species following acute exposure. Acute metaldehyde poisoning is often encountered in pets, especially dogs and cats. Onset of signs of metaldehyde toxicosis usually appear within 15 min to a few hours after ingestion. Clinical signs of maximal severity occur within 1–3 h of ingestion. In general, toxicity signs of mild metaldehyde poisoning include anxiety, hyperesthesia, foaming at the mouth, and muscle twitching. Signs of severe poisoning include hypotension and tachycardia, panting, nystagmus, mydriasis, dehydration, hyperthermia, respiratory depression, convulsion, continuous tonic seizures, coma, ataxia, respiratory failure, and death. Not every poisoned animal may show all these signs. In dogs, hemorrhagic gastroenteritis, hypersalivation, and vomiting are also seen. Clinical signs in cats are similar to those described for dogs, but nystagmus is more prevalent in cats (Beasley, 1999).

It is a common observation that exposed pets either die within a few hours from an early episode of acute toxicity or within a few days from liver, kidney, and respiratory failure. Symptoms of metaldehyde poisoning in domesticated and wild mammals include inability to stand,

blindness, change in respiratory rate, dehydration, excessive sweating and salivation, seizures and sudden death (Grant and Schuman, 1993).

In practical situations, chronic toxicity is very rare. Usually, dosages which are not toxic when given singly do not cause illness when repeated (Dreisbach, 1987; Knowles, 1991). In a 2-year chronic toxicity study (three-generation reproductive studies) in rats, changes in liver enzyme activity, and increased liver and ovary weight were found at dietary doses of about 12.5 mg/kg/day; 50% of female rats given this dose showed paralysis. At higher doses, metaldehyde is known to adversely affect reproduction and the survival rate of offspring (Sax, 1984). Pregnancy exacerbates the toxicity of metaldehyde.

In cattle, sheep, and horses, mild poisoning is evidenced by hypersalivation, ataxia, and hyperpnea. In severe poisoning, symptoms include convulsions, sweating, tachycardia, and muscle spasms. Death occurs within the first 48 h of accidental ingestion, and is attributable to respiratory failure (Von Burg and Stout, 1991). Pathological lesions can be found in liver, kidney, lungs, and GI tract.

Metaldehyde-related poisonings and deaths have been reported in avian species (Baker, 1967; Reece *et al.*, 1985; Andreasen, 1993). A minimal lethal dose of 500 mg/kg body weight for chickens and 300 mg/kg body weight for ducks has been determined. The clinical signs in avians include excitability, incoordination, tremors, muscle spasms, torticollis, diarrhea, and difficult or rapid breathing. Death of geese and ducks has been reported following accidental exposure. Metaldehyde is considered practically nontoxic to aquatic organisms.

From experimental studies, there is no evidence that metaldehyde is teratogenic (Verschuren *et al.*, 1975), mutagenic (Quinta and Martire, 1981), or carcinogenic (Verschuren *et al.*, 1975).

Diagnosis of metaldehyde poisoning can be established based on a history of exposure and clinical signs. Confirmation can be made by demonstrating metaldehyde/acetaldehyde in vomitus or GI tract content, blood, or urine. The odor of acetaldehyde in stomach content is suggestive of metaldehyde exposure. Although pathological findings are nonspecific, lesions can be found in liver, kidney, lungs, and GI tract.

## TREATMENT

There is no specific antidotal treatment for metaldehyde poisoning. So, treatment rests with supportive and symptomatic measures that usually consist of (1) removal of remaining metaldehyde from the GI tract, (2) prevention of absorption by activated charcoal, (3) control of seizures, (4) re-establishing ventilation and administer of oxygen, and (5) restoring fluid and electrolyte balance to correct acidosis. Administration of 2–5% sodium bicarbonate solution can reduce the conversion of metaldehyde to acetaldehyde.

In the case of dogs, if the patient is asymptomatic, immediately induce vomiting by syrup of ipecac (one teaspoon) or a mixture of hydrogen peroxide and water (one tablespoon), followed by gastric lavage using milk or water. The milk helps decrease the further absorption of the metaldehyde. Simple osmotic diuresis is helpful. Administration of activated charcoal (1–4 g/kg, p.o.) is found to be beneficial, as it prevents further absorption of metaldehyde from intestine. If necessary, charcoal treatment at half the original dose can be repeated at 6–8 h interval. Charcoal treatment has been found to reduce absorption of metaldehyde by more than 45% (Shintani *et al.*, 1999). Cathartics can also be used with activated charcoal to assist in removing the metaldehyde from the intestinal tract. Gastric lavage and cathartics are found to be effective treatment for up to 12–24 h after metaldehyde ingestion. Muscle twitch and spasms in dogs and cats can be controlled by methocarbamol (55–220 mg/kg, i.v.). Seizures can be treated by using diazepam (0.5–2 mg/kg, i.v.; repeat if necessary) or sodium phenobarbital (30 mg/kg, i.v.). Urinary acidosis can be corrected by the administration of sodium bicarbonate. In an experimental study conducted on mice, administration of diazepam (10 mg/kg, i.p.) or clonidine HCl (0.5 mg/kg, i.p.) decreased the toxicity of metaldehyde (100 mg/kg, p.o.). Diazepam at low dose (0.5 mg/kg body weight) was without any protective effect.

In the case of pigs, barbiturates can be used to anesthetize poisoned animals for a period of 6–12 h, so as to allow excretion of metaldehyde and its metabolites.

## CONCLUSIONS

Most of the time, metaldehyde poisoning is acute in nature and results from accidental or malicious activity. The poisoning is characterized by neurological signs, such as violent convulsions and seizures. Without timely and aggressive treatment, death ensues due to respiratory failure. Since no specific antidote is available, treatment relies upon supportive and symptomatic therapies.

## ACKNOWLEDGMENTS

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## REFERENCES


Andreasen JR (1993) Metaldehyde toxicosis in ducklings. *J Vet Diagn Invest* **5**: 500–1.

Baker JR (1967) Metaldehyde poisoning in geese. *Vet Rec* **81**: 448–9.

Beasley VR (1999) Toxicants associated with CNS stimulation or seizures. *A Systems Affected Approach to Veterinary Toxicology*. University of Illinois, College of Veterinary Medicine, Urbana, IL pp. 94–7.

Booze TF, Oehme FW (1985) Metaldehyde toxicity. A Review. *Vet Hum Toxicol* **27**: 11–19.

Booze TF, Oehme FW (1986) An investigation of metaldehyde and acetaldehyde toxicities in dogs. *Fund Appl Toxicol* **6**: 440–6.

Dobler LK (2003) Metaldehyde toxicosis. *Vet Med* **March**: 213–15.

Dreisbach RH (1987) *Handbook of Poisoning*, 12th edn. Appleton and Lange, Norwalk CT, 185 pp.

Grant WM, Schuman JS (1993) *Toxicology of the Eye*, 4th edn. Charles C. Thomas, Springfield, IL.

Knowles CO (1991) Miscellaneous pesticides. In *Handbook of Pesticide Toxicology*, Hayes WJ, Laws ER (eds). Academic Press, New York, pp. 10–186.

Moody JP, Inglis FG (1992) Persistence of metaldehyde during acute molluscicide poisoning. *Vet Hum Toxicol* **11**: 361–2.

NLM, U.S. National Library of Medicine (1995) *Hazardous Substances Databank*, 10-9. NLM, Bethesda, MD.

Olson K (1999) *Poisoning and Overdose*, 3rd edn. Appleton and Lange, Paramount Publishing Business and Professional Group.

Plumlee KH (2001) Pesticide toxicosis in the horse. *Vet Clin North Am (Equine Pract)* **17**: 496–7.

Puschner B (2001) Metaldehyde. In *Small Animal Toxicology*, Peterson ME, Talcott PA (eds). Saunders WB, Philadelphia, PA, pp. 553–62.

Quinta I, Martire G (1981) Screening of 24 pesticides by *Salmonella* microsomes assay. *Mutation Res* **85**: 265.

Reece RL, Scott PC, Forsyth WM, *et al.* (1985) Toxicity episodes involving agricultural chemicals and other substances in birds in Victoria, Australia. *Vet Rec* **117**: 525–7.

RSC (1987) *The Agrochemical Handbook*, 2nd edn. Royal Society of Chemistry, Nottingham, UK.

Sax NI (1984) *Dangerous Properties of Industrial Materials*, 6th edn. VanNostrand Reinhold Co., New York.

Shintani S, Goto K, Endo Y, Iwamoto C, Ohata K (1999) Adsorption effects of activated charcoal on metaldehyde toxicity in rats. *Vet Hum Toxicol* **41**: 15–18.

Verschuren HG, Dentonkekler EM, Bertiverns JM, Helleman PW, Van Esch GJ (1975) Long-term toxicity and reproduction studies with metaldehyde in rats. *Toxicology* **4**: 97–115.

Von Burg R, Stout T (1991) Toxicology update: metaldehyde. *J Appl Toxicol* **11**: 377–8.

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# Part 7

## Rodenticides and Avicides

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C H A P T E R

# 48

# Anticoagulant rodenticides

*Michael J. Murphy*

## INTRODUCTION

The origin of oral anticoagulant therapy and anticoagulant rodenticides traces back to investigations of moldy sweet-clover poisoning in the 1920s. This disease of cattle in Wisconsin was characterized by high mortality and internal bleeding. Investigations revealed that the diseased cattle had been fed moldy sweet-clover hay.

An association between vitamin K and coagulopathies was made in the 1930s (Dam, 1935; Fieser *et al.*, 1939). Soon, thereafter Prof. Link reported the discovery of dicoumarol in the moldy hay (Last, 2002). Naturally occurring coumarin in sweet-clover hay is converted by fungi to dicoumarol. Dicoumarol was found to be the causative agent of the disease, so the elements needed for the disease were coumarin-containing plant material plus mold growth. Subsequently a range of molecules were synthesized. One named warfarin became the most popular (Duxbury and Poller, 2001). Warfarin takes its name, in part, from the Wisconsin Alumni Research Foundation. Warfarin and dicoumarol found application as both oral anticoagulants and as rodenticides. Sweet clover requires the action of molds to form dicoumarol, Giant fennel does not.

Giant fennel, *Ferula communis*, grows in Mediterranean countries. It has a naturally occurring anticoagulant effect. An association between the plant and anticoagulation was first reported in the 1950s (Costa, 1950a, b; Carta, 1951). It was further investigated in Italy (Mazzetti, 1957; Corticelli and Deiana, 1957; Corticelli *et al.*, 1957; Cannava, 1958) then Israel (Shlosberg and Egyed, 1983). The anticoagulant activity of the plant in Morocco has recently been reviewed (Lamnaouer, 1999).

Five coumarins and eleven daucane derivatives have been isolated from *F. communis* (Arnoldi *et al.*, 2004). Previously identified chemicals included allohedycaryol (Zhabinskii *et al.*, 1996), fercoperol (Miski *et al.*, 1986), and ferulenol.

The toxicity of ferulenol in rats, mice, and sheep has been reported. Ferulenol is a 4-hydroxycoumarin with the expected anticoagulation effects (Fraigui *et al.*, 2002). The single oral $LD_{50}$ of ferulenol in albino mice is 2100 (Fraigui *et al.*, 2002). This value is quite similar to oral $LD_{50}$ values of 1650 and 2000 for rats and mice, respectively using fessoukh, the resinous gum of *Ferula* (Fraigui *et al.*, 2001). This supported prior studies in rats (Aragno *et al.*, 1988; Tagliapietra *et al.*, 1989).

Anticoagulation activity of the plant in sheep was reported in 1985 (Shlosberg and Egyed, 1985). Subsequently, ferulenol has been measured in the serum of sheep experimentally dosed with 600 g of powdered plant material (Tligui and Ruth, 1994). Ferulenol was detected at 6 h after dosing and for about 12 h after cessation of dosing. The prothrombin time (PT) was elevated to 6 times normal about 70 h after the last dose and returned to normal by day 5 (Tligui and Ruth, 1994; Tligui *et al.*, 1994).

*Ferula* has also been examined for chemotherapy (Poli *et al.*, 2005), antimycobacterial (Appendino *et al.*, 2004; Mossa *et al.*, 2004), and microtubule effects (Bocca *et al.*, 2002) and testicular and epididymal changes in rams (Gil *et al.*, 2002) much like the warfarin and other oral anticoagulants now have. It is found in Morocco (Fraigui *et al.*, 2001), Israel (Shlosberg and Egyed, 1983), and Italy (Tagliapietra *et al.*, 1989).

The phrase oral anticoagulants normally refer to these chemicals when used therapeutically. The oral

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anticoagulants are briefly discussed before the detailed discussion of the application of the dicoumarol progeny as anticoagulant rodenticides.

Warfarin and its congeners are still used as therapeutic agents. Oral anticoagulants available therapeutically in Europe include warfarin, phenprocoumaron, and nicoumalone – also called acenocoumarol (Shetty *et al.*, 1993). Oral anticoagulants are used therapeutically to reduce thromboembolic events. Warfarin examples include a reduction in catheter-related thrombosis (Guidry *et al.*, 1991; Magagnoli *et al.*, 2006), early venous thrombosis after operations (Calnan and Allenby, 1975; Pan *et al.*, 2005), including hip surgery, atrial fibrillation (Middlekauff *et al.*, 1995; Reiffel, 2000), and myocardial infarction (Asperger and Jursic, 1970). Dicoumarol is also used in the therapy of thrombotic occlusion of intracoronary stents (Alonso Martin *et al.*, 1997), prosthetic valves (Dalla, 1994), and other types of venous thromboses (Piovella *et al.*, 1995; Ferlito, 1996) or thrombophlebitis (Byrne, 1970; Creutzig, 1993).

Warfarin may also reduce the recurrence of malignant melanoma (Thornes *et al.*, 1994) or small cell lung cancer (Aisner *et al.*, 1992). This anti-metastatic activity may be associated with distribution of metastatic cells on thrombi (McNiel and Morgan, 1984; Smith *et al.*, 1988).

Low-dose warfarin therapy has been adopted by the US, Europe, and World health Organization (Duxbury and Poller, 2001). Internationalized Normalized Ratio (INR) is now used to measure anticoagulation (Duxbury and Poller, 2001).

Oral anticoagulants have been used for decades and a number of adverse events have been recognized. Most are related to drug interactions (Dayton and Perel, 1971). Although controversy exists as to whether acetaminophen induces an adverse effect on warfarinized patients in therapeutic doses (Toes *et al.*, 2005). The acetaminophen metabolite NAPQI (*N*-acetyl-para-benzoquinoneimine) seems to interfere with vitamin-K-dependent $\gamma$-carboxylase. Such interference may lead to potentiation of the anticoagulant effect of warfarin (Thijssen *et al.*, 2004). COX-2 inhibitors may interfere with control of warfarin anticoagulation in elderly patients (Savage, 2005) and rifampin may interfere with warfarin metabolism due to induction of P-450 activity (Strayhorn *et al.*, 1997; Finch *et al.*, 2002). Inconsistent anticoagulation has been recognized in generic versus prescription warfarin (Burns, 1999) and a single case of hemothorax with eosinophilia has been reported in a warfarin overdose (Nasilowski and Krenke, 2002).

Complementary or alternative medical therapies may also interfere with warfarin metabolism (Tumova, 2000; Wood *et al.*, 2003). For example, Passionflower, hydroalcoholic extracts, juniper, and verbena contain vitamin $K_1$ so they can lessen the anticoagulation effect of warfarin therapy (Argento *et al.*, 2000). On the other hand, genoderma, japonicum, papaw, *Salvia miltiorrhiza*, ginseng, devil's claw, garlic, quinine, ginkgo, ginger, red clover, and horse chestnut may reinforce the anticoagulant effect of warfarin by various mechanisms (Argento *et al.*, 2000).

A "coumarin-induced hepatitis" has been reported a number of times in warfarinized patients (Rehnqvist, 1978; Bint and Burtt, 1980; Tanaka *et al.*, 1985; Hohler *et al.*, 1994; Matsukawa *et al.*, 1994; Ehrenforth *et al.*, 1999; Bamanikar and Hiremath, 2002; Biagini *et al.*, 2006). Liver damage has also been reported in a brushtail possum (*Trichosurus vulpecula*) dosed with the anticoagulant rodenticide pindone (Jolly *et al.*, 1994). Pindone has also been associated with an increased frequency of sex chromosome loss in *Drosophila melanogaster* (Santoro *et al.*, 1993). The remainder of the chapter is devoted to the application of the chemical progeny of dicoumarol to pest control.

# BACKGROUND

## Anticoagulant rodenticides

In the 1940s, a small British pharmaceutical company suggested that dicoumarol might have rodenticidal properties. Trials carried out by Armour and Barnett (1950) confirmed the idea and started the era of anticoagulant rodenticides. Warfarin was the first anticoagulant rodenticide introduced into the market shortly after World War II and became widely used in many countries. Other anticoagulant compounds with potency similar to that of warfarin were also synthesized. These early anticoagulant rodenticides have often been called "first-generation anticoagulant rodenticides." These first-generation compounds generally have moderate toxicity, with acute $LD_{50}$ values ranging from 10 to 50 mg/kg body wt. (Table 48.1). The first-generation compounds often needed continuous bait exposure for rodent control.

Many rodent species developed a resistance to warfarin (Jackson *et al.*, 1975) presumably due to continued exposure and widespread use. Consequently, new chemical structures were synthesized and used as anticoagulant rodenticides. These newer compounds are generally more toxic than warfarin with acute $LD_{50}$s of 0.2–3.9 mg/kg body wt. For example, a bait concentration of only 50 ppm of brodifacoum is adequate to give control in a single feeding for most rodents and non-commensal species (Matolesy *et al.*, 1988). These newer compounds were called "second-generation anticoagulant rodenticides" and are often now referred to as "superwarfarins" in the contemporary medical literature (Chong *et al.*, 1986; Greeff *et al.*, 1987; Swigar *et al.*, 1990; Wallace *et al.*, 1990; Routh *et al.*, 1991; Wilton, 1991; Exner *et al.*, 1992; Rauch *et al.*, 1994; Hui *et al.*, 1996; Tecimer and Yam, 1997; Gallo, 1998; Chua and Friedenberg, 1998; Pavlu *et al.*, 2005; Sharma and Bentley, 2005; Dolin *et al.*, 2006).

TABLE 48.1 The oral $LD_{50}$ values (mg/kg body wt.) of some anticoagulant rodenticides

| Animals | Bromadiolone | Brodifacoum | Difenacoum |
|---|---|---|---|
| Rat (acute) | 0.65 | 0.27 | 1.8 |
| Rat (chronic) | (0.06–0.14) x 5 | (0.05–0.08) x 5 | 0.15 x 5 |
| Mouse | 0.99 | 0.4 | 0.8 |
| Rabbit | 1.0 | 0.2 | 2.0 |
| Pig | 3.0 | 10.0 | 80.0 |
| Dog | 10.0 | 3.5 | 50.0 |
| Cat | 25.0 | 25.0 | 100.0 |
| Chicken | 5.0 | 10.0–20.0 | 50.0 |
| Guinea pig | 2.8 | – | – |
| Opossum | – | 0.17 | – |
| Sheep | – | 10.0 | 100.0 |

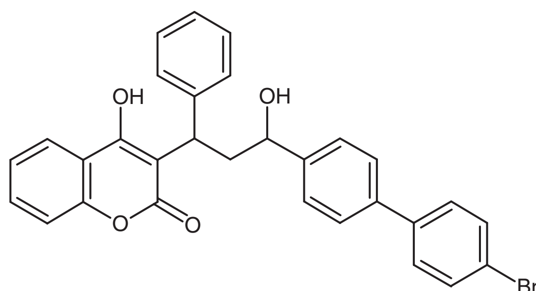
Anticoagulant rodenticides are also categorized by chemical structure. The chemical structure of the currently marketed products fits in one of two chemical classes:

1 *Hydroxycoumarins*: This group of compounds have a 4-hydroxycoumarin ring with different side-chain substituents at the 3-position. Commonly used anticoagulant rodenticides in this group are bromadiolone, brodifacoum, coumafuryl, coumatetralyl, difenacoum, and warfarin.
2 *Indanediones:* This group of compounds has a 1,3 indanedione structure with different side-chain substituents at the 2-position. The most common anticoagulant rodenticides in this group are chlorophacinone and diphacinone. Examples of each of these compounds are briefly summarized.

A few representative chemicals are described below. A number of other chemicals have now been developed.

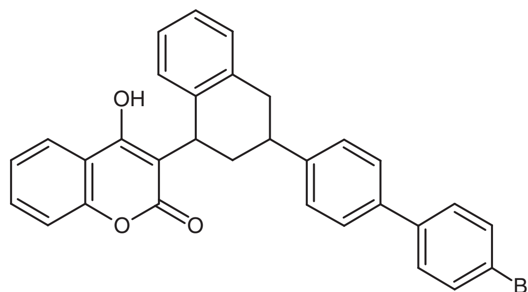
### 1 Hydroxycoumarins

*A Bromadiolone*




Bromadiolone {3-(3-(4′-bromobiphenyl-4-yl)-3-hydroxy-1-phenyl propyl)-4-hydroxycoumarin} was synthesized and marketed by the French company Lipha SA during the mid-1970s. It is used widely for control of commensal and field rodents in many countries. Technical grade bromadiolone is 97% pure. It is a yellowish powder and stable up to 2000°C (Chalermchaikit, 1992). It is very soluble in dimethylformamide (730 g/l), but less soluble in ethyl acetate (25 g/l), and ethanol (8.2 g/l), and sparingly soluble in water (0.019 g/l). Bromadiolone is considered more palatable to rodents than most other anticoagulants. Its concentration in baits is usually 50 ppm (Chalermchaikit *et al.*, 1993). Although bromadiolone is considered a "second-generation anticoagulant rodenticide", some resistance problems have been reported with *Rattus norvegicus* and *Mus musculus* in the UK and Denmark (Rowe *et al.*, 1981).

*B Brodifacoum*




Brodifacoum {3-(3-(4′-bromobiphenyl-4-yl)-1,2,3,4-tetrahydro naphth-1-yl)-4-hydroxycoumarin} is one of the newer and more potent second-generation anticoagulant rodenticides. It was introduced in 1977 by Sorex Ltd. of London, then developed by the Imperial Chemicals Incorporated (ICI) Plant Protection Division (Chalermchaikit *et al.*, 1993).

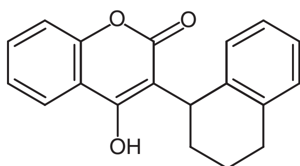
Pure brodifacoum is off-white to fawn colored powder with a solubility of 6–20 g/l in acetone, 3 g/l in chloroform, 0.6–6 g/l in benzene, and less than 10 mg/l water. It is very stable in the environment with no loss after 30 days exposure to direct sunlight (Chalermchaikit *et al.*, 1993).

Brodifacoum has been marketed in several countries for the control of a wide range of rodent pest species. It is available as a 0.005% pellet for rat and house mouse control, a smaller 0.001% pellet for field rodent control, and as 29-g wax blocks for sewer rat control. It is the only anticoagulant rodenticide found to produce 100% mortality in most rodent species after only a 24-h dose

(Chalermchaikit *et al.*, 1993). Brodifacoum was effective against warfarin-resistant rats and mice in 1984, but the possibility of resistance has been raised (Lund, 1984).

There is variation in the susceptibility of species to brodifacoum. The average pig, sheep, or chicken must consume considerable quantities of prepared bait to be endangered. However, dogs are susceptible and are commonly exposed to potentially toxic quantities of brodifacoum (Chalermchaikit *et al.*, 1993).

*C Coumatetralyl*

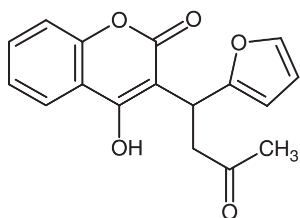



Coumatetralyl {3-(alpha-tetralyl)-4-hydroxycoumarin} was introduced by Bayer AG with the trademark name of Racumin. It has been used for commensal control in many countries. It is formulated as a dry bait (0.0375%), a liquid bait of its sodium-salt, and a 0.75% tracking dust (Chalermchaikit *et al.*, 1993).

Pure coumatetralyl is a colorless powder which is stable at temperatures below 150ºC. Its solubility is 20–50 g/l in propan-2-ol, 50–100 g/l in methylene dichloride, and 4 mg/l in water. The acute and chronic $LD_{50}$'s to *R. norvegicus* are 16.5 and 0.3 mg/kg for five consecutive doses, respectively. Chickens are quite resistant to coumatetralyl, with a chronic $LD_{50}$ of 50 mg/kg for eight consecutive doses. Signs did not appear in fish until the concentration of coumatetralyl reached 1000 mg/l in water (Chalermchaikit *et al.*, 1993).

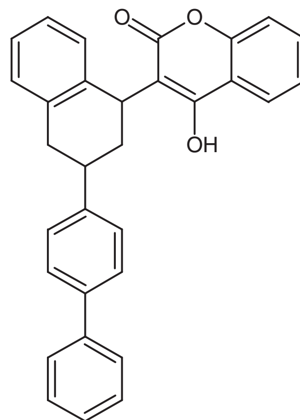
In spite of its low toxicity, it is reported to be a little more effective than warfarin against *R. norvegicus*, apparently due to a higher palatability. Coumatetralyl was introduced after the detection of warfarin-resistant rat populations and showed considerable success for a number of years, but resistant pests have been reported in the UK and Denmark (Rowe and Redfern, 1968).

*D Coumafuryl*



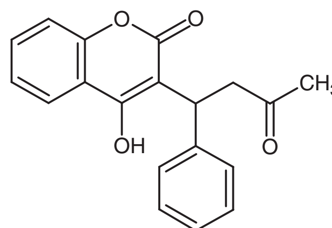

Coumafuryl {3-(alpha-acetonylfurfuryl)-4-hydroxycoumarin} is a German anticoagulant, introduced in 1952, and is used at 0.025–0.05% in baits. Its toxicity is considered equal to warfarin for *R. norvegicus* but slightly less efficient against *M. musculus*. The chronic $LD_{50}$ in *R. norvegicus* is 1.4 mg/kg for five repeated doses. Cats and dogs seem to be almost as susceptible as rats, with dogs being killed by 2 mg/kg for five repeated doses and cats by 10 mg/kg for four repeated doses (Chalermchaikit *et al.*, 1993).

*E Difenacoum*




Difenacoum {3-(3-*p*-diphenyl-1,2,3,4-hydronaphth-1-yl)-4-hydroxycoumarin} was synthesized in the UK and marketed in 1975 by Sorex Ltd. under the trademark "Neosorexa", and by ICI Plant Protection Division under the trademark "Ratak" as a 0.005% pelleted bait, and as a wax block. Pure difenacoum is an off-white powder with a solubility of greater than 50 g/l in acetone, 600 mg/l in benzene, and less than 10 mg/l in water. It is more toxic than warfarin, but less palatable. Difenacoum is still effective against many populations of warfarin-resistant rats (Desideri *et al.*, 1979), but resistance may be developing in the UK (Greaves *et al.*, 1982).

*F Warfarin*



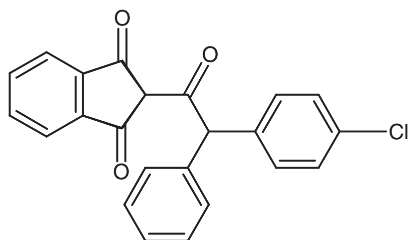

Warfarin {3-(a-acetonylbenzyl)-4-hydroxycoumarin} was the first anticoagulant rodenticide introduced shortly after

World War II after development by the Wisconsin Alumni Research Foundation. Warfarin is still used widely, especially for the control of *R. norvegicus* in areas where resistance has not developed. In its racemic form, warfarin is colorless and crystalline, insoluble in water, but readily soluble in acetone, dioxane, and moderately soluble in alcohols. Warfarin is formulated as dry bait (0.005–0.05%) as well as a liquid bait, based on the sodium salt, and a tracking dust (0.5–1.0%). It is generally applied as the S-isomer, which has a toxicity 10 times greater than the R-isomer. The acute and chronic $LD_{50}$s for *R. norvegicus* are around 10–12 and 0.75 mg/kg for five repeated doses, respectively (Colvin and Wang, 1974).

Warfarin is sometimes combined with an antibacterial agent, sulfaquinoxaline, in order to reduce the bacterial production of vitamin K in the rat intestine, but the effectiveness of this combination has not been proven. Warfarin is considered one of the safest anticoagulants, as far as domestic and other non-target animals are concerned. Serious resistance problems have been reported in Europe. It has recently been evaluated against sewer rats in London (Channon *et al.*, 2000).

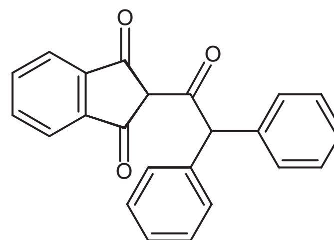
### 2 Indanedione group

*A Chlorophacinone*




Chlorophacinone {2-(alpha-4-chlorophenyl-a-phenylacetyl)-1,3-indandione} was first introduced during the mid-1960s by Lipha S.A. of France, at concentrations of 0.05% in baits and 0.2% in tracking dust. Pure chlorophacinone is a yellow crystalline solid which is very soluble in acetone, ethanol, ethyl acetate, but is sparingly soluble in water. It is quite stable and resistance to weathering. Chlorophacinone does not induce "bait-shyness" and is compatible with cereals, fruits, roots, and other potential bait substances. Its acute $LD_{50}$s in *R. norvegicus* is about 20.5 mg/kg which is less toxic than warfarin, but it has a stronger initial effect on rats and mice. For control of house mice populations, a prolonged feeding period is needed. Chlorophacinone is not effective against warfarin-resistant rodents (Chalermchaikit *et al.*, 1993).

*B Diphacinone*




Diphacinone (2-diphenylacetyl-1,3-indandione) is an old anticoagulant rodenticide, introduced by Vesicol Chemical Corp. and the Upjohn Co. It has been produced and used primarily in the US as a 0.005% dry or liquid bait. Pure diphacinone is a yellow powder which is very soluble in chloroform (204 g/kg), toluene (73 g/kg), xylene (50 g/kg), and acetone (29 g/kg), but sparingly soluble in water (0.30 g/l). It will decompose in water due to sunlight. The acute $LD_{50}$s in *R. norvegicus* are 22.7 mg/kg in females and 43.3 mg/kg in males. It is more toxic than warfarin to rats, mice, and dogs, but its palatability is somewhat lower. Diphacinone is not effective against warfarin-resistant rodents (Chalermchaikit *et al.*, 1993).

The anticoagulant rodenticides are marketed to have efficacy against a number of target pest species. These species are listed below by chemical compounds.

## Target species

### Brodifacoum

One day of feeding on a 0.005% brodifacoum or difenacoum bait is successful in controlling the lesser bandicoot rat (*Bandicota bengalensis*) in Burma (Brooks *et al.*, 1980). Within 3 days, a 0.005% brodifacoum bait gave complete control of golden hamsters (*Mesocricetus auratus*) resistant to 0.005% warfarin and difenacoum (Bradfield and Gill, 1984). A 20-ppm brodifacoum bait gave virtually complete control of Norway rats (*R. norvegicus*) in 21–73 days after other treatments had failed (Greaves *et al.*, 1982). At a 0.005% bait concentration brodifacoum was more effective at 8 days in controlling *Meriones shawi* than warfarin at 0.025%, coumatetralyl at 0.0375%, difenacoum at 0.005%, and bromadiolone at 0.005% (Gill and Redfern, 1983). Brodifacoum's efficacy has also been shown for *Tatera indica* (Rehman and Ahmad, 1983), *T. indica*, *Nesokia indica*, *B. bengalensis* (Greaves and Rehman, 1977), field mice *Mus booduga* Gray (Balasubramanyam *et al.*, 1984) *Rattus rattus* and *B. bengalensis* (Deobhankar, 1985) eight rodent species (Lund, 1981, 1988), the Egyptian spiny mouse (*Acomys cahirinus*) (Mahmoud and Redfern, 1981), non-resistent (Mosterd and Thijssen, 1991), and warfarin-resistant rats (Rennison and Dubock, 1978). A number of early studies

were conducted under the designation WBA 8119 (Rowe and Bradfield, 1976; Rowe *et al.*, 1978). A number of different rodenticides have been tested for efficacy against rodents in cucumber (*Cucumis sativus*) plantings (Sabhlok *et al.*, 1997).

### *Bromadiolone*

Bromadiolone has been effective against warfarin-resistant rats and mice (Redfern and Gill, 1980). It is effective against the Norway rat (*R. norvegicus*) (Richards, 1981; Kamil, 1987). Bromadiolone residues have been examined in tissues of *Arvicola terreteris* (Giraudoux *et al.*, 2006) and coypu (*Myocastor coypus*) (Jeantet *et al.*, 1991) after field use. Its effects on the breeding performance of house mice have also been investigated (Twigg and Kay, 1995).

### *Chlorophacinone*

The efficacy of chlorophacinone against mice, voles, and squirrels has been established. At a 25-ppm bait concentration, chlorophacinone is more effective than coumachlor in controlling common mice (*M. musculus*) in Egypt (Mesban *et al.*, 2003). It can control common voles (*Microtus arvalis*) (Nikodemusz *et al.*, 1981), palm squirrels (*Funambulus pennanti*) (Mathur and Prakash, 1980), and house mice (*M. musculus*) (Lund, 1971).

### *Coumafuryl*

Coumafuryl is more effective on *R. rattus*, *R. norvegicus*, and *B. bengalensis* than fumarin and warfarin when used in liquid form (Renapurkar, 1982). It was effective in controlling the cotton rat (*Sigmodon hispidus*) at concentrations used to control *R. rattus* and *R. norvegicus* (Gill and Redfern, 1980), and *Mastomys natalensis* (Gill and Redfern, 1979).

### *Coumatetralyl*

Median survival time was 4.7 and 11.2 days in *B. bengalensis* and *R. rattus* exposed to 0.0375% coumatetralyl bait (Chopra and Parshad, 1985).

### *Diphacinone*

Diphacinone has been shown to control rats (Elias and Johns, 1981), vampire bats (Burns and Bullard, 1979, 1980; Thornton, 1980), *B. bengalensis* (Brooks *et al.*, 1980), and coyotes (Szuber and Diechtiar, 1968; Sterner, 1979).

### *Difenacoum*

Fifty percent of male mice exposed to 0.5 mg difenacoum/kg body wt. died within 9 days, whereas no female mice died (Winn *et al.*, 1989). Norway rats (*R. norvegicus*) fed 25 ppm difenacoum bait for 5, 10, or 20 days had whole carcass residues of 0.52–0.74 mg/kg body wt. with the higher amount being present after the longer feeding period (Rennison and Hadler, 1975; Redfern and Gill, 1980; Atterby *et al.*, 2005). A symptom-dependent taste aversion of the brown rat (*R. norvegicus*) has been reported because the taste aversion is only present when signs are present in the animal (Smith *et al.*, 1994).

### *Flocoumafen*

Flocoumafen has been demonstrated to control *R. rattus*, *B. bengalensis* (Parshad and Chopra, 1986), *R. norvegicus* (Buckle, 1986), and the house mouse *(M. musculus)* (Rowe *et al.*, 1985a). Flocoumafen gave a quicker and equally effective kill of 68 mice *(M. musculus*) exposed to a 0.005% oatmeal bait when compared to difenacoum, bromadiolone, and brodifacoum at the same bait concentrations (Rowe *et al.*, 1985a). A non-biliary intestinal elimination of flocoumafen has been reported in rats (Huckle *et al.*, 1989) and metabolic and toxicological studies have been reported (Veenstra *et al.*, 1991). Accumulation of flocoumafen in rats after repeated exposure has been examined (Huckle *et al.*, 1988).

### *Flupropadine*

Flupropadine is nearly as effective as calciferol/warfarin in control of the house mouse (*M. musculus*) (Rowe *et al.*, 1985a).

### *Warfarin*

The efficacy of warfarin has been evaluated against squirrels (Chambers and Chambers, 1983) and a host of other species.

## Non-target species

Unfortunately non-target species may also be exposed to anticoagulant rodenticides. Anticoagulant rodenticides are a potential hazard to all species of mammals and birds. The environmental, avian, and wildlife species so exposed are summarized briefly before the discussion of non-target exposure in humans and domestic animals below.

### *Environmental*

Anticoagulant rodenticides may be detected in water, soil, and invertebrates. A method of detecting warfarin in water has recently been reported (Badia and Diaz-Garcia, 1999), perhaps since anticoagulant rodenticides are used in rice paddies (Baskaran *et al.*, 1995) and accidental discharges of brodifacoum bait may occur in fresh water or a marine environment (Primus *et al.*, 2005). The toxicity of anticoagulant rodenticides in soil may be related to the portion not bound to humic acid (Andre *et al.*, 2005). Testing for the halogenated biphenyl side chain has been suggested as a way to determine soil exposure to rodenticides (Townsend *et al.*, 1995).

Diphacinone has been detected in snails and slugs in Hawaii. It ranged from 0.8 to 2.5 ppm in *Oxychilus* sp. snails, from 1.3 to 4.0 in *Deroceras laeve* slugs, and up to 1.8 ppm in *Limax maximus* slugs (Primus *et al.*, 2006).

### Wildlife

Anticoagulant rodenticides have been detected in polecats and mink in the wild. Recently, difenacoum and bromadiolone were detected in 35% of male (13 of 37) and 38% of female (5 of 13) polecats (*Mustela putorius*) collected in England and Wales in areas where the baits had been used (Shore *et al.*, 2003). Spatial and temporal residues in polecats in Britain have also been reported (Shore *et al.*, 2003). A previous study found difenacoum in 7 of 24 livers (Shore *et al.*, 1996). In France, populations of the free-ranging European mink (*Mustela lutreola*) have declined. Investigators found bromadiolone and chlorophacinone residues in the livers of four species of free-ranging mink and raised the question of the risk to European mink from anticoagulant rodenticides (Fournier-Chambrillon *et al.*, 2004). See also the risks of brodifacoum in non-target birds and animals (Eason *et al.*, 2002). Secondary poisoning of fox after broadcast of anticoagulant rodenticides for voles has been proposed (Raoul *et al.*, 2003). Bromadiolone toxicosis of coypu has also been reported (Morin *et al.*, 1990).

### Avian

Anticoagulant rodenticide toxicosis of birds has been reported throughout the United States, and in Australia (Reece *et al.*, 1985). Brodifacoum and difenacoum residues have been detected in a number of non-target avian species. Offspring of turkey vultures (*Cathartes aura*) died after being fed brodifacoum-poisoned mice in a zoo setting. Similar residues have been detected in other carnivorous birds including *Dacelo novae-guinae* and *Tockus deckeni*.

Ten percent of barn owls collected in England had detectable residues of difenacoum or brodifacoum in their liver. Liver concentrations of difenacoum and brodifacoum ranged from 5 to 106 ng/g and 19 to 515 ng/g, respectively. Mice were fed difenacoum and brodifacoum baits and died in 2–11 days. Poisoned mice were fed to barn owls for 1, 3, or 6 days. All six owls fed difenacoum mice survived and coagulation times returned to normal in 5–23 days. Four of six owls fed brodifacoum-dosed mice died 6–17 days after feeding. Dead owls had 630–1250 ng/g brodifacoum in their livers (Newton *et al.*, 1990). A white-winged wood duck has been treated for brodifacoum toxicity (James *et al.*, 1998) and secondary poisoning of stone martens (*Martes foina*) fed bromadiolone poisoned mice (Lund and Rasmussen, 1986) has been suspected.

Brodifacoum has also been implicated in poisoning of non-target wildlife in 80% of 55 animals investigated in the New York area. Diphacinone, bromadiolone, chlorophacinone, and coumatetralyl were also implicated. Exposure of raptors including great-horned owls (*Bubo virginianus*) and red-tailed hawks (*Buteo jamaicensis*) constituted half the cases, with gray squirrels (*Scirurus carolinensis*), raccoons (*Procyon lotor*), and white-tailed deer (*Odocoileus virginianus*) constituting the others (Stone *et al.*, 1999).

Broadcast diphacinone has also been implicated in avian toxicoses. Poouli is an endangered avian species in Hawaii. For example, a 3% and an 8% probability of mortality have been determined for adult and juvenile Poouli after a 5-day exposure to broadcast diphacinone. Diphacinone residues in snails apparently increase the risk of mortality in juvenile birds (Johnston *et al.*, 2005).

Diphacinone, dicoumarol, and pival have all been shown to increase the PT in chicks (Charles *et al.*, 1966). Bromadiolone was detected both in fox (*Vulpes vulpes*) and buzzards (*Buteo buteo*) in France (Berny *et al.*, 1997).

Chlorophacinone has also been examined. A "no observed effect" concentration of chlorophacinone in feed is 1 mg chlorophacinone/kg feed in Japanese quail (*Coturnix japonica*) (Riedel *et al.*, 1990). Emaciated California quail (*Callopepla californica*) were found to have impacted crops due to parafinized chlorophacinone pellets attributed to the paraffin, since PT were normal (Blus *et al.*, 1985). Chlorophacinone has also been studied in captive kestrels (Radvanyi *et al.*, 1988). A 100% mortality occurred in 1-week-old chicks fed coumestral in wood–straw mats (Munger *et al.*, 1993).

## Mechanism of action

The biochemical mechanism, metabolism, and kinetics of the anticoagulant rodenticides are summarized. The mechanism of action of all anticoagulant rodenticides is similar to that of warfarin, i.e. inhibition of vitamin $K_1$ epoxide reductase (Park *et al.*, 1979; Leck and Park, 1981; Breckenridge *et al.*, 1985). In the coagulation cascade, the clotting factors II, VII, IX, and X must bind calcium ions to be active in clot formation. The $Ca^{2+}$-binding ability requires converting glutamyl residues on these clotting factors to γ-carboxyl glutamyl residues by the process of carboxylation. This carboxylation uses vitamin $K_1$ hydroquinone as a cofactor. This vitamin-K-dependent carboxylase reaction converts vitamin $K_1$ hydroquinone to its epoxide form, vitamin $K_1$ 2,3-epoxide. In the normal cycle, vitamin $K_1$ 2,3-epoxide is reduced to the original vitamin $K_1$ (phylloquinone) by enzyme epoxide reductase, and is thus recycled (Figure 48.1). Anticoagulant rodenticides produce their effect by interfering with the enzyme vitamin $K_1$ epoxide reductase, resulting in the depletion of vitamin $K_1$ and subsequently impairing the synthesis of normal clotting factors II, VII, IX, and X (Craciun *et al.*, 1997, 1998). Clinical coagulopathy soon follows the depletion of

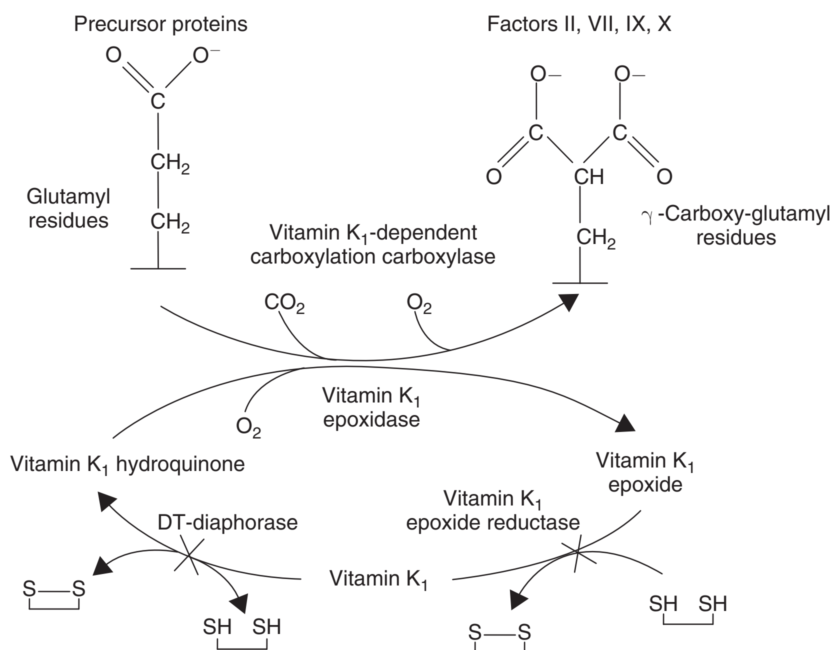



**FIGURE 48.1** Liver metabolism of vitamin K. The dithiol-dependent vitamin $K_1$ epoxide reduction and vitamin $K_1$ (phylloquinone) reduction are the metabolic steps inhibited by anticoagulant rodenticides.

vitamin $K_1$ in the liver. Since these clotting factors in the dog have plasma half-lives of 41, 6.2, 13.9, and 16.5 h, respectively, a lag time of 3 to 5 days is commonly observed between ingestion of a bait and the onset of clinical signs (Jackson and Suttie, 1977; Suttie, 1986; Murphy and Gerken, 1989). The interrelationship of vitamin K, prothrombin, γ-carboxyglutamic acid are reviewed in Stenflo (1978). The interaction of warfarin and vitamin K are reviewed in Suttie (1990).

### *Biochemistry*

Microsomal vitamin-K-dependent carboxylase, vitamin K epoxidase, vitamin $K_1$ epoxide reductase, and cytosolic vitamin K reductase (DT-diaphorase) are involved in vitamin K reduction (Hildebrandt and Suttie, 1982). The physiologically important site of action of the anticoagulant rodenticides is vitamin $K_1$ epoxide reductase (Hildebrandt and Suttie, 1982). Anticoagulant rodenticides act by inhibiting vitamin $K_1$-2,3 epoxide reductase and consequently the synthesis of clotting factors II, VII, IX, and X. S-warfarin and difenacoum are more potent in complete inhibition of clotting factor synthesis than racemic warfarin, R-warfarin, or brodifacoum (Breckenridge *et al.*, 1985).

The greater potency and duration of action of the long acting or "superwarfarins" has been attributed to (1) a greater affinity for vitamin $K_1$-2,3-epoxide reductase, (2) ability to inhibit the vitamin $K_1$ epoxide cycle at more than one point, (3) hepatic accumulation, and (4) unusually long-half-lives due to lipid solubility and enterohepatic circulation (Watt *et al.*, 2005).

As it turns out, rat liver has two pathways for vitamin K reduction. One is responsible for the therapeutic effect of vitamin $K_1$ therapy. This pathway is DT-diaphorase (EC.1.6.99.2) and a microsomal dehydrogenase that has 3.6-fold higher activity with nicotinamide adenine dinucleotide hydrogen (NADH) than with nicotinamide adenine dinucleotide phosphate hydrogen (NADPH). It is not a cytochrome P-450 or cytochrome-b5 reductase (Wallin, 1986) Although, dicumerol, warfarin and diphenadione inhibit NAD(P)H in rat liver *in vitro*, only dicumerol inhibited the enzyme in rats dosed *in vivo* (Schor *et al.*, 1983).

Pathway I is inactive in warfarin and difenacoum intoxicated rats. Vitamin $K_1$ epoxide reductase was also inactive, so this may be part of Pathway I *in vivo*. Pathway II mediates the therapeutic effect of vitamin $K_1$ and resulting carboxylation *in vitro* (Wallin, 1986).

Vitamin K and vitamin $K_1$ epoxide can be measured in serum (Bjornsson *et al.*, 1978, 1979; Donnahey *et al.*, 1979) and tissue. A number of vitamin K detection methods have been reported (Williams *et al.*, 1972; Haroon *et al.*, 1980, 1986, 1987; Haroon and Hauschka, 1983; Hart *et al.*, 1984; including detection in human plasma (Langenberg and Tjaden, 1984) and serum (Lefevere *et al.*, 1979) using high-pressure liquid chromatography (HPLC) with electrochemical detection (Takani and Suttie, 1983) or fluorimetric detection in liver (Usui *et al.*, 1989). The mode of action of vitamin K has been reviewed (Olson, 1966).

Because anticoagulant rodenticides inhibit the vitamin $K_1$ reductase reaction (Pelz *et al.*, 2005) vitamin $K_1$ epoxide is elevated and vitamin $K_1$ is reduced. For example, diphenadione (Mount and Kass, 1989) and warfarin-exposed dogs (Carlisle and Blaschke, 1981) have elevated vitamin $K_1$ epoxide after subcutaneous vitamin $K_1$ administration.

Also, vitamin $K_1$ concentrations are low to non-detectable in rats 24 h after exposure to difenacoum (Winn *et al.*, 1987). Measurement of the epoxide in serum has been proposed as a method to detect surreptitious exposure (Bechtold and Jahnchen, 1979; Bechtold *et al.*, 1983). The disposition of vitamin K in anticoagulant rodenticide poisoning was examined some years ago (Park *et al.*, 1984).

### *Resistance*

Resistance to anticoagulant rodenticides is largely transmitted as an autosomal dominant trait. Initial investigators postulated that a genetic mutation in the resistant rodents produced an enzyme epoxide reductase with a reduced binding affinity for warfarin and similar anticoagulants that resulted in resistance to warfarin (Bell and Caldwell, 1973; Zimmerman and Matschiner, 1974; Hadler and Shadbolt, 1975; Misenheimer *et al.*, 1994). This theory seems to have been born out.

Eight different mutations in the vitamin K reductase reaction have been identified in laboratory strains of brown rats and house mice, and in wild caught brown rats. Five mutations are of only two amino acids. Resistance to warfarin is largely influenced by mutations at Tyr139 (Pelz *et al.*, 2005). Previously the genomic assignment of the warfarin-resistant locus (Kohn and Pelz, 1999) and a gene-anchored map of the rat warfarin-resistant locus (Kohn and Pelz, 2000) were reported. These followed on reports of the biochemical basis of warfarin and bromadiolone resistence in house mice (Misenheimer *et al.*, 1994). However, warfarin-resistant rats are not resistant to coumatetralyl, so more than on mechanism of resistence may be found (Bell *et al.*, 1976).

Feeding of difenacoum and menadione (vitamin $K_3$) for 4 days is currently used as a test to determine the degree of resistance of laboratory Norway rats (*R. norvegicus*) and wild rats to difenacoum (Gill *et al.*, 1993). Chemical differences of the side-chains at the 3-position of the second-generation 4-hydroxycoumarins may provide a point of attachment to a lipophilic site on the epoxide reductase enzyme, where warfarin and other first-generation rodenticides do not bind strongly (Hadler and Shadbolt, 1975). This increased binding increases their toxicity and may reduce the development of resistance in rodents.

An absolute requirement for vitamin $K_1$ in Danish Norway rats (*R. norvegicus*) (Markussen *et al.*, 2003) and others resistant to warfarin remains as another theory (Trivedi *et al.*, 1988).

### *Antioxidant*

The anticoagulant rodenticides and oral anticoagulants are also of interest in cancer research. In part, because catalyzing obligatory two-electron reductions of quinones to hydroquinones, NAD(P)H: quinone reductase (QR1) protects cells against the deleterious effects of redox cycling of quinones and their ability to deplete glutathione and produce neoplasia (Dinkova-Kostova and Talalay *et al.*, 2000). DT-diaphorase and coenzyme Q appear to have antioxidant and prooxidant functions in quinone metabolism (Cadenas, 1995; Beyer, 1994).

### *Metabolism*

Warfarin is metabolized by CYP 2C9 and 2C19 in humans (Goldstein, 2001; Brandon *et al.*, 2005). The CYP 2C9 enzyme has several inherited polymorphisms (Kirchheiner and Brockmoller, 2005). Chlorophacinone elimination may be enhanced by phenobarbital administration (Burocoa *et al.*, 1989; Lagrange *et al.*, 1999) perhaps due to CYP induction. The effect of phenobarbitone on vitamin $K_1$ metabolism (Wilson and Park, 1984) can be compared to that of the rabbit (Winn *et al.*, 1988).

## Toxicokinetics

### *Bioavailability*

Most anticoagulant rodenticide toxicoses occur after oral exposure. However, a diphacinone-induced coagulopathy has been reported after dermal exposure to a liquid preparation (Spiller *et al.*, 2003). A quite unusual case is exposure to brodifacoum after donation and transplantation of multiple organs (Ornstein *et al.*, 1999).

The oral bioavailability of warfarin, chlorophacinone, and bromadiolone were estimated at 79%, 92%, and 88% respectively, in sheep. These anticoagulants degraded by about 15% over 24 h in rumen extracts (Berny *et al.*, 2006). The bioavailability of warfarin is influenced by dietary protein (Barber and Colvin, 1980).

### *Distribution*

Sixty percent of $^{14}C$ labeled diphacinone is eliminated in feces and 10% in urine over 4 days in mice and 8 days in rats (Yu *et al.*, 1982). Tissue distribution indicated that liver had the most $^{14}C$ activity, with the lowest amounts in brain, muscle, blood, and fat (Yu *et al.*, 1982). The disposition and pharmacodynamic properties of brodifacoum have also been characterized in rats (Bachmann and Sullivan, 1983). Similarly, 30% of $^{14}C$ labeled flocoumafen is eliminated in feces and less than 3% in urine within 3 days in rats (Huckle *et al.*, 1988). About 60% of $^{14}C$ flocoumafen is liable to beta-glucuronidase and most radioactivity is found unchanged in the liver. Elimination is biphasic with rapid phase of 5 days, then a prolonged phase of 100 days in Japanese quail (Huckle *et al.*, 1989).

Alteration of parental behavior was speculated to be a cause of 54% lethality in offspring in the face of 40% lethality of lactating female albino rats dosed with coumatetralyl (Marchini and Turillazzi, 1978). However, passage into milk is an alternative theory.

### *Elimination*

The different chemical structures give different elimination kinetics for the various anticoagulant rodenticides. Elimination kinetics is estimated from human or animal clinical cases in many instances. Such cases are presented for the 4-hydroxycoumarins warfarin, brodifacoum, difenacoum, bromadiolone, difethialone, and chlorophacinone.

Warfarin has a terminal half-life of 5.6 + 0.7 h with a mono-exponential decay (Breckenridge *et al.*, 1985). Brodifacoum and difenacoum have a bi-exponential decay of 60 ± 1.9 and 83 ± 10 h, respectively, in rabbits (Breckenridge *et al.*, 1985). Although an estimated median half-life of brodifacoum elimination has been estimated to be 2.4 days in 7 dogs (Robben *et al.*, 1998), the data may reflect the first elimination phase. Plasma half-life for brodifacoum was xxx in a zzz (Hollinger and Pastoor, 1993).

A 41-year-old man had a terminal half-life of 11.7 days for difenacoum after covert administration of an unknown amount of the drug (McCarthy *et al.*, 1997). A second individual had a peak serum concentration of 600 ng/ml of difenacoum (Butcher *et al.*, 1992).

A peak serum concentration of 440 μg/l then an elimination half-life of 140 h was reported in a 55-year-old man who ate an unknown amount of bromadiolone (Grobosch *et al.*, 2006). Elimination half-lives of 2.2 and 3.2 days have been reported in two dogs with difethialone exposure (Robben *et al.*, 1998). A half-live of 5 days is reported in sheep dosed with pindone (Robinson *et al.*, 2005).

### *Duration*

Despite reported elimination half-lives, the duration of anticoagulant effect provides an indication of the clinically relevant treatment times. Brodifacoum and difenacoum cases seem to have the longest duration of anticoagulant effect in animals and humans.

Rabbits are anticoagulated 6 weeks after oral exposure to 1 mg/kg body wt. brodifacoum (Park and Leck, 1982). Although clotting times were prolonged for 7 months after a person's exposure to an unknown amount of difenacoum (McCarthy *et al.*, 1997), elevated concentrations of vitamin $K_1$ 2,3-epoxide were detected for 18 months after exposure of two factory workers to brodifacoum and difenacoum despite normal PTs (Park *et al.*, 1986).

Duration of treatment has also been reported for bromadiolone and chlorophacinone cases, and pindone-dosed sheep. A patient exposed to bromadiolone had to be treated for 6 months (Haug *et al.*, 1992). An 18-year-old woman's PT normalized 7 weeks after deliberate ingestion of 100 mg chlorophacinone (Vogel *et al.*, 1988). Pindone has been detected for up to 14 days, in blood, 17 days in fat, and 39 days in liver of sheep dosed with it (Robinson *et al.*, 2005).

## Toxicity

### *Occurrence*

Anticoagulant rodenticides are the most common rodenticide exposure of dogs (Murphy and Hornfeldt) and the most common toxin seen in many US veterinary practices (Beasley and Trammel, 1989). Dogs suspected of anticoagulant rodenticide poisoning in the Netherlands had brodifacoum (19), bromadiolone (14), difenacoum (8), difethialone (6), and chlorophacinone (1) (Robben *et al.*, 1997). Assessment of potential toxicity of pindone for domestic animals has also been made (Martin *et al.*, 1991).

### *Dose*

Humans: A 25-year-old man attempted suicide by eating four 42-g boxes of 0.005% brodifacoum bait and succeeded in developing a coagulopathy (Kruse and Carlson, 1992). A 33-year-old man ate 1875 mg of chlorophacinone (Lagrange *et al.*, 1999) and an 18-year-old female deliberately ingested 100 mg chlorophacinone (Vogel *et al.*, 1988) and became anticoagulated. Sheep dosed orally with 10, 3, or 2 mg/kg body wt. pindone also developed coagulopathies (Robinson *et al.*, 2005). Coumatetralyl poisoning has been reported in pigs (Dobson, 1973) and pest species (Rowe and Redfern, 1968; Greaves and Ayres, 1969).

Rodenticides may be more toxic when repeatedly ingested over several days than when an equal amount is consumed in a single feeding (Dorman, 1990). Susceptibility may be greater in hypoprothrombinemic juveniles or animals with malabsorption syndromes. Also the concurrent administration of highly protein-bound drugs, e.g. phenylbutazone, aspirin, or disease states, such as chronic renal disease may increase the susceptibility of individuals to anticoagulant rodenticide poisoning (Beasley and Buck, 1983; Mount *et al.*, 1985).

## Diagnosis

### *History*

A clinical diagnosis of anticoagulant rodenticide poisoning is most often dependent on a history of exposure, or clinical signs, evidence of a coagulopathy, and response to vitamin $K_1$ therapy. The most pragmatic approach for determining the specific anticoagulant rodenticide involved is to read the product package. This approach alone is not definitive because as much as 25% of anticoagulant rodenticide intoxicated dogs do not have the anticoagulant in serum the owners suspect (Murphy).

Most anticoagulant rodenticide toxicoses is accidental, however some is intentional (Mack, 1994) in both humans and animals. Intent may become a legal issue in malicious

poisoning cases. Spontaneous hemorrhage after accidental brodifacoum exposure in a child is known to occur rarely (Watts *et al.*, 1990; Travis *et al.*, 1993). Iatrogenic cases in adults occur with more frequency (Weitzel *et al.*, 1990; Waien *et al.*, 2001; Walker and Beach, 2002) and combinations of anticoagulant rodenticides and glass (Tsutaoka *et al.*, 2003) or ethylene glycol (Seidelmann *et al.*, 1995). Human bromadiolone toxicosis has recently been reported in China (Shi *et al.*, 2005) and is known elsewhere (Shanberge, 1988). Consequently, a history of exposure or lack thereof, is not always present or reliable.

### *Clinical signs*

A history of exposure to anticoagulant rodenticides is not always available, so the toxicity must be inferred from the clinical signs. Clinical signs have been reported in a number of human and animal cases. Humans have dosed themselves or their children (Babcock *et al.*, 1993). Most clinical signs are related to the coagulopathy. Animals exposed to toxic doses of anticoagulant rodenticides remain asymptomatic until depletion of the active clotting factors occur. Therefore, clinical signs generally do not develop until 1–2 days (Dorman, 1990) or 3 to 5 days (Murphy and Gerken, 1989) postingestion.

A 55-year-old man had red sputum after ingesting an unknown amount of bromadiolone (Grobosch *et al.*, 2006). Epistaxis, gingival bleeding, widespread bruising, hematomas, hematuria, menorrhea, GI bleeding, rectal bleeding, and hemorrhage into any body organ and anemia are reported (Watt *et al.*, 2005). A 36-year-old woman had abdominal pain, hematuria, and red blood in her stool with diffuse cutaneous hematomas after ingesting difenacoum for several weeks. She had hemoperitoneum, and urethral hematoma on tomography (Barlow *et al.*, 1982; Berry *et al.*, 2000; Terneu *et al.*, 2003), and diffuse alveolar hemorrhage (Barnett *et al.*, 1992).

A 51-year-old woman who later admitted chronic ingestion of difenacoum was admitted with hemoperitoneum and intramural hematoma of the small intestine (Soubiron *et al.*, 2000). Epistaxis, hematoma, purpuria, and bruising have also been observed in a 41-year-old man with difenacoum (McCarthy *et al.*, 1997). Frank hematuria (Butcher *et al.*, 1992), neck pain, and cervical hematoma have also been observed in humans with diphenacoum exposure (Nighoghossian *et al.*, 1990). Acquired coagulopathy has been reported due to anticoagulant rodenticide exposure (Humphry, 1989; Huic *et al.*, 2002).

Human pleural, pericardial, mediastinal, and subarachnoid hemorrhages are reported with brodifacoum exposure (Kruse and Carlson, 1992). Cases of chlorophacinone poisoning in humans have also been reported (Murdoch, 1983; Dusein *et al.*, 1984). Hemoperitoneum from brodifacoum overdose has been observed (Morgan *et al.*, 1996). A number of other cases of brodifacoum (Braithwaite, 1982; Corke, 1997; Stanziale *et al.*, 1997; Casner, 1998; Bruno *et al.*, 2000), bromadiolone (Chow *et al.*, 1992), and other anticoagulant rodenticide exposure are present in the human literature (Ross *et al.*, 1992).

Clinical signs in animals are largely from canine cases (Woody *et al.*, 1992). Sometimes the only clinical signs in anticoagulant poisoned animals are dyspnea, lethargy, or anorexia but more often depression, weakness, pallor, and ventral hematomas are present (DuVall *et al.*, 1989). In addition, pulmonary edema, pleural effusion, pericardial effusion (Schulman *et al.*, 1986), intratracheal hemorrhage (McGuire *et al.*, 1999), thymic hemorrhage (Elsinghorst, 2003), laryngeal obstruction (Peterson and Streeter, 1996), pericardial effusion, and cardiac tamponade (Petrus and Henik, 1999), renal subcapsular hemorrhage (Radi and Thompson, 2004), and hematometra (Padgett *et al.*, 1998) have been reported.

Additional cases of diphacinone (Schulman *et al.*, 1986; Troy, 1988) and brodifacoum toxicosis (McSporran and Phillips, 1983; Booth, 1989; Grayson, 1982; Baker *et al.*, 2002) in dogs are reported. Horses have been exposed experimentally (Boermans *et al.*, 1991) and in the field (McConnico *et al.*, 1997) to these rodenticides. Brodifacoum may have been observed in neonatal puppies (Munday and Thompson, 2003) and has been successfully treated in a pregnant bitch (Hornfeldt and Phearman, 1996).

In clinical settings, prolonged bleeding from injection sites is usually noticed. A few clinical signs are reported that are not directly attributable to the coagulopathy. A paradoxical venous thrombosis was reported in person with chlorophacinone exposure (Papin *et al.*, 2006). Death, reduced breeding performance, stillborn, and non-viable lambs, as well as reduced sperm motility of rams is reported in sheep dosed with pindone (Robinson *et al.*, 2005).

### *Coagulopathy*

Evidence of a coagulopathy is the second element of the diagnosis. Coagulation tests are normally run on live animals and a necropsy on dead animals to support the presence of a coagulopathy. The basic mechanisms of clotting have been reviewed (Seegers, 1969).

Hematology: The anticoagulant rodenticides reduce activity of factors II, VII, IX, and X in circulation. The one-stage prothrombin time (OSPT) for evaluating factor VII is the most sensitive tool for early diagnosis because factor VII has the shortest half-life of the vitamin $K_1$ dependent clotting factors, namely about 6.2 h in dogs. Activated partial thromboplastin time (APTT) tests for all coagulation factors except factor VII, are usually used in conjunction with OSPT. Activated coagulation time (ACT) is used in the same way as the APTT. ACT is easiest to use in a clinic setting since it only requires

diatomaceous earth tubes and a heater block or water bath (Byrne, 1970).

Laboratory test results of abnormal prolonged OSPT, APTT, and ACT in presence of normal thrombin time (TT), fibrinogen, circulating fibrin degradation products (FDPs), and platelet counts is consistent with anticoagulant rodenticide poisoning. However, animals with severe anemia may have elevated FDPs and reduced platelet counts.

The diagnostic protocol based on these coagulation factor evaluation tests (OSPT, APTT, ACT) cannot differentiate between short- and long-acting anticoagulant rodenticide poisoning. The ability to recognize long-acting anticoagulants is critical, since therapeutic success may be based on the duration of vitamin $K_1$ treatment. The ability to identify the specific anticoagulant rodenticide involved using analytical chemistry is discussed below.

Coagulation testing is not always indicated after minor exposure. Of 110 children ingesting anticoagulant rodenticides, 8 had prolonged PTs. Seventeen percent (6 of 34) were prolonged 48 h after exposure while only 1.9% (2 of 104) at 24 h after exposure. 24 and 48 h after exposure is recommended time to check for PTs in children exposed (Smolinske *et al.*, 1989). Routine measurement of the INR is unnecessary in young children because the amounts ingested are invariably small (Watt *et al.*, 2005).

Although some bias in pediatric brodifacoum exposure data (Osterhoudt and Henretig, 2003) is suspected, prospective studies of acute, unintentional, pediatric superwarfarin ingestions managed without (Ingels *et al.*, 2002) or with (Smolinske *et al.*, 1989) decontamination have been conducted. Some argue against the need for a PT in unintentional pediatric superwarfarin exposures (Mullins *et al.*, 2000). See also cases of acute pediatric brodifacoum ingestions (Shepherd *et al.*, 2002). Although less treatment is often better (Kanabar, 2002) for accidental pediatric brodifacoum exposures, some pediatric cases have coagulopathies despite early treatment (Montanio *et al.*, 1993).

In all other cases, the INR should be measured 36–48 h post exposure. If the INR is normal at this time, even in the case of long-acting formulations, no further action is required (Watt *et al.*, 2005).

The most significant elevation of PT, partial thromboplastin time (PTT) and proteins induced by vitamin K antagonism (PIVKA) was observed 72 h after a single dose of diphacinone of 2 mg/kg in ground squirrels (*Spermophilus beecheyi*) although elevations were seen at 24 h (Whisson and Salmon, 2002). The increase in ACT, APTT, and OSPT are often used to support a clinical diagnosis of anticoagulant rodenticide poisoning in dogs (Dorman, 1990). Coagulation factor synthesis may be inhibited for up to 30 days in diphacinone-exposed dogs (Mount and Feldman, 1983).

PT should be checked 48 h after stopping vitamin $K_1$ therapy to detect any recurrence of coagulopathy (Chataigner *et al.*, 1989). PT times in sheep were stable in samples stored at 0, 20, and 30 degrees for 24 h (Shlosberg and Egyed, 1985).

Vitamin-K-dependent factor activity has been suggested for rapid identification of surreptitious brodifacoum poisoning (Miller *et al.*, 2006).

#### ***Necropsy/autopsy***

Pleural, pericardial, mediastinal, and subarachnoid hemorrhages have been reported in humans with brodifacoum exposure (Kruse and Carlson, 1992).

Hemoperitoneum, hemothorax, and pulmonary hemorrhage are the most common necropsy findings in dogs and cats with anticoagulant rodenticide residues in liver (DuVall *et al.*, 1989). Pulmonary edema, pleural effusion, pericardial effusion (Schulman *et al.*, 1986), and intratracheal hemorrhage are reported as well (McGuire *et al.*, 1999).

Many cases of anticoagulant poisoning are subacute in nature, but sudden death may occur as the result of acute internal hemorrhage. Evidence of external hemorrhage, such as melena, epistaxis, hematemesis, hematuria, gingival bleeding, or excessive bleeding from an open wound, may or may not be seen. However, internal hemorrhage involving the lungs, pleural and or peritoneal cavities, and facial planes are commonly reported (Murphy and Gerken, 1989; DuVall *et al.*, 1989). Massive tracheal and esophageal hemorrhage 2 days after bait ingestion is reported in a dog (Stowe *et al.*, 1983). In New Zealand, where brodifacoum was used in wild rabbit population control, 43 rabbits carcasses were found with massive hemorrhage in the abdominal cavity (52%), thoracic cavity (17%), and the remaining 31% of cases were found with hemorrhage of muscles, cecum, stomach, kidney, mesentery, and placenta of pregnant does (Rammell *et al.*, 1984). Postmortem findings in warfarin poisoning have been described by Dakin (1968).

##### *Response to vitamin $K_1$ treatment*

Remission of the coagulopathy 24 h after vitamin $K_1$ treatment supports a clinical diagnosis of a vitamin $K_1$ responsive coagulopathy (Tvedten, 1989). A clinical diagnosis is not sufficient in all cases. Some cases require that an etiological diagnosis be made. Analytical chemistry testing is required in such cases.

#### ***Analytical chemistry***

A number of analytical methods have been reported for detecting anticoagulant rodenticides in various matrices. Fluorimetric and GC methods have been commonly used to detect warfarin in serum (O'Reilly *et al.*, 1962; Corn and Berberich 1967; Lewis *et al.*, 1970; Welling *et al.*, 1970; Mildha *et al.*, 1974; Vessell and Shivley, 1974; Fasco *et al.*, 1977; Hanna *et al.*, 1978; Lee *et al.*, 1981).

Warfarin-specific methods were generally not adequate for the anticoagulant rodenticides, so a number of other methods were developed. These include thin layer chromatography (TLC), HPLC, mass spectroscopy (MS), and antibody-mediated tests. Coumarin anticoagulant rodenticides were initially detected using TLC (Lau-Cam and Chu-Fong, 1972; Mallet *et al.*, 1973). A high-performance TLC method with an estimated detection limit of 200 ppb and 87% recovery from liver has recently been reported (Berny *et al.*, 1995).

Early HPLC methods were focused on an individual chemical. For example, methods to detect chlorophacinone in formulations (Grant and Pike, 1979; Vigh *et al.*, 1981), brodifacoum in serum (Murphy and Gerken, 1989), brodifacoum (Koubek *et al.*, 1979; Keiboom and Rammel, 1981; Hoogenboom and Rammell, 1983; Ray, *et al.*, 1989), bromadiolone (Hunter *et al.*, 1988; Subbiah *et al.*, 2005), chlorophacinone (Hunter *et al.*, 1984), difethiolone (Goldade *et al.*, 1998), and difenacoum (Mundy and Machin, 1977) in tissue have been reported. Then a method was developed to look for all the anticoagulant rodenticides on the market at a time. It succeeded in extracting and detecting eight anticoagulant rodenticides in serum and liver using fluorescence and ultraviolet (UV) detection. Samples are extracted with acetonitrile then cleaned up on solid phase columns. Four hydroxycoumarins are detected by fluorescence with excitation at 318 nm and emission at 390 nm. The indanediones are detected at 285 nm. An extraction recovery of 75% for serum and 69% from liver was reported. Hydroxycoumarins may be detected down to about 1 ng/ml of serum and 1 ng/g of liver, and indanediones down to 10 ng/ml of serum and 10 ng/g of liver (Felice and Murphy, 1989; Felice *et al.*, 1991; Chalermchaikit *et al.*, 1993). Another HPLC method for detecting brodifacoum in serum and liver using difenacoum as the internal standard has been reported (O'Bryan and Constable, 1991). Method for simultaneous detection of five superwarfarin rodenticides in human serum has been described by Kuijpers *et al.* (1995). Other serum methods have been reported with detection limits of 3–12 ng/ml for fluorescence and 20–75 ng/ml for UV detection (Mura *et al.*, 1992; Kuijpers *et al.*, 1995; McCarthy *et al.*, 1997; Feng *et al.*, 1999).

Tissue methods include a solid phase cartridge extraction from liver with a recoveries ranging from 52% for difenacoum to 78% for warfarin. The limit of detection is 10 ppb for warfarin and difenacoum, and 110 ppb for chlorophacinone (Jones, 1996; Fauconnet *et al.*, 1997; Addison, 1982).

HPLC methods have also been published to distinguish cis and trans isomers of difenacoum with detection limits of 5 ng/ml (Kelly *et al.*, 1993). An early interesting approach was using a post-column pH shift to enhance fluorescence detection (Hunter, 1985; Hunter *et al.*, 1988). Several HPLC methods have also bee reported (AOAC, 1976a, b; Mundy and Machin, 1982; Hunter 1983a, b) for diphacinone (Bullard *et al.*, 1975, 1976) with fluorescence detector for bromadiolone (Deepa and Mishra, 2005), brodifacoum (Fu *et al.*, 2006), brodifacoum in tissues (Hagenboom and Rammell, 1983), difenacoum (Hadler and Shadbolt, 1975), and determination of Rozol in parafinized formulations (Kawano and Chang, 1980), and bromadiolone in tissues (Nahas, 1986).

A recent method uses diode array detector (Yang *et al.*, 2001). An interesting new method uses HPLC to detect anticoagulant rodenticides in soft drinks (Dimuccio *et al.*, 1991).

An ion pair liquid chromatography method has been reported to detect chlorophacinone and diphacinone in oats (Primus *et al.*, 1998).

Contemporary confirmatory methods use MS. Most recently liquid chromatography–electrospray ionization–mass spectroscopy (LC-ESI-MS) has been reported for the analysis of 10 anticoagulant rodenticides with a limit of quantitation of about 5 ug/l (Grobosch *et al.*, 2006). Other recent methods use LC MS-MS for unknown drugs including warfarin (Marquet *et al.*, 2003) and LC-ESI-MS and HPLC-UV to detect anticoagulant rodenticides as low as 20 ng on column (Mesmer and Flurer, 2000). One of the earlier MS methods used a direct probe technique to detect indanedione residues in food animals (Braselton *et al.*, 1992).

A cell culture/ELISA assay has recently been developed to detect anticoagulant rodenticides in treated grain (Lawley *et al.*, 2006). A prior immunoassay was developed to detect diphacinone and chlorophacinone (Mount *et al.*, 1988). Enantiomers of warfarin, coumachlor, and coumafuryl can be separated chromatographically (Armstrong *et al.*, 1993).

Serum concentration of dogs with anticoagulant rodenticide poisoning ranged from less than 10–851 ng/l for brodifacoum, difethialone, and difenacoum (Robben *et al.*, 1998).

## Treatment

General, supportive, and specific treatment areas are available for anticoagulant rodenticide toxicosis. Prospective study of the outcome of patients with excessive warfarin exposure is described by Hylek *et al.* (2000).

### *General*

Emetic, adsorbent, and cathartic therapies are indicated if the ingestion of the anticoagulant rodenticide has occurred within the last few hours. Peak serum concentrations of brodifacoum occur 2 h after oral dosing, however so the coagulation status of the animal should be monitored at 24 and 48 h after exposure.

Clinical coagulopathy normally occurs 2–5 days after oral exposure, so emetics and cathartic at the time of

presentation are not normally indicated. Oral-activated charcoal therapy however, may be useful for those chemicals that undergo enterohepatic circulation.

### *Supportive*

Recommendations for humans with anticoagulant rodenticide toxicosis have recently been reported by Watt *et al.* (2005). If active bleeding occurs, prothrombin complex concentrate (which contains factors II, VII, IX, and X) 50 units/kg, or recombinant-activated factor VII 1.2–4.8 mg or fresh frozen plasma 15 ml/kg if no concentrate is available, and phytomenadione 10 mg intravenous (i.v.) (100 μg/kg body wt. in a child) should be given. If there is no active bleeding and the INR is $<$ or $=4.0$, no treatment is required; if INR is $>$ or $=4.0$ phytomenadione 10 mg should be administered i.v. (Watt *et al.*, 2005).

Animals with severe clinical bleeding or markedly reduced packed cell volumes (PCVs) should receive fresh plasma or blood transfusions because of the 4–8 h often required to increase clotting factor activity following vitamin $K_1$ therapy (Chalermchaikit *et al.*, 1993). These recommendation are consistent with those of others (McCarthy *et al.*, 1997; Soubiron *et al.*, 2000; Terneu *et al.*, 2003).

### *Specific*

Vitamin $K_1$ therapy is recommended in humans (McCarthy *et al.*, 1997; Soubiron *et al.*, 2000; Terneu *et al.*, 2003) and animals with elevated coagulation times after exposure to anticoagulant rodenticides (Murphy *et al.*, 1989; Robben *et al.*, 1998; Mount *et al.*, 1982). Vitamin $K_1$ (phylloquinone) is the most effective form for the treatment of anticoagulant rodenticide intoxication because of its immediate availability for the synthesis of new clotting factors (Chalermchaikit *et al.*, 1993).

The pharmacological half-live of vitamin $K_1$ is $1.7 + 0.1$ h in rabbits dosed with brodifacoum (Park and Leck, 1982). Prothrombin activity reaches peak improvement 4 h after administration of vitamin $K_1$ to rabbits anticoagulated with brodifacoum or difenacoum (Park and Leck, 1982).

The duration of vitamin $K_1$ treatment is prolonged (Butcher *et al.*, 1992) for the longer-acting chemicals. It may be required for up to 2 weeks in diphacinone-exposed dogs (Mount and Feldman, 1983). Daily doses of vitamin $K_1$ in the range of 0.25–2.5 mg/kg for 1 week are recommended for exposure to short-acting rodenticides, and 2.5–5.0 mg/kg for 3–4 weeks is recommended for exposure to long-acting rodenticides (Mount *et al.*, 1985). Prolonged anticoagulation in rat poisoning has been reported by Jones *et al.* (1984) and Lipton and Klass (1984).

The two most commonly recommended routes of vitamin $K_1$ administration are oral and subcutaneous. Intramuscular injections in a hypoprothrombinemic animal can produce hematoma and i.v. administration of vitamin $K_1$ has been associated with anaphylaxis, therefore these routes are discouraged in the therapeutic regimen (Clark and Halliwell, 1963).

Vitamin $K_1$ therapy may be reduced in a step-wise manner as long as the PT remains normal (Robben *et al.*, 1998). The length of treatment is presently decided by evaluation of OSPT values for 2 days after the cessation of vitamin $K_1$ administration. If prolonged OPST is found, treatment is commonly continued for another week, but if it remains normal for 5–6 days, the vitamin $K_1$ treatment is usually terminated (Murphy *et al.*, 1989).

### *Diagnosis*

Diagnostic approach to the bleeding patient has been described by Johnstone (1989). A diagnostic protocol should utilize more than one coagulation test, since it is necessary to differentiate rodenticide poisoning from other coagulopathies, such as disseminated intravascular coagulopathy, congenital factor deficiencies, hyperviscosity syndromes, platelet deficiencies or functional defects, von Willebrand's disease, and canine ehrlichiosis. Hypovitaminosis K associated bleeding has been reported in cats with malabsorption syndrome (Edwards and Russell, 1987).

## REFERENCES

Addison JB (1982) Improved method for HPLC determination of chlorophacinone in mouse tissue. *J Assoc Off Anal Chem* **65**: 1299–301.

Aisner J, Goutsou M, Maurer LH, Cooper R, Chahinian P, Carey R, Skarin A, Slawson R, Perry MC, Green MR (1992) Intensive combination chemotherapy, concurrent chest irradiation, and warfarin for the treatment of limited-disease small-cell lung cancer: a Cancer and Leukemia Group B pilot study. *J Clin Oncol* **10**(8): 1230–6.

Alonso Martin JJ, Duran Hernandez JM, Gimeno de Carlos F, de la Fuente Galan L, Munoz San Jose JC, Fernandez-Aviles F (1997) [Post-implant antithrombotic treatment after intracoronary stents. thrombotic occlusion]. *Rev Esp Cardiol* **50**(Suppl. 2): 31–43.

Andre C, Guyon C, Thomassin M, Barbier A, Richert L, Guillaume YC (2005) Association mechanism between a series of rodenticide and humic acid: a frontal analysis to support the biological data. *J Chromatogr B Analyt Technol Biomed Life Sci* **820**(1): 9–14.

AOAC (1976a) Determination of chlorophacinone by ultraviolet spectroscopy, Chlorophacinone EPA-1, May 1977, *Supplement to EPA Manual of Chemical Methods for Pesticides and Devices*, AOAC, Arlington, VA.

AOAC (1976b) Determination of diphacinone by high pressure liquid chromatography using paired-ion chromatography, Diphacinone EPA-2, May 1978, *Supplement to EPA Manual of Chemical Methods for Pesticides and Devices*, AOAC, Arlington, VA.

Appendino G, Mercalli E, Fuzzati N, Arnoldi L, Stavri M, Gibbons S, Ballero M, Maxia A (2004)Antimycobacterial coumarins from the sardinian giant fennel (Ferula communis). *J Nat Prod* **67**(12): 2108–10.

Aragno M, Tagliapietra S, Nano GM, Ugazio G (1988) Experimental studies on the toxicity of Ferula communis in the rat. *Res Commun Chem Pathol Pharmacol* **59**(3): 399–402.

Argento A, Tiraferri E, Marzaloni M (2000) [Oral anticoagulants and medicinal plants. An emerging interaction] *Ann Ital Med Int* **15**(2): 139–43. (in Italian).

Armour CJ, Barnett SA (1950) The action of dicoumarol on laboratory and wild rats and its effect on feeding behavior. *J Hyg* (*Cambridge*) **48**: 158–71.

Armstrong DW, Reid III GL, Hilton ML, Chang CD (1993) Relevance of enantiomeric separations in environmental science. *Environ Pollut* **79**(1): 51–8.

Arnoldi L, Ballero M, Fuzzati N, Maxia A, Mercalli E, Pagni L (2004) HPLC-DAD-MS identification of bioactive secondary metabolites from Ferula communis roots. *Fitoterapia* **75**(3–4): 342–54.

Asperger Z, Jursic M (1970) [Prolonged administration of anticoagulants after myocardial infarct] *Lijec Vjesn* **92**(3): 369–74. (in Croatian).

Atterby H, Kerins GM, MacNicoll AD (2005) Whole-carcass residues of the rodenticide difenacoum in anticoagulant-resistant and susceptible rat strains (*Rattus norvegicus*). *Environ Toxicol Chem* **24**(2): 318–23.

Babcock J, Hartman K, Pedersen A, Murphy M, Alving B (1993) Rodenticide-induced coagulopathy in a young child. A case of Munchausen syndrome by proxy. *Am J Pediatr Hematol Oncol* **15**(1): 126–30.

Bachmann KA, Sullivan TJ (1983) Disposition and pharmacodynamic characteristics of brodifacoum in warfarin-sensitive rats. *Pharmacology* **27**: 281–8.

Badia R, Diaz-Garcia ME (1999)Cyclodextrin-based optosensor for the determination of warfarin in waters. *J Agric Food Chem* **47**(10): 4256–60.

Baker JT, Graversen CH, Files JE (2002) Brodifacoum toxicity. *J Miss State Med Assoc* **43**(4): 106–7.

Balasubramanyam M, Christopher MJ, Purushotham KR (1984) Laboratory trials of three anticoagulant rodenticides for use against the Indian field mouse, Mus booduga Gray. *J Hyg (London)* **93**(3): 575–8.

Bamanikar A, Hiremath S (2002) Hepatotoxic reaction to warfarin in a recovering hepatitis patient with hypoalbuminenia. *J Assoc Phys India* **50**: 1456.

Barber DL, Colvin Jr HW (1980) Influence of dietary protein on the response of rats receiving toxic levels of warfarin. *Toxicol Appl Pharmacol* **56**(1): 8–15.

Barlow AM, Gay AL, Park BK (1982) Difenacoum (Neosorexa) poisoning. *Br Med J (Clin Res Ed)* **285**(6341): 541.

Barnett VT, Bergmann F, Humphrey H, Chediak J (1992) Diffuse alveolar hemorrhage secondary to superwarfarin ingestion. *Chest* **102**(4): 1301–2.

Baskaran J, Kanakasabai R, Neelanarayanan P (1995) Evaluation of two rodenticides in the paddy fields during Samba and Thaladi seasons. *Indian J Exp Biol* **33**(2): 113–21.

Beasley VR, Buck WB (1983) Warfarin and other anticoagulant poisoning. In *Current Veterinary Therapy VIII*, Kirk RW (ed.). WB Saunders, Philadelphia, PA, pp. 101–6.

Beasley VR, Trammel HL (1989)Incidence of poisoning in small animals. In *Current Veterinary Therapy X*, Kirk RW (ed.). WB Saunders, Philadelphia, PA, pp. 97–113.

Bechtold H, Jahnchen E (1979) Quantitative analysis of vitamin K1 and vitamin K1 2,3 epoxide in plasma by electron capture gas–liquid chromatography. *J Chromatogr* **164**: 85–90.

Bechtold H, Trenk D, Jahnchen E, Meinertz T (1983) Plasma vitamin K12,3-epoxide as diagnostic aid to detect surreptitious ingestion of oral anticoagulant drugs. *Lancet* **1**: 596–7.

Bell RG, Caldwell PT (1973) Mechanism of warfarin resistance: warfarin and the metabolism of vitamin K1. *Biochemistry* **12**: 1759–62.

Bell RG, Caldwell PT, Holm EE (1976) Coumarins and the vitamin K–K epoxide cycle. Lack of resistance to coumatetralyl in warfarin-resistant rats. *Biochem Pharmacol* **25**(9): 1067–70.

Berny PJ, Buronfosse T, Lorgue G (1995) Anticoagulant poisoning in animals: a simple new high-performance thin-layer chromatographic (HPTLC) method for the simultaneous determination of eight anticoagulant rodenticides in liver samples. *J Anal Toxicol* **19**(7): 576–80.

Berny PJ, Buronfosse T, Buronfosse F, Lamarque F, Lorgue G (1997) Field evidence of secondary poisoning of foxes (*Vulpes vulpes*) and buzzards (*Buteo buteo*) by bromadiolone, a 4-year survey. *Chemosphere* **35**(8): 1817–29.

Berny PJ, de Oliveira LA, Videmann B, Rossi S (2006) Assessment of ruminal degradation, oral bioavailability, and toxic effects of anticoagulant rodenticides in sheep. *Am J Vet Res* **67**(2): 363–71.

Berry RG, Morrison JA, Watts JW, Anagnost JW, Gonzalez JJ (2000) Surreptitious superwarfarin ingestion with brodifacoum. *South Med J* **93**(1): 74–5.

Beyer RE (1994) The relative essentiality of the antioxidative function of coenzyme Q – the interactive role of DT-diaphorase. *Mol Aspects Med* **15**(Suppl.): 117–29.

Biagini CP, Boissel E, Borde F, Bender VE, Bouskila M, Blazy F, Nicaise L, Mignot A, Cassio D, Chevalier S (2006) Investigation of the hepatotoxicity profile of chemical entities using Liverbeads (R) and WIF-B9 *in vitro* models. *Toxicol In Vitro* **20**(6): 1051–9.

Bint AJ, Burtt I (1980) Adverse antibiotic drug interactions. *Drugs* **20**(1): 57–68.

Bjornsson TD, Swezey SE, Meffin RJ, Swezey SE, Blascke TF (1978) Quantitation of radiolabeled vitamin K and vitamin K epoxide in plasma by high pressure liquid chromatography. *Thromb Hemost*, **39**: 466–73.

Bjornsson TD, Meffin RJ, Swezey SE, Blascke TF (1979) Effects of clofibrate and warfarin alone and in combination on the disposition of vitamin K1. *J Pharmacol Exp Ther* **210**: 322–6.

Blus LJ, Henny CJ, Grove RA (1985) Effects of pelletized anticoagulant rodenticides on California quail. *J Wildl Dis* **21**(4): 391–5.

Bocca C, Gabriel L, Bozzo F, Miglietta A (2002) Microtubule-interacting activity and cytotoxicity of the prenylated coumarin ferulenol. *Planta Med* **68**(12): 1135–7.

Boermans HJ, Johnstone I, Black WD, Murphy M (1991) Clinical signs, laboratory changes and toxicokinetics of brodifacoum in the horse. *Can J Vet Res* **55**(1): 21–7.

Booth K (1989) Brodifacoum poisoning in a dog. *NZ Vet J* **37**(2): 74–5.

Bradfield AA, Gill JE (1984) Laboratory trials of five rodenticides for the control of Mesocricetus auratus Waterhouse. *J Hyg (London)* **93**(2): 389–94.

Braithwaite GB (1982) Vitamin K and brodifacoum. *J Am Vet Med Assoc* **181**(6): 531–4.

Brandon EF, Meijerman I, Klijn JS, den Arend D, Sparidans RW, Lazaro LL, Beijnen JH, Schellens JH (2005) *In-vitro* cytotoxicity of ET-743 (Trabectedin, Yondelis), a marine anti-cancer drug, in the Hep G2 cell line: influence of cytochrome P450 and phase II inhibition, and cytochrome P450 induction. *Anticancer Drugs* **16**(9): 935–43.

Braselton Jr WE, Neiger RD, Poppenga RH (1992) Confirmation of indandione rodenticide toxicoses by mass spectrometry/mass spectrometry. *J Vet Diagn Invest* **4**(4): 441–6.

Breckenridge AM, Cholerton S, Hart JA, Park BK, Scott AK (1985) A study of the relationship between the pharmacokinetics and the pharmacodynamics of the 4-hydroxycoumarin anticoagulants warfarin, difenacoum and brodifacoum in the rabbit. *Br J Pharmacol* **84**(1): 81–91.

Brooks JE, Htun PT, Naing H (1980) The susceptibility of *Bandicota bengalensis* from Rangoon, Burma to several anticoagulant rodenticides. *J Hyg (London)* **84**(1): 127–35.

Bruno GR, Howland MA, McMeeking A, Hoffman RS (2000) Long-acting anticoagulant overdose: brodifacoum kinetics and optimal vitamin K dosing. *Ann Emerg Med* **36**(3): 262–7.

Buckle AP (1986) Field trials of flocoumafen against warfarin-resistant infestations of the Norway rat (*Rattus norvegicus* Berk.). *J Hyg (London)* **96**(3): 467–73.

Bullard RW, Holguin G, Peterson JE (1975) Determination of chlorophacinone and diphenadione residues in biological materials. *J Agric Food Chem* **23**(1): 72–4.

Bullard RW, Thompson RD, Holguin G (1976) Diphacinone residues in tissues of cattle. *J Agric Food Chem* **24**: 261–3.

Burns M (1999) Management of narrow therapeutic index drugs. *J Thromb Thrombol* **7**(2): 137–43.

Burns RJ, Bullard RW (1979) Diphacinone residue from whole bodies of vampire bats: a laboratory study *Bull Pan Am Health Organ* **13**(4): 365–9.

Burns RJ, Bullard RW (1980) Residues of diphacinone in cadavers of vampire bats: a laboratory study. *Bol Oficina Sanit Panam* **88**(5): 396–401 (in Spanish).

Burocoa Ch, Mura P, Robert R, Boinot C, Bouset S, Piriou A (1989) Chlorophacinone intoxication, a biological and toxicological study. *Clin Toxicol* **27**: 78–89.

Butcher GP, Shearer MJ, MacNicoll AD, Kelly MJ, Ind PW (1992) Difenacoum poisoning as a cause of haematuria. *Hum Exp Toxicol* **11**(6): 553–4.

Byrne JJ (1970) Thrombophlebitis in pregnancy. *Clin Obstet Gynecol* **13**(2): 305–20.

Cadenas E (1995) Antioxidant and prooxidant functions of DT-diaphorase in quinone metabolism. *Biochem Pharmacol* **49**(2): 127–40.

Calnan JS, Allenby F (1975) The prevention of deep vein thrombosis after surgery. *Br J Anaesth* **47**(2): 151–60.

Cannava A (1958) Is 3-methyl-4-hydroxycoumarin the active principal to which we attribute the hypoprothrombinizing action of Ferula communis. *Boll Chim Farm* **97**(4): 207–12.

Carlisle DM, Blaschke TF (1981) Vitamin K1 epoxide and warfarin interrelationships in dog. *Biochem. Pharmacol* **30**: 2931–6.

Carta A (1951) Ferulosis; isolation of the substance with hypoprothrombinemizing action from the galbanum of Ferula communis. *Boll Soc Ital Biol Sper* **27**(5): 690–3.

Casner PR (1998) Superwarfarin toxicity. *Am J Ther* **2**: 117–20.

Chalermchaikit T, Felice LJ, Murphy MJ (1993) Simultaneous determination of eight anticoagulant rodenticides in blood serum and liver. *J Anal Toxicol* **17**(1): 56–61.

Chambers CM, Chambers PL (1983) Warfarin and the grey squirrel. *Arch Toxicol* Suppl. **6**: 214–21.

Channon D, Cole M, Cole L (2000) A long-term study of *Rattus norvegicus* in the London borough of Enfield using baiting returns as an indicator of sewer population levels. *Epidemiol Infect* **125**(2): 441–5.

Charles OW, Dilworth BC, Bushong Jr RD, Day EJ (1966) The effect of dicumarol, diphacinone and pivalyl upon blood prothrombin time of chicks. *Poult Sci* **45**(2): 387–93.

Chataigner D, Garnier R, Elmalem J, Efthymiou ML (1989) Prolonged hypocoagulability following the ingestion of anticoagulant raticides. *Ann Med Interne (Paris)* **139**(8): 537–41. French.

Chong LL, Chau WK, Ho CH (1986) A case of 'superwarfarin' poisoning. *Scand J Haematol* **36**(3): 314–5.

Chopra G, Parshad VR (1985) Evaluation of coumatetralyl against two predominant murid species. *J Hyg (London)* **94**(3): 327–30.

Chow EY, Haley LP, Vickars LM, Murphy MJ (1992) A case of bromadiolone (superwarfarin) ingestion. *CMAJ* **147**(1): 60–2.

Chua JD, Friedenberg WR (1998) Superwarfarin poisoning. *Arch Intern Med*. **158**(17): 1929–32.

Clark WT, Halliwell REW (1963) The treatment with vitamin K preparation of warfarin poisoning in dogs. *Vet Rec* **75**: 1210–3.

Colvin Jr HW, Wang WL (1974) Toxic effects of warfarin in rats fed different diets. *Toxicol Appl Pharmacol* **28**(3): 337–48.

Corke PJ (1997) Superwarfarin (brodifacoum) poisoning. *Anaesth Intensive Care* **25**(6): 707–9.

Corn M, Berberich R (1967) Rapidfluorometric assay for plasma warfarin. *Clin Chem* **13**: 126–31.

Corticelli B, Deiana S (1957) Electrophoretic behavior of serous and plasmatic proteins of the rabbit poisoned by Ferula communis. *Boll Soc Ital Biol Sper* **33**(5): 625–8.

Corticelli B, Deiana S, Palmas G (1957) Protective and antihemorrhagic effects of vitamin K-1 in poisoning by *Ferula communis*. *Boll Soc Ital Biol Sper* **33**(5): 629–31.

Costa A (1950a) Hemorrhagic diathesis from juice of the roots of Ferula communis; behavior of the V factor of Owren. *Boll Soc Ital Biol Sper* **26**(7): 1043–4.

Costa A (1950b) Hemorrhagic diathesis from the juice of the roots of Ferula communis; behavior of the fibrinogen. *Boll Soc Ital Biol Sper* **26**(7): 1041–2.

Craciun AM, Groenen-van Dooren MM, Vermeer C (1997) Nutritional vitamin K-intake and urinary gamma-carboxyglutamate excretion in the rat. *Biochim Biophys Acta* **1334**(1): 44–50.

Craciun AM, Groenen-van Dooren MM, Thijssen HH, Vermeer C (1998) Induction of prothrombin synthesis by K-vitamins compared in vitamin K-deficient and in brodifacoum-treated rats. *Biochim Biophys Acta* **1380**(1): 75–81.

Creutzig A (1993) Thrombophlebitis – basic principles of treatment. *Z Kardiol* **82**(Suppl. 2): 41–7 (in German).

Dakin G (1968) Post-mortem toxicological findings in a case of warfarin poisoning. *Vet Rec* **83**(25): 664.

Dalla Volta S (1994) Valvular prosthesis: indications and updated protocols for thrombosis prophylaxis. *Cardiologia* **39**(12 Suppl. 1): 331–40 (in Italian).

Dam H (1935) The antihemorrhagic vitamin of the chick. *Biochem J* **29**: 1273–85.

Dayton PG, Perel JM (1971) Physiological and physicochemical bases of drug interactions in man. *Ann NY Acad Sci* **179**: 67–87.

Deepa S, Mishra AK (2005) Fluorescence spectroscopic study of serum albumin–bromadiolone interaction: fluorimetric determination of bromadiolone. *J Pharm Biomed Anal* **38**(3): 556–63.

Deobhankar PB (1985) Field evaluation of Brodifacoum against *Rattus rattus* and *Bandicota bengalensis* in Bombay. *J Commun Dis* **17**(2): 151–61.

Desideri D, Aldighieri R, Le Louet M, Tardieu A (1979) Murine resistance to anticoagulants in the port of Marseille Response to difenacoum. *Bull Soc Pathol Exot Filiales* **72**(3): 278–83 (in French).

Dimuccio A, Camoni I, Vergori L, Dommarco R, Attard Barbini D, Vergori F, Ausili A, Santilio A (1991) Screening for coumatetralyl in soft drinks by solid-matrix extraction and high-performance liquid chromatography with diode-array detection. *J Chromatogr* **553**(1–2): 305–9.

Dinkova-Kostova AT, Talalay P (2000) Persuasive evidence that quinone reductase type 1 (DT diaphorase) protects cells against the toxicity of electrophiles and reactive forms of oxygen. *Free Radic Biol Med* **29**(3–4): 231–40.

Dobson KJ (1973) Coumatetralyl poisoning in pigs and effectiveness of vitamin K1. *Aust Vet J* **49**(2): 98–100.

Dolin EK, Baker DL, Buck SC (2006) A 44-year-old woman with hematemesis and cutaneous hemorrhages as a result of superwarfarin poisoning. *J Am Osteopath Assoc* **106**(5): 280–4.

Donnahey PL, Burt VT, Rees HH, Pennock JF (1979) High performance liquid chromatography of menaquinone-4,
2,3-epoxyme-naquinone-4, demethyl/menaquinone4 and related compounds. *J Chromatogr* **170**: 272–7.

Dorman DC (1990) Anticoagulant, cholecalciferol, and bromethalin-based rodenticides. In Toxicology of selected pesticides, drugs, and chemicals. *Vet Clin N Am Small Anim Pract* **20**: 339–52.

Dusein P, Manigand G, Taillandier J (1984) Severe, prolonged hypoprothrombinemia following poisoning by chlorophacinone. *Presse Med* **13**(30): 1845 (in French).

DuVall MD, Murphy MJ, Ray AC, Reagor JC (1989) Case studies on second-generation anticoagulant rodenticide toxicities in nontarget species. *J Vet Diagn Invest* **1**(1): 66–68.

Duxbury BM, Poller L (2001) The oral anticoagulant saga: past, present, and future. *Clin Appl Thromb Hemost* **7**(4): 269–75.

Eason CT, Murphy EC, Wright GR, Spurr EB (2002) Assessment of risks of brodifacoum to non-target birds and mammals in New Zealand. *Ecotoxicology* **11**(1): 35–48.

Edwards DF, Russell RG (1987) Probable vitamin K-deficient bleeding in two cats with malabsorption syndrome secondary to lymphocytic-plasmacytic enteritis. *J Vet Intern Med* **1**: 97–101.

Ehrenforth S, Schenk JF, Scharrer I (1999) Liver damage induced by coumarin anticoagulants. *Semin Thromb Hemost* **25**(1): 79–83.

Elias DJ, Johns BE (1981) Response of rats to chronic ingestion of diphacinone. *Bull Environ Contam Toxicol* **27**(4): 559–67.

Elsinghorst TA (2003) First cases of animal diseases published since 2000. 1. *Dogs Vet Q* **25**(3): 112–23.

Exner DV, Brien WF, Murphy MJ (1992) Superwarfarin ingestion. *CMAJ* **146**(1): 34–5.

Fasco MJ, Piper lJ, Kaminsky LS (1977) Biochemical applications of a quantitative HPLC assay of warfarin and its metabolites. *J Chromatogr* **131**: 365–73.

Fauconnet V, Pouliquen H, Pinault L (1997) Reversed-phase HPLC determination of eight anticoagulant rodenticides in animal liver. *J Anal Toxicol* **21**(7): 548–53.

Felice LJ, Murphy MJ (1989) The determination of the anticoagulant rodenticide brodifacoum in blood serum by liquid chromatography with fluorescence detection. *J Anal Toxicol* **13**: 299–31.

Felice LJ, Chalermchaikit T, Murphy MJ (1991) Multicomponent determination of 4 hydroxycoumarin anticoagulant rodenticides in blood serum by liquid chromatography with fluorescence detection. *J Anal Toxicol* **15**: 126–9.

Feng SZ, Zhou HZ, Li YL, Wang FL, Sun J, Liu Y (1999) [SPE analysis of 4 rodenticides in whole blood and liver by HPLC]. *Fa Yi Xue Za Zhi* **15**(1): 21–2 (in Chinese).

Ferlito S (1996) Main antithrombotic drugs in the therapy and prevention of arterial and venous thrombosis. *Minerva Cardioangiol* **44**(6): 299–312 (in Italian).

Fieser LF, Campbell WP, Fry EM, Gates Jr MD (1939) Naphthoquinones of vitamin K1 type of structure. *J Am Chem Soc* **61**: 3216–23.

Finch CK, Chrisman CR, Baciewicz AM, Self TH (2002) Rifampin and rifabutin drug interactions: an update. *Arch Intern Med* **162**(9): 985–92.

Fournier-Chambrillon C, Berny PJ, Coiffier O, Barbedienne P, Dasse B, Delas G, Galineau H, Mazet A, Pouzenc P, Rosoux R, Fournier P (2004) Evidence of secondary poisoning of free-ranging riparian mustelids by anticoagulant rodenticides in France: implications for conservation of European mink (*Mustela lutreola*). *J Wildl Dis* **40**(4): 688–95.

Fraigui O, Lamnaouer D, Faouzi MY, Cherrah Y, Tijjane M (2001) Acute and chronic toxicity of fessoukh, the resinous gum of Ferula communis L, compared to warfarin. *Vet Hum Toxicol* **43**(6): 327–30.

Fraigui O, Lamnaouer D, Faouzi MY (2002) Acute toxicity of ferulenol, a 4-hydroxycoumarin isolated from *Ferula communis* L. *Vet Hum Toxicol* **44**(1): 5–7.

Fu ZH, Huang XX, Xiao HR (2006) [Determination of serum brodifacoum with high performance liquid chromatography]. *Zhonghua Lao Dong Wei Sheng Zhi Ye Bing Za Zhi* **24**(5): 295.

Gallo J (1998) Brodifacoum. *Anaesth Intens Care* **26**(6): 708–9.

Gil MC, Gomez L, Roy TJ, Prieto L, Pena FJ, Garcia L (2002) Testicular and epididymal changes in rams following intoxication by Ferula communis. *Vet Rec* **150**(1): 24–5.

Gill JE, Redfern R (1979) Laboratory test of seven rodenticides for the control of *Mastomys natalensis*. *J Hyg (London)* **83**(2): 345–52.

Gill JE, Redfern R (1980) Laboratory trials of seven rodenticides for use against the cotton rat (*Sigmodon hispidus*). *J Hyg (London)* **85**(3): 443–50.

Gill JE, Redfern R (1983) Laboratory tests of seven rodenticides for the control of *Meriones shawi*. *J Hyg (London)* **91**(2): 351–7.

Gill JE, Kerins GM, Langton SD, MacNicoll AD (1993) The development of a blood clotting response test for discriminating between difenacoum-resistant and susceptible Norway rats (*Rattus norvegicus*, Berk.). *Comp Biochem Physiol C* **104**(1): 29–36.

Giraudoux P, Tremollieres C, Barbier B, Defaut R, Rieffel D, Bernard N, Lucot E, Berny P (2006) Persistence of bromadiolone anticoagulant rodenticide in Arvicola terrestris populations after field control. *Environ Res* **102**(3): 291–8.

Goldade DA, Primus TM, Johnston JJ, Zapien DC (1998) Reversed-phase ion-pair high-performance liquid chromatographic quantitation of difethialone residues in whole-body rodents with solid-phase extraction cleanup. *J Agric Food Chem* **46**(2): 504–8.

Goldstein JA (2001) Clinical relevance of genetic polymorphisms in the human CYP2C subfamily. *Br J Clin Pharmacol* **52**(4): 349–55.

Grant RG Pike RK (1979) High pressure liquid chromatographic determination of chlorophacinone in formulations. *J Assoc Off Anal Chem* **62**: 1001–3.

Grayson JL (1982) Brodifacoum poisoning in a dog. *N Z Vet J*. **37**(4): 173; author reply 173.

Greaves JH, Ayres P (1969) Some rodenticidal properties of coumatetralyl. *J Hyg (London)* **67**(2):311–5.

Greaves JH, Rehman AB (1977) The susceptibility of *Tatera indica*, *Nesokia indica* and *Bandicota bengalensis* to three anticoagulant rodenticides. *J Hyg (London)* **78**(1): 75–84.

Greaves JH, Shepherd DS, Quy R (1982) Field trials of second-generation anticoagulants against difenacoum-resistant Norway rat populations. *J Hyg (London)* **89**(2): 295–301.

Greeff MC, Mashile O, MacDougall LG (1987) Superwarfarin (bromodialone) poisoning in two children resulting in prolonged anticoagulation. *Lancet* **2**(8570): 1269.

Grobosch T, Angelow B, Schonberg L, Lampe D (2006) Acute bromadiolone intoxication. *J Anal Toxicol* **30**(4): 281–6.

Guidry JR, Raschke RA, Morkunas AR (1991) Toxic effects of drugs used in the ICU. Anticoagulants and thrombolytics. Risks and benefits. *Crit Care Clin* **7**(3): 533–54.

Hadler M, Shadbolt RS (1975) Novel 4-hydroxycoumarin anticoagulants active against resistant rats. *Nature* **253**: 275–7.

Hagenboom JJL, Rammell CG (1983) Improved HPLC method for determining brodifacoum in animal tissues. *Bull Env Cont Tox* **31**: 239–44.

Hanna S, Rosen M, Eisenberger P, Rasero L, Lachman L (1978) GLC determination of warfarin in human plasma. *J Pharm Sci*. **67**: 84–6.

Haroon Y, Hauschka PV (1983) Application of high-performance liquid-chromatography to assay phylloquinone (vitamin K1) in rat liver. *J Lipid Res* **24**: 481–4.

Haroon Y, Shearer MJ, Barkhan P (1980) Resolution of phylloquinone (vitamin K1), phylloquinone 2,3-epoxide, 2-chloro-phylloquinone and their geometric isomers by high-performance liquid chromatography. *J Chromatogr* **200**: 293–9.

Haroon Y, Bacon DS, Sadowski JA (1986) Liquid-chromatography determination of vitamin K1 in plasma, with fluorometric detection. *Clin Chem* **32**: 1925–9.

Haroon Y, David SB, Sadowski JA (1987) Chemical reduction system for the detection of phylloquinone (vitamin K1) and menaquinones (vitamin K2). *J Chromatogr* **384**: 383–9.

Hart JAD, Haynes BP, Park BK (1984) A study of factors which determine the pharmacological response to vitamin K in coumarin anticoagulated rabbit. *Biochem Pharm* **33**: 3013–29.

Haug B, Schjodt-Iversen L, Rygh J (1992) Poisoning with long-acting anticoagulants. *Tidsskr Nor Laegeforen* **112**(15): 1958–60 (in Norwegian).

Hildebrandt EF, Suttie JW (1982) Mechanism of coumarin action: sensitivity of vitamin K metabolizing enzymes of normal and warfarin-resistant rat liver. *Biochemistry* **21**(10): 2406–11.

Hohler T, Schnutgen M, Helmreich-Becker I, Mayet WJ, Mayer zum Buschenfelde KH (1994) Drug-induced hepatitis: a rare complication of oral anticoagulants. *J Hepatol* **21**(3): 447–9.

Hollinger BR, Pastoor TP (1993) Case management and plasma half-life in a case of brodifacoum poisoning. *Arch Intern Med* **153**(16): 1925–8.

Hoogenboom JJ, Rammell CG (1983) Improved HPLC method for determining brodifacoum in animal tissues. *Bull Environ Contam Toxicol* **31**(2): 239–43.

Hornfeldt CS, Phearman S (1996) Successful treatment of brodifacoum poisoning in a pregnant bitch. *J Am Vet Med Assoc* **209**(10): 1690–1.

Huckle KR, Hutson DH, Warburton PA (1988) Elimination and accumulation of the rodenticide flocoumafen in rats following repeated oral administration. *Xenobiotica* **18**(12): 1465–79.

Huckle KR, Morrison BJ, Warburton PA (1989) The percutaneous fate of the rodenticide flocoumafen in the rat: role of non-biliary intestinal excretion. *Xenobiotica* **19**(1): 63–74.

Hui CH, Lie A, Lam CK, Bourke C (1996) "Superwarfarin" poisoning leading to prolonged coagulopathy. *Foren Sci Int* **78**(1): 13–18.

Huic M, Francetic I, Bakran I, Macolic-Sarinic V, Bilusic M (2002) Acquired coagulopathy due to anticoagulant rodenticide poisoning. *Croat Med J* **43**(5): 615–7.

Humphry NF (1989) Anticoagulant rodenticides. *Med J Aust* **150**(12): 727–8.

Hunter K (1983a) Determination of coumarin anticoagulant rodenticide residues in animal tissue by high-performance liquid chromatography. II. Fluorescence detection using ion-pair chromatography. *J Chromatogr*. **270**: 277–83.

Hunter K (1983b) Determination of coumarin anticoagulant rodenticides in animal tissue by HPLC. I. Fluorescence detection using post-column techniques. *J Chromatogr* **270**: 267–76.

Hunter K (1984) Reversed-phase ion-pair liquid chromatographic determination of chlorophacinone residues in animal tissues. *J Chromatogr* **299**: 405–14.

Hunter K (1985) High-performance liquid chromatographic strategies for the determination and confirmation of anticoagulant rodenticide residues in animal tissues. *J Chromatogr* **321**(2): 255–72.

Hunter K, Sharp EA, Newton A (1988) Determination of diastereoisomers of bromadiolone, an anticoagulant rodenticide, in animal tissues by high-performance liquid chromatography. *J Chromatogr* **435**(1): 83–95.

Hylek EM, Chang YC, Skates SJ, Hughes RA, Singer DE (2000) Prospective study of the outcomes of ambulatory patients with excessive warfarin anticoagulation. *Arch Intern Med* **160**(11): 1612–17.

Ingels M, Lai C, Tai W, Manning BH, Rangan C, Williams SR, Manoguerra AS, Albertson T, Clark RF (2002) A prospective study of acute, unintentional, pediatric superwarfarin ingestions managed without decontamination. *Ann Emerg Med* **40**(1): 73–8.

Jackson WB, BrooksJE, Bowerman AM (1975) Anticoagulant resistance in Norway rats. *Pest Contr* **43**: 14–23.

Jackson CM, Suttie JW (1977) Recent developments in understanding the mechanism of vitamin K and vitamin K-antagonist drug action and the consequences of vitamin K action in blood coagulation. *Progr Haematol* **10**: 333–59.

James SB, Raphael BL, Cook RA (1998) Brodifacoum toxicity and treatment in a white-winged wood duck (*Cairina scutulata*). *J Zoo Wildl Med* **29**(3): 324–7.

Jeantet AY, Truchet M, Naulleau G, Martoja R (1991) Effects of bromadiolone on some organs and tissues (liver, kidney, spleen, blood) of coypu (Myocastor coypus). *C R Acad Sci III* **312**(4): 149–56 (in French).

Johnston JJ, Pitt WC, Sugihara RT, Eisemann JD, Primus TM, Holmes MJ, Crocker J, Hart A (2005) Probabilistic risk assessment for snails, slugs, and endangered honeycreepers in diphacinone rodenticide baited areas on Hawaii, USA. *Environ Toxicol Chem* **24**(6): 1557–67.

Johnstone TB (1989) Diagnostic approach to the bleeding patient. In *Current Veterinary Therapy X*, Kirk RW (ed.). WB Saunders, Philadelphia, PA, pp. 436–42.

Jones A. (1996) HPLC determination of anticoagulant rodenticide residues in animal livers. *Bull Environ Contam Toxicol* **56**(1): 8–15.

Jones EC, Growe GH, Naiman SC (1984) Prolonged anticoagulation in rat poisoning. *J Am Med Assoc* **252**: 3005–7.

Jolly SE, Eason CT, Frampton C, Gumbrell RC (1994) The anticoagulant pindone causes liver damage in the brushtail possum (*Trichosurus vulpecula*). *Aust Vet J* **71**(7): 220.

Kamil N (1987) Kinetics of bromadiolone, anticoagulant rodenticide, in the Norway rat (*Rattus norvegicus*). *Pharmacol Res Commun* **19**(11): 767–75.

Kanabar D, Volans G (2002) Accidental superwarfarin poisoning in children – less treatment is better. *Lancet* **360**(9338): 963.

Kawano Y, Chang W (1980) Spectrophotometric determination of Rozol in paraffinized formulations. *J Ass Off Anal Chem* **63**: 996–8.

Keiboom PJ, Rammel CG (1981) Detection of brodifacoum in animal tissues by High performance liquid chromatography. *Bull Environ Toxicol* **26**: 674–8.

Kelly MJ, Chambers J, MacNicoll AD (1993) Simple and rapid method for the determination of the diastereomers of difenacoum in blood and liver using high-performance liquid chromatography with fluorescence detection. *J Chromatogr* **620**(1): 105–12.

Kirchheiner J, Brockmoller J (2005) Clinical consequences of cytochrome P450 2C9 polymorphisms. *Clin Pharmacol Ther* **77**(1): 1–16.

Kohn MH, Pelz HJ (1999) Genomic assignment of the warfarin resistance locus, Rw, in the rat. *Mamm Genom* **10**(7): 696–8.

Kohn MH, Pelz HJ (2000) A gene-anchored map position of the rat warfarin-resistance locus, Rw, and its orthologs in mice and humans. *Blood* **96**(5): 1996–8.

Koubek KG, Ussary JP, Saulsee RE (1979) High performance liquid chromatographic determination of the rodenticide brodifacoum in rat tissue. *J Assoc Off Anal Chem* **62**: 1297–301.

Kruse JA, Carlson RW (1992) Fatal rodenticide poisoning with brodifacoum. *Ann Emerg Med* **21**(3): 331–6.

Kuijpers EA, den Hartigh J, Savelkoul TJ, de Wolff FA (1995) A method for the simultaneous identification and quantitation of five superwarfarin rodenticides in human serum. *J Anal Toxicol* **19**(7): 557–62.

Lagrange F, Corniot AG, Titier K, Bedry R, Pehourcq F (1999) Toxicological management of chlorophacinone poisoning. *Acta Clin Belg* Suppl. **1**: 13–16.

Lamnaouer D (1999) Anticoagulant activity of coumarins from *Ferula communis* L (review). *Therapie* **54**(6): 747–51 (in French).

Langenberg JP, Tjaden UR (1984) Determination of (endogenous) vitamin K1 in human plasma by reversed-phase HPLC using fluorometric detection after post-column electro-chemical reduction. *J Chromatogr* **305**: 61–72.

Last JA (2002) The missing link: the story of Karl Paul link. *Toxicol Sci* **66**(1): 4–6.

Lau-Cam CA, Chu-Fong I (1972) Thin-layer chromatography of coumarin anticoagulant rodenticides. *J Pharm Sci* **61**: 1303–6.

Lawley WJ, Charlton AJ, Hughson EJ, Grundy HH, Brown PM, Jones A (2006) Development of a cell culture/ELISA assay to detect anticoagulant rodenticides and its application to analysis of rodenticide treated grain. *J Agric Food Chem* **54**(5): 1588–93.

Leck JB, Park BK (1981) A comparative study of the effects of warfarin and brodifacoum on the relationship between vitamin K1 metabolism and clotting factor activity in warfarin-susceptible and warfarin-resistant rats. *Biochem Pharmacol* **30**: 123–8.

Lee SH, Field LR, Howard WN, Trager WF (1981) High performance liquid chromatographic separation and fluorescence detection of warfarin and its metabolites by postcolumn acid base manipulation. *Anal Chem* **53**: 467–71.

Lefevere MF, Leenheer de AP, Claeys AE (1979) High performance liquid chromatographic assay of vitamin K in human serum. *J Chromatogr* **186**: 749–62.

Lewis RJ, Ilnicki LP, Carlstrom M (1970) The assay of warfarin in plasma or stool. *Biochem Med* **4**: 376–82.

Lipton RA, Klass EM (1984) Human ingestion of a superwarfarin rodenticide resulting in prolonged anticoagulant effect. *J Am Med Assoc* **252**: 3004–5.

Lund M (1971) The toxicity of chlorophacinone and warfarin to house mice (*Mus musculus*). *J Hyg (London)* **69**(1): 69–72.

Lund M (1981) Comparative effect of the three rodenticides warfarin, difenacoum and brodifacoum on eight rodent species in short feeding periods. *J Hyg (London)* **87**(1): 101–7.

Lund M (1984) Resistance to the second-generation anticoagulant rodenticides. *Proceedings of the 11th Vertebral Pesticide Conference*, Sacramento, CA, p. 89.

Lund M, Rasmussen AM (1986) Secondary poisoning hazards in stone martens (*Martes foina*) fed bromadiolone-poisoned mice. *Nord Vet Med* **38**(4): 241–3.

Lund M (1988) The resistance to anticoagulant. Danish Pest Inf Lab Annu Report., Lyngby, Denmark. 1972: p. 57. Cited in: Lund M. Anticoagulant rodenticides. In *Rodent Pest Management*, Prakash I (ed.). CRC Press Inc, Boca Raton, FL, pp.342–51.

Mack RB (1994) Not all rats have four legs. Superwarfarin poisoning. *NC Med J* **55**(11): 554–6.

Magagnoli M, Masci G, Castagna L, Pedicini V, Poretti D, Morenghi E, Brambilla G, Santoro A (2006) Prophylaxis of central venous catheter-related thrombosis with minidose warfarin in patients treated with high-dose chemotherapy and peripheral-blood stem-cell transplantation: retrospective analysis of 228 cancer patients. *Am J Hematol* **81**(1): 1–4.

Mahmoud W, Redfern R (1981) The response of the Egyptian spiny mouse (*Acomys cahirinus*) and two other species of commensal rodents to anticoagulant rodenticides. *J Hyg (London)* **86**(3): 329–34.

Mallet V, Surette D, Brun GL (1973) Detection of naturally fluorescent pesticides on silica gel layers. *J Chromatogr* **79**: 217–22.

Marchini S, Turillazzi PG (1978) Effect of an anticoagulant rodenticide on the female albino rat with offspring. *Parassitologia* **20** (1–3): 59–70 (in Italian).

Markussen MD, Heiberg AC, Nielsen R, Leirs H (2003) Vitamin K requirement in Danish anticoagulant-resistant Norway rats (*Rattus norvegicus*). *Pest Manag Sci* **59**(8): 913–20.

Marquet P, Saint-Marcoux F, Gamble TN, Leblanc JC (2003) Comparison of a preliminary procedure for the general unknown screening of drugs and toxic compounds using a quadrupole-linear ion-trap mass spectrometer with a liquid chromatography-mass spectrometry reference technique. *J Chromatogr B Analyt Technol Biomed Life Sci* **789**(1): 9–18.

Martin GR, Sutherland RJ, Robertson ID, Kirkpatrick WE, King DR, Hood PJ (1991) Assessment of the potential toxicity of a poison for rabbits, pindone (2-pivalyl 1, 3 indandione), to domestic animals. *Aust Vet J* **68**(7): 241–3.

Mathur RP, Prakash I (1980) Laboratory evaluation of anticoagulant-treated baits for control of the northern palm squirrel, *Funambulus pennanti* Wroughton. *J Hyg (London)* **85**(3): 421–6.

Matolesy Gy, Nadasy M, Andriaska V. (1988) Rodenticides In *Pesticide Chemistry*. Elsevier Science Publishing Co, Amsterdam, Netherlands, pp. 261–71.

Matsukawa R, Uemura S, Fukuchi S, Tsuruta Y, Murakami S (1994) Thrombosed St. Jude Medical prosthesis with drug induced hepatitis due to warfarin potassium – a case report. *Nippon Kyobu Geka Gakkai Zasshi* **42**(3): 413–5 (in Japanese).

Mazzetti G, Cappelletti GA (1957) Effect of the active principle of Ferula communis on blood coagulation; thromboelastographic study. *Arch Sci Med (Torino)* **104**(3): 236–45.

McCarthy PT, Cox AD, Harrington DJ, Evely RS, Hampton E, Al-Sabah AI, Massey E, Jackson H, Ferguson T (1997) Covert poisoning with difenacoum: clinical and toxicological observations. *Hum Exp Toxicol* **16**(3): 166–70.

McConnico RS, Copedge K, Bischoff KL (1997) Brodifacoum toxicosis in two horses. *J Am Vet Med Assoc* **211**(7): 882–6.

McGuire NC, Williams J, Marks SL (1999) What is your diagnosis? Rodenticide poisoning in a dog. *J Am Vet Med Assoc* **214**(8): 1157–8.

McNiel NO, Morgan Jr LR (1984) Effects of sodium warfarin and sodium heparin plus anticancer agents on growth of rat C6 glioma cells. *J Natl Cancer Inst* **73**(1): 169–76.

McSporran KD, Phillips CA (1983) Brodifacoum poisoning in a dog. *New Zeal Vet J* **31**(10): 185–6.

Mesban HA, Tayeb EH, Mourad AK, Younis LK, el Zaher MA, Aly MT (2003) Toxicology and histopathology of some rodenticides and palatable food items combinations on the common mice *Mus musculus var. albus* in Egypt. *Commun Agric Appl Biol Sci* **68**(4 Pt B): 771–87.

Mesmer MZ, Flurer RA (2000) Determination of chlorophacinone and diphacinone in commercial rodenticides by liquid chromatography-UV detection and liquid chromatography-electrospray ionization mass spectrometry. *J Chromatogr A* **891**(2): 249–55.

Middlekauff HR, Stevenson WG, Gornbein JA (1995) Antiarrhythmic prophylaxis vs warfarin anticoagulation to prevent thromboembolic events among patients with atrial fibrillation. A decision analysis. *Arch Intern Med* **155**(9): 913–20.

Mildha KK, McGitveray IJ, Cooper JK (1974) GLC determination of plasma levels of warfarin. *J Pharm Sci* **63**: 1725–9.

Miller MA, Levy PD, Hile D (2006) Rapid identification of surreptitious brodifacoum poisoning by analysis of vitamin K-dependent factor activity. *Am J Emerg Med* **24**(3): 383.

Misenheimer TM, Lund M, Baker EM, Suttie JW (1994) Biochemical basis of warfarin and bromadiolone resistance in the house mouse, Mus musculus domesticus. *Biochem Pharmacol* **47**(4): 673–8.

Miski M, Mabry TJ, Bohlmann F (1986) Fercoperol, an unusual cyclic-endoperoxynerolidol derivative from *Ferula communis subsp. communis*. *J Nat Prod* **49**(5): 916–8.

Montanio CD, Wruk KM, Kulig KW, Riggs BS, Rumack BH (1993) Acute pediatric warfarin (Coumadin) ingestion: toxic effects despite early treatment. *Am J Dis Child* 147(6):609–610.

Morgan BW, Tomaszewski C, Rotker I (1996) Spontaneous hemoperitoneum from brodifacoum overdose. *Am J Emerg Med* **14**(7): 656–9.

Morin MF, Merlet N, Naulleau G, Dore M (1990) Primary toxicity of bromadiolone on the coypu. *Bull Environ Contam Toxicol* **44**(4): 595–601.

Mossa JS, El-Feraly FS, Muhammad I (2004) Antimycobacterial constituents from Juniperus procera, Ferula communis and Plumbago zeylanica and their in vitro synergistic activity with isonicotinic acid hydrazide. *Phytother Res* **18**(11): 934-–7.

Mosterd JJ, Thijssen HH (1991) The long-term effects of the rodenticide, brodifacoum, on blood coagulation and vitamin K metabolism in rats. *Br J Pharmacol* **104**(2): 531–5.

Mount ME,Feldman BF (1983) Mechanism of diphacinone rodenticide toxicosis in the dog and its therapeutic implications. *Am J Vet Res* **44**(11): 2009–17.

Mount ME, Kass PH (1989) Diagnostic importance of vitamin K 1 and its epoxide measured in serum of dogs exposed to an anticoagulant rodenticide. *Am J Vet Res* **50**: 1704–9.

Mount ME, Feldman BF, Buffington T (1982) Vitamin K and its therapeutic importance. *J Am Vet Med Assoc* **180**(11): 1354–6.

Mount ME, Woody MJ, Murphy MJ (1985) The anticoagulant rodenticides. In *Current Veterinary Therapy IX*, Kirk RW (ed.). WB Saunders, Philadelphia, PA, pp. 156–65.

Mount ME, Kurth MJ, Jackson DY (1988) Production of antibodies and development of an immunoassay for the anticoagulant, diphacinone. *J Immunoass* **9**(1): 69–81.

Mullins ME, Brands CL, Daya MR (2000) Unintentional pediatric superwarfarin exposures: do we really need a prothrombin time? *Pediatrics* **105**(2): 402–4.

Munday JS, Thompson LJ (2003) Brodifacoum toxicosis in two neonatal puppies. *Vet Pathol* **40**(2): 216–9.

Mundy DE, Machin AF (1977) Determination of the rodenticide difenacoum in biological materials by high-pressure liquid chromatography with confirmation of identity by mass spectrometry. *J Chromatogr* **139**(2): 321–9.

Mundy DE, Machin AF (1982) The multi-residue determination of coumarin-based anticoagulant rodenticides in animal materials by HPLC. *J Chromatogr* **234**: 427–35.

Munger LL, Su JJ, Barnes HJ (1993). Coumafuryl (Fumarin) toxicity in chicks. *Avian Dis* **37**(2): 622–4.

Mura P, Piriou A, Papet Y, Lochon D, Reiss D (1992) Rapid high-performance liquid chromatographic assay of chlorophacinone in human serum. *J Anal Toxicol* **16**(3): 179–81.

Murdoch DA (1983) Prolonged anticoagulation in chlorphacinone poisoning. *Lancet* **1**(8320): 355–6.

Murphy MJ, Gerken DF (1989) The anticoagulant rodenticides. In *Current Veterinary Therapy X*, Kirk RW (ed.). WB Saunders, Philadelphia, PA, pp. 143–6.

Murphy MJ, Ray AC, Bailey EM (1989) A high-performance liquid chromatographic method for the detection of brodifacoum in serum. *Vet Hum Toxicol* **31**: 228–31.

Nahas K (1986) Analysis of bromadiolone (an anticoagulant rodenticide) in plasma, liver and kidney of the rat. *J Chromatogr* **369**(2): 445–8.

Nasilowski J, Krenke R (2002) Hemothorax with high number of eosinophils following warfarin overdose. *Pneumonol Alergol Pol* **70**(9–10): 496–503.

Newton I, Wyllie I, Freestone P (1990) Rodenticides in British barn owls. *Environ Pollut* **68**(1–2): 101–17.

Nighoghossian N, Ruel JH, Ffrench P, Froment JC, Trouillas P (1990) Cervicodorsal subdural hematoma caused by coumarinic rodenticide poisoning. *Rev Neurol (Paris)* **146**(3): 221–3.

Nikodemusz E, Nechay G, Imre R (1981) Histopathological changes resulting by some pesticides in the common vole (*Microtus arvalis pallas*). *Acta Vet Acad Sci Hung* **29**(3): 317–26.

O'Bryan SM, Constable DJ (1991). Quantification of brodifacoum in plasma and liver tissue by HPLC. *J Anal Toxicol* **15**(3): 144–7.

O'Reilly RA, Aggeler PM, Hoag MS, Leong L (1962) Studies on the coumarin anticoagulant drugs: the assay of warfarin and its biological application. *Thromb Diath Haemorrh* **8**: 82–6.

Olson RE (1966) Studies on the mode of action of vitamin K. *Adv Enzyme Regul* **4**: 181–96.

Ornstein DL, Lord KE, Yanofsky NN, Cornell CJ, Zacharski LR (1999) Successful donation and transplantation of multiple organs after fatal poisoning with brodifacoum, a long-acting anticoagulant rodenticide: case report. *Transplantation* **67**(3): 475–8.

Osterhoudt KC, Henretig FM (2003) Bias in pediatric brodifacoum exposure data. *Pediatr Emerg Care* **19**(1): 62.

Padgett SL, Stokes JE, Tucker RL, Wheaton LG (1998) Hematometra secondary to anticoagulant rodenticide toxicity. *J Am Anim Hosp Assoc* **34**(5): 437–9.

Pan K, Xia LG, Chen XC, Zhong KL, Jiang HX (2005) Diagnosis and treatment of mesenteric venous thrombosis early after operation. *Zhonghua Wei Chang Wai Ke Za Zhi* **8**(1): 50–2.

Papin F, Clarot F, Vicomte C, Gaulier JM, Daubin C, Chapon F, Vaz E, Proust B (2006) Lethal paradoxical cerebral vein thrombosis due to suspicious anticoagulant rodenticide intoxication with chlorophacinone. *Forensic Sci Int*. May 20.

Park BK, Leck JB (1982) A comparison of vitamin K antagonism by warfarin, difenacoum and brodifacoum in rabbit. *Biochem Pharmacol* **31**: 3635–9.

Park BK, Leck JB, Wilson A, Breckenridge AM (1979) Investigation of anticoagulants and vitamin K1 in the rabbit. In *Vitamin K Metabolism and Vitamin K dependent Protein*, Suttie JW (ed.). *Proceedings of the 8th Steenbock Symposium*. University of Wisconsin, Madison, pp. 348–53.

Park BK, Scott AK, Wilson AC, Haynes BP, Breckenridge AM (1984) Plasma disposition of vitamin K1 in relation to anticoagulant poisoning. *Br J Clin Pharmacol* **18**(5): 655–62.

Park BK, Choonara IA, Haynes BP, Breckenridge AM, Malia RG, Preston FE (1986) Abnormal vitamin K metabolism in the presence of normal clotting factor activity in factory workers exposed to 4-hydroxycoumarins. *Br J Clin Pharmacol* **21**(3): 289–93.

Parshad VR, Chopra G (1986) The susceptibility of *Rattus rattus* and *Bandicota bengalensis* to a new anticoagulant rodenticide, flocoumafen. *J Hyg (London)* **96**(3): 475–8.

Pavlu J, Harrington DJ, Voong K, Savidge GF, Jan-Mohamed R, Kaczmarski R (2005) Superwarfarin poisoning. *Lancet* **365**(9459): 628.

Pelz HJ, Rost S, Hunerberg M, Fregin A, Heiberg AC, Baert K, MacNicoll AD, Prescott CV, Walker AS, Oldenburg J, Muller CR (2005) The genetic basis of resistance to anticoagulants in rodents. *Genetics* **170**(4): 1839–47.

Peterson J, Streeter V (1996) Laryngeal obstruction secondary to brodifacoum toxicosis in a dog. *J Am Vet Med Assoc* **208**(3): 352–4; discussion 354–355.

Petrus DJ, Henik RA (1999) Pericardial effusion and cardiac tamponade secondary to brodifacoum toxicosis in a dog. *J Am Vet Med Assoc* **215**(5): 647–8.

Piovella F, Siragusa S, Barone M, Beltrametti C, Carbone S, Vicentini L, Ascari E (1995) Secondary prophylaxis of venous thromboembolism: rational use of oral anticoagulants. *Haematologica* **80**(Suppl. 2): 87–91.

Poli F, Appendino G, Sacchetti G, Ballero M, Maggiano N, Ranelletti FO (2005) Antiproliferative effects of daucane esters from *Ferula communis* and *F. arrigonii* on human colon cancer cell lines. *Phytother Res* **19**(2): 152–7.

Primus T, Wright G, Fisher P (2005) Accidental discharge of brodifacoum baits in a tidal marine environment: a case study. *Bull Environ Contam Toxicol* **74**(5): 913–9.

Primus TM, Griffin DL, Volz SA, Johnston JJ (1998) Reversed-phase ion-pair liquid chromatographic determination of chlorophacinone and diphacinone in steam-rolled oat baits and steam-rolled oat/wax baits. *J AOAC Int* **81**(2): 349–57.

Primus TM, Kohler DJ, Johnston JJ (2006) Determination of diphacinone residues in Hawaiian invertebrates. *J Chromatogr Sci* **44**(1): 1–5.

Radi ZA, Thompson LJ (2004) Renal subcapsular hematoma associated with brodifacoum toxicosis in a dog. *Vet Hum Toxicol* **46**(2): 83–4.

Radvanyi A, Weaver P, Massari C, Bird D, Broughton E (1988) Effects of chlorophacinone on captive kestrels. *Bull Environ Contam Toxicol* **41**(3): 441–8.

Rammell CG, Cotter M, Williams JM, Bell J (1984) Brodifacoum residues in target and non-target animals following rabbit poisoning trials. *New Zeal J Exp Agric* **12**: 107–11.

Raoul F, Michelat D, Ordinaire M, Decote Y, Aubert M, Delattre P, Deplazes P, Giraudoux P (2003) Echinococcus multilocularis: secondary poisoning of fox population during a vole outbreak reduces environmental contamination in a high endemicity area. *Int J Parasitol* **33**(9): 945–54.

Rauch AE, Weininger R, Pasquale D, Burkart PT, Dunn HG, Weissman C, Rydzak E.(1994) Superwarfarin poisoning: a significant public health problem. *J Commun Health* **19**(1): 55–65.

Ray AC, Murphy MJ, DuVa11 MD, Reagor MD (1989) Determination of brodifacoum and bromadiolone residues in rodent and canine liver. *Am J Vet Res* **50**: 546–500.

Redfern R, Gill JE (1980) Laboratory evaluation of bromadiolone as a rodenticide for use against warfarin-resistant and non-resistant rats and mice. *J Hyg (London)* **84**(2): 263–8.

Reece RL, Scott PC, Forsyth WM, Gould JA, Barr DA (1985) Toxicity episodes involving agricultural chemicals and other substances in birds in Victoria, Australia. *Vet Rec* **117**(20): 525–7.

Rehman AB, Ahmad SI. (1983). Comparative haematological studies on Tatera indica with three anticoagulant compounds. *J Pak Med Assoc* **33**(8): 203–7.

Rehnqvist N (1978) Intrahepatic jaundice due to warfarin therapy. *Acta Med Scand* **204**(4): 335–6.

Reiffel JA (2000) Drug choices in the treatment of atrial fibrillation. *Am J Cardiol* **85**(10A): 12D–19D.

Renapurkar DM (1982) A comparative assessment of efficacy of three anticoagulant rodenticides. *J Hyg Epidemiol Microbiol Immunol* **26**(2): 125–30.

Rennison BD, Dubock AC (1978) Field trials of WBA 8119 (PP 581, brodifacoum) against warfarin-resistant infestations of *Rattus norvegicus*. *J Hyg (London)* **80**(1): 77–82.

Rennison BD, Hadler MR (1975) Field trials of difenacoum against warfarin-resistant infestations of *Rattus norvegicus*. *J Hyg (London)* **74**(3): 449–55.

Richards CG (1981) Field trials of bromadiolone against infestations of warfarin-resistant *Rattus norvegicus*. *J Hyg (London)* **86**(3): 363–7.

Riedel B, Grun G, Clausing P (1990) The subacute and subchronic toxicity of chlorophacinone in Japanese quail (*Coturnix c. japonica*). *Arch Exp Veterinarmed* **44**(3): 341–6 (in German).

Robben JH, Mout HC, Kuijpers EA (1997) Anticoagulant rodenticide poisoning in dogs in the Netherlands. *Tijdschr Diergeneeskd* **122**(17): 466–71 (in Dutch).

Robben JH, Kuijpers EA, Mout HC (1998) Plasma superwarfarin levels and vitamin K1 treatment in dogs with anticoagulant rodenticide poisoning. *Vet Q* **20**(1): 24–7.

Robinson MH, Twigg LE, Wheeler SH, Martin GR (2005) Effect of the anticoagulant, pindone, on the breeding performance and survival of merino sheep, Ovis aries. *Comp Biochem Physiol B Biochem Mol Biol* **140**(3): 465–73.

Ross GS, Zacharski LR, Robert D, Rabin DL (1992) An acquired hemorrhagic disorder from long-acting rodenticide ingestion. *Arch Intern Med* **152**(2): 410–2.

Routh CR, Triplett DA, Murphy MJ, Felice LJ, Sadowski JA, Bovill EG (1991) Superwarfarin ingestion. *Am J Hematol* **36**: 50–4.

Rowe FP, Bradfield A (1976) Trials of the anticoagulants rodenticide WBA 8119 against confined colonies of warfarin-resistant house mice (*Mus musculus* L.). *J Hyg (London)* **77**(3): 427–31.

Rowe FP, Redfern R (1968) Comparative toxicity of the two anticoagulants, coumatetralyl and warfarin, to wild house mice (*Mus musculus* L.). *Ann Appl Bioi* **62**: 355–61.

Rowe FP, Swinney T, Plant C (1978) Field trials of brodifacoum (WBA 8119) against the house mouse (*Mus musculus* L.). *J Hyg (London)* **81**(2): 197–201.

Rowe FP, Plant CJ, Bradfield A (1981) Trials of the anticoagulant rodenticides bromadiolone and difenacoum against the house mouse (*Mus musculus* L.). *J Hyg (London)* **87**(2): 171–7.

Rowe FP, Bradfield A, Swinney T (1985a) Pen and field trials of a new anticoagulant rodenticide flocoumafen against the house mouse (*Mus musculus* L.). *J Hyg (London)* **95**(3): 623–7.

Rowe FP, Bradfield A, Swinney T (1985b) Pen and field trials of flupropadine against the house mouse (*Mus musculus* L.). *J Hyg (London)* **95**(2): 513–8.

Sabhlok VP, Pasahan SC, Kumar P, Singal RK (1997) Evaluation of different rodenticidal baits against rodent population in cucumber (*Cucumis sativus*) crop fields. *Indian J Exp Biol* **35**(6): 670–2.

Santoro P, Parisi G, Copetti S (1993) Mutagenic effect of pindone on *D. melanogaster*. *Boll Soc Ital Biol Sper* **69**(4): 237–41 (in Italian).

Savage R (2005). Cyclo-oxygenase-2 inhibitors: when should they be used in the elderly? *Drugs Aging* **22**(3): 185–200.

Schor NA, Huddleson RL, Kane GM, Lee G (1983) Effects of the administration of anticoagulants on the activity of the enzyme-reduced NAD(P)H dehydrogenase in rat livers, hepatomas and precarcinomatous rat liver lesions. *Enzyme* **30**(4): 244–51.

Schulman A, Lusk R, Lippincott CL, Ettinger SJ (1986) Diphacinone-induced coagulopathy in the dog. *J Am Vet Med Assoc* **188**(4): 402–5.

Seegers WH (1969) Blood clotting mechanisms: three basic reactions. *Annu Rev Physiol* **31**: 269–94.

Seidelmann S, Kubic V, Burton E, Schmitz L (1995) Combined superwarfarin and ethylene glycol ingestion. A unique case report with misleading clinical history. *Am J Clin Pathol* **104**(6): 663–6.

Shanberge JN (1988) Bromodialone poisoning. *Lancet* **1**(8581): 363–4.

Sharma P, Bentley P (2005) Of rats and men: superwarfarin toxicity. *Lancet* **365**(9459): 552–3.

Shepherd G, Klein-Schwartz W, Anderson BD (2002) Acute, unintentional pediatric brodifacoum ingestions. *Pediatr Emerg Care* **18**(3): 174–8.

Shetty HG, Woods F, Routledge PA (1993) The pharmacology of oral anticoagulants: implications for therapy. *J Heart Valve Dis* **2**(1): 53–62.

Shi HP, Liu Y, Ma DY (2005) One case of acute severe bromadiolone poisoning. *Zhonghua Lao Dong Wei Sheng Zhi Ye Bing Za Zhi* **23**(6): 469–70. Chinese.

Shlosberg A, Egyed MN (1983) Examples of poisonous plants in Israel of importance to animals and man. *Arch Toxicol* **6**: (Suppl). 194–6.

Shlosberg A, Egyed MN (1985) Experimental Ferula communis (giant fennel) toxicosis in sheep. *Zentralbl Veterinarmed A* **32**(10): 778–84.

Shore RF, Birks JD, Freestone P, Kitchener AC (1996) Second-generation rodenticides and polecats (*Mustela putorius*) in Britain. *Environ Pollut* **91**(3): 279–82.

Shore RF, Birks JD, Afsar A, Wienburg CL, Kitchener AC (2003) Spatial and temporal analysis of second-generation anticoagulant rodenticide residues in polecats (*Mustela putorius*) from throughout their range in Britain, 1992–1999. *Environ Pollut* **122**(2): 183–93.

Smith GF, Neubauer BL, Sundboom JL, Best KL, Goode RL, Tanzer LR, Merriman RL, Frank JD, Herrmann RG. (1988). Correlation of the *in vivo* anticoagulant, antithrombotic, and antimetastatic efficacy of warfarin in the rat. *Thromb Res* **50**(1): 163–74.

Smith P, Inglis IR, Cowan DP, Kerins GM, Bull DS (1994) Symptom-dependent taste aversion induced by an anticoagulant rodenticide in the brown rat (*Rattus norvegicus*). *J Comp Psychol* **108**(3): 282–90.

Smolinske SC, Scherger DL, Kearns PS, Wruk KM, Kulig KW, Rumack BH (1989) Superwarfarin poisoning in children: a prospective study. *Pediatrics* **84**(3): 490–4.

Soubiron L, Hantson P, Michaux I, Lambert M, Mahieu P, Pringot J (2000) Spontaneous haemoperitoneum from surreptitious ingestion of a rodenticide. *Eur J Emerg Med* **7**(4): 305–7.

Spiller HA, Gallenstein GL, Murphy MJ (2003) Dermal absorption of a liquid diphacinone rodenticide causing coagulaopathy. *Vet Hum Toxicol* **45**(6): 313–4.

Stanziale SF, Christopher JC, Fisher RB (1997) Brodifacoum rodenticide ingestion in a patient with shigellosis. *South Med J* **90**(8): 833–5.

Stenflo J (1978) Vitamin K, prothrombin, and gamma-carboxyglutamic acid. *Adv Enzymol Relat Areas Mol Biol* **46**: 1–31.

Sterner RT (1979) Effects of sodium cyanide and diphacinone in coyotes (*Canis latrans*): applications as predacides in livestock toxic collars. *Bull Environ Contam Toxicol* **23**(1–2): 211–7.

Stone WB, Okoniewski JC, Stedelin JR (1999) Poisoning of wildlife with anticoagulant rodenticides in New York. *J Wildl Dis* **35**(2): 187–93.

Stowe CM, Metz AL, Arendt TD, Schulman J (1983) Apparent brodifacoum poisoning in a dog. *J Am. Vet. Med.Assoc* **182**(8): 817–8.

Strayhorn VA, Baciewicz AM, Self TH (1997) Update on rifampin drug interactions, III. *Arch Intern Med* **157**(21): 2453–8.

Subbiah D, Kala S, Mishra AK (2005) Study on the fluorescence characteristics of bromadiolone in aqueous and organized media and application in analysis. *Chemosphere* **61**(11): 1580–6.

Suttie JW (1986) Vitamin K-dependent carboxylase and coumarin anticoagulant action. In *Prothrombin and Other Vitamin K Proteins (Vol 2)*, Seegers WH, Walz DA (eds). CRC Press, FL, pp. 17–47.

Suttie JW (1990) Warfarin and vitamin K. *Clin Cardiol* **13**: VI-16–8.

Swigar ME, Clemow LP, Saidi P, Kim HC (1990) "Superwarfarin" ingestion. A new problem in covert anticoagulant overdose. *Gen Hosp Psychiatr* **12**(5): 309–12.

Szuber T, Diechtiar M (1968) Studies of the effectiveness of the anticoagulant rodenticide diphacinone (2-diphenylacetyl-1,3-indiandone) using the conditioning method. *Rocz Panstw Zakl Hig* **19**(3): 343–53 (in Polish).

Tagliapietra S, Aragno M, Ugazio G, Nano GM (1989) Experimental studies on the toxicity of some compounds isolated from *Ferula communis* in the rat. *Res Commun Chem Pathol Pharmacol* **66**(2): 333–6.

Takani U, Suttie JW (1983) High performance liquid chromatography-reductive electrochemical detection analysis of serum transphylloquinone. *Anal Biochem* **133**: 63–7.

Tanaka N, Matsushita E, Morimoto H, Kobayashi K, Hattori N (1985) Toxic hepatitis induced by cardiovascular agents. *Nippon Rinsho* **43**(6): 1172–5 (in Japanese).

Tecimer C, Yam LT (1997) Surreptitious superwarfarin poisoning with brodifacoum. *South Med J* **90**(10): 1053–5.

Terneu S, Verhelst D, Thys F, Ketelslegers E, Hantson P, Wittebole X (2003) An unusual cause of abdominal pain. *Acta Clin Belg* **58**(4): 241–4.

Thijssen HH, Soute BA, Vervoort LM, Claessens JG (2004) Paracetamol (acetaminophen) warfarin interaction: NAPQI, the toxic metabolite of paracetamol, is an inhibitor of enzymes in the vitamin K cycle. *Thromb Haemost* **92**(4): 797–802.

Thornes RD, Daly L, Lynch G, Breslin B, Browne H, Browne HY, Corrigan T, Daly P, Edwards G, Gaffney E, *et al*. (1994) Treatment with coumarin to prevent or delay recurrence of malignant melanoma. *J Cancer Res Clin Oncol* **120**(Suppl.): S32–34.

Thornton H (1980) An ingenious method of destroying the vampire bats which transmit rabies to cattle. *Cent Afr J Med* **26**(9): 207–9.

Tligui N, Ruth GR (1994) Ferula communis variety brevifolia intoxication of sheep. *Am J Vet Res* **55**(11): 1558–63.

Tligui N, Ruth GR, Felice LJ (1994) Plasma ferulenol concentration and activity of clotting factors in sheep with *Ferula communis* variety brevifolia intoxication. *Am J Vet Res* **55**(11): 1564–9.

Toes MJ, Jones AL, Prescott L (2005) Drug interactions with paracetamol. *Am J Ther* **12**(1): 56–66.

Townsend MG, Entwisle P, Hart AD (1995) Use of two halogenated biphenyls as indicators of non-target exposure during rodenticide treatments. *Bull Environ Contam Toxicol* **54**(4): 526–33.

Travis SF, Warfield W, Greenbaum BH, Molokisher M, Siegel JE (1993) Spontaneous hemorrhage associated with accidental brodifacoum poisoning in a child. *J Pediatr* **122**(6): 982–4.

Trivedi LS, Rhee M, Galivan JH, Fasco MJ (1988) Normal and warfarin-resistant rat hepatocyte metabolism of vitamin K 2,3-epoxide: evidence for multiple pathways of hydroxyvitamin K formation. *Arch Biochem Biophys* **264**(1): 67–73.

Troy GC (1988) Diphacinone toxicity, von Willebrand's Disease, and Ehrlichia canis in a dog. *Vet Clin North Am Small Anim Pract* **18**(1): 255–7.

Tsutaoka BT, Miller M, Fung SM, Patel MM, Olson KR (2003) Superwarfarin and glass ingestion with prolonged coagulopathy requiring high-dose vitamin K1 therapy. *Pharmacotherapy* **23**(9): 1186–9.

Tumova L (2000) Interactions between herbal medicines and drugs. *Ceska Slov Farm* **49**(4): 162–167.

Tvedten H (1989) Hemostatic abnormalities. In *Small Animal Clinical Diagnosis by Laboratory Methods*, Willard MD, Tvedten H, Turnwald GA (eds). WB Saunders, Philadelphia, PA, pp. 86–102.

Twigg LE, Kay BJ (1995) The effect of sub-lethal doses of bromadiolone on the breeding performance of house mice (*Mus domesticus*). *Comp Biochem Physiol C Pharmacol Toxicol Endocrinol* **110**(1): 77–82.

Usui U, Nishjmura N, Kobayashi N, Okanoue T, Kimoto M, Ozawa K (1989) Measurement of vitamin K in human liver by gradient elution high performance liquid chromatography using platinum-black catalyst reduction and fluorometric detection. *J Chromatogr* **489**: 291–301.

Veenstra GE, Owen DE, Huckle KR (1991) Metabolic and toxicological studies on the anticoagulant rodenticide, flocoumafen. *Arch Toxicol* **14**: (Suppl). 160–5.

Vesell ES, Shivley CA (1974) Liquid chromatographic assay of warfarin: similarity of warfarin half-lives in human subjects. *Science* **184**: 466–8.

Vigh Gy, Varga-Puchony l, Papp-Hites E, Hlavay J (1981) Determination of chlorophacinone in formulations by reversed-phased ion-pair chromatography. *J Chromatogr* **214**: 335–41.

Vogel JJ, de Moerloose P, Bouvier CA, Gaspoz J, Riant P (1988) Prolonged anticoagulation following chlorophacinone poisoning. *Schweiz Med Wochenschr* **118**(50): 1915–7 (in French).

Waien SA, Hayes Jr D, Leonardo JM (2001) Severe coagulopathy as a consequence of smoking crack cocaine laced with rodenticide. *N Engl J Med* **345**(9): 700–1 [erratum in *N Engl J Med* Dec 20; **345**(25): 1860].

Walker J, Beach FX (2002) Deliberate self-poisoning with rodenticide: a diagnostic dilemma. *Int J Clin Pract* **56**(3): 223–4.

Wallace S, Worsnop C, Paull P, Mashford ML (1990) Covert self poisoning with brodifacoum, a superwarfarin. *Aust New Zeal J Med* **20**(5): 713–5.

Wallin R (1986) Vitamin K antagonism of coumarin anticoagulation. A dehydrogenase pathway in rat liver is responsible for the antagonistic effect. *Biochem J* **236**(3): 685–93.

Watt BE, Proudfoot AT, Bradberry SM, Vale JA (2005) Anticoagulant rodenticides. *Toxicol Rev* **24**(4): 259–69.

Watts RG, Castleberry RP, Sadowski JA (1990) Accidental poisoning with a superwarfarin compound (brodifacoum) in a child. *Pediatrics* **86**(6): 883–7.

Weitzel IN, Sadowski JA, Furie BC, Morosee R, Kim H, Mount ME, Murphy MJ, Furie B (1990) Hemorrhagic disorder caused by surreptitious ingestion of long acting vitamin K antagonist/rodenticide, brodifacoum. *Blood* **76**: 2555–9.

Welling PG, Lee KP, Khanna U, Wagner JG (1970) Comparison of plasma concentrations of warfarin measured by both simple extraction and thin-layer liquid chromatographic methods. *J Pharm Sci* **59**: 1621–5.

Whisson DA, Salmon TP (2002) Effect of diphacinone on blood coagulation in Spermophilus beecheyi as a basis for determining optimal timing of field bait applications. *Pest Manag Sci* **58**(7): 736–8.

Williams RC, Schmit JA, Henry RA (1972) Quantitative analysis of the fat-soluble vitamins by high-speed liquid chromatography. *J Chromatogr Sci* **10**: 494–501.

Wilson AC, Park BK (1984) The effect of phenobarbitone pretreatment on vitamin K1 disposition in the rat and rabbit. *Biochem Pharmacol* **33**(1): 141–6.

Wilton NM (1991) Superwarfarins as agents of accidental or deliberate intoxication. *Aust New Zeal J Med* **21**(4): 491.

Winn MJ, Clegg JA, Park BK (1987) An investigation of sex-linked differences to the toxic and to the pharmacological actions of difenacoum: studies in mice and rats. *J Pharm Pharmacol* **39**(3): 219–22.

Winn MJ, Cholerton S, Park BK (1988) An investigation of the pharmacological response to vitamin K1 in the rabbit. *Br J Pharmacol* **94**(4): 1077–84.

Winn MJ, White PM, Scott AK, Pratt SK, Park BK (1989) The bioavailability of a mixed micellar preparation of vitamin K1, and its procoagulant effect in anticoagulated rabbits. *J Pharm Pharmacol* **41**(4): 257–60.

Wood MJ, Stewart RL, Merry H, Johnstone DE, Cox JL (2003) Use of complementary and alternative medical therapies in patients with cardiovascular disease. *Am Heart J* **145**(5): 806–12.

Woody BJ, Murphy MJ, Ray AC, Green RA (1992) Coagulopathic effects and therapy of brodifacoum toxicosis in dogs. *J Vet Intern Med* **6**(1): 23–8.

Yang SY, Pan GM, Meng GF, Zhang DM (2001) Study of diphacinone in biological samples by high performance liquid chromatography/diode array detector. *Se Pu* **19**(3): 245–7 (in Chinese).

Yu CC, Atallah YH, Whitacre DM (1982) Metabolism and disposition of diphacinone in rats and mice. *Drug Metab Dispos* **10**(6): 645–8.

Zhabinskii VN, Minnaard AJ, Wijnberg JB, de Groot A (1996) Relative and absolute configuration of allohedycaryol. Enantiospecific total synthesis of its enantiomer. *J Org Chem* **61**(12): 4022–7.

Zimmerman A, Matschiner JT (1974) Biochemical basis of hereditary resistance to warfarin in the rat. *Biochem Pharmacol* **23**: 1033–40.

C H A P T E R

# 49

# Non-anticoagulant rodenticides

*Ramesh C. Gupta*

## STRYCHNINE

### Introduction

Strychnine is an alkaloid derived from the seeds and bark of a tree, *Strychnos nux-vomica*, that is native to Southeast Asia (India, West Indies, Sri Lanka, Indonesia) and Australia. Strychnine is also found in *Strychnos ignatii.* Strychnine is a white, odorless, crystalline powder, which has chemical formula $C_{21}H_{22}N_2O_2$ and molecular weight of 334.41. Its structural formula is shown in Figure 49.1.

Nux-vomica/strychnine has been used for at least five centuries for both pests and people. Its major use is as a pesticide (rodenticide, avicide, and insecticide), but it is also used as a therapeutic agent in human ailments (laxative, appetizer, and central nervous system (CNS) stimulant). In addition, strychnine in small amounts is known to be added to lysergic acid diethylamide (LSD), heroin, cocaine, and other "street drugs", and has been known to cause poisoning (O'Callaghan *et al.*, 1982). In the 1960s, strychnine was the title of a song by the Garage Rock Band "The Sonics", which includes the lines: "Some folks like the water, some folks like the wine; I like the taste of straight strychnine". Strychnine has many names and is sold under various trade names including Boomer-Rid, Certox, Dog-button, Dolco mouse Ceral, Gopher Bait, Gopher Gitter, Kwik-kill, Stricnina, Mole death, Mouse-nots, Mouse-rid, Mouse-tox, Ro-dex, Strychnos, Sanaseed, and others. Strychnine poisoning in animals occurs from ingestion of baits designed for use against rodents. The most common domestic animal to be affected is the dog, either through accidental ingestion or intentional poisoning.

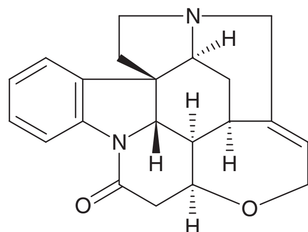



**FIGURE 49.1** Structural formula of strychnine.

### Background

Strychnine was first discovered in 1818 by two French scientists (Joseph-Bienaime Caventou and Pierre-Joseph Pelletier) in the *Saint ignatii* beans. *S. ignatii* is a woody climbing shrub found in the Philippines. In general, the beans have the same properties as the species *S. nux-vomica*. Strychnine is also found in other species of *Strychnos* (*Strychnos colubrine*, and *Strychnos tieute*), and is accompanied by another alkaloid brucine. The ripe seeds of *Strychnos* look like flattened discs, which are very hard and covered with satiny hairs. The properties of strychnine are substantially those of the nux-vomica. Strychnine is an extremely toxic alkaloid primarily used to kill rodents, moles, predatory animals, and birds, or trapping fur-bearing animals. Pharmaceutically, strychnine is an unjustifiable component of traditional tonics, cathartic pills, and CNS stimulant.

### Toxicokinetics

Following ingestion, strychnine is readily absorbed from the gastrointestinal (GI) tract, but mainly from the small

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intestine. Soon after absorption, strychnine readily distributes to various tissues within 5 min (Reynolds, 1982). Strychnine is readily metabolized in the liver. In fact, the metabolism is rapid enough that approximately two lethal doses can be given over 24 h without cumulative effects. Its half-life has been reported to be about 10 h in humans. Elimination of strychnine is also rapid, as its unchanged residue can be detected in the urine within few minutes of exposure. The elimination constant ($K_{el} = 0.07\,h^{-1}$) indicates that 7% of the strychnine in the serum at any one moment would be eliminated in 1 h (Edmunds *et al.*, 1986). Following exposure to a sublethal dose of a strychnine, about 50% of the dose is eliminated within 6 h (Boyd *et al.*, 1983), 10–20% within 24 h, and almost complete in 48–72 h (Cooper, 1974).

## Mechanism of action

Pharmacologically, because of its bitter taste, strychnine strongly stimulates salivary and gastric secretions. This increases appetite and as a result strychnine has been used for a long time to counteract the loss of appetite associated with illnesses.

Strychnine is a potent convulsant. It increases reflex excitability in the spinal cord and that results in a loss of the normal inhibition of spread of motor cell stimulation, so that all muscles contract simultaneously. Strychnine causes excitation of all parts of the CNS. It increases the level of neuronal excitability by interfering with inhibitory influences on the motor neurons. The site of mechanism of action of strychnine is the post-synaptic membrane. The convulsant action of strychnine is due to interference with the post-synaptic inhibition that is mediated by the amino acid glycine. Glycine is an inhibitory transmitter to motor neurons and interneurons in the spinal cord. Strychnine acts as a selective competitive antagonist to block the inhibitory effects of glycine at the glycine receptors. Studies indicate that strychnine and glycine interact with the same receptor, but at different sites. There is also evidence of an increase in brain levels of glutamic acid, an amino acid that acts as a transmitter for excitatory nerve impulses that excite muscle contraction. The result of these effects is that skeletal muscles become hyperexcitable. With a little sound or touch, uncontrollable convulsions and seizures become eminent, followed by suffocation and death. Death occurs due to respiratory failure.

## Toxicity

### *Animals*

Strychnine has been studied for acute toxicity in many species, and all species that were tested were found susceptible. Strychnine is an extremely poisonous substance with an $LD_{50}$ of 2.3 mg/kg in rats (Ward and Crabtree, 1942), 2 mg/kg in mouse (Prasad *et al.*, 1981), 0.6 mg/kg in rabbits (Flury and Zernik, 1935), and 0.5 mg/kg in cats and dogs (Moraillon and Pinault, 1978). An approximate lethal dose for a dog is 0.75 mg/kg body wt. So, 5 g bait having 0.3% strychnine could be enough to kill a 20 kg dog. Toxicity data of strychnine for other species are given in Table 49.1.

Presently, strychnine is used as rodenticide, avicide, and insecticide. Among animals, poisoning occurs with greatest frequency in dogs due to accidental ingestion or malicious intent. The onset of signs can occur within 15–30 min or occasionally 60 min after oral exposure, depending on whether the stomach is empty or full. The clinical signs are associated with CNS effects. Onset of signs includes restlessness, anxiety, muscle twitching, and stiffness of the neck. The poisoned dogs usually show the signs of mydriasis, tonic convulsions, contractions of striated muscles, seizures, opisthotonus, and death. The animal becomes sensitive to touch, sound, noise, or any other sudden change in the environment. A minor stimulation can trigger violent convulsions. Muscular contractions are easily triggered by external stimuli, accompanied by hypothermia, lactic acidosis, and rhabdomyolysis.

Although strychnine has no direct effects on skeletal muscles, all voluntary muscles contract simultaneously. The increase in muscle tone is caused by the central action of strychnine. The most powerful effects are seen on the muscles of joints. Respiratory muscles (diaphragm, thoracic, and abdominal) contract, respiration ceases, and eventually death ensues due to respiratory failure.

Birds poisoned by strychnine exhibit the signs of ataxia, ruffled feathers, wing droop, salivation, tremors, and convulsions. Death occurs due to respiratory failure. On postmortem, lesions are only observed in the lungs, i.e., pinpoint hemorrhages resulting from death due to asphyxia. Rigor mortis occurs shortly after death and persists for days. Occasionally, wildlife species are also inadvertently poisoned by strychnine.

There is no evidence of cumulative toxicity from strychnine (Gosselin *et al.*, 1984). From animal studies there is no

**TABLE 49.1 Acute toxicity data of strychnine**

| Species | Route of administration | $LD_{50}$ (mg/kg) |
|---|---|---|
| Rat | Oral | 16.00 |
| | i.p. | 2.50 |
| | s.c. | 1.20 |
| | i.v. | 0.96 |
| Mice | Oral | 2.00 |
| | i.p. | 0.98 |
| | s.c. | 0.474 |
| | i.v. | 0.41 |
| Duck | Oral | 3.00 |
| Pigeon | Oral | 21.00 |

NIOSH (1983–1984).

evidence that strychnine has potential for reproductive and developmental toxicity, and mutagenic and carcinogenic activity.

#### *Humans*

Humans are very sensitive to strychnine poisoning. Exposure to strychnine usually occurs through ingestion or inhalation. It has been reported that the lethal oral dose is 1.5–2.0 mg/kg body wt. (Gosselin *et al.*, 1984). The fatal dose is usually in the range of 100–200 mg, but as little as 30 mg in adults and 15 mg in children has proved fatal. Onset of clinical signs such as muscle spasms at the neck and head can occur within 10–20 min after exposure. Then muscle spasms spread to every muscle in the body, are nearly continuous, and get worse at the slightest stimulus. The condition progresses in intensity and frequency until the backbone arches continually. Seizures are the major symptoms of strychnine poisoning and are caused by excitation of all parts of the CNS. Death occurs from paralysis of the respiratory center and muscle. Rigor mortis sets in immediately, with the eyes left wide open.

The major identifying clinical features of strychnine poisoning through ingestion are severe painful spasms of the neck, back, and limbs, and convulsions with an intact sensorium. In severe cases, symptoms progress to coma, although tachycardia and hypertension are common effects (Edmunds *et al.*, 1986; Flomenbaum, 2002). The muscle contractions caused by strychnine produce characteristic contractions of the body, arched backward so that only the heals and the top of the head touch the ground, and of the face, a fixed grin known as the *risus sardonicus*.

The prognosis for strychnine poisoning is good if the patient's condition can be maintained over the first 6–12 h (Boyd *et al.*, 1983). If the convulsions are not treated, the patient may die within 1–3 h after exposure to a fatal dose (Perper, 1985). The diaphragm, chest, and abdominal muscles are in a sustained spasm and breathing becomes difficult. The occurrence of hypoxia and cyanosis is usually followed by death.

#### *Diagnosis*

Diagnosis of strychnine poisoning is based on: (1) history of exposure to a strychnine bait or presence of cracked corn in the digestive tract, (2) clinical signs of tetanic convulsions, seizures, hypersensitivity to external stimuli, and muscle stiffness, and (3) chemical identification of strychnine in the stomach content, blood, urine, or visceral organs (liver and kidney). Strychnine residue can be detected and quantified using gas chromatography-flame ionization detector (GC-FID) or gas chromatography-mass spectrometry (GC-MS). It is important to mention that strychnine causes elevation of serum enzymes, including glutamic oxaloacetic transaminase (GOT), creatine phosphokinase (CPK), and lactate dehydrogenase (LDH). In addition, lactic acidosis, hyperkalemia, and leukocytosis are the characteristic laboratory findings. In differential diagnosis, tetanus must be ruled out.

### Treatment

There is no specific antidote for strychnine poisoning, so treatment rests with symptomatic and supportive therapies. Seizures need to be controlled as soon as possible with diazepam or phenobarbital. Artificial respiration can be used for apnea. Once the seizures are controlled, detoxification can be performed using gastric lavage with potassium permanganate. Given activated charcoal with saline cathartic. Animal should be kept in a quite environment, and should be protected from any secondary sensory input.

### Conclusion

Strychnine is an extremely toxic plant alkaloid, which is primarily used as rodenticide. Due to inadvertent use or malicious intent the poisoning is frequently encountered in dogs. Onset of clinical signs appears very quickly, and the poisoning is characterized by convulsions, seizures, and hypersensitivity to any external stimulus. Treatment is symptomatic and warranted immediately.

## BROMETHALIN

### Introduction

Chemically, bromethalin is a diphenylamine compound with an appearance of a pale, odorless, and solid crystalline powder. It has a chemical formula of $C_{14}H_{17}Br_3F_3N_3O_4$ with a molecular weight of 577.93. Its chemical structure is shown below in Figure 49.2. It is commonly used as rodenticide for the control of rats and mice. Bromethalin has many other names and is sold under the brand names such as Assault, Trounce, Vengeance, and Wipe Out.

### Background

Bromethalin was developed for use against warfarin-resistant rodents. It is a restricted use rodenticide used in and around buildings and sewers, and inside transportation and cargo vehicles. It is extremely toxic and only a single dose of bromethalin is needed to cause death in rats and mice. Bromethalin is marketed in a 0.01% formulation (2.84 mg of bromethalin per ounce of bait) and comes in bait pellets, bars, and packs. It is important to mention that

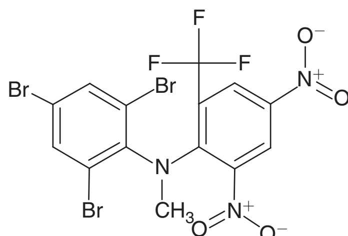


**FIGURE 49.2** Structural formula of bromethalin.

bromethalin cannot be distinguished from other rodent baits by color or appearance alone. Secondary poisoning usually occurs in dogs and cats that eat a mouse killed by bromethalin.

## Toxicokinetics

Toxicokinetic information on bromethalin is available from laboratory animals, like rats. Bromethalin is rapidly absorbed following oral ingestion. Plasma concentration peaks in about 4 h. The metabolism takes place in the liver, where bromethalin undergoes *N*-demethylation, forming des-methyl bromethalin. This metabolite is toxic, as its $LD_{50}$ is 7.5 mg/kg body wt. Plasma half-life of bromethalin is about 6 days (Dorman *et al.*, 1990a), suggesting slow elimination. Excretion occurs mainly in bile, as it enters in enterohepatic circulation.

## Mechanism of action

Bromethalin is a neurotoxicant and affects the CNS. It uncouples oxidative phosphorylation in the mitochondria thereby decreasing ATP synthesis. With a marked decrease in ATP, very little energy is available to maintain $Na^+$, $K^+$-ATPase pumps. As a result, cells lose their ability to maintain osmotic control, $Na^+$ is retained intracellularly, and the cells swell with water. This leads to fluid accumulation within myelin sheaths and vacuolation of the nervous system, resulting in nerve conduction impairment. Damage to neuronal axons and increased intracranial pressure occurs, which is followed by convulsions, paralysis, and death (Dorman *et al.*, 1992).

## Toxicity

Bromethalin is a single-dose rodenticide. It is classified as very highly toxic if swallowed or inhaled, and highly toxic if absorbed through skin. The acute oral $LD_{50}$ of bromethalin is 2.38–5.6 mg/kg in bait is for dog and 0.4–0.71 mg/kg in bait for cats (Dorman *et al.*, 1990a). Its $LD_{50}$ is 2 mg/kg in mice, 5 mg/kg in rats, 13 mg/kg in rabbits, and 0.25 mg/kg in pigs.

In field cases, most of the time poisoning occurs in pets including dogs and cats. Signs of bromethalin appear within 10 h to several days after exposure and may last up to 12 days. In most cases the poisoning is acute in nature characterized by cerebral edema and paralysis of the hind limbs. In general, clinical signs in pets include severe muscle tremors, hyperexcitability, hyperesthesia (hypersensitivity to touch), and seizures. Symptoms with mild exposure to bromethalin occur with slow progress in several days, and include loss of ability to bark, loss of appetite, vomiting, depression, lethargy, tremors, paralysis, lateral recumbency, coma, and death. Following exposure to a large dose of bromethalin animals can show the signs of muscle tremors and seizures, hyperexcitability, ataxia and paddling, hyperthermia, potential loss of vocalization, loss of tactile sensation, and forelimb extensor rigidity (Schiff–Sherrington posture), and death within 2–4 days. Death can occur with a low or high dose, and is usually caused by respiratory paralysis. Poisoned dogs show the signs of tremors, ataxia, depression, tachypnea, hyper-reflexia of the hind limbs, loss of vocalization, recumbency, anorexia, vomiting, and death (Dorman *et al.*, 1990b). Poisoned cats exhibit the signs of ataxia, seizures, vocalization, rigidity, decreased proprioception, abdominal distension, recumbency, depression, and death (Dorman *et al.*, 1990c, 1992). Other signs of poisoning may include generalized seizures, head pressing, hyperesthesia, coma, hyperexcitability, ataxia, extensor rigidity, nystagmus, hyperthermia, cyanosis, miosis, and drooling (Moorman, 2001). Overall, cats are much more sensitive than dogs to bromethalin.

Histopathological changes have been described in dogs receiving a single oral dose of bromethalin (6.25 mg/kg). Histologic lesions included diffuse white matter spongiosis, mild microgliosis, optic nerve vacuolization, mild thickening of Bowman's capsule, and occasional splenic megakaryocytes. Ultramicroscopic examination of mid-brain stem revealed occasional swollen axons, intramyelinic vacuolization, and myelin splitting at the intraperiod line (Dorman *et al.*, 1990a). These authors have also reported histopathological changes in cats induced by bromethalin (Dorman *et al.*, 1992). In brief, ultrastructural changes include separation of myelin lamellae at the interperiod lines with the formation of intramyelinic vacuoles (intramyelinic edema), rupture and coalescence of intramyelinic vacuoles into larger extracellular spaces (spongy change), and pronounced cytosolic edema of astrocytes and oligodendroglial cells. Histopathology of the brain and spinal cord of rodents receiving multiple low or sublethal doses of bromethalin revealed a spongy degeneration of the white matter which was shown upon ultramicroscopic examination to be intramyelinic edema (Van Lier and Cherry, 1988).

According to the World Health Organization and US Environmental Protection Agency, bromethalin is considered carcinogenic.

### *Diagnosis*

Diagnosis of bromethalin poisoning is based on history of exposure to bromethalin bait, clinical signs, and identification of bromethalin in bait, GI content, brain, and visceral organs. Residue of bromethalin or its major metabolite (desmethyl bromethalin) can be quantified using gas chromatography (GC) coupled with electron capture (ECD) (Dorman *et al.*, 1990a), high-pressure liquid chromatography (HPLC) coupled with ultraviolet detector or with negative-ion atmospheric pressure chemical ionization (APCI) – mass spectrometric (MS) detection (Mesmer and Flurer, 2001). The highest concentrations of bromethalin are found in fat, liver, kidney, and brain (Dorman *et al.*, 1990a). Differential diagnosis should rule out for lead, ethylene glycol, organophosphates, strychnine, metaldehyde, zinc phosphide, and tremorgenic mycotoxins.

## Treatment

There is no specific antidote for bromethalin poisoning. Symptoms can be treated with corticosteroids, but clinical studies indicate that symptoms return as soon as the corticosteroids are discontinued. Emesis should be induced using apomorphine or 3% hydrogen peroxide, if the animal is not exhibiting the signs of convulsions and seizures. Alternatively, perform gastric lavage and give activated charcoal with saline cathartic. Activated charcoal needs to be repeated if the dog or cat is exposed to a large dose of bromethalin. The animal needs to be monitored and treated for cerebral edema. Intravenous (i.v.) fluids can be administered with great caution so as not to worsen cerebral edema. Diazepam or barbiturate can be given for controlling seizures.

## Conclusion

Bromethalin is a commonly used rodenticide, which is encountered in poisoning in dogs and cats. Bromethalin exerts toxicity by uncoupling oxidative phosphorylation in mitochondria, thereby decreasing ATP synthesis. CNS is the target organ, and the toxicity is characterized by cerebral edema, convulsions, and paralysis. There is no specific antidote, so treatment is symptomatic and supportive.

# CHOLECALCIFEROL

## Introduction

Cholecalciferol is a form of vitamin D, also called vitamin $D_3$ that is commonly used as rodenticide. Vitamin $D_3$ is a

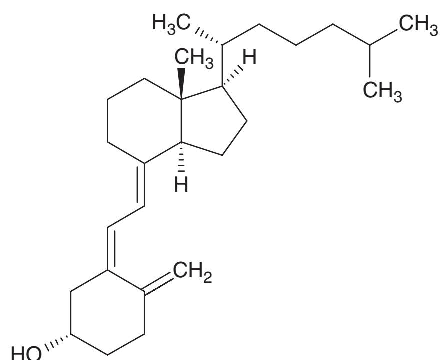


**FIGURE 49.3** Structural formula of cholecalciferol.

secosteroid and structurally similar to other steroids, such as cholesterol, testosterone, and cortisol. It has chemical formula $C_{27}H_{44}O$ with a molecular weight 384.64. Its structural formula is shown above in Figure 49.3. Cholecalciferol has other names and is marketed as a rodenticide under the brand names Quintox, True Grit Rampage, Ortho Rat-B-Gone; and as a feed additive, Viactive.

## Background

Cholecalciferol is a rodenticide which is used in and around buildings, and inside of transport vehicles. Vitamin $D_3$, as such, does not have significant biological activity, but in two steps, it is metabolized in the body to make an active form. In liver, cholecalciferol is hydroxylated to 25-hydroxycholecalciferol (calcifedion) by the enzyme 25-hydroxylase. In kidney, 25-hydroxycholecalciferol serves as a substrate for 1-$\alpha$-hydroxylase, forming 1,25-dihydroxycholecalciferol (calcitriol), which is the biologically active form. Cholecalciferol is formulated in the granular form (0.075% bait) and has been found very effective against Norway rats, roof rats, and house mice.

## Toxicokinetics

Very little information is available on toxicokinetics of cholecalciferol. Cholecalciferol is transported in blood bound to carrier proteins. The major carrier protein is vitamin-D-binding protein. The half-life of 25-hydroxycholecalciferol is several weeks, while that of 1,25-dihydroxycholecalciferol is just a few hours.

## Mechanism of action

Ingestion of cholecalciferol-containing bait is known to cause a marked increase in calcium level in blood and tissues. Cholecalciferol not only increases the absorption

of calcium but also mobilizes calcium and phosphorus from bones to circulation. High calcium causes heart problems and bleeding secondary to mineralization of the vessels, kidneys, stomach wall, and lungs. Mineralization of the kidney leads to renal failure and death. Cholecalciferol and its metabolites exert their effects by binding to vitamin D receptors in tissues. 1,25-dihydrocholecalciferol is the most metabolically active form that binds to the vitamin D receptors 500 times greater than 25-hydroxycholecalciferol and 1000 times greater than cholecalciferol.

## Toxicity

Cholecalciferol is of low toxicity to mammalian species, as it is classified as a Class III toxic chemical. The oral $LD_{50}$ of cholecalciferol in rats is 43.6 mg/kg and in mice is 42.5 mg/kg. The dermal $LD_{50}$ in rabbits is 2000 mg/kg. Studies suggest that cholecalciferol is of low toxicity to birds (oral $LD_{50}$ = >2000 mg/kg in mallard ducks and dietary $LC_{50}$ = 4000 ppm in mallard ducks, and 2000 ppm in bobwhite quail).

Pets, such as dogs are poisoned by ingesting rodenticide bait, while the farm animals are affected by overdose of additive vitamin $D_3$ in the feed. Signs and symptoms of poisoning are similar to hypercalcemia, such as anorexia, fatigue, headache, itching, weakness, nausea, vomiting, and diarrhea. In acute cases, cholecalciferol causes severe polyneuropathy. Dogs poisoned with cholecalciferol-containing rodenticide bait usually show the signs of depression, anorexia, vomiting, bloody diarrhea, cardiac irregularities, hypertension, seizures, and death. In dogs, signs of poisoning may occur with as little as 2 mg/kg dose, and death may occur with 10 mg/kg dose of cholecalciferol.

## Treatment

Decontamination, including induction of emesis, gastric lavage, and administration of activated charcoal, is beneficial. Administration of i.v. fluids helps in reducing serum calcium levels by increasing urine production and calcium excretion. Biphosphonate pamidronate disodium is used to decrease the serum calcium levels. Seizures can be controlled by diazepam or barbiturates. Treatment needs to be continued for 2–3 weeks, since the elimination half-life of cholecalciferol is more than 2 weeks. Poisoned animals should receive the feed devoid of vitamin $D_3$.

## Conclusion

Cholecalciferol is a single- or multiple-dose rodenticide, which has low toxicity to mammalian and avian species. It produces toxicity by marked increases in calcium and mineralization of tissues. Once the animals develop severe signs, they usually die.

# RED SQUILL

## Introduction

Red squill, which is also known as sea onion, is obtained in the powder form from a plant *Urginea maritima.* The plant is native to the Mediterranean region. It resembles to onion whose bulb extracts and dried powders have been used for the control of rodents since the 13th century. Although red squill has many alkaloids, scilliroside is the most toxic and provides rodenticidal activity. It has chemical formula $C_{32}H_{44}O_{12}$ with a molecular weight of 620.7. Its structural formula is shown below in Figure 49.4. The compound is sparingly soluble in water and thermostable. Scilliroside is formulated in the powder form (Dethdiet) and liquid extract (Rodine), and has many other names, including Sea squill, Scilla maritima, Silmurin, Silmine, Sea onion, Squill, etc. It was demonstrated long ago by Winton (1927b) that only red squill has rodenticide activity, and not the white squill. The red squill is mixed in baits and applied at a 10% concentration and mixed with meat, fish, and cereals (Thomson, 1991–1992).

## Background

Red squill bulbs were an ancient source of rodenticide products replaced later by warfarin and other modern anti-coagulant rodenticides. Since rats have developed resistance to such products there is now renewed interest in the red squill. Studies suggested that the white squill-drug is used as a diuretic, stimulant, and expectorant,

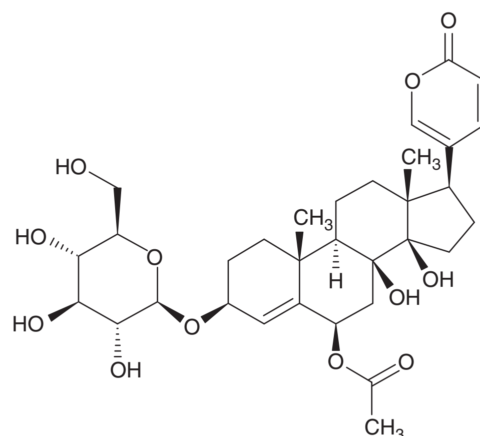


**FIGURE 49.4** Structural formula of scilliroside.

while the red squill is used mostly as a rat poison. Many glycosides and aglycones have been isolated from the red squill bulb. Krenn *et al.* (1994) and Pascual-Villalobos (2002) isolated five different bufadienolides as pure substances (proscillaridin A, scillaren A, scilliroside, gamma-bufotalin, and scillirosidin). Scilliroside is the most toxic bufadienolide glycoside (Verbiscar *et al.*, 1986a, b).

Scilliroside has an emetic property. So, if rodents ingest a product-containing scilliroside, because they are incapable of vomiting, they develop glycoside intoxication and pulmonary edema. However, the compound has rarely been associated with toxicity in humans because humans are capable of vomiting and exhibit poor GI absorption.

## Toxicokinetics

Scilliroside is inefficiently absorbed from the GI tract. Its metabolism is not well studied, though the unmetabolized scilliroside is rapidly excreted in the urine.

## Mechanism of action

Red squill bulbs contain many glycosides, but scilliroside is the major component which has rodenticidal property. The compound is cardiotoxic similar to digitalis, i.e., injection of scilliroside induces cardiac impulse condition and arrhythmias. In very early experiments, red squill preparations administered to rats induced convulsions and paralysis (Winton, 1927a). Female rats succumb to doses of red squill, at half the dose that required to kill males.

## Toxicity

The red squill plant (mainly the bulb) contains scilliroside, a highly toxic bufadienolide glycoside. It adversely affects cardiovascular and CNS, causing convulsions and death. The oral $LD_{50}$ of scilliroside is 0.7 mg/kg in male rats and 0.43 mg/kg in female rats; and 0.35 mg/kg in mice. Studies revealed that pigs and cats survived 16 mg/kg, and fowls survived 400 mg/kg (Worthing, 1983). It is classified as Class I, i.e., highly toxic chemical.

Red squill contains several compounds that have emetic properties. Due to poor GI absorption and decreased potency, red squill has seldom been associated with toxicity in humans, dogs, cats, and pigeons. However, rats and mice are unable to vomit, and they die within a few hours after ingesting lethal dose of scilliroside.

In toxic doses, red squill produces inflammation of the GI and genito-urinary tracts, manifested by nausea, vomiting, abdominal pain, and purging. Other signs of poisoning include convulsions, hypothermia, enfeebled circulation, blurred vision, and sometimes death. Convulsions are seen in humans, as they have been observed in rats. Higher doses of red squill can cause serious heart rhythm alterations resulting in death. Farm animals require large quantities for intoxication. There are field cases, where dogs, cats, and pigs have been poisoned. Signs of poisoning include vomiting, ataxia, and hyperesthesia, followed by paralysis, depression, or convulsions. Cardiac arrest occurs due to bradycardia and cardiac arrhythmias. Generally, animals exposed to a sublethal dose can recover in less than 48 h.

## Treatment

Treatment is based on symptomatic and supportive therapies. In case a significant amount of red squill is retained in the stomach, decontamination (gastric lavage with saline cathartic) is rewarding. The patient needs to be monitored for cardiac arrhythmias and conduction disturbances. Atropine sulfate subcutaneous (s.c.) at 6–8 h intervals may prevent cardiac arrest. Phenytoin at 35 mg/kg, TID, should be given to dogs to suppress arrhythmias.

## Conclusion

Red squill is a botanical rodenticide and exerts toxicity due to cardiac effects. Treatment is symptomatic and supportive.

# FLUOROACETATE

## Introduction

Sodium fluoroacetate (compound 1080) is an extremely toxic white powder that has been used as rodenticide around the world. The compound has chemical formula $C_2H_2FO_2.Na$ with a molecular weight 100.02. Its structural formula is shown in Figure 49.5. It is used to control rats, mice, squirrels, prairie dogs, foxes, wolves, dogs, coyotes, and rabbits. The compound is also used to control brush-tail possums, deers, wild pigs, wallabies, and rooks. It is very toxic to birds, domestic animals, and carnivores. Secondary poisoning is very common in birds and carnivores from eating poisoned carcasses.

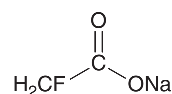


**FIGURE 49.5** Structural formula of sodium fluoroacetate.

Sodium fluoroacetate has many other names and sold under the trade names, such as Nissol, 1080 gel, 1080 paste, 1080 solution, Tenate, Tenate 1080, etc. The commercial products are provided with a black dye called nigrosine (0.5%).

## Background

Sodium fluoroacetate was discovered by German military chemists during World War II. But later on, American chemists discovered its use as rodenticide. The name "1080" refers to the catalog number of the poison, which became its brand name. The compound is an extremely toxic substance, which is commonly used as rodenticide. Inhalation of dust or swallowing can also be fatal. The compound can also be absorbed through cuts or abrasions in the skin and lead to poisoning.

It is important to mention that sodium fluoroacetate is also formed naturally in about 40 plants that are native to Australia, Brazil, and Africa, after fluoride uptake from soil, water, or air. Some of the examples of plants that contain sodium fluoroacetate are *Dichapentalum cymosum*, *D. toxicarum*, *Chailletia toxicaria*, *Gastrolobium grandiflorum* (poison peas), *Oxylobium parviflorum*, and *Acacia georginae*. Consumption of these plants has resulted in many serious cases of livestock poisoning and high stock losses (Oelrichs and McEwan, 1962). Poisoning has also been documented in field workers exposed to fluoroacetate (Suh *et al.*, 1970).

## Toxicokinetics

Sodium fluoroacetate (1080) is rapidly absorbed from the GI tract. Dust formulations are easily absorbed by inhalation, which is not usually the route for poisoning cases; 1080 is not readily absorbed through intact skin but it can be absorbed in the case of cuts, abrasions, or dermatitis (Brockman *et al.*, 1955). It is reported that sublethal doses of compound 1080 are completely metabolized and excreted in 4 days.

The bioavailability of sodium fluoroacetate appears to be similar for oral, injected, and inhaled doses. Dermal absorption is lower since a s.c. $LD_{50}$ is 10 to 15-fold higher than the oral dose. Distribution studies suggest that the plasma levels of sodium fluoroacetate are twice that of tissues. Sheep receiving sodium fluoroacetate (0.1 mg/kg) contained the residue in plasma, kidney, heart, muscle, spleen, and liver as 0.098, 0.057, 0.052, 0.042, 0.026, and 0.021 (Eason *et al.*, 1993). The plasma $t_{1/2}$ is found to be 3.6–6.9 h in goats, 6.6–13.3 h in sheep, 1.1 h in rabbits, and 1.6–1.7 h in mice (Eason *et al.*, 1993; Gooneratne *et al.*, 1995). Metabolism of fluoroacetate (i.e., defluorination) takes place in the liver. Fluoroacetate and fluorocitrate salts are excreted mainly in the urine.

## Mechanism of action

In the body, the fluoroacetate (1080) is converted to fluorocitrate which is a potent inhibitor of the enzyme aconitase in the tricarboxylic acid cycle (Krebs cycle) (Elliot and Kalnitsky, 1996). As a result, the elevated levels of citrate in blood become observable 30 min and maximum levels at 4 h after administration. It has been shown that citrate levels are directly influenced by thyroid hormone, i.e., free $T_3$ (Maruo *et al.*, 1992). Accumulation of citrate causes the toxicity due to reduction of ATP levels by inhibiting energy production in most cells of the body, leading to a slow and painful death as the body "suffocates from within". Compound 1080 causes damage to tissue of high energy needs, such as brain, heart, lungs, and fetus.

Accumulated levels of citrate cause chelation of divalent metal ions, especially $Ca^{2+}$. Depletion of these ions in CNS may be responsible for seizures in certain species (Hornfeldt and Larson, 1990).

Fluoroacetate/fluorocitrate also affects activities of other enzymes, including mitochondrial citrate carriers, pyruvate dehydrogenase kinase (Taylor *et al.*, 1977), succinate dehydrogenase (Mehlman, 1967), glutamine synthetase, phosphofructokinase (Godoy and del Carmen Villarruel, 1974), and ATP-citrate lyase (Rokita and Walsh, 1983).

## Toxicity

Most species are susceptible to fluoroacetate (1080), however, rodents and dogs are the most susceptible species. The oral $LD_{50}$ of 1080 in rats is 0.1–0.22 mg/kg, mice 0.1 mg/kg, rabbits is 0.34 mg/kg, and guinea pigs is 0.3 mg/kg. The oral $LD_{50}$ of this compound in the house sparrow, red winged blackbird, starling, and golden eagle are 3.0, 4.22, 2.37, and 1.25–5 mg/kg, respectively. Measured $LD_{50}$ of this rodenticide in mammalian wildlife, such as in mule deer is 0.22–0.44 mg/kg, male ferrets 1.41 mg/kg, and in bears 0.5–1.0 mg/kg.

In general, fluoroacetate is very toxic to mammalian, bird, and wildlife species, while it is of low toxicity to fish. Toxicity of 1080 is different according to route of exposure, i.e., ingestion or inhalation, and symptoms vary widely among species. Species have been categorized into four groups according to symptomatology (Chenoweth, 1949):

1. *Rabbit, goat, horse, sheep, and spider monkey*: CNS effects are not observed, and death is due to cardiac effects with ventricular fibrillation.
2. *Cat, pig, rhesus monkey, and human*: Heart and CNS are affected, death usually resulting from respiratory failure

during convulsions, but occasionally due to ventricular fibrillation.

3 *Dog and guinea pig*: Epileptiform convulsions predominate, with death being due to cessation of respiratory activity following running movements like those of strychnine poisoning.

4 *Rat and hamster*: Respiratory depression and delayed bradycardia is the main feature.

In general, 1080 produces convulsions, involuntary urination, vomiting, and ventricular fibrillation. The onset of symptoms of poisoning usually appears between 30 min and 4 h after exposure. The common symptoms are vomiting, involuntary hyper-extension of the limbs, convulsions, and finally cardiac and respiratory failure. Dogs usually show CNS signs, such as convulsions and uncontrollable running, while sheep and cattle show predominantly cardiac signs. Dogs appear to be highly sensitive to fluoroacetate, and mass poisonings of dogs eating contaminated poultry have been documented (Egyed, 1979).

The main target organs affected are the CNS, cardiovascular, and respiratory system. This causes metabolic derangement that includes alteration in transaminase, calcium, and glucose levels apart from acidosis and renal failure.

Clinic effects are associated with neurological and cardiac systems. CNS effects include tremulousness, hallucinations, convulsions, and respiratory depression. Cardiac effects include arrhythmias, ventricular fibrillation, and cardiac arrest. If the patient survives the first 24 h after ingestion of sodium fluoroacetate, recovery is favorable. Acute exposure often results in complete recovery or death. Of course, in some cases, exposure results in cardiac damage.

Severity of signs is found to be dose related. The oral route is the most important in cases of poisoning of 1080. Dust formulations are easily absorbed by inhalation, which is not usually the route for poisoning cases; 1080 is not readily absorbed through intact skin, but it can be absorbed in the case of cuts and dermatitis.

The fluorocitric acid is itself highly toxic, and therefore sodium fluoroacetate can cause secondary poisoning, i.e., poisoning in an organism which has consumed a part of an organism already poisoned.

In humans, one-fourth of a gram of sodium fluoroacetate could kill a grown man. The onset of signs such as nausea, mental apprehension followed by epileptiform convulsions, can occur 30 min to 2 h after oral ingestion. After a period of several hours, ventricular fibrillation occurs, which is followed by cardiac arrest and death.

The development of tolerance to increasing doses of fluoroacetate has been reported in rats and mice, whereby a dose of 0.5 mg/kg protects rats against a dose of 5 mg/kg for a period of 48 h (Chenoweth, 1949). The mechanism of fluoroacetate resistance in certain species is not well understood, but rate of defluorination does not appear to play a significant role (Mead *et al.*, 1985).

Studies suggest that sodium fluoroacetate has no carcinogenic, mutagenic, and teratogenic potential.

### *Diagnosis*

Diagnosis is based on evidence of exposure, clinical signs, necropsy findings, and chemical confirmation. Samples for chemical confirmation should include suspected bait, vomitus, stomach content, liver, and kidney. Testing for 1080 should be performed on the vomited stomach contents. In case of ruminants, rumen content needs to be analyzed for fluoroacetate. Significant elevation of citric acid levels in blood and kidney is a reliable biochemical marker of fluoroacetate or fluorocitrate poisoning (Bosakowski and Levin, 1986). Hyperglycemia, hypocalcemia, and hypokalemia are characteristic laboratory findings. Other metabolic/biochemical changes include metabolic acidosis resulting from a build up of citric acid, lactic acid, and ammonium in blood and organs. Metabolic acidosis is also associated with elevated serum creatinine and transaminase levels.

Differential diagnosis should include lead, strychnine, chlorinated hydrocarbons, and plant alkaloids.

## Treatment

There is no specific antidote for 1080 poisoning. Use of glyceryl monoacetate has shown some positive results, as it provides acetate ions to allow continuation of the cellular respiration process which the 1080 has disrupted. Other symptomatic and supportive measures following decontamination procedures (to prevent further absorption by activated charcoal) include use of anti-convulsants, muscle relaxants, and mechanical ventilation. It is important to mention that induction of emesis is contraindicated because of potential arrhythmias and convulsions. Special attention should be paid to stabilize cardiac and CNS functions.

Acetate and ethanol have been found potentially effective in mice, guinea pigs, and rabbits, but not in dogs (Tourtellotte and Coon, 1950). A combination of calcium gluconate and sodium succinate has proven effective in mice (Omara and Sisodia, 1990). Calcium chloride has antidotal effects in cats (Roy *et al.*, 1980).

## Conclusion

Fluoroacetate converts to fluorocitrate, which is an extremely toxic metabolite. Symptoms vary markedly between species. For example, carnivores exhibit more signs related to CNS, herbivores related to cardiac effects, and omnivores show signs of both CNS and cardiac. Treatment relies upon symptomatic and supportive measures.

# ALPHA-NAPHTHYL THIOUREA

## Introduction

Alpha-naphthyl thiourea (ANTU) is a colorless, odorless, crystalline powder, which is exclusively used as rodenticide. Technical product is gray powder. It has chemical formula $C_{11}H_{10}N_2S$ with a molecular weight of 220.28, and its structural formula is shown in Figure 49.6.

ANTU has several other names, such as alpha-naphthyl thiocarbamide, 1-naphthyl-thiourea, N(1-naphthyl)-2 thiourea, Alrato, Anturat, Bantu, Dirax, Krysid, Rat-tu, and Rattrack, etc.

## Toxicokinetics and mechanism of action

Following ingestion, ANTU is rapidly absorbed from the GI tract. ANTU has a specific effect on the capillaries of the lungs leading to the rapid development of pleural effusion and pulmonary edema. This results in anorexia and respiratory failure.

## Toxicity

All animals are susceptible to ANTU, but lethal doses differ widely. Lethal doses of ANTU (expressed in terms of mg/kg) in various species are as follows: rats (3), dogs (10), pigs (25), horses (30), cows (50), cats (75), and fowl (2500). The acute oral $LD_{50}$ of ANTU in rats is 6 mg/kg and in dogs 0.38 mg/kg.

Clinical signs of poisoning due to ANTU include vomiting, abdominal pain, dyspnea, shortness of breath, seizures, bluish discoloration, coarse pulmonary rales, pulmonary edema, and liver damage.

In a time course study, ANTU at the dose rate of 5 mg/kg induced lung edema in adult albino rats (Vivet *et al.*, 1983). After 6 h, pulmonary extravascular water increased by 50% in ANTU-treated rats and the volume of the pulmonary effusion reached 3.4 ± 0.1 ml. The most characteristic feature is the absence of hypoxemia in the ANTU-treated rats. The absence of hypoxemia is common with normobaric oxygen. ANTU can produce hyperglycemia of 3 times normal in 3 h.

Chronic sublethal exposure to ANTU may cause enlarged thyroid gland (goiter) and interfere with normal thyroid function.

It is important to mention that repeated sublethal doses in rats leads to the development of tolerance so that resistance to several lethal doses is developed (Peoples, 1970).

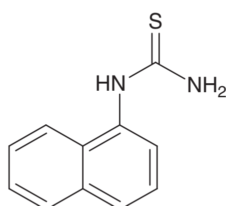

**FIGURE 49.6** Structural formula of ANTU.

### *Diagnosis*

Diagnosis of ANTU poisoning is based on evidence of rodenticide exposure, clinical signs, lung and liver damage, and chemical confirmation. ANTU can be quantified using HPLC.

## Treatment

There is no specific antidote. So, treatment relies upon symptomatic and supportive measures. Induce vomition if the patient is not showing convulsions and seizures.

Decontamination procedure includes administration of activated charcoal.

# ZINC PHOSPHIDE

## Introduction

Zinc phosphide is an inorganic compound with appearance of gray crystalline powder and a decaying fish or garlic odor. It has chemical formula $Zn_3P_2$ with a mol. wt. of 258.1. Its structural formula is shown in Figure 49.7. Zinc phosphide was first synthesized in 1740 and was first used as a rodenticide in 1911–1912 by the Italians. But in the United States it was not used until 1939. In the 1970s, there was a renewed interest in zinc phosphide for rodent control in agriculture. Presently, zinc phosphide is used worldwide for the control of a range of animals, including mice, rats, voles, ground squirrels, prairie dogs, moles, muskrats, rabbits, and gophers. Pelleted zinc phosphide is sold extensively under the brand names such as Arrex, Blue-ox, Gopha-rid, Kil-rat, Mous-con, Phosvin, Pollux, Ratol, Ridall, Rodenticide AG, Zinc-tox, and ZP. It is also used on crop/non-crop areas, including lawns, golf courses, and around wetlands.

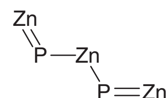

**FIGURE 49.7** Structural formula of zinc phosphide.

## Background

The history of use of zinc phosphide as a rodenticide dates back almost a century. In the early years, technical zinc phosphide and highly concentrated paste were sold in shops and used by consumers to make up their own baits for rodents. This led to use of the technical material for suicide or accidental deaths in small children and pets.

Zinc phosphide is recognized as the slowest acting of the commonly used rodenticides. It can cause toxicity and death in rodents after one feeding, if adequate dose is consumed. But bait must be continuously available to be effective. In fact, zinc phosphide is recommended as the rodenticide of choice because it is fairly specific for rodents and the secondary poisoning is rare, except in dogs and cats.

Presently, rodenticide-grade zinc phosphide usually comes as a black powder containing 75% of zinc phosphide and 25% of antimony potassium tartrate, an emetic to cause vomiting if the material is accidentally ingested by humans or domestic animals.

## Toxicokinetics

Zinc phosphide can be absorbed into the body by oral ingestion, inhalation, and through damaged skin. Following oral ingestion, zinc phosphide reacts in the stomach and intestine with water and hydrochloric acid to liberate phosphine gas. Metabolism of zinc phosphide can occur via oxidation of the phosphorus to various phosphorus oxyacids or via reduction of the phosphorus to phosphine gas. Zinc phosphide excretes in the urine either as a hypophosphite or as dissolved phosphine. The presence of strong reducing substance in the urine is a common feature of poisoning with zinc phosphide. Other metabolites include phosphoric acid and phosphate. Phosphine gas is also exhaled from the lungs.

## Mechanism of action

The overall toxicity of zinc phosphide is due to both zinc and phosphine gas, but it is primarily from phosphine. Phosphine can enter the blood stream and adversely affects the lungs, liver, kidneys, heart, and CNS. Phosphine causes CNS depression, irritation of the lungs, and damage to the blood vessels and erythrocyte membranes, and eventually cardiovascular collapse and irritation of the alimentary tract. Zinc phosphide also causes damage to the liver and kidney.

## Toxicity

Zinc phosphide is an extremely toxic compound. The oral $LD_{50}$ in rats is 41 mg/kg body wt. In sheep the $LD_{50}$ ranges from 60 to 70 mg/kg. It is very toxic to cows, sheep, goats, pigs, rabbits, and other animals. Following oral ingestion, both zinc phosphide and phosphine are absorbed from the GI tract, although the majority of acute effects are caused by phosphine. Zinc phosphide causes damage to the liver and kidney. Phosphine causes CNS depression, irritation of the lungs, and damage to the liver, kidney, heart, and CNS. Death occurs as a result of heart failure, and more commonly pulmonary edema, although there are reports of heart failure accompanied by kidney damage. Following a large dose, death usually occur within an hour, while with smaller doses, death can occur between 4 and 72 h. Symptoms usually appear after 20–25 min. Animals are prostrated with deep slow respiration, finally terminating in convulsions. It is important to mention that following repeated exposure, cumulative effects occur in the liver, kidney, and lungs.

In humans, the symptoms of acute toxicity include nausea, shock, weak heartbeat, low blood pressure, and loss of consciousness. Other symptoms include vomiting, diarrhea, cyanosis, restlessness, and fever. There are documented cases of adults dying from massive doses of the zinc phosphide (4–5 g), while others have survived acute exposure of as high as 25–100 g of zinc phosphide if vomiting occurred early and absorption was limited (Ecobichon, 1991). The inhalation of zinc phosphide or phosphine gas also results in acute toxicity.

Zinc phosphide is highly toxic to wild animals and birds, and freshwater fish. The most sensitive bird species which have been evaluated are geese ($LD_{50}$, 7.5 mg/kg). Pheasants, morning doves, quails, mallard ducks, and horned larks are very susceptible to this rodenticide. The fish species which have been found susceptible to zinc phosphide include bluegill sunfish, rainbow trout, and carp.

### *Diagnosis*

Diagnosis of zinc phosphide in animals is based on detection of zinc phosphide, phosphine, and zinc. At necropsy, stomach content smells like acetylene. Zinc phosphide level at or above 50 ppm in stomach content is considered significant and is indicative of zinc phosphide poisoning.

## Treatment

There is no specific antidote and treatment is mainly symptomatic. Vomiting should be induced as soon as possible after ingestion, followed by the administration of activated charcoal. Sodium bicarbonate can be given orally to stop liberation of phosphine gas. Calcium gluconate and sodium lactate can be given i.v. to combat acidosis.

## Conclusions

Zinc phosphide is a rodenticide of high toxicity. At acidic pH in stomach, zinc phosphide generates phosphine gas which is responsible for the majority of toxic effects. There is no specific antidote and treatment is symptomatic.

## REFERENCES


Bosakowski T, Levin AA (1986) Serum citrate as a peripheral indication of fluoroacetate and fluorocitrate toxicity in rats and dogs. *Toxicol Appl Pharmacol* **85**: 428–36.

Boyd RE, Brennan PT, Deng JF, Rochester DF, Spyker DA (1983) Strychnine poisoning. *Am J Med* **74**: 507–12.

Brockman JL, McDowell AW, Leeds WG (1955) Fatal poisoning with sodium fluoroacetate. *J Am Med Assoc* **159**: 1529–32.

Chenoweth MB (1949) Monofluoroacetic acid and related compounds. *J Pharmacol Exp Ther* **102**: 21–49.

Cooper P (1974) *Poisoning by Drugs and Chemicals*. Alchemist Publications, London, pp. 193–94.

Dorman DC, Simon J, Harlin KA, Buck WB (1990a) Diagnosis of bromethalin toxicosis in the dog. *J Vet Diagn Invest* **2**: 123–8.

Dorman DC, Parker AJ, Buck WB (1990b) Bromethalin toxicosis in the dog. Part I. Clinical effects. *J Am Anim Hosp Assoc* **26**: 589–94.

Dorman DC, Parker AJ, Dye JA, Buck WB (1990c) Bromethalin toxicosis in the cat. *Progr Vet Neurol* **1**: 189–96.

Dorman DC, Zachary JF, Buck WB (1992) Neuropathologic findings of bromethalin toxicosis in cat. *Vet Pathol* **29**: 139–44.

Eason CT, Gooneratne R, Fitzgerald H, Wright G, Frampton C (1993) Persistence of sodium monofluoroacetate in livestock animals and risk to humans. *Hum Exp Toxicol* **13**(2): 119–22.

Ecobichon DJ (1991) Toxic effects of pesticides. In *Casarett and Doull's Toxicology: The Basic Science of Poisons*, 4th edn. Amdur MO, Doull J, Klaassen CD (eds). Pergamon Press, New York.

Edmunds M, Sheehan TM, Van't Hoff W (1986) Strychnine poisoning: clinical and toxicological observations. *J Toxicol Clin Toxicol* **24**: 245–55.

Egyed MN (1979) Mass poisoning in dogs due to meat contaminated by sodium fluoroacetate or fluoroacetamide: special reference to the differential diagnosis. *Fluoride* **12**(2): 76–84.

Elliot WB, Kalnitsky G (1996) Mechanism for fluoroacetate inhibition. Govt. Reports Announcements & Index (GRA&I), Issue 02.

Flomenbaum NE (2002) Rodenticides. In *Goldfrank's Toxicologic Emergencies*, 7th edn. Goldfrank LR, Flomentaum ME, Lewin NE, Howland MA, Hoffman R (eds). McGraw Hill, New York, NY, pp. 1379–92.

Flury F, Zernik F (1935) Zusammenstellung der toxischen und letalen dosen for die gebräuchlichsten gifte und versuchstiere. *Abder Hand Biol Arbeitsmethod* **4**: 1289–422.

Godoy HM, del Carmen Villarruel M (1974) Myocardial adenine nucleotides, hexose phosphates and inorganic phosphate, and the regulation of phosphofructokinase activity during fluoroacetate poisoning in the rat. *Biochem Pharmacol* **23**: 3179–89.

Gooneratne SR, Eason CT, Dickson CJ, Fitzgerald H, Wright G (1995) Persistence of sodium monofluoroacetate in rabbits and risk to non-target species. *Hum Exp Toxicol* **14**: 212–16.

Gosselin RE, Smith RP, Hodge HC (1984) *Clinical Toxicology of Commercial Products*, 5th edn. Williams and Wilkins C. Baltimore, MD, pp. 375–9.

Hornfeldt CS, Larson AA (1990) Seizures induced by fluoroacetic acid and fluorocitric acid may involve chelation of divalent cations in the spinal cord. *Eur J Pharmacol* **179**: 307–13.

Krenn L, Kopp B, Deim A, Robien W, Kubelka W (1994) About the bufadienolide complex of red squill. *Planta Medica* **60**: 63–69.

Maruo T Katayama K, Barnea ER, Mochizuki M (1992) A role for thyroid hormone in the induction of ovulation and corpus luteum function. *Horm Res* **37**(Suppl. 1): 12–18.

Mead RJ, Moulden DL, Twigg LE (1985) Significance of sulfhydryl compounds in the manifestation of fluoroacetate toxicity to the rat, brush-tailed possum, woylie and western grey kangaroo. *Aust J Biol Sci* **38**: 139–9.

Mehlman MA (1967) Inhibition of pyruvate carboxylation by fluorocitrate in rat kidney mitochondria. *J Biol Chem* **243**: 1919–25.

Oelrichs PB, McEwan T (1962) The toxic principle of *Acacia georginae*. *Queensland J Agr Sci* **19**: 1–16.

Omara, F., and Sisodia, C.S. (1990). Evaluation of potential antidotes for sodium fluoroacetate in mice. *Vet Hum Toxicol* **32**(5): 427–31.

Mesmer MZ, Flurer RA (2001) Determination of bromethalin in commercial rodenticides found in consumer product samples by HPLC-UV-Vis spectrophotometry and HPLC-negative-ion APCI-MS. *J Chromatogr Sci* **39**: 49–53.

Moorman M (2001). Bromethalin – It's not what you think. *Vet Techn* pp. 484–6.

Moraillon R, Pinault L (1978) Diagnostic et traitment d'intoxications courantes des carnivores. *Rec Med Vet* **154**: 137–50.

NIOSH (1983–1984) Registry of toxic effects of chemical substances. Cumulative supplement to the 1981–2 edition, Maryland, Advanced Engineering and Planning Corp., Inc. pp. 1738–9.

O'Callaghan WG, Joyce N, Counihan HE, Ward M, Lavelle P, O'Brien E (1982) Unusual strychnine poisoning and its treatment – report of eight cases. *Br Med J* **185**: 478.

Pascual-Villalobos MJ (2002) Anti-insect activity of bufadienolides from *Urginea maritima.* In *Trends in New Crops and New Uses.* Janick J, Whipkey A (eds). ASHS Press, Alexandria, VA, pp. 564–6.

Peoples SA (1970) The pharmacology of rodenticides. *Vertebrate Pest Conference Proceedings Collection*. *4th Vertebrate Pest Conference*, pp. 1–18.

Perper JA (1985). Fatal strychnine poisoning. A case report and a review of literature. *J Forensic Sci* **30**: 1248–55.

Prasad CR, Patnaik GK, Gupta RC, Arand N, Dhawan BN (1981) Central nervous system stimulant activity of *N*-3-(chromene3carbonyl)4-aminopyridine(compound 69/224). *Indian J Exp Biol* **19**: 1075–6.

Reynolds JEF (1982) *Martindale the Extra Pharmacopoeia*. The Pharmaceutical Press, London, pp. 319–20, 995–1000.

Rokita SE, Walsh C (1983) Turnover and inactivation of bacterial citrate lyase with 2-fluorocitrate and 2-hydroxycitrate stereoisomers. *Biochemistry* **22**: 2821–8.

Roy A, Taitelman U, Bursztein S (1980) Evaluation of the role of ionized calcium in sodium fluoroacetate (1080) poisoning. *Toxicol Appl Pharmacol* **56**: 216–20.

Suh D, Kim K, Hong D, Hong S (1970) Acute intoxication due to agricultural chemicals. *Taeham Naekwa Hakkoe Chapci* **13**(3): 197–206.

Taylor WM, D'Costa M, Angel A, Halperin ML (1977) Insulin-like effects of fluoroacetate on lipolysis and lipogenesis in adipose tissue. *Can J Biochem* **55**: 982–7.

Thomson WT (1991–1992) *Agricultural Chemicals Book III. Fumigants, Growth Regulators, Seed Safeners, Repellents, Fish Toxicants, Bird Toxicants, Pheromones, Rodenticides, and Others*. Thomson Publication, Fresno, CA, pp. 157–8.

Tourtellotte WW, Coon JM (1950) Treatment of fluoroacetate poisoning in mice and dogs. *J Pharmacol Exp Ther* **101**: 82–91.

Van Lier RB, Cherry LD (1988) The toxicity and mechanism of action of bromethalin: a new single-feeding rodenticide. *Fundam Appl Toxicol* **11**: 664–72.

Verbiscar AJ, Patel J, Banigan TF, Schatz RA (1986a) Scilliroside and other Scilla compounds in red squill. *J Agr Food Chem* **34**: 973–9.

Verbiscar AJ, Banigan TF, Gentry HS (1986b) Recent research on red squill as a rodenticide. Salmon TP (ed.). In *Proceedings of the 25th Vertebrate Pest Conference*, University of California, Davis, CA, pp. 51–56.

Vivet P, Brun-Pascaud M, Mansour H, Pocidolo JJ (1983) Non-hypoxaemic pulmonary edema induced by alpha-naphthyl thiourea in the rat. *Br J Exp Pathol* **64**: 361–6.

Ward JC, Crabtree DG (1942) Strychnine X. Comparative accuracies of stomach tube and intraperitoneal injection methods of bioassays. *J Am Pharm Assoc Scientific Edn* **31**: 113–15.

Winton FR (1927a) The rat-poisoning substance in red squill. *J Pharmacol Exp Ther* **31**: 123–36.

Winton FR (1927b) A contrast between the actions of red and white squills. *J Pharmacol Exp Ther* **31**: 137–44.

Worthing CR (1983) *The Pesticide Manual. A World Compendium*, 7th edn. The British Crop Protection Council. Croydon, England, pp. 695.

CHAPTER

# 50

# Avitrol

*Ramesh C. Gupta*

## INTRODUCTION

Avitrol (4-aminopyridine) is white, odorless, crystalline sand-like material. It has a chemical formula of $C_5H_6N_2$ with a molecular weight of 94.1 kDa. Its chemical structure is shown below in Figure 50.1.

4-aminopyridine was developed by Phillips Petroleum Company and marketed in 1963 as an avicide under the name "Avitrol". It was also considered bird repellent. Presently, it is a product of Avitrol Corp. (Tulsa, Oklahoma). Avitrol has many other names, including 4-AP, 4-pyridylamine, 4-pyridamine, and amino 4-pyridine. Presently, avitrol is used to control overpopulation of certain birds that are considered as pest birds, including pigeons, red-winged blackbirds, blackbirds, cowbirds, grackles, sparrows, starlings, gulls, crows, and others.

Depending upon the bird species to be controlled, the formulation can be prepared in grain/corn or bread. Avitrol is available in various formulations, such as grain baits (0.5%, 1%, and 3%), powder concentrate (25% and 50%), and powder mix (50% and 25% concentrate). Presently, according to EPA, grain bait formulations of 4-aminopyridine are in toxicity Class III, while powder concentrate formulations are in toxicity Class I. Avitrol is intend to make few birds in the flock sick, i.e., the sick birds thrash around and scare off the other birds. In higher doses, avitrol can not only be toxic or lethal to birds, but it can also be lethal to non-target species. This chapter describes toxicity of avitrol in birds, mammals, and fish.

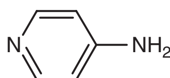


**FIGURE 50.1** Structural formula avitrol.

## BACKGROUND

Scientists have been seeking an ideal way to control pest birds that destroy crops, feed/grain meant for livestock, and ruin the look of beautiful buildings and monuments. A number of chemicals have been investigated for their ability to repel birds (Clark, 1998; Dolbeer *et al.*, 1998; Stevens and Clark, 1998). Presently, avitrol (4-aminopyridine) is the most popular avicide which is registered at the EPA for control of certain pest birds that feed on cattle feed lots, field corn, wheat, sorghum, sunflowers, peanuts, pecans, grain, feed processing plants, etc. Avitrol is also used to drive away birds from monuments, airports, warehouse premises, and other public buildings (Thomson, 1991–1992). Upon ingestion, in certain birds avitrol causes utter vocalization and physical distress (i.e., hyperactive), which acts as an area repellency to the remainder of the flock, so-called "flock frightening syndrome" (Spyker *et al.*, 1980; Meister *et al.*, 1984). Avitrol is toxic to birds, mammals, and fish. Wildlife feeding on treated bait can also be killed. In recent years, use of avitrol has been banned in many cities, including New York, NY; San Francisco, CA; and Boulder, CO, because of its toxicity to non-target species. Many field cases of avitrol poisoning have been reported in birds, domestic, wildlife, and zoo animals (Schafer, 1972; Schafer *et al.*, 1974; Nelson *et al.*, 1976; Ray *et al.*, 1978; Spyker *et al.*, 1980; Frank *et al.*, 1981; Nicholson and Prejean, 1981; Conover, 1994).

## TOXICOKINETICS

Like other aminopyridines, 4-aminopyridine is rapidly absorbed from the gastrointestinal (GI) tract into circulation.

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The compound is readily metabolized in the liver and metabolites are excreted in urine. About 90% of the administered dose, following intravenous (i.v.) or oral administration, excretes in the urine.

## MECHANISM OF ACTION

In acute toxicity, central nervous system (CNS) is the target organ, while in chronic toxicity CNS and liver are the target organs. 4-aminopyridine blocks potassium ion channels and increases acetylcholine (ACh) levels at the synapses and neuromuscular junctions (Rowan, 1985). This results in hyperactivity, convulsions, and seizures. In acute toxicity, birds and animals suffer immensely. Avitrol impairs bird's nervous system, and as a result birds become disoriented and exhibit erratic flight and tremors and violent convulsions for hours before they finally succumb to death. Avitrol also causes excess methemoglobin formation, which is unable to carry oxygen to tissues and that leads to respiratory distress.

## TOXICITY

Avitrol is highly toxic to all species of birds. It is also toxic to mammals, humans, fish, and wildlife. The oral acute $LD_{50}$ values (expressed as mg/kg body weight) for avitrol are reported to be as 10–12 (chickens), 8 (gulls), 3 (crows), 5.6 (pheasants), 15 (bobwhite quail), 8 (mourning dove), and 3.8–4 (sparrows), 5–6 (starlings), 4–7 (pigeons), 9 (blackbirds), and 3.2 (boat-tailed grackle) (NPCA, 1972). The oral acute $LD_{50}$ value in rats is 20 mg/kg (Meister *et al.*, 1984) and in dogs is 3.7 mg/kg (NPCA, 1972). The dermal $LD_{50}$ of technical grade 4-aminopyridine in rabbits is 326 mg/kg (NPCA, 1972).

Avitrol produces toxicity in birds in a dose-dependent manner, i.e., birds exposed with a low dose exhibit utter distress calls, while with higher dose they become incapacitated and die. Birds exposed to avitrol usually show onset of signs within 5–15 min and signs of severe intoxication for a period of 30–60 min. Poisoned birds become disoriented and exhibit erratic flight. Eventually, they are unable to stand or sit in sternal position, and show tremors and convulsions for a brief period to hours (Conover, 1994). In a case report, Bischoff *et al.* (2001) described the primary signs of poisoning in crows as frequent vocalization, CNS abnormalities, and inability to fly or walk. "Downed" birds either die or recover within 1–15 h (NPCA, 1972). Intoxicated birds with avitrol that react and alarm a flock usually die.

It is important to mention that non-target species including mammals are equally sensitive to the toxicity of avitrol. In mammals, avitrol produces symptoms like epileptic seizures. Doses near $LD_{50}$ exert a usual sequence of symptoms including hyperexcitability, salivation, tremors, muscle incoordination, convulsions, and cardiac and respiratory failure. Most of these symptoms are associated with hypercholinergic activity. In general, onset of signs occurs within 10–15 min and death occurs within 15 min to 4 h. It needs to be mentioned that predators such as raptors, foxes, hawks, cats, and dogs die from secondary poisoning after feeding on the dead or dying birds. Secondary poisoning has also been found in endangered birds, including red-tailed hawks and peregrine falcons, as they died by ingesting the remains of pigeons and other birds poisoned with avitrol.

In humans, avitrol is known to cause seizures. Exposed individuals exhibit weakness, diaphoresis, altered mental status, and hypertension. Other symptoms include thirst, nausea, dizziness, weakness, ataxia, tremors, dyspnea, and tonic–clonic convulsions. Avitrol also produces metabolic acidosis, leukocytosis, and elevations of serum enzymes (GOT, LDH, and alkaline phosphatase) as notable laboratory findings. Death occurs due to respiratory failure.

Dermal exposure to avitrol can lead to systemic intoxication or general overall poisoning. Very few chronic studies have been done and no conclusive findings are reported. Presently, studies are not available to suggest that avitrol has any potential for mutagenic, teratogenic, or carcinogenic activity.

In fish, avitrol has been found to be moderately toxic (Extoxnet). Fish become increasingly sensitive with increased exposure. The $LC_{50}$ ranges from 4 mg/l (in soft water) to 2.43 mg/l (in hard water) in channel cat fish. The $LC_{50}$ is 3.40 mg (in soft water) to 3.2 mg/l (in hard water) in bluegill.

Diagnosis of avitrol poisoning is based on history of exposure, clinical signs, pathologic findings, and residue determination of 4-aminopyridine in crop, GI content, or tissues. The crop and GI content have the highest content of 4-aminopyridine. Small residue can also be found in the liver, heart, muscle, kidney, brain, and lung. The residue of 4-aminopyridine can be determined by TLC, HPLC, or GC/MS.

## TREATMENT

Pancuronium is a pharmacologic antidote and is recommended in severely poisoned human patients. Propranolol appears to block some of the cardiac toxicity (such as cardiac arrhythmias) of 4-aminopyridine. Seizures can be treated with diazepam (0.1 mg/kg, i.v.). In severe cases,

phenobarbital or phenytoin can be given, if no response to diazepam. In case of avitrol ingestion, general symptomatic and supportive treatment includes emesis, gastric lavage, activated charcoal, and cathartic sodium thiosulfate. Bicarbonate should be added to the fluids to treat acidosis.

## CONCLUSIONS

Avitrol is commonly used to deter pest birds from roosting and nesting. The compound is toxic to all species of birds. It is also toxic to mammals, birds, and fish. Secondary poisoning is very common in non-target birds and dogs. Avitrol produces toxicity by affecting CNS, and as a result, toxic signs are of hyperactivity, such as convulsions and seizures.

## REFERENCES

Bischoff K, Morgan S, Chelsvig J, Spencer D (2001) 4-aminopyridine poisoning of crows in the Chicago area. *Vet Human Toxicol* **43**: 350–2.

Clark L (1998) Review of bird repellents. *Proceedings of the 18th Vertebrate Pest Conference*, Davis, CA, pp. 330–7.

Conover MR (1994) Behavioral responses of red-winged blackbirds (*Agelaius phoeniceus*) to viewing a nonspecific distress by 4-aminopyridine. *Pestic Sci* **41**: 13–19.

Dolbeer RA, Seamans TW, Blackwell BF, *et al.* (1998) Anthraquinone formulation (Flight Control$^{TM}$) shows promise an avian feeding repellent. *J Wildl Manag* **62**: 1558–64.

Extoxnet: http://extoxnet.orst.edu/pips/4-aminop.htm.

Frank R, Sirons GJ, Wilson D (1981) Residues of 4-aminopyridine in poisoned birds. *Bull Environm Contam Toxicol* **26**: 389–92.

Meister RT, Berg GL, Sine C, Meister S, Poplyk J (1984) *Farm Chemical Handbook.* Meister Publications Co, Willoughby, OH.

Nelson HA, Decker RA, Oshiem DA (1976) Poisoning in zoo animals with 4-aminopyridine. *Vet Human Toxicol* **30**: 118–20.

Nicholson SS, Prejean CJ (1981) Suspected 4-aminopyridine toxicosis in cattle. *J Am Vet Med Assoc* **178**: 1277.

NPCA (1972) *National Pest Control Association*. Technical Release-Avitrol. No. 5-72, Elizabeth, NJ.

Ray AC, Dwyr JN, Fambro GW, *et al.* (1978) Clinical signs and chemical confirmation of 4-aminopyridine poisoning in horses. *Am J Vet Res* **39**: 329–31.

Rowan MJ (1985) Central nervous system toxicity evaluation in vitro: neurophysiological approach. In *Neurotoxicology*, Blum K, Manzo L (eds). Marcel Dekker Inc., New York, pp. 596–8.

Schafer EW (1972) The acute oral toxicity of 369 pesticidal, pharmaceutical and other chemicals to wild birds. *Toxicol Appl Pharmacol* **21**: 315–30.

Schafer EW, Brunton RB, Lockyer NF (1974) Hazards to animals feeding on blackbirds killed with 4-aminopyridine baits. *J Wildl Manage* **38**: 424–6.

Spyker DA, Lynch C, Shabanowits J, *et al.* (1980) Poisoning with 4-aminopyridine: report of 3 cases. *Clin Toxicol* **64**: 487–9.

Stevens GR, Clark L (1998) Bird repellents: development of avian-specific tear gases for resolution of human–wildlife conflicts. *Int Biodeterior Biodegrad* **42**: 153–60.

Thomson WT (1991–1992) *Agricultural Chemicals-Book-III*. Miscellaneous Agricultural Chemicals. Fumigants, growth regulators, repellents, fish toxicants, bird toxicants, pheromones, rodenticides, and others. Thomson Publ., Fresno, CA, pp. 114–15.

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# Part 8

# Herbicides and Fungicides

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CHAPTER

# 51

# Toxicity of herbicides

*P.K. Gupta*

## INTRODUCTION

Herbicides are phytotoxic chemicals used for destroying various weeds or inhibiting their growth. They have variable degree of specificity. Some, for example paraquat, kill all green plants, whereas phenoxy compounds are specific for certain group of plants. The worldwide consumption of herbicides is almost 48% of the total pesticide usage. The consumption of herbicides in developing countries is low because weed control is mainly done by hand weeding (Gupta, 2004). Many of the earlier chemicals used as herbicides include sulfuric acid, sodium chlorate, arsenic trioxide, sodium arsenate, petroleum oils, etc. Iron and copper sulfate or sodium borate were generally hard to handle and/or were toxic, relatively non-specific, or phytotoxic to the crop as well as the unwanted plant life if not applied at exactly the proper time. The biochemical differences in plant make it possible to design herbicides that have selective toxicity potential against various species of plants. In the past two decades, the herbicides have represented by the most rapidly growing section of the pesticide industry due in part to (i) movement into monoculture practices and (ii) mechanization of agricultural practices because of increased labor costs. The result has been a plethora of chemically diverse structures rivaling the innovative chemistry so as to develop synthetic organic herbicides that are quite selective for specific plants and have low mammalian toxicity. The aim is to protect desirable crops and obtain high yields by selectively eliminating unwanted plant species, thereby reducing the competition for nutrients.

Most of the animal health problems that result from exposure to herbicides are due to their improper use or careless disposal of containers. Very few problems occur when these chemicals are used properly. However, there is increased concern about the effects of herbicides on animal health because of run-off from agricultural applications and entrance into drinking water supply (Gupta, 1986, 1988).

## BACKGROUND

The first discovery in the field of selective weed control was the introduction of 2,4-dinitro-*o*-cresol (DNOC) in France in 1933. This is very toxic to mammals and can cause bilateral cataract in man. In 1934, phenoxy herbicides were developed and 2,4-dichlorophenoxyacetic acid (2,4-D) was introduced as a potent herbicide (Gupta, 1985). During World War II, considerable effort was directed toward the development of effective, broad-spectrum herbicides with a view to both increasing food production and finding potential chemical warfare agents (Gupta, 1989). One chemical class of phenoxy derivatives including the acids, salts, amines, and esters were the first commercially available products evolving from this research in 1946. This class of herbicides has been in continuous, extensive, and uninterrupted use since 1947 and is the most widely used family of herbicides. The chemical class of herbicides deserving particular attention is the bipyridyl group, specially paraquat and diquat. Weidel and Russo first described the structure of paraquat in 1882. In 1933, Michaelis and Hill discovered its redox properties and called the compound methyl viologen. Its herbicidal properties were discovered by ICI in 1955 and it became commercially available in 1962 (Smith, 1997).

Ureas and thioureas are a group of herbicides used for general weed control in agricultural and non-agricultural practices. The first urea herbicide, *N*,*N*-dimethyl-*N*′ (chlorophenyl)-urea was introduced in 1952 by Du Pont

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under the common name of monuron. In subsequent years, many more derivatives of this class of compounds have been marketed (Liu, 2001).

Protopyrinogen oxidase (Protox)-inhibiting herbicides have been used since 1960s and now represent a relatively large and growing segment of the herbicide market. Nitrofen was the first Protox-inhibiting herbicide to be introduced for commercial use in 1964. This diphenyl ether (DPE) herbicide was eventually recognized as a relatively weak inhibitor of Protox, but was a lead compound of an entire class of structurally related herbicides that were much more active. Subsequently several DPE herbicides have been successfully commercialized (Nandihalli *et al.*, 1992; Anderson *et al.*, 1994).

Subsitituted aniline, an alachlor herbicide was registered and introduced in 1967 for the pre-plant or pre-emergent control of a broad spectrum of grass, sedge, and broad leaf weeds (Heydens *et al.*, 2001). Recently inhibitors of aromatic acid biosynthesis herbicides (organic phosphorus) such as glyphosate, broad-spectrum, non-selective, post-emergent, systemic herbicide with activity on essentially all annual and perennial plants have been developed. Monsanto discovered the herbicidal properties of glyphosate in 1970, and the first commercial formulation was introduced in 1974, under the Roundup brand name. There are other triazine and triazole herbicides, which are extensively used in agriculture in the United States and other parts of the world for more than 40 years (Steven and Summer, 1991). The triazines inhibit photosynthesis (Gysin and Knuesli, 1960). Dicamba is another organic (benzoic) acid herbicide that acts by mimicking the effects of auxins (i.e. natural plant growth hormones), causing enhanced but uncontrolled growth rates, alterations in plant function homeostasis, and death (Harp, 2001). Another class of synthetic chemical compounds called the imidazolinone herbicides was discovered in the 1970s, with the first US patent awarded in 1980 for imazamethabenz-methyl. New families of herbicides introduced since 1970s account for increasing shares of use and include bipyridyl (paraquat), bentazon, fenaxalactogen, oxyfluorfen, clomazone, clorpyralid, fluazifop, norfluorazon, etc. Now the use of newer compounds having low toxicity is quite common (Osteen and Padgitt, 2002).

## TOXICOKINETICS

Toxicokinetics is associated with the absorption, distribution, metabolism, and excretion of drugs and xenobiotics. Toxicokinetics studies provide important data on the amount of toxicant delivered to a target as well as species-, age-, and gender-specific metabolism. Animals are exposed to herbicides of different chemical classes. They may be ingested or absorbed through the skin or the respiratory system. Different factors regulate their absorption, distribution, metabolism, and excretion (Gupta, 1985). In general, liver is the primary site for biotransformation and may include activation as well as detoxification reactions through cytochrome P450-dependent monooxygenase system, the flavin-containing monooxygenase, esterases, and a variety of transferases, most notably the glutathione (GSH) *S*-transferases (Hodgson and Meyer, 1997).

Absorption of phenoxy acid derivatives occurs rapidly from stomach and intestines and peak levels reach in 10 min to 24 h depending on species, dose, and chemical form. Following oral exposure to 2,4-D plasma half-lives, range from 3.5 to 18 h approximately. The compound is protein bound *in vivo* and is rapidly distributed to the liver, kidneys, and brain. 2,4-D is not metabolized to reactive intermediates, does not accumulate in tissues, and is excreted predominantly as the parent compound. The salts and esters of 2,4-D undergo acid and/or enzymatic hydrolysis to form 2,4-D acid, may be small amounts are conjugated with glycine or taurine. Excretion can be markedly enhanced by ion trapping using alkaline agents, as most of these herbicides are organic acids (Erne, 1966a, b; Pelletier, *et al.*, 1989).

Another organic acid herbicide dicamba is rapidly and non-selectively distributed to most of the organs. Ninety per cent excretion is through urine and small amounts in feces. Dicamba is mostly unmetabolized but may be conjugated with glucuronic acid or glycine. Elimination occurs rapidly, and there is no evidence of bioaccumulation in mammalian system (Harp, 2001).

Bipyridyl derivative paraquat is rapidly but incompletely absorbed from the gastrointestinal (GI) tract of laboratory animals and humans, with plasma concentration of 20–90 min and poorly absorbed through contact skin. Distribution studies show higher radioactivity in Type I and Type II epithelial cells and the Clara cells of the rodent and human lungs, which are the major target cells for paraquat toxicity (Smith, 1997). The presence of radioactive has also been detected in choroid plexus, muscle, and melanin in addition to excretory pathways such as the proximal tubules of the kidney, urine, liver, gall bladder, and intestinal contents of the mouse indicating some biliary excretion (Waddell and Marlowe, 1980). Paraquat is taken up into the brain via the neural amino acid transporter, thus may be a factor in the etiology of Parkinson's disease. The amount of paraquat excretion in feces corresponds to 60–70% of the ingested dose (Van Dijck *et al.*, 1975). Paraquat is very poorly metabolized and bulk is excreted unchanged in the urine and feces. The transport mechanism for organic cations in renal proximal tubular cells is not fully understood, however, presence of two membrane proteins, organic cation transporter 1 (OCT1) and organic cation transporter 2 (OCT2) have been isolated from rat kidney. The OCT1 located at the basolateral membrane will transport tetra ethyl ammonium, and this can be inhibited by other organic cations such as quinine. The OCT2 stimulates the uptake of

tetra ethyl ammonium and this can be markedly inhibited by cimetidine. The transport of paraquat can be blocked by the addition of the divalent cation quinine, cimetidine, and to a lesser extent tetra ethyl ammonium suggesting that paraquat may be transported by both transport systems, an electro neutral organic cation/$H^+$ exchange and P-glycoprotein (Chan *et al.*, 1998). A schematic representation of the proposed transport systems for paraquat across renal tubular is shown in Figure 51.1.

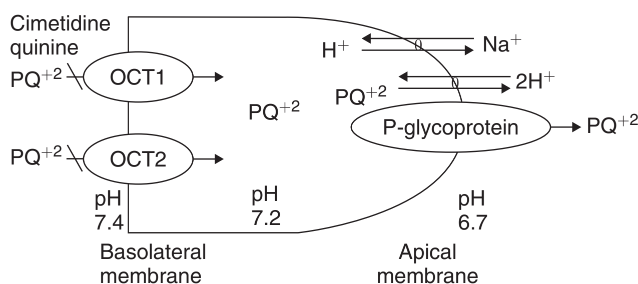



**FIGURE 51.1** A schematic representation of the proposed transport systems for paraquat across renal tubular cells. The transporters are OCT1 and OCT2 at the basolateral membrane and P-glycoprotein and the cation/$H^+$ exchange system at the brush border membrane. From Chan *et al.* (1998). Reproduced with permission.

Unlike paraquat, diquat does not accumulate in the lungs, however, the presence is observed in liver, kidney, plasma, and adrenal gland. Diquat does not enter the brain (Rose *et al.*, 1976). Following oral administration 90–98% of the dose is eliminated via the urine (Daniel and Gage, 1966). Metabolism studies indicate some unidentified metabolites of diquat in the urine of rabbits and guinea pigs. In rat, diquat monopyridone has been identified in the feces, at about 5% of an oral dose, while diquat–dipyridone has been detected in urine. These results indicate that diquat, probably is metabolized by GI bacteria (JMPR, 1993).

Ureas and thioureas such as diuron and linuron are mainly metabolized by dealkalization of the urea methyl groups. Hydrolysis of diuron to 3,4-dichloroaniline and oxidation to 3,4-dichlorophenol as well as dehydroxylation at carbon 2 and/or 6 of the benzene ring, have also been reported. The predominant metabolite of diuron in urine is *N*-(3,4-dichlorophenyl)-urea. Diuron is partially excreted unchanged in feces and urine (Boehme and Ernst, 1965; Hodge *et al.*, 1967). The storage of diuron does not occur in tissues.

Organo phosphorus herbicides such as glyphosate and glufosinate are poorly absorbed both orally and via dermal route. There is rapid elimination, these are not biotransformed and do not accumulate in tissues. More than 70% of an orally administered dose of glyphosate is rapidly eliminated through feces and 20% through urine. The main metabolite of glyphosate is aminomethylphosphonic acid (AMPA); the AMPA is of no greater toxicological concern than its parent compound (JMPR, 2004).

The Proton class of oxidase inhibitor herbicides is either not readily absorbed and/or are rapidly degraded by metabolism and/or excreted. The mammalian metabolites are similar to photochemical degradation products. In mammals, there are remarkable species differences in the levels of porphyrin accumulation resulting from exposure to Protox inhibitors. There is no bioaccumulation risk in animals. Metabolism of Protox inhibitors have been studied in a number of species, including rats, rabbits, goats, sheep, cattle, and chicken. In general, the metabolic degradation of these compounds by animals includes nitro reduction, de-esterification, and conjugation to GSH, cysteine, and carbohydrates. Most of the metabolites are excreted in urine, with small amounts excreted in feces and milk. In chickens, approximately 95% of the metabolites are eliminated in excreta, with small amounts (0.09%) eliminated in the eggs (Hunt *et al.*, 1977; Leung *et al.*, 1991).

The carboxyester group of the triazolinone herbicide carfentrazone ethyl is initially metabolized to a carboxylic acid group. Other metabolites identified in rats and lactating goats include hydroxymethylpropionic acid and cinnamic acid derivatives, which are further metabolized to yield a benzoic acid derivative (Aizawa and Brown, 1999).

Substituted anilines are well absorbed in rats orally. The dermal penetration in monkeys is relatively slow. The metabolism of alachlor in rats is complex due to extensive biliary excretion, intestinal microbial metabolism, and enterohepatic circulation of metabolites. The main route of excretion is urine and feces, and nearly 90% of the dose is eliminated in 10 days. The metabolism in rats and mice is similar, however, there are significant quantitative differences between the two species. In contrast, alachlor is metabolized in monkeys to a limited number of GSH and glucuronide conjugates which are excreted primarily via kidney. Excretion in monkeys is more rapid than rodents with approximately 90% being excreted in the urine within 48 h. Alachlor metabolites undergo biliary excretion and hepatic circulation in rodents, whereas, biliary excretion is limited in monkeys. In rats, acetochlor is rapidly metabolized to several polar metabolites and more than 95% are quickly excreted in urine and feces. The metabolites are the result of the mercapturic acid pathway formed by initial GSH conjugation. As in case of alachlor, acetochlor and propachlor also undergo glucuronide conjugate, and enterohepatic circulation (Millburn, 1975; Heydens *et al.*, 2001).

Dimethenamid, an amide derivative is slowly but well absorbed after oral administration (90% in rats) and is extensively metabolized in rats. The maximum concentration in blood is not achieved until about 72 h. Excretion is primarily via bile. By 168 h after treatment, an average of 90% of the administered dose is eliminated. In rats, the concentration of radioactivity in blood decreases more slowly than in other tissues and is associated with specific binding to globin (not in human). Metabolism is primarily via the GSH conjugation pathway, but racemic dime-thenamid is

also metabolized by cytochrome P450 enzymes via reductive dechlorination, oxidation, hydroxylation, *O*-demethylation, and cyclization pathways, as well as conjugation with glucuronic acid. Unchanged dimethenamid in excreta accounts for only 1–2% of the administered dose, more than 40 metabolites have been detected (JMPR, 2005).

In rats, the triazolopyrimidine compounds are rapidly absorbed and urinary elimination is rapid with half-lives ranging from 6 to 12 h. The excretion is mainly through urine and small amounts are excreted in feces. The only metabolite present is the 4-OH phenyl derivative and/or oxidation product. In metosulam toxicity, demethylation of the 3-methyl moiety of the phenyl ring and of the 3-methoxy moiety of the pyridine ring and other conjugation products of the parent material have been observed. Other minor metabolites include hydroxylated products of the pyridine ring, though the position of hydroxylation has not been identified. Due to rapid elimination, there is little potential to accumulate in the tissues (Hanley and Billington, 2001).

## MECHANISM OF ACTION

There are a number of biochemical changes or free radical-mediated processes; some may also be produced by other mechanisms that have been used to assess tissue injury. For example, the loss of tissue GSH may reflect alkylation reactions, not oxidation. Furthermore, the some free radical-mediated changes that may cause injury are also the result of injury. This is exemplified by the phenomenon of lipid peroxidation that is invoked as a toxic mechanism in many situations, but also occurs subsequent to cell death and membrane lysis. Under most of the situations, it becomes difficult to pin point the exact mechanism of action. The mechanism of action of phenoxy derivatives, triazines, triazolopyrimidines, imidazolinones, dinitroaniline, and many other classes of herbicides is not precisely known. However, phenoxy compounds are known to depress ribonuclease synthesis, uncouple oxidative phosphorylation, and increase the number of hepatic peroxisomes. The relationship of these biochemical changes to clinical effect is not clear. In dogs these herbicides may directly affect muscle membranes (Sandhu and Brar, 2000). Herbicides such as 2,4-D, 2,4,5-T, and dicamba act as peroxisome proliferators. Oxadiazinon causes hepatic porphyria in both mice and rats. The phenyl urea herbicides linuron and monuron are rodent liver carcinogens. The chloroacetanilide and metolachlor have shown weak hepatocarcinogenicity in female rats and is non-genotoxic suggesting a tumor-promoting action. The dinitro compounds markedly stimulate respiration while simultaneously impair adenosine triphosphate synthesis. The main toxic action is uncoupling of oxidative phosphorylation, converting all cellular energy in the form of heat and causing extreme hyperthermia. In addition, the gut flora in ruminants is able to further reduce the dinitro compounds to diamine metabolites which are capable of inducing methemoglobinemia.

The available information on substituted anilines indicates that there is non-genotoxic mechanism of action and lack of relevance to human for the nasal turbinate, stomach, and/or thyroid oncogenic effects produced in rats. The data support grouping for alachlor, acetochlor, and butachlor with respect to a common mechanism of toxicity for nasal turbinate and thyroid tumors, and alachlor and butachlor for stomach tumors (Heydens *et al.*, 2001).

The mechanism of action of paraquat and diquat is very similar at the molecular level and involves cyclic reduction–oxidation reactions, which produce reactive oxygen species and depletion of nicotinamide adenine dinucleotide phosphate hydrogen (NADPH). However, the critical target organ differs with the two compounds, so that the mammalian toxicity is quite different. While both herbicides affect kidneys, paraquat is selectively taken up in the lungs. Paraquat causes pulmonary lesions as a result of Type I and Type II pneumocytosis. The primary event in the mechanism of toxicity within cells is its ability to undergo a single electron reduction from the cation to form a free radical that is stable in the absence of oxygen. If oxygen is present, a concomitant reduction of oxygen takes place to form superoxide anion ($O_2^-$). Superoxide radical in turn is non-enzymatically converted to singlet oxygen, which attacks polyunsaturated lipids associated with cell membranes to form lipid hydroperoxides. Lipid hydroperoxides are normally converted to non-toxic lipid alcohols by the selenium-containing GSH-dependent enzyme, GSH peroxidase. Selenium deficiency, deficiency of GSH, or excess lipid hydroperoxides allows the lipid hydroperoxides to form lipid-free radicals. Lipid hydroperoxides are unstable in the presence of trace amounts of transition metal ions and decompose to free radicals, which in turn cause further peroxidation of polyunsaturated lipid in a process that is slowed down by vitamin E. Peroxidation of the membranes could, in turn, cause cellular dysfunction and hence lead to cell damage or death (Smith, 1997). A schematic diagram incorporating these elements of the mechanism of paraquat toxicity is shown in Figure 51.2.

The mechanism of action of diquat differs somewhat from paraquat because it undergoes alternate reduction followed by reoxidation, a process known as redox recycling. Like paraquat, diquat can redox cycle, the major difference being that diquat can more readily accept an electron than paraquat (Gage, 1968). The major target organs are the GI tract, the liver, and the kidneys. Unlike paraquat, diquat shows no special affinity for the lung and does not appear to involve the same mechanism that selectively concentrates paraquat in the lung (Rose and Smith, 1977).

Glyphosate, a member of phosphonomethyl amino acid group selectively inhibits the enzyme 5-enolpyruvoylshikimate 3-phosphate synthetase. The enzyme plays

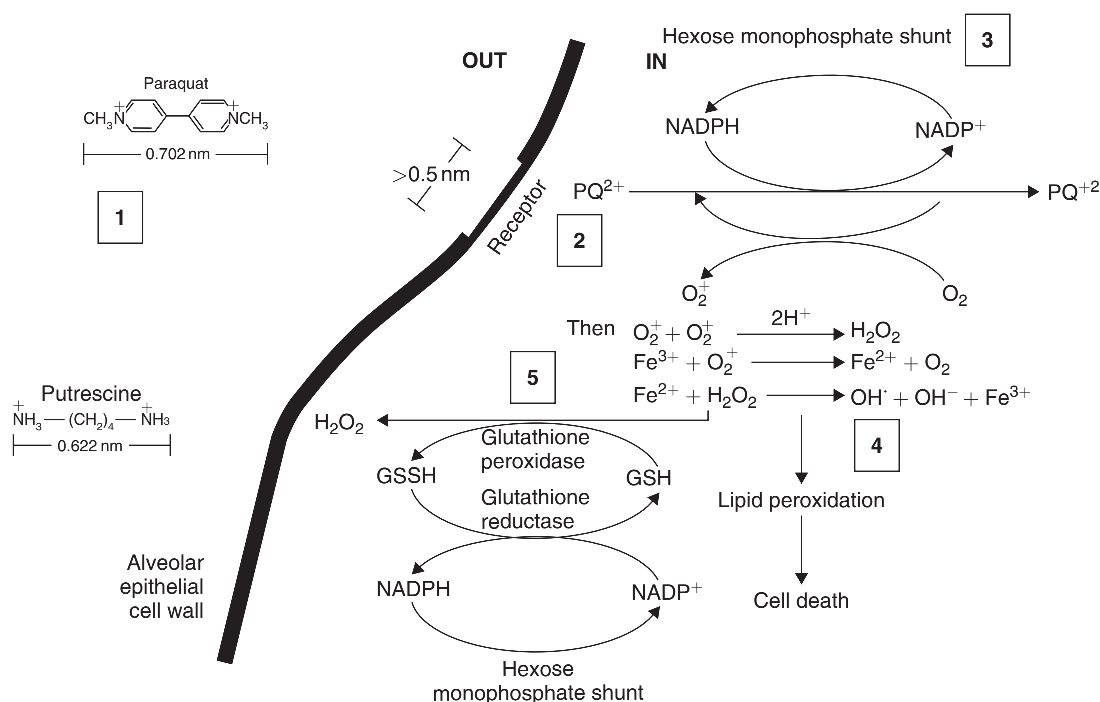



**FIGURE 51.2** Schematic representation of mechanism of toxicity of paraquat. (1) Structure of paraquat and putrescine; (2) putative accumulation receptor; (3) redox cycling of paraquat utilizing NADPH, (4) formation of hydroxyl radical ($OH^{\bullet}$) leading to lipid peroxidation; and (5) detoxication of $H_2O_2$ via GSH reductase peroxidase couple, utilizing NADPH. From Smith (1997). Reproduced with permission.

a key role in the biosynthesis of the intermediate, chorismate, necessary for the synthesis of the essential amino acids phenylalanine, tyrosine, and tryptophan. This aromatic amino acid biosynthesis pathway is found in plants as well as fungi and bacteria but not in insects, birds, fish, mammals, and humans, thus providing a specific selective toxicity to plant species (Franz *et al.*, 1997). Another compound of this group, glufosinate acts by inhibiting the enzyme glutamine synthetase in animals. Glutamine synthetase in mammals is involved in ammonia homeostasis in many organs and the glutamine–glutamate shunt between gamma-aminobutyrate and glutamate in the central nervous system (CNS). However, the enzyme is normally working at a small fraction of its capacity, and considerable inhibition is required in mammals before ammonia levels increase. Hence this does not lead to a problem in ammonia metabolism, the mammals obviously compensating by using other metabolic pathways (Hack *et al.*, 1994; JMPR, 2000).

## TOXICITY

There are more than 200 active ingredients used as herbicides, however, some of them are believed to be obsolete or discontinued for use. Out of these, several of them have been evaluated for their toxic potential and acceptable daily intake has been recommended by Joint Meeting on Pesticide Residues (IPCS, 2002). The herbicides may be classified by their chemical structure but this is not very enlightening because of overlapping biological effects of a variety of chemical structures. The second method of classification is on the basis of their use such as how and when the agents are applied. For example, pre-planting herbicides are applied to the soil before a crop is seeded; pre-emergent herbicides are applied to the soil before the usual time of appearance of the unwanted weeds. Post-emergent herbicides are applied to the soil after the germination of the crops and/or weeds. Another way of classification is based on their mechanism of action in plants; their action is referred to selective (toxic to some species), contact (direct contact), or translocated (through absorption via the soil or through the foliage into the plant xylem and phloem). WHO has recommended classification of pesticides by their acute hazardous nature. Individual products are classified according to the oral or dermal toxicity of technical products, and its physical state. The comparative toxicity of selected herbicides by hazards along with their $LD_{50}$ values in rats is summarized in Table 51.1.

These values are intended to be for the guide only because the toxicity of herbicides may be due to the presence of

**TABLE 51.1 WHO recommended category (hazardous nature) of major chemical classes of technical grade herbicides with representative examples of acute $LD_{50}$ values in rats**

| Chemical class | Hazardous class | $LD_{50}$ (mg/kg b.w.) |
|---|---|---|
| **Phenoxy acid derivatives** | | |
| 2,4-D | II | 375 |
| 2,4,5-T | O | – |
| Dichlorprop or 2,4-DP | III | 800 |
| 2,4-DB | III | 700 |
| Dalapon | U | 9330 |
| MCPB | III | 680 |
| MCPA | III | 700 |
| Mecoprop | III | 930 |
| Mecoprop-P | III | 1050 |
| Silvex or fenprop | O | – |
| **Bipyridyl derivatives** | | |
| Paraquat | II | 150 |
| Diquat | II | 231 |
| **Ureas and thioureas (phenyl or substituted ureas)** | | |
| Chlorbromuron | U | >5000 |
| Chlorotoluron | U | – |
| Diuron | U | 3400 |
| Fenuron | U | 6400 |
| Fenuron-TCA | U | 4000 |
| Fluometuron | U | >8000 |
| Flupyrsulfuron | U | >5000 |
| Isoproturon | III | 1800 |
| Linuron | U | 4000 |
| Metobromuron | U | 2500 |
| Metoxuron | U | >3200 |
| Monolinuron | U | 2250 |
| Monuron | O | – |
| Monuron-TCA | O | – |
| Neburon | U | >10000 |
| Noruron | O | – |
| Siduron | U | >7500 |
| Tebuthiuron | III | – |
| Thidiazuron | U | >4000 |
| **Organic phosphorus/phosphonomethyl amino acids or inhibitors of aromatic acid biosynthesis** | | |
| Glyphosate | U | 4230 |
| Glufosinate | III | 1625 |
| **Protoporphyrinogen oxidase inhibitors** | | |
| Nitrofen | O | – |
| Oxadiazon | U | >8000 |
| **Triazines and triazoles** | | |
| *Symmetrical triazines* | | |
| Simazine | U | >5000 |
| Atrazine | U | c2000 |
| Propazine | U | 5000 |
| Cyanazine | O | – |
| Ametryn | III | 1110 |
| Prometryn | U | 3150 |
| Terbutryn | U | 2400 |
| Prometon | U | 2980 |
| *Asymmetrical triazines* | | |
| Metribuzin | II | 322 |
| **Subsituted anilines** | | |
| Alachlor | III | 930 |
| Acetochlor | III | 2950 |
| Butachlor[d] | U | 3300 |
| Metolachlor[d] | III | 2780 |
| Propachlor | III | 1500 |

TABLE 51.1 (Continued)

| Chemical class | Hazardous class | $LD_{50}$ (mg/kg b.w.) |
|---|---|---|
| **Amides and acetamides** | | |
| Bensulide[d] | II | 270 |
| Dimethenamid-P | II | 429 |
| Propanil | III | c1400 |
| **Dinitro compounds** | | |
| Binapacryl | O | – |
| DNOC | Ib | 25 |
| Dinoterb | Ib | 25 |
| Dinoseb | O | – |
| **Triazolopyrimidines** | | |
| Cloransulam-methyl | U | >5000 |
| Diclosulam | U | >5000 |
| Flumetsulam | U | >5000 |
| Metosulam | U | >5000 |
| **Imidazolinones** | | |
| Imazapyr | U | >5000 |
| Imazamethabenzmethy | U | >5000 |
| Imazethapyr | U | >5000 |
| Imazaquin | U | >5000 |
| **Benzoic acids** | | |
| Chloramben | U | 5620 |
| Dicamba | III | 1707 |
| Napalam | U | 8200 |
| **Carbamate and thiocarbamate compounds** | | |
| Asulam | U | > 4000 |
| Chlorpropham | U | > 5000 |
| Butylate[d] | U | > 4000 |
| EPTC[d] | II | 1652 |
| Di-allate | O | – |
| Pebulate[d] | II | 1120 |
| Terbutol | | |
| Thiobencarb[d] | II | 1300 |
| Triallate[d] | III | 2165 |
| Vernolate[d] | II | 1780 |
| **Methyl uracil compounds** | | |
| Bromacil | U | 5200 |
| Terbacil | U | > 5000 |
| **Polycyclic alkanoic acids** | | |
| Diclofop | III | 565 |
| Fenoxaprop ethyl | O | – |
| Fenthiaprop | O | – |
| Fluazifop | O | – |
| Haloxyfop | II | 393 |
| **Sulfonylureas** | | |
| Chlorsulfuron | U | 5545 |
| Sulfometuron | U | >5000 |
| Metsulfuron methyl | U | >5000 |
| Primisulfuron | U | 8200 |
| **Dinitroaniline** | | |
| Trifluralin | U | >10,000 |
| Tridiphane | O | – |
| **Nitriles** | | |
| Ioxynil | II | 110 |
| Bromoxynil | II | 190 |

Ib: Highly hazardous; II: moderately hazardous; III: slightly hazardous; U: unlike to present acute hazard in normal use; O: obsolete or discontinued for use as herbicide; d: liquid; “c”: before $LD_{50}$ reflects variable values. Compiled from IPCS (2002) and JMPR (2005).

other ingredients (such as surfactants, emulsifiers, etc.) present in the formulation. The details of guidelines for their hazard evaluation and their classification have already been summarized (IPCS, 2002; Gupta, 2006). With a few exceptions, most of the newly developed chemicals have a low order of toxicity to mammals. However, some herbicides are known to have adverse effects on developing embryo. A list of selected herbicides having teratogenic potential is summarized in Table 51.2. In this chapter, herbicides classified by their chemical nature and mechanisms by which they exert biological effects have been reviewed.

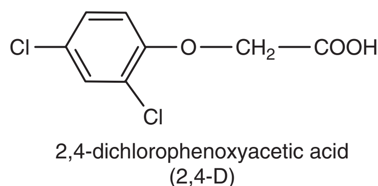
## Inorganic herbicides

Substances such as sodium arsenite, arsenic trioxide, sodium chlorate, ammonium sulfamate, borax, and many others were formerly used on a large scale. Indeed the Romans are reputed to have sterilized the soil of Carthase with common salt after the Romans' victory in 146 BC. The disadvantage with such herbicides from agricultural point of view, is that they are non-selective so their use has declined due to availability of better and selective organic preparations (Marrs, 2004).

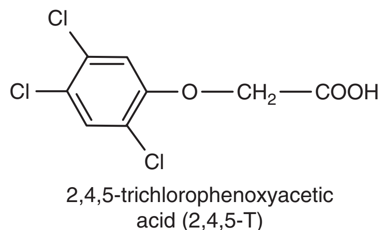
## Phenoxy acid derivatives

This class of herbicides include 2,4-D {2,4-dichlorophenoxyacetic acid}, 2,4,5-T {2,4,5-trichlorophenoxyacetic acid}, 2,4-DB {4-(2,4-dichlorophenoxy) butyric acid}, dalapon, dichlorprop or 2,4-DP {2-(2-methyl-4-chlorophenoxy) propionic acid}, mecoprop (MCPP), {2-(4-chloro-2-methylphenoxy) propionic acid}, MCPA {2-methyl-4-chlorophenoxyacetic acid}, and Silvex {2-(2,4,5-trichlorophenoxy) propionic acid}. The phenoxy derivatives are no longer the agents of choice because of formation of chlorinated dibenzofurans and dibenzodioxins, particularly 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), as a consequence of poorly monitored manufacturing practices. Some formulations of 2,4,5-T contain dioxin contaminants that increase the toxicity of technical grade herbicides and therefore the safe use of phenoxy herbicides has been questioned. The published reports indicate the possibility of occurrence of three rare forms of cancer (Hodgkin's disease, soft tissue carcinoma, and non-Hodgkin's lymphoma) in workers exposed to these herbicides (Kennepohl and Munro, 2001). However, they are still used in developing countries around the world, their toxicology cannot be ignored. The structural formulae of selected phenoxy derivatives are given as below.

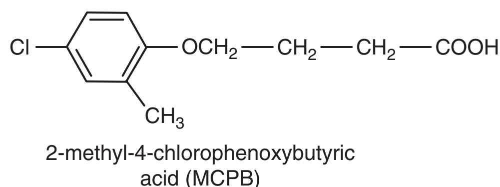
A tremendous amount of mammalian toxicity data has been collected over the past 50 years. As a group, these are essentially non-toxic and acute oral/dermal exposure to phenoxy herbicides is slight to moderately hazardous in



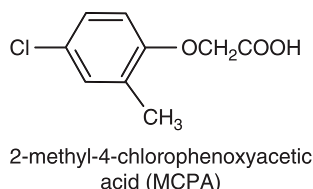
2,4-dichlorophenoxyacetic acid (2,4-D)



2,4,5-trichlorophenoxyacetic acid (2,4,5-T)



2-methyl-4-chlorophenoxybutyric acid (MCPB)



2-methyl-4-chlorophenoxyacetic acid (MCPA)

normal use (Table 51.1). Dermal irritation in rabbits is considered slight for the acid form of 2,4-D and minimal for the salt and ester forms. Eye irritation in rabbits, on the other hand, is severe for the acid and salt forms, but minimal for the ester. The oral $LD_{50}$ for 2,4-D and 2,4,5-T in dogs ~100 mg/kg b.w. The dog is more sensitive and may develop myotonia, ataxia, posterior weakness, vomiting, bloody diarrhea, and metabolic acidosis because of difficulty in the renal elimination of such organic acids (Gehring *et al.*, 1976). Kidney effects consisting of reduced cytoplasmic eosinophilia of the epithelial cells lining and some convoluted tubules have been reported in dog. 2,4-D does not produce any testicular damage or induce any abnormal reproductive disorders, however, some workers have reported their teratogenic potential in animals (Table 51.2). The group of compounds neither induces adverse effects in the nervous system nor has any potential to induce cancer or mutagenicity in laboratory animals (Yano *et al.*, 1991a, b; Munro *et al.*, 1992).

Phenoxy acid derivatives can potentiate the toxic effects of some plants. They may increase the nitrate content of some plant species and can increase the palatability of certain toxic plants (Sudan grass), thus increasing poisoning risks from these plants. Dogs are the most sensitive animals, whereas pigs, sheep, cattle, and poultry are less susceptible (Table 51.3).

**TABLE 51.2 Selected list of herbicides having teratogenic potential in experimental animals**

| | |
|---|---|
| 2,4-D; 2,4,5-T alone or in combination | Malformations/teratogenic (mice, rats) |
| Dichlorprop | Teratogenic (mice), affect postnatal behavior (rats) |
| MCPA | Teratogenic and embryotoxic (rats), teratogenic (mice) |
| Mecoprop | Malformations (mice only) |
| Monolinuron | Cleft palate (mice) |
| Linuron | Malformations (rats) |
| Buturon | Cleft palate, increased fetal mortality (mice) |
| Atrizine | Disruption of ovarian cycle and induced repetitive pseudopregnancy (rats, at high doses) |
| Propachlor | Slight teratogenic (rats) |
| *Dinitro compounds* | |
| Dinoseb[a] | Multiple defects (mice, rabbits) |
| Dinoterb | Skeletal malformations (rats), skeletal, jaw, head, and visceral (rabbits) |
| Chlorpropham | Malformations or other developmental toxicity (mice) |
| Nitrofen[a] | Malformations (mice, rats, hamsters) |

a: Obsolete. Compiled from Schardein (2000).

**TABLE 51.3 TD of phenoxy acid derivatives in animals**

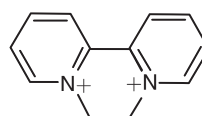
| | Species | mg/kg b.w. |
|---|---|---|
| *Phenoxy acid and its sodium salt* | | |
| $LD_{50}$ | Chickens | 547 |
| | Dogs | 100–800 |
| LD | Pigs | 500 |
| | Hens | 380–765 |
| TD | Pigs | 100 |
| | Calves | 200 |
| *Butyl glycol ester* | | |
| TD | Cattle | 250 for 3 days |
| | Sheep | 250 for 2 days |
| *Amine salts* | | |
| TD | Cattle | 250 for 10 days |
| | Sheep | 250 for 10 days |
| | | 500 for 7 days |

Compiled from Lorgue *et al.* (1996).

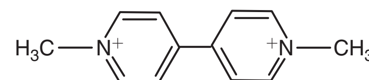
Animals can tolerate repeated oral doses of phenoxy herbicides without showing significant signs of toxicity; suggesting that there is very little cumulative effect on target organs. At high dosages, two types of clinical symptoms, i.e. related to GI tract and/or nervous system have been observed. GI signs include anorexia, rumen atony, diarrhea, ulceration of oral mucosa, bloat, and rumen stasis in cattle and vomiting, diarrhea, salivation, etc. in dogs and pigs. Neuromuscular signs include depression and muscular weakness in cattle, and ataxia, posterior weakness (particularly pelvic limbs), and periodic clonic spasms (at high doses) in dogs. Silvex is unusual for this group because it is very toxic and small doses (2–6 mg/kg b.w.) may cause ill effects in dogs (Sandhu and Brar, 2000).

## Bipyridyl derivatives

This chemical class of herbicides includes paraquat (1,1′-dimethyl-4,4′-bipyridylium dichloride) and diquat (1,1′-ethylene-2,2′-bipyridylium dibromide).



Diquat



Paraquat

Paraquat is usually formulated as dichloride salt (also known as methyl viologen). The bis (methyl sulfate) salt is no longer commercialized. Paraquat is a non-selective, and is a fast-acting contact herbicide. This compound is the most toxic of the commonly used herbicides and the toxicity varies in different animals depending upon the formulation and species used. The toxic doses of paraquat and diquat in rats range from 150 to 231 mg/kg body wt. and this class of herbicides is classified as moderately hazardous (Table 51.1). Mice are less sensitive than the rats to orally administered paraquat, while guinea pigs, cats, monkeys, and rabbits are more susceptible (Murray and Gibson, 1972; Bus *et al.*, 1976a, b; Nagata *et al.*, 1992; Lorgue *et al.*, 1996). The cattle and sheep are more sensitive than other species. The comparative toxic oral doses (TD) of paraquat and diquat in different species are summarized in Table 51.4.

As indicated previously, paraquat and diquat have somewhat different mechanisms of action. Diquat exerts most of its harmful effects in the GI tract. The major cause of death after paraquat is due to lung damage. Rabbits, however, do not show signs of respiratory distress. Immediate toxic effects include convulsions or depression and in-coordination, gastroenteritis, and finally difficult respiration due to pulmonary edema and alveolar fibrosis (2–7 days). Animals that survive first few days develop dehydration, pallor or cyanosis, tachycardia, tachypnea, harsh respiratory sounds, and emphysema or pneumomediastinum. Studies have shown that mice lacking copper/zinc superoxide dismutase show a marked increase in sensitivity to paraquat (Ho *et al.*, 1998).

On long-term exposure, there is progressive pulmonary fibrosis and increased respiratory distress. The morphological changes seen in animals include degeneration and

**TABLE 51.4 TD of bipyridyl derivatives in animals**

| | Species | mg/kg b.w. |
|---|---|---|
| *Paraquat* | | |
| $LD_{50}$ | Dogs | 25–50 |
| | Cats | 35 |
| | Monkeys | 50–70 |
| | Cattle | 35–50 |
| | Chickens | 110–360 |
| LD | Sheep | 8–10 |
| | Pigs | 75 |
| *Diquat* | | |
| $LD_{50}$ | Dogs | 100–200 |
| | Cats | 35–50 |
| | Cattle | 20–40 |
| | Chickens | 200–400 |

Compiled from Lorgue *et al*. (1996).

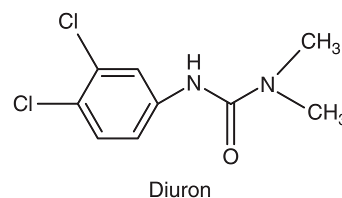
vacuolization of pneumocytes, damage to Type I and Type II alveolar epithelial cells, destruction of the epithelial membranes, and proliferation of fibrotic cells. The animals die as a consequence of reduced gas exchange and development of severe hypoxia.

Gross lesions include pulmonary congestion, edema, and hemorrhages. Lingual ulcers may be seen. Other findings include failure of lungs to collapse when chest is opened and areas of hemorrhages, fibrosis, and atelectasis. Microscopic lesions include necrosis of Type I alveolar epithelial cells followed by progressive alveolar and intestinal fibrosis and alveolar emphysema. Renal proximal tubular degeneration and moderate centrilobular hepatic degeneration may also be seen (Smith, 1997). It has neither carcinogenic nor mutagenic potential, however, high doses injected into pregnant rats and mice on various days of gestation may cause significant maternal toxicity but does not produce teratogenic effects (Bus and Gibson, 1975).

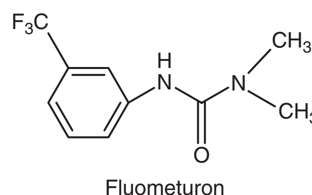
Diquat is formulated as the dibromide salt and is slightly less toxic to dogs than paraquat (Table 51.4). After chronic exposure, the major target organs are the GI tract, the liver, and the kidneys, however, lungs are not affected (Hays, 1982). Presence of cataracts in both dogs and rats has been observed. Similar signs of toxicity have been seen in mice, guinea pigs, rabbits, dogs, and monkeys. On histopathology injury to the lining of the stomach and GI tract has been observed but these are not life threatening (Clark and Hurst, 1970; Cobb and Grimshaw, 1979). Diquat has no effect on fertility, is not teratogenic, and only produces fetotoxicity at doses that are maternally toxic. In the multigeneration study, at high-doses cataract has been observed in rats (Lock and Wilks, 2001).

## Ureas and thioureas

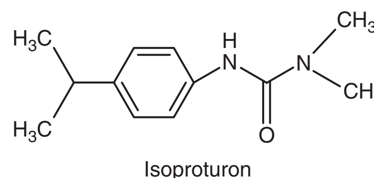
The ureas and thioureas (polyureas) are available under different names such as diuron, fluometuron, isoproturon, linuron, buturon, chlorbromuron, chlortoluron, chloroxuron, difenoxuron, fenuron, methiuron, metobromuron, metoxuron, monuron, neburon, parafluron, siduron, tebuthiuron, tetrafluron, and thidiazuron. Of these, diuron and fluometuron are the most commonly used in the United States, whereas isoproturon is mostly used in other countries including India.



Diuron



Fluometuron



Isoproturon

In general, polyureas have low acute toxicity and are unlikely to present any hazard in normal use, except tebuthiuron, which may be slightly hazardous (Table 51.1). In general, the cattle are more sensitivity to polyurea herbicides than sheep, cats, and dogs. The comparative toxic values of polyurea herbicides are summarized in Table 51.5.

Diuron and Monuron are potent inducers of hepatic metabolizing enzymes compared to those polyurea herbicides with one or no halogen substitutions (chlortoluron and isoproturon). Male rats are more sensitive than females to the enzyme-inducing activity of diuron and can lead to detoxication of EPN and *O*-demethylation of *p*-nitro anisole. *N*-demethylation of aminopyrine increases for 1–3 weeks, then return to normal (Hodgson and Meyer, 1997). Recovery from diuron intoxication is quick (within 72 h) and no signs of skin irritation or dermal sensitization have been reported in guinea pigs. After repeated administration, hemoglobin levels and erythrocyte counts are significantly reduced, while methemoglobin concentration and white blood cell counts are increased. Increased pigmentation (hemosiderin) in the spleen is seen histopathologically. Linuron in sheep causes erythrocytosis and leucocytosis with hypohemoglobinemia and hypoproteinemia, hematuria and ataxia, enteritis, degeneration of the liver, muscular dystrophy. In chickens it leads to loss of weight, dyspnea, cyanosis, and diarrhea. It is non-toxic to fish (Lorgue *et al.*, 1996). Fluometuron is less toxic than diuron. In sheep, depression, salivation, grinding of teeth, chewing

TABLE 51.5 TD of ureas and thioureas in animals

| | Species | mg/kg b.w. |
|---|---|---|
| *Diuron* | | |
| TD | Cattle | 100 for 10 days |
| | Sheep | 250 or 100 for 2 days |
| | Chickens | 50 for 10 days |
| *Linuron* | | |
| | Dogs | 100–200 |
| | Cats | 35–50 |
| | Cattle | 20–40 |
| | Chickens | 200–400 |
| *Tebuthiuron* | | |
| TD | Cats | 200 |
| | Dogs | 50 per day for 3 months (anorexia and weight loss reported) |

No deaths were reported in dogs, chickens, quail, or ducks at a dosage of 500 mg/kg b.w. of tebuthiuron. Compiled from Lorgue *et al.* (1996).

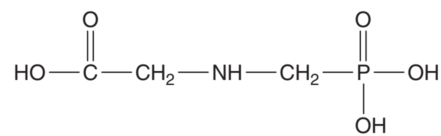
movements of the jaws, mydriasis, dyspnea, in-coordination of movements, and drowsiness is commonly seen. On histopathology severe congestion of red pulp with corresponding atrophy of the white pulp of spleen and depletion of the lymphocyte elements have been reported (Mehmood *et al.*, 1995). The acute $LD_{50}$ of isoproturon in rats is almost similar to diuron and does not produce any overt signs of toxicity, except at very high doses. Single oral dose of isoproturon in mice may produce some neurotoxic effects at very high doses and may reduce spontaneous and forced locomotor activity (Sarkar and Gupta, 1993a, b).

Polyurea herbicides have been suspected to have some mutagenic effects but do not have carcinogenic potential (Liu, 2001). In general, the compounds do not cause developmental and reproductive toxicity, except monolinuron, linuron, and buturon are known to cause some teratogenic abnormalities in experimental animals (Table 51.2).

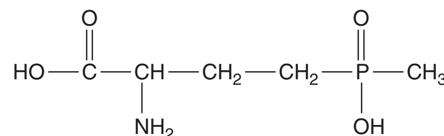
## Phosphonomethyl amino acids or inhibitors of aromatic acid biosynthesis

Two organophosphorus compounds glyphosate (Roundup, Vision) {*N*-(phosphonomethyl)glycine} and glufosinate (Basta) {*N*-(phosphonomethyl)homoalanine} are broad-spectrum, non-selective systemic herbicides. While they exist as free acids but due to their slow solubility they are marketed as the isopropyl amine or trimethylsulfonium salts of glyphosate and the ammonium salt of glufosinate.

Glyphosate has low acute oral toxicity in mice and rats and is unlikely to pose acute hazard in normal use (Table 51.1). The $LD_{50}$ of trimethylsulfonium salt is 750 mg/kg b.w. The animals most affected are cattle, sheep, and dogs. Dogs and cats show eye, skin, and upper respiratory tract signs when exposed during or subsequent to an application to weeds or grass. Nausea vomiting, staggering, and hind



Glyphosate acid



Glufosinate

leg weakness have been reported in dogs and cats that were exposed to fresh chemical on treated foliage (Susan, 2003). Glyphosate is not a dermal irritant and does not induce photosensitization; formulations can cause severe occupational contact dermatitis. Glyphosate is an ocular irritant in the rabbit and human with minor to moderate conjunctival irritation and slight iritis that usually disappears within 48 h after exposure (Acquavella *et al.*, 1999; JMPR, 2004). Common signs of toxicity include loss of body weight, diarrhea, and serum chemistry changes. In rats, it may lead to increased incidence of cataract and degenerative ocular lens changes, alterations in the parotid and sub maxillary glands, and increased liver weights. Formulations of glyphosate can cause intoxication in human beings, which may be due to the presence of surfactants, polyoxyethyleneamine. This class of surfactants has been associated with hemolysis and with GI and CNS effects (Talbot *et al.*, 1991). There is no evidence of mutagenic or carcinogenic potential of glyphosate. It does not have adverse effect on reproductive performance in animals, except at very high doses that elicits maternal toxicity (JMPR, 2004).

The acute oral toxicity of glufosinate is low and is slightly more hazardous than glyphosate (Table 51.1). Common signs of toxicity include CNS excitation and hypothermia in animals (Ebert *et al.*, 1990; Hack *et al.*, 1994). Glufosinate is not considered to be mutagenic, teratogenic, or carcinogenic, except in whole-embryo culture. Teratogenic effects in mice have been observed, the effect being apoptosis in the neuroepithelium of developing embryo (Watanabe, 1997). Glufosinate ammonium formulation has been involved in a number of poisoning cases (cardiovascular and CNS adverse effects) may be due to surfactant-induced penetration into the CNS (Watanabe and Sano, 1998).

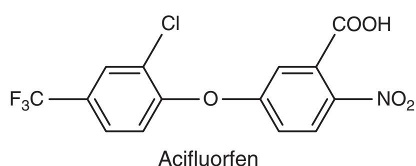
## Protoporphyrinogen oxidase inhibitors

Earlier the protoporphyrinogen oxidase (Protox)-inhibiting herbicides were often termed “DPE-type herbicides” and almost all of the Protox inhibitors were DPEs. This nomenclature led to some confusion in herbicide classification, because other DPE herbicides have an entirely

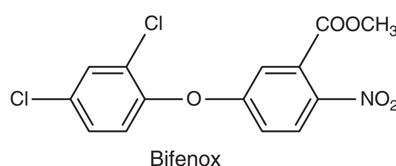
different molecular site of action (i.e. inhibition of acetyl CoA carboxylase). Now many other structurally related Protox inhibitors have been commercialized. In general, the newer products are more potent Protox inhibitors, resulting in lower application rates than the older herbicides of this class. Some of them appear to be analogs of the substrate or a substrate/product transition state of the enzyme (Reddy *et al.*, 1998).

Protox inhibitors may be DPE or non-DPE Protox inhibitors. Amongst DPE Protox inhibitors, nitrofen was the first Protox-inhibiting herbicide introduced in 1964. Although many of the older commercialized DPEs have a *p*-nitro phenyl substitution, newer DPE-like herbicides commonly contain *p*-trifluoromethyl phenyl substitutions. After the first generation of Protox inhibitors (with the exception of oxadiazon), which was based on the DPE, numerous other non-oxygen-bridged compounds (non-DPE Protox inhibitors) with the same site of action (carfentrazone, JV 485 and oxadiargyl) have been commercialized (Dayan *et al.*, 2001). The structural formulae of commercially available DPE, DPE-type, and non-DPE Protox-inhibiting herbicides are given as below.

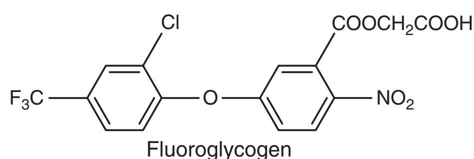
Protox inhibitors have little acute toxicity and are unlikely to pose any acute hazard in normal use (Table 51.1).



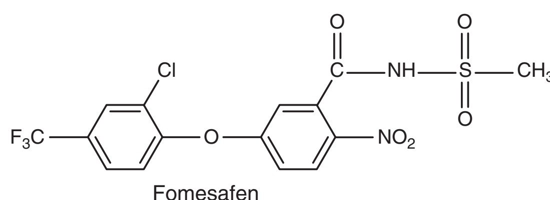
Acifluorfen



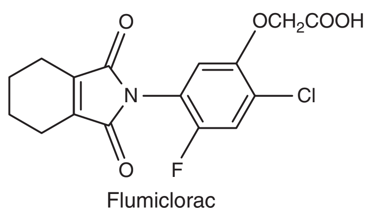
Bifenox



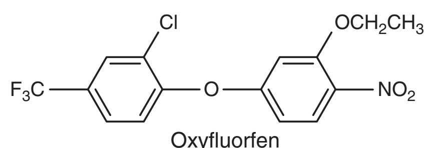
Fluoroglycogen



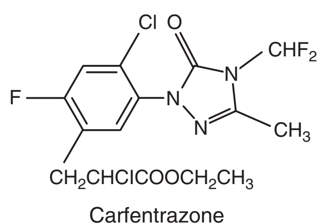
Fomesafen



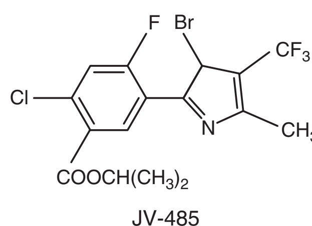
Flumiclorac



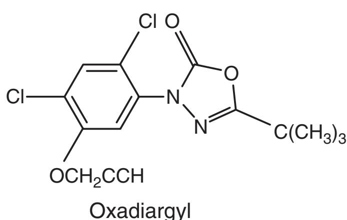
Oxyfluorfen



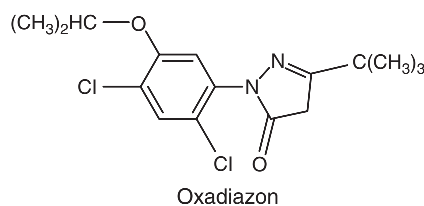
Carfentrazone



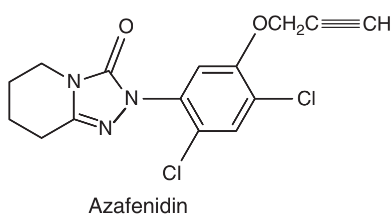
JV-485



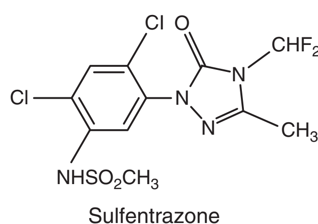
Oxadiargyl



Oxadiazon



Azafenidin

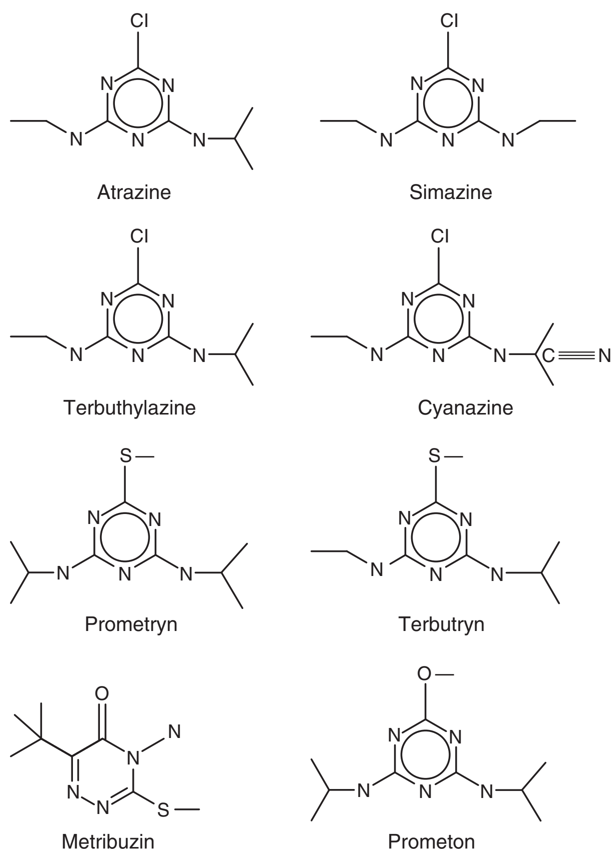


Sulfentrazone

These compounds increase the porphyrin levels in animals when administered orally and the porphyrin levels return to normal within a few days. Rats and mice are sensitive and Variegate porphyria-like symptoms can be generated in mice with high doses of Protox inhibitors. Majority of these compounds are neither mutagenic nor carcinogenic in nature and the development toxicity correlates with Protox accumulation (Dayan *et al.*, 2001).

## Triazines and triazoles

Triazines and triazoles have been used extensively as selective herbicides for more than 40 years. These herbicides are inhibitors of photosynthesis and include both the asymmetrical and symmetrical triazines. Examples of symmetrical triazines are chloro-*S*-triazines (simazine, atrazine, propazine, and cyanazine); the thiomethyl-*S*-triazines (ametryn, prometryn, terbutryn), and the methoxy-*S*-triazine (prometon). The commonly used asymmetrical triazine is metribuzin. The structures of symmetrical and asymmetrical triazines are given as below.



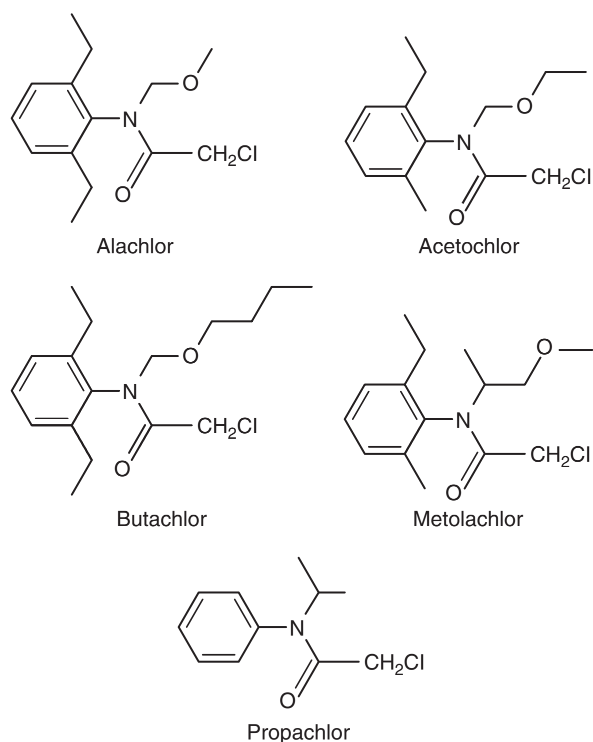

These herbicides have low oral toxicity and are unlikely to pose acute hazards in normal use, except ametryn and metribuzin, which may be slight to moderately hazardous (Table 51.1). They are neither irritant to the skin or eye, nor are skin sensitizers. The exceptions are atrazine, which is skin sensitizer and cyanazine, which is toxic by the oral route. But sensitivity of sheep and cattle to these herbicides is appreciably high. The oral $LD_{50}$ for rats is >5 g/kg b.w. The main symptoms are anorexia, hemotoxia, hypothermia, locomotor disturbances, irritability, tachypnea, and hypersensitivity (Sandhu and Brar, 2000). Doses of 500 mg/kg of simazine or 30 mg/kg atrazine for 30–60 days are lethal to sheep. Deaths have been reported in sheep and horses grazing triazine-treated pasture 1–7 days after spraying. Cumulative effects are not seen. Metribuzin is slightly more toxic than simazine but does not produce any harmful effects in dogs fed at 100 ppm in the diet. Simazine is excreted in milk, so it is of public health concern (Susan, 2003). Atrazine is more toxic to rats, but comparatively less toxic to sheep and cattle than simazine. When cultured human cells are exposed to atrazine, splenocytes are damaged, bone marrow cells are not affected. This class of herbicides is liver microsomal enzyme inducer and is converted to *N*-dealkylated derivatives. In contrast to simazine, it is not excreted in milk. Triazines seem to have no potential to be mutagenic or to produce carcinogenicity in animals. The exception is cyanazine, which is more acutely toxic, week mutagenic, and results in developmental toxicity, presumably because of the presence of cyano moiety (Hodgson and Meyer, 1997).

## Substituted anilines

Substituted anilines are used as systemic herbicides. The commonly used herbicides are alachlor, acetochlor, butachlor, metolachlor, and propachlor. The structural formulae of selected ones are given as below.

This class of herbicides is slightly hazardous, except butachlor, which is not likely to pose any hazard (Table 51.1). The compounds are non-irritant to eye, slight-to-moderate skin irritant, and produce skin sensitization in guinea pigs. Lower doses in rats and dogs do not produce any adverse effects, however, long-term exposure to dogs cause liver toxicity, hepatotoxicity, and spleenic effects. The ocular lesions produced by alachlor is considered to be unique to the Long–Evans rat because the response has not been observed in other strains of rats, mice, or dogs. This effect has not been observed in human beings involved in manufacture of alachlor. At high oral doses, it may lead to maternal and fetal toxicity but may not cause any adverse effect on reproduction. It is neither teratogenic nor produces any microbial genotoxicity. Alachlor has the potential to produce thyroid tumors and adenocarcinomas of the stomach and nasal turbinates of Long–Evans rats and in the lungs of CD-1 mice. It is considered to be a human carcinogen (Ahrens, 1994; Monsanto, 1997a, b).

Long-term exposure of acetochlor to rats has no adverse effects on reproductive performance in animals. Acetochlor is converted into a rat-specific metabolite that




may be related to the nasal tumors, thus posing no genetic or carcinogenic hazard to humans (Ashby *et al.*, 1996). Butachlor does not adversely affect reproductive performance or pup survival. It is non-genotoxic and there is no carcinogenic potential in animals. Metolachlor can increase the incidence of liver tumors in rats and has been classified as possible human carcinogen (Monsanto, 1991; Wilson and Takei, 1999).

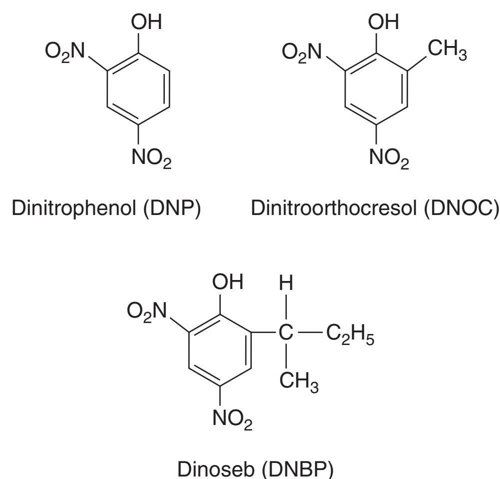
As compared to other substituted anilines, propachlor is severely irritating to the eye and slightly irritating to the skin. Propachlor produces skin sensitization in guinea pigs. High doses of propachlor produce erosion, ulceration and hyperplasia of the mucosa, and herniated mucosal glands in the pyloric region of stomach, hypertrophy, and necrosis of the liver in rats. In dogs, there is poor diet palatability, which results in loss of weight and poor consumption of food. Propachlor may produce slight development or adverse reproductive effects (Table 51.2). It is not genotoxic or clastogenic in mammals. However, there is evidence that it produces benign hepatic tumors in male mice (Heydens *et al.*, 2001).

## Amides and acetamides

The commonly used amides and acetamides include bensulide, dimethenamid-P, and propanil and are slightly to moderately hazardous in normal use (Table 51.1). Dimethenamid is a racemic mixture of the M and P stereoisomers, whereas P isomer has useful herbicidal activity. Both substances produce only mild reversible skin and eye irritation and skin sensitization in guinea pigs. Comparison of racemic dimethenamid with dimethenamid-P indicates that there is little difference in their toxicological profiles. The signs of toxicity in mice, rats, and dogs are similar, with reduced body-weight gain and liver enlargement with induction of liver xenobiotics metabolizing enzyme. There is strong binding to hemoglobin in rats, but this has no relevance to humans. Dimethenamid-P and racemic dimethenamid produce very similar effects in the liver of rats. Both substances neither produce any signs of neurotoxicity nor adverse effects in developmental toxicity or on reproductive performance. Dimethenamid can reduce fetal body weights but is not teratogenic. There is no compound-related mutagenic or carcinogenic potential (JMPR, 2005).

## Dinitrophenol compounds

Several substituted dinitrophenols alone or as salts such as DNP (2,4-dinitrophenol), DNOC (dinitro-*o*-cresol), and dinoseb {2-(1-methylpropyl)-4,6-dinitro} are used as insecticides, fungicides, acaricides, and herbicides. The main source of poisoning in animals are human negligence in removing the preparation if it spills, in disposing off the containers and preventing animals access to treated fields. The structural formulae of DNP, DNOC, and dinoseb are given as under.




In general, the dinitro compounds are not very water-soluble and are highly hazardous to animals (Table 51.1). The oral acute $LD_{50}$ of DNOC in mice, guinea pigs, rabbits, hens, dogs, pigs, and goats ranges from 25 to 100 mg/kg b.w. (Table 51.6). In sheep, dosage of 25 mg/kg/day causes toxicosis in 2–4 days. Clinical signs include fever, dyspnea, acidosis, oliguria, muscular weakness, tachycardia and convulsions, followed by coma and death with a rapid onset of rigor mortis. Abortions have been reported in sows. In cattle and ruminants, methemoglobinemia, intravascular hemolysis, and hemoproteinemia have been observed. Cataract can occur with chronic dinitrophenol intoxication.

TABLE 51.6 TD of DNOC and dinoseb in animals

| | Species | mg/kg b.w. |
|---|---|---|
| *DNOC* | | |
| $LD_{50}$ | Hens | 26 |
| | Dogs | 50 |
| | Pigs | 50 |
| | Goats | 100 |
| TD | Cattle | 2–50 |
| | Sheep | 20–50 |
| LD | Sheep | 25 for 5 days |
| *Dinoseb* | | |
| $LD_{50}$ | Hens | 26 |
| TD | Cattle | 25 for 8 days |
| | Sheep | 25 for 10 days |

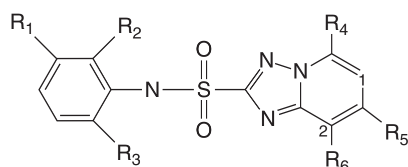
Compiled from Lorgue *et al.* (1996).

Exposure to these compounds may cause yellow staining of skin, conjunctiva, or hair (Lorgue *et al.*, 1996).

## Triazolopyrimidine herbicides

Triazolopyrimidine herbicides include cloransulam-methyl, diclosulam, florasulammethyl, flumetsulam, and metosulam. The generic structure of the triazolopyrimidine herbicides connected to a substituted phenyl ring through a sulfonamide bridge is given as under. The substituents of the various members of this class are summarized in Table 51.7.

The acute oral toxicity of triazolopyrimidine herbicides is very low. On repeated exposure, the primary organs are the kidney (rat and mouse), liver (rat, mouse, and dog), and thyroid (rat). Slight decreases in red cell parameters and urine-specific gravity in males, and slightly increased cecal and liver weights, slight to moderate hypertrophy of collecting renal tubule epithelial cells and/or slight vacuolization of proximal tubular epithelium, hepatocellular vacuolation, and slight thyroid follicular hypertrophy have been observed (Hanley and Billington, 2001). In most of the cases the histopathological changes represent adaptive responses. Analysis of $^{14}C$ activity of dog eyes indicated that this organ is a target of toxicity in dog, exhibited an affinity for the radiotracer not seen in other species (Timchalk *et al.*, 1996). No adverse effects on neurotoxicity, reproductive performance, mutagenic abnormalities have been observed. The compound has no carcinogenic potential in humans (EPA, 1997a, b).




Generic structure of the triazolopyrimidine herbicides

## Imidazolinones

Imidazolinone herbicides include imazapyr, imazamethabenzmethyl, imazapic, imazethapyr, imazamox, and imazaquin. These are selective broad-spectrum herbicides discovered in 1970s. The structural formulae of this class of herbicides are given as below.

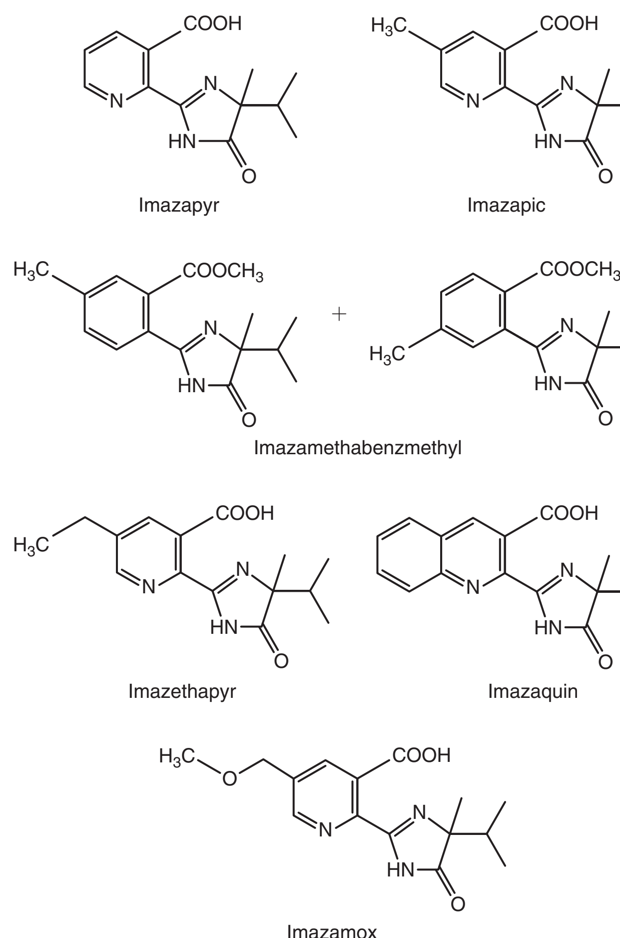



TABLE 51.7 Substituents of triazolopyrimidine sulfonamide herbicides

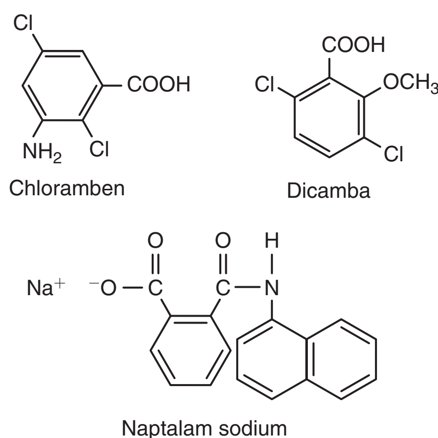
| | 1 | 2 | $R_1$ | $R_2$ | $R_3$ | $R_4$ | $R_5$ | $R_6$ |
|---|---|---|---|---|---|---|---|---|
| Cloransulam-methyl | N | C | H | $CO_2CH_3$ | Cl | $CO_2CH_3$ | F | H |
| Diclosulam | N | C | H | Cl | Cl | $OCH_2CH_3$ | F | H |
| Florasulam | N | C | H | F | F | $OCH_3$ | H | F |
| Flumetsulam | C | N | H | F | F | H | $CH_3$ | – |
| Metosulam | C | N | $CH_3$ | Cl | Cl | $OCH_3$ | $OCH_3$ | – |

From Hanley and Billington (2001). Reproduced with permission © 2001.

These herbicides are relatively non-toxic (Table 51.1). Result from primary eye irritation studies ranges from no irritation (imazaquin) to slightly irritating (imazamethabenzmethyl) and to moderately irritating (imazapic and imazethapyr), showing complete recovery within 7 days post-dosing. The rabbit primary irritation study with imazapyr showed irreversible irritation. Toxicological effects of imidazolinone herbicides are slight-to-moderate skeletal myopathy and/or slight anemia in dogs occurring in the 1-year dietary toxicity studies with three structurally similar imidazolinones (imazapic, imazaquin, and imazethapyr). There is no evidence of any adverse effect on reproductive performance and on fetal abnormalities in the rat and the rabbit. Neither mutagenicity nor any carcinogenicity has been reported in either of these species (Hess *et al.*, 2001).

## Benzoic acids

The herbicides in this group include chloramben, dicamba, and naptalam. These have a low order of toxicity and the $LD_{50}$ values in rats are summarized in Table 51.1. The structural formulae of selected herbicides are given as under.




In practice, dicamba is often combined with other herbicides and is used to control a wide spectrum of weed control. The signs and lesions are similar to those described for the chlorophenoxy acids. Poisoning after normal use has not been reported in domestic animals. The compound does not show any adverse effects in a three-generation study in rats (Harp, 2001).

Dicamba induces peroxisomal enzymes in rat liver and causes transcription upregulation of the peroxisome proliferated-activated receptor. Dicamba induces tumors in rats, which may be due to its action as peroxisome proliferators, however, exact implications of these findings are not clear and may require further study (Espandiari *et al.*, 1998).

## Carbamates, thiocarbamates, and dithiocarbamate compounds

The compounds in this category include derivatives of carbarnic acid (asulam, barban, chlorpropham, chlorbufam, karbutilate, and phenmedipham), derivatives of thiocarbarnic acid (butylate, cycloate, di-allate, EPTC, molinate, and tri-allate) and derivatives of dithiocarbarnic acid (metham-sodium). These herbicides have low-to-moderate toxicity in rats and do not pose acute hazards (Table 51.1). They are used at low concentrations, and poisoning problems have not been reported. In general, these herbicides do not produce skin or eye irritation. With repeated exposure, there is possibility of alopecia for some time after ingestion. The toxic doses of asulam and di-allate in animals are summarized in Table 51.8.

**TABLE 51.8 TD of asulam and di-allate in animals**

| | Species | mg/kg b.w. |
|---|---|---|
| *Asulam* | | |
| $LD_{50}$ | Rabbits | >2000 |
| | Chickens | >2000 |
| | Dogs | >5000 |
| *Di-allate* | | |
| $LD_{50}$ | Dogs | 510 |
| TD | Chickens | 150 for 10 days or 250 for 7 days |
| | Cattle | 25 for 5 days or 50 for 3 days |
| | Sheep | 25 for 5 days or 50 for 3 days |

Compiled from Lorgue *et al.* (1996).

In ruminants, di-allate results in anorexia, ataxia, muscular contractions, exhaustion, prostration, and alopecia in sheep which is an indication of chronic poisoning. Thiobencarb has induced toxic neuropathies in neonatal and adult laboratory rats. It appears to increase permeability of the blood–brain barrier. The non-specific lesions include hepatic, renal and pulmonary congestion, enteritis, ascites, and hydrothorax (Susan, 2003).

## Others

Bromacil and terbacil are commonly used methyluracil compounds. These compounds can cause mild toxic signs at levels of 50 mg/kg b.w. in sheep, 250 mg/kg in cattle, and 500 mg/kg in poultry when given daily for 8–10 days. Signs of toxicity include bloat, in-coordination, depression, and anorexia. Toxic doses of bromacil can be hazardous,

TABLE 51.9 TD of bromacil in animals

| Species | mg/kg b.w. |
|---|---|
| Cattle | 250 |
| Chickens | 500 for 10 days |
| Sheep | 50 for 10 days or |
| Sheep | 250 for 8 days |

Compiled from Lorgue *et al.* (1996).

especially for sheep, but no field cases of toxicity have been reported (Table 51.9).

The nitrile herbicides, ioxynil and bromoxynil, may uncouple and/or inhibit oxidative phosphorylation. Ioxynil, presumably due its iodine content, causes enlargement of the thyroid gland in the rat (Marrs, 2004). Members of polycyclic alkanoic acids (diclofop, fenoxaprop, fenthiaprop, fluazifop, haloxyfop) have moderately low toxicity, whereas, haloxyfop-methyl is an exception having high toxicity. They tend to be more toxic, if exposure is dermal. The dermal $LD_{50}$ of diclofop in rabbits is only 180 mg/kg (Susan, 2003). Some members of amide group such as bensulide and propanil are used as plant growth regulators, and some of them are more toxic than others (Table 51.1). A lethal dose of bensulide for dogs is ~200 mg/kg.

The prominent clinical sign is anorexia; other signs and lesions are not definitive and are similar to those of chlorophenoxy acid poisoning. Hemolysis, methemoglobinemia, and immunotoxicity have occurred after experimental exposure to propanil (Lorgue *et al.*, 1996). The toxicity of sulfonylureas (chlorsulfuron, sulfometuron, metsulfuron, chloremuron, kensulfuron) appears to be quite low (Susan, 2003).

A number of substances are used as defoliants in agriculture: sulfuric acid to destroy potato haulms and two closely related trialkylphosphorothioate (DEF and merphos) to defoliate cotton. A notable feature of the later is that they produce organophosphate-induced delayed neuropathy in hens (Baron and Kohnson, 1964). Chlomequat is used as growth regulator on fruit trees. The signs of toxicity in experimental animals indicate that it is partial cholinergic agonist (JMPR, 2000).

## TREATMENT

The successful management of herbicide poisoning depends on: (1) the clinicians' understanding of the mechanism of herbicide toxicity and applying them to the treatment options, (2) accurate diagnosis and assessment of the severity of intoxication, (3) maintenance of vital body functions and adequate clinical monitoring, (4) minimization of further absorption of the compound, and (5) appropriate use of specific treatment. Since majority of the herbicides have relatively low acute and chronic toxicity, therefore, no attempt has been made to find out any antidote for intoxication. However, a great deal of controversy over purported chronic effects of phenoxy derivatives such as 2,4-D and 2,4,5-T, due to the potential for contamination of 2,4,5-T with the highly toxic and unwanted by-product commonly referred to as dioxin, has surfaced. Treatment is usually symptomatic and supportive. Intravenous fluid should be given to promote diuresis. Adsorbents and drugs, which aid in restoration of liver functions, are recommended. Similarly, there is no specific treatment for bipyridyl herbicide poisoning. The therapy of bipyridyl poisoning may be focused on prevention of absorption from the GI tract. Paraquat binds tightly to diatomaceous clay. Oral administration of adsorbents such as bentonite or Fuller's earth (clay) along with a cathartic such as magnesium sulfate may be helpful. Toxicity of paraquat is enhanced by selenium/vitamin E deficiency, oxygen, and low tissue GSH peroxidase activity. Therefore, vitamin E and selenium with supportive therapy may be useful in early stages of intoxication. Excretion of bipyridyl compounds may be accelerated by forced diuresis induced by mannitol infusion and furosemide administration. Oxygen therapy and fluid therapy are contraindicated (Clark, 1971; Smith *et al.*, 1974).

An effective antidote for dinitrophenol compounds is not known. Affected animals should be cooled and sedated to help control hyperthermia. Phenothiazine tranquilizers are contraindicated, however, diazepam can be used to calm the animal. Atropine sulfate, aspirin, and antipyretics should not be used but use physical cooling measures, e.g. cool baths or sponging and keeping animal in a shaded area are advocated. Intravenous administration of large doses of sodium bicarbonate (in carnivores) solutions, parenteral vitamin A, and intense oxygen therapy, where possible may be useful. If toxin is ingested and the animal is alert, emetic should be administered; if the animal is depressed, gastric lavage should be performed. Treatment with activated charcoal should follow. Dextrose-saline infusions in combination with diuretics and tranquillizers (not barbiturates) are very useful. In ruminants, for methemoglobinemia, methylene blue solution, and administration of ascorbic acid are useful (Lorgue *et al.*, 1996).

## CONCLUSION

Herbicides are used routinely to control noxious plants. Most of these chemicals, particularly the more recently developed synthetic organic herbicides are quite selective for specific plants and have low toxicity for mammals; other less selective compounds (e.g. arsenicals, chlorates,

dinitrophenols) are more toxic to animals. Most animal health problems result from exposure to excessive amounts of herbicides because of improper or careless use or disposal of containers. The residue potential for most of these chemicals is low. However, some areas of herbicidal toxicity such as the isozyme specificity for metabolism, induction, and inhibition in interaction of herbicides with hepatic P450, flavin-containing monooxygenase-dependent oxidations, and phase I and phase II enzymes need special attention because these are not clear. A number of other questions such as what interactions at portals of entry and site of toxic action take place? Can this information help to understand the mechanism of action? In addition, during reproduction, physiological changes occur in virtually every maternal organ systems as consequence of, and in order to support, the rapid growth of the fetus and reproductive tissues. These changes may have a profound effect on the toxicokinetics of the chemical. For example, 2,4,5-T, is more slowly eliminated in mice as gestation progresses due to increased tissue residence, resulting in potentially higher fetal exposure during the later stages of pregnancy. Indeed, clearance of chemicals from the developing fetus appears to be progressively decreased during gestation in rats. It is, therefore, a challenge to predict maternal or embryo/fetal exposure at various times during pregnancy. A good understanding of pregnancy-related anatomical and physiological changes in animals and their potential impact on the chemical kinetic is required.

## REFERENCES


Acquavella JF, Weber JA, Cullen MR, Cruz OA, Martens MA, Holden LR, Riordan S, Thompson M, Farmer DR (1999) Human ocular effects from self-reported exposure to roundup herbicides. *Hum Exp Toxicol* **18**: 479–86.

Ahrens WH (1994) *Herbicide Handbook of the Weed Society of America*, 7th edn. Weed Science Society America, Champaign, IL, USA.

Aizawa K, Brown HM (1999) Mechanism and degradation of porphyrin biosynthesis inhibitor herbicide. In *Peroxidizing Herbicides*, Boger P, Wakbayashi K (eds). Spring-Verlag, Berlin, pp. 371–83.

Anderson RJ, Norris AE, Hess FD (1994) Synthetic organic chemicals that act through the porphyrin pathway. *Am Chem Soc Symp Ser* **559**: 18–33.

Ashby J, Kier L, Wilson AGE, Green T, Lefevre PA, Tinwell H, Willis GA, Heydens WF, Clapp MJL (1996) Evaluation of the potential carcinogenicity and gene toxicity to humans of the herbicide acetochlor. *Hum Exp Toxicol* **15**: 702–35.

Baron RL, Kohnson CH (1964) Neurological distuption produced in hens by two organophosphate esters. *Br J Pharmacol* **23**: 295–304.

Boehme C, Ernst W (1965) The mechanism of urea–herbicides in the rat: Diuron and Linuron. Food Cosmet. *Toxicology* **3**: 797–802 (in German).

Bus JS, Gibson JE (1975) Postnatal toxicity of chronically administered paraquat in mice and interactions with oxygen and bromobenzene. *Toxicol Appl Pharmacol* **33**: 461–70.

Bus JS, Aust SD, Gibson JE (1976a) Paraquat toxicity: Proposed mechanism of action involving lipid peroxidation. *Environ Health Persp* **16**: 139–46.

Bus JS, Cagen SZ, Olgard M, Gibson JE (1976b) Paraquat toxicity: A mechanism of paraquat toxicity in mice and rats. *Toxicol Appl Pharmacol* **35**: 501–13.

Chan BSH, Lazzaro VA, Seale JP, Duggin GG (1998) The renal excretory mechanisms and the role of organic cations in modulating the renal handling of paraquat. *Pharmacol Therapeut* **79**: 193–203.

Clark DG (1971) Inhibition of the absorption of paraquat from the gastrointestinal tract by absorbents. *Br J Ind Med* **28**: 186–8.

Clark DG, Hurst EW (1970) The toxicity of diquat. *Br J Ind Med* **27**: 51–5.

Cobb LM, Grimshaw P (1979) Acute toxicity of oral diquat (1,1-ethylene-2,2′-bipyridinium) in cynomolgus monkeys. *Toxicol Appl Pharmacol* **51**: 277–82.

Daniel JW, Gage JC (1966) Absorption and excretion of diquat and paraquat in rats. *Br J Ind Med* **23**: 133–6.

Dayan FE, Romagni JG, Duke SO (2001) Protophyrinogen oxidase inhibitors. In *Hand Book of Pesticide Toxicology*, 2nd edn, vol. 2. Robert Krieger (ed.). Academic Press, USA, pp. 1529–41.

Ebert E, Leist KH, Mayer D (1990) Summary of safety evaluation of toxicity study of Glufosinate ammonium. *Food Chem Toxicol* **28**: 339–49.

EPA (Environmental Protection Agency) (1997a). Cloransulam-methyl: pesticide fact sheet. OPPTS 7501C.

EPA (Environmental Protection Agency) (1997b). Cloransulam-methyl: pesticide tolerances. 40 CR 180. Federal Register 62 (182). 49158, (Friday, November 20, 1998).

Erne K (1966a) Distribution and elimination of chlorinated phenoxyacetic herbicides in animals. *Acta Vet Scand* **7**: 240–56.

Erne K (1966b) Studies on animal metabolism of phenoxyacetic herbicides. *Acta Vet Scand* **7**: 264–71.

Espandiari P, Ludewig G, Glauert HP, and Robertson LW (1998) Activation of hepatic NF-kB by the herbicide Dicamba (2-methoxy-3,6,dichlorobenzoic acid) in female and male rats. *J Biochem Mol Toxicol* **12**: 339–44.

Franz JE, Mato MK, Sikorski JA (1997) *Glyphosate: A Unique Global herbicide* ACS Monograph No. 189. American Chemical Society, Washington, DC, USA.

Gage JC (1968) The action of paraquat and diquat on the respiration of liver cell fractions. *Biochem J* **109**: 757–61.

Gehring PJ, Watanabe PG, Blau GE (1976) Pharmacokinetic studies in evaluation of the toxicological and environmental hazard of chemicals. In *New Concepts of in Safety Evaluation*, Mehlman MA, Shapoiro RE, Blumenthal LL (eds). Wiley, New York, pp. 195–270.

Gupta PK (1985) Pesticides. In *Modern Toxicology: Adverse Effects of Xenobiotics*, Gupta PK, Salunkhe DK (eds). vol. II. Metropolitan Book Co. Pvt Ltd, New Delhi, pp. 1–60.

Gupta PK (1986) *Pesticides in the Indian Environment*. Interprint. New Delhi.

Gupta PK (1988) *Veterinary Toxicology*. Cosmo Publications, New Delhi.

Gupta PK (1989) Pesticide production in India: An over view. In *Soil Pollution and Soil Organisms*, Mishra PC (ed.). Ashish Publishing House, New Delhi, pp. 1–16.

Gupta PK (2004) Pesticide exposure – Indian scene. *Toxicology* **198**: 83–90.

Gupta PK (2006) WHO/FAO guidelines for cholinesterase-inhibiting pesticide residues in food. In *Toxicology of Organophosphate and Carbamate Compounds*, Gupta RC (ed.). Elsevier, USA, pp. 643–54.

Gysin H, Knuesli E (1960) Chemistry and herbicidal properties of triazine derivatives. In *Advances in Pest Control Research*, Metcalf R (ed.). vol. III. Wiley Interscience, New York, pp. 289–358.

Hack R, Ebert E, Ehling G (1994) Glufosinate ammonium – some aspects of its mode of action in mammals. *Food Chem Toxicol* **32**: 461–70.

Hanley Jr TR, Billington R (2001) Toxicology of triazolopyrimidine herbicides. In *Hand Book of Pesticide Toxicology*, 2nd edn, vol. 2. Robert Krieger (ed.). Academic Press, USA, pp. 1653–65.

Harp P (2001) Dicamba. In *Hand Book of Pesticide Toxicology*, 2nd edn, vol. 2. Robert Krieger (ed.). Academic Press, USA, pp. 1639–40.

Hays Jr WJ (1982) *Pesticide Studies in Man*. Williams and Wilkins, Baltimore, USA.

Hess FG, Harris JE, Pendino K, Ponnock K (2001) Imidazolinones. In *Hand Book of Pesticide Toxicology*, 2nd edn, vol. 2. Robert Krieger (ed.). Academic Press, USA, pp. 1641–51.

Heydens WF, Lamb IC, Wilson AGE (2001) Chloracetanilides. In *Hand Book of Pesticide Toxicology*, 2nd edn, vol. 2. Robert Krieger (ed.). Academic Press, USA, pp. 1543–58.

Ho YS, Magnenat JL, Gargano M, Cao J (1998) The nature of antioxidant defense mechanism: a lesson from transgenic mice. *Environ Health Perspt* **106**: 1219–28.

Hodge HC, Downs WL, Panner BS, Smith DW, Maynard EA (1967) Oral toxicity and metabolism of Diuron (*N*-3,4-dichlorophenyl-*N*′,*N*′-dimethylurea) in rats and dogs. *Food Cosmet Toxicol* **5**: 513–31.

Hodgson E, Meyer SA (1997) Pesticides. In *Comprehensive Toxicology-Hepatic and Gastrointestinal Toxicology*, vol. 9. Gleen, Sipes, Charlene A, McQueen A, Jay Gandolfi (eds). Pergamon, Elsevier Science, Inc., USA, pp. 369–87.

Hunt LM, Chamberlain WF, Gilbrert BN, Hopkins DE, Gingrich AR (1977) Absorption, excretion, and metabolism of nitrofen by a sheep. *J Agric Food Chem* **25**: 1062–5.

IPCS (2002) *The WHO Recommended Classification of Pesticides by Hazard and Guidelines to Classification 2002–2003.* WHO, Geneva.

JMPR (1993) Pesticide residues in food. Evaluation Part II. Toxicological. *Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and a WHO Expert Group on Pesticide Residues.* WHO/PCS/94.4, Geneva, 1994.

JMPR (2000) Pesticide residues in food. Evaluation: Part II. Toxicological. *Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and a WHO Expert Group on Pesticide Residues*. WHO/PCS/01.3, Geneva, 2001.

JMPR (2004) Pesticide residues in food. *Report of the Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and a WHO Expert Group on Pesticide Residues*. FAO Plant Production and Protection Paper, 178, Rome.

JMPR (2005) Pesticide residues in food. *Report of the Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and a WHO Expert Group on Pesticide Residues.* FAO Plant Production and Protection Paper, 179, Rome.

Kennepohl E, Munro IC (2001) Phenoxy herbicides (2,4-D). In *Hand Book of Pesticides*, 2nd edn, vol. 2. Robert Krieger (ed.). Academic Press, USA, pp. 1623–38.

Leung LY, Lyga JW, Robinson RA (1991) Mechanism and distribution of the experimental triazolinone herbicide sulfentrazone in the rat, goat and hen. *J Agric Food Chem* **39**: 1509–14.

Liu J (2001) Phenylurea herbicides In *Hand Book of Pesticide Toxicology*, 2nd edn, vol. 2. Robert Krieger (ed.). Academic Press, USA, pp. 1521–7.

Lock EA, Wilks MF (2001) Diquat. In *Hand Book of Pesticide Toxicology*, 2nd edn, vol. 2. Robert Krieger (ed.). Academic Press, USA, pp. 1605–21.

Lorgue G, Lechenet J, Riviere A (1996) *Clinical Veterinary Toxicology*, (English version by M.J.Chapman). Blackwell Science, UK.

Marrs TC (2004) Toxicology of herbicides. In *Pesticide Toxicology and International Regulation*. Marrs TC, Bryan Ballantyne (eds). John Wiley & Sons, Ltd, UK, pp. 305–45.

Millburn P (1975) Excretion of xenobiotics compounds in bile. In *The Hepatobiology System: Fundamental and Pathological Mechanisms* Taylor W (ed.). Plumen, New York, pp. 109.

Mehmood OSA, Ahmed KE, Adam SEI, Idris OF (1995) Toxicity of cotoran (Fluometuron) in desert sheep. *Vet Human Toxicol* **37**: 214–6.

Monsanto (1991) *Material Data Sheet: Butachlor Technical*. Monsanto Company, St. Louis, USA.

Monsanto (1997a) *Material Data Sheet: Alachlor Technical*. Monsanto Company, St. Louis, USA.

Monsanto (1997b) *Material Data Sheet: Acetochlor Technical*. Monsanto Company, St. Louis, USA.

Munro IC, Carlo GL, Orr JC, Sund KG, Wilson RM, Kennepohl F, Lynch BS, Jabinske M, Lee NL (1992) A comprehensive, integrated review and evaluation of the scientific evidence relating to the safety of the herbicide 2,4-D. *J Am Coll Toxicol* **11**: 559–664.

Murray RE, Gibson JE (1972) A comparative study of paraquat intoxication in rats, guinea pigs and monkeys. *Exp Med Pathol* **17**: 317–25.

Nagata T, Kono I, Masaoka T, Akahori F (1992) Acute toxicological studies on paraquat pathological findings in beagle dogs following single subcutaneous injections. *Vet Human Toxicol* **34**: 105–12.

Nandihalli UB, Duke MV, Duke SO (1992) Quantitative structure activity relationships of protoporphyrinogen oxidase inhibiting diphenyl ether herbicides. *Pestic Biochem Physiol* **43**: 193–211.

Osteen CD, Padgitt M (2002) Economic issues of agricultural pesticide use and policy in the United States. In *Pesticides in Agriculture and the Environment*, Wheeler W.B (ed.). Marcel Dekker, Inc. USA, pp. 59–95

Pelletier O, Ritter L, Caron J, Somers D (1989) Disposition of 2,4-dichlorophenoxyacetic and dimethylamine salt by Fischer 344 rats dosed orally and dermally. *J Toxicol Environ Health* **28**: 221–34.

Reddy KN, Dayan FE, Duke SO (1998) QSAR analysis of protoporphyrinogen oxidase inhibitors. In *Comparative QSAR*, Devillers J (ed.). Tailor and Francis, London, pp. 197–234.

Rose MS, Smith LL (1977) The relevance of paraquat accumulation by tissues. In *Biochemical Mechanism of Paraquat Toxicity,* Autor AP (ed.), Academic Press, USA, pp. 71–91.

Rose MS, Lock EA, Smith LL, Wyatt I (1976) Paraquat accumulation. Tissue and species specificity. *Biochem Pharmacol* **25**: 419–23.

Sandhu HS, Brar RS (2000) *Text Book of Veterinary Toxicology*, Kalyani Publishers, Ludhiana, India.

Sarkar SN, Gupta PK (1993a) Feto-toxic and teratogenic potential of substituted phenylurea herbicide, isoproturon in rats. *Indian J Exp Biol* **31**: 280–2.

Sarkar SN, Gupta PK (1993b) Neurotoxicity of isoproturon, a substituted phenylurea herbicide in mice. *Indian J Exp Biol* **31**: 977–81.

Schardein JL (2000) *Chemically Induced Birth Defects*, 3rd edn, Marcel Dekker, Inc, New York.

Smith, Lewis L (1997) Paraquat. In *Comprehensive Toxicology – Toxicology of the Respiratory System*, vol. 8, Gleen, Sipes, Charlene A, McQueen A , Jay Gandolfi (eds). Pergamon, Elsevier Science, Inc., USA, pp. 581–9.

Smith, Lewis L, Wright A, Rose MS (1974) Effective treatment of paraquat poisoning in rats and its relevance to the treatment of paraquat poisoning in man. *Br Med J* **4**: 569–71.

Steven JT, Summer DD (1991) Herbicides. In *Handbook of Pesticide Toxicology*, vol. III. Hayes WJ, Laws ER (eds). Academic Press, USA, pp. 1317–408.

Susan EA (2003) *The Merck Veterinary Manual*, 8th edn. Merck & Company Inc., USA.

Talbot AR, Shiaw MH, Haung JS (1991) Acute poisoning with a glyphosate-surfactant herbicide (Roundup). A Review of 93 cases. *Hum Exp Toxicol* **10**: 1–8.

Timchalk C, Dryzga MD, Johnson KA, Eddy SL, Freshour NL, Kropscott BE, Nolan RJ (1996) Comparative pharmacokinetics of $^{14}$C metosulam (N[2,6-dichloro-3-methylphenyl]-5,7-dimethoxy-1,2,4-triazolo-[1,5a]-pyrimidine-2-sulfonamide) in rats, mice and dogs. *J Appl Toxicol* **17**: 9–21.

Van Dijck A, Macs RA, Drost RH, Douze JMC, Van Heyst ANP (1975) Paraquat poisoning in man. *Arch Toxicol* **35**: 129–36.

Waddell WJ, Marlowe C (1980) Tissue and cellular disposition of paraquat in mice. *Toxicol Appl Pharmacol* **56**: 127–40.

Watanabe T (1997) Apoptosis induced by glufosinate ammonium in the neuro-epithelium of developing mouse in culture. *Neurosci Lett* **222**: 17–20.

Watanabe T, Sano T (1998) Neurological effects of glufosinate poisoning with a brief review. *Hum Exp Toxicol* **17**: 35–9.

Wilson AGE, Takei AS (1999) Summary of toxicology studies with butachlor. *J Pestic Sci* **25**: 75–83.

Yano BL, Cosse PF, Atkin L, Corley RA (1991a) *2,4-D Isopropylamine Salt (2,4-D IPA): A 13-Week Dietary Toxicity Study in Fischer 344 Rats.* HET M-004725-006. Dow Elanco, Indiapolis, USA.

Yano BL, Cosse PF, Markham DA, Atkin L (1991b) *2,4-D Tri-Isopropylamine Salt (2,4-D IPA): A 13-Week Dietary Toxicity Study in Fischer 344 Rats.* K-008866-006. Dow Elanco. Indiapolis, USA.

CHAPTER

52

# Toxicity of fungicides

*P.K. Gupta and Manoj Aggarwal*

## INTRODUCTION

Fungicides are agents which are used to prevent or eradicate fungal infections from plants or seeds. In agriculture, they are used to protect tubers, fruits and vegetables during storage or are applied directly to ornamental plants, trees, field crops, cereals and turf grasses. Numerous substances having widely varying chemical constituents are used as fungicides (Gupta, 1988). Fungicides have been classified according to chemical structures or have been categorized agriculturally and horticulturally according to the mode of action (Ballantyne, 2004). According to the mode of application, fungicides are grouped as foliar, soil and dressing fungicides. *Foliar fungicides* are applied as liquids or powders to the aerial green parts of plants, producing a protective barrier on the cuticular surface and systemic toxicity in the developing fungus. *Soil fungicides* are applied as liquids, dry powders or granules, acting either through the vapor phase or by systemic properties. *Dressing fungicides* are applied to the post-harvest crop as liquids or dry powders to prevent fungal infestation of the crop, particularly if stored under less than optimum conditions of temperature and humidity. Thus effective fungicides must be protective, curative or eradicative and should possess the following properties: (1) low toxicity to the plant/animal but high toxicity to the particular fungus; (2) activity *per se* or ability to convert itself (by plant or fungal enzymes) into a toxic intermediate; (3) ability to penetrate fungal spores or the developing mycelium to reach the site of action; (4) low ecotoxicity and (5) formation of a protective, tenacious deposit on the plant surface that will be resistant to weathering by sunlight, rain and wind (Phillips, 2001). With a few exceptions, most of the newly developed chemicals have a low order of toxicity to mammals. Public concern has focused on the positive mutagenicity tests obtained with many fungicides and the predictive possibility of both teratogenic and carcinogenic potential. The estimated quantity of fungicides used on the major crops increased by 2.3 times between 1964 and 1997. Shares of quantity declined for inorganics (primarily copper compound) and dithiocarbamates since the 1960s but increased to 90% for captan, chlorothalonils and other organic materials. The share of newer groups, such as benzimidazoles, conazoles, dicarboximides and metal organic compounds accounted for about 10% of quantity (Osteen and Padgitt, 2002). In this chapter, fungicides have been discussed using chemical classification system.

## BACKGROUND

The earliest fungicides were inorganic materials like sulfur, lime, copper and mercury compounds. The use of element sulfur as a fungicide was recommended as early as 1803. The mercury-containing fungicides have been responsible for many deaths or permanent neurological disability. Some of the earlier inorganic metallic fungicides have been withdrawn in many countries because of their toxicity and adverse environmental effects (Ballantyne, 2004). Another compound hexachlorobenzene (HCB) was extensively used from the 1940s through the 1950s as a fungicidal dressing applied to seed grains as a dry powder. Between 1955 and 1959, an epidemic of poisoning occurred in Turkey and resulted in a syndrome, called black sore, and caused deaths of over 4000 individuals. While this agent has largely fallen by the wayside, it is still being used in developing countries. It is a highly toxic compound and can lead to severe skin manifestations including hypersensitivity (Hays, 1982; Gupta, 1985a). Since

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then many compounds have been developed and used to control fungal diseases in plants, seeds and produce. Carbamic acid derivatives including ethylenebisdithiocarbamates (EBDCs) are a group of fungicides that have been used widely throughout the world since 1940s. The important members of this class include mancozeb, maneb, metiram, zineb and nabam. All the members have an EBDC backbone with different metals associated with the individual compounds. Captan, folpet and captafol have been in use for over 50 years. These compounds belong to chloroalkylthiodicarboximide class of fungicides due to the presence of chlorine, carbon and sulfur in side-chain. Related compounds associated with this fungicide class are dichlofluanid and tolylfluanid. These later two compounds have a fluorine atom substituted for one of the terminal chlorine atoms. Another compound chlorothalonil, a halogenated benzonitrile fungicide, was first registered for use as an agrochemical in the United States in 1966. Chlorothalonil also has wider biocidal applications including its use in paints and lubricant fluids. The benzimidazole fungicides – benomyl and carbendazim – have been in use for over 35 years. Recently anilinopyrimidines, a new class of fungicides (cyprodinil, mepanipyrim and pyrimethanil), has been introduced (cyprodinil by Ciba in France in 1993) for application on cereal grains (Ollinger *et al.*, 2001).

## TOXICOKINETICS

Toxicokinetic studies provide important data on the amount of toxicant delivered to a target as well as species-specific metabolism. Animals are exposed to fungicides either through ingestion or are absorbed through the skin or the respiratory system. Different factors regulate their absorption, distribution, metabolism and excretion. In general, the liver is the primary site for biotransformation and may include activation as well as detoxification reactions (Gupta, 1986). Some fungicides do not undergo any metabolism and bind with other active binding sites. The aryl organomercurials, methyl or ethyl mercury chloride are poorly excreted and tend to accumulate in muscle, brain and other tissues, while the aryl organomercurials, phenyl mercury is more readily excreted via the kidney and less likely to accumulate in brain and muscles. Similarly, HCB possesses all the properties of chemical stability, slow degradation and biotransformation, environmental persistence, bioaccumulation in adipose tissue and organs containing a high content of lipid membranes (Ecobichon, 2001). The newly introduced class of fungicides is rapidly absorbed, metabolized and excreted and does not accumulate in tissues, whereas some of them are partially absorbed from gastrointestinal tract (GIT). For example absorption of chlorothalonil from the GIT is of the order of 30–32% of the administered dose. At least 80% of the administered dose is excreted in feces within 96 h. Highest concentrations are observed in the kidneys, approximately 0.1% of the dose. In this case gut microflora play a role in the disposition and metabolism in the rats. Glutathione conjugation plays a central role in the metabolism and subsequent complex metabolic processing of these conjugates results in selective renal uptake and urinary excretion of thiol-derived metabolites. Hepatic glutathione levels are decreased and renal glutathione levels are elevated. The depletion of hepatic glutathione is considered a direct consequence of glutathione conjugation within the liver utilizing tissue resources. The increase in renal glutathione content is more difficult to explain but may be a consequence of urinary excretion of glutathione conjugates (Parsons, 2001).

The captan is rapidly degraded to 1,2,3,6-tetrahydrophthalimide (THPI) and thiophosgene (via thiocarbonyl chloride) in the stomach before reaching the duodenum. THPI has a half-life of 1–4 s and thiophosgene is detoxified by reaction with cysteine or glutathione and is rapidly excreted. No captan is detected in blood or urine. It is, therefore, unlikely that these compounds or even thiophosgene would survive long enough to reach systemic targets such as the liver, uterus or testes. Due to the rapid elimination, meat, milk or eggs from livestock/poultry would be devoid of the parent materials. Humans appear to metabolize captan in a similar manner to other mammals (Krieger and Thongsinthusak, 1993; JMPR, 2004). Cyprodinil, a fungicide of anilinopyrimidine class, is rapidly absorbed from GIT into systemic circulation in rats. Approximately 48–68% of the administered dose is excreted in the urine, whereas 29–47% is found in the feces. Total excretions reach 92–97% of the administered dose within 48 h. Cyprodinil is almost completely metabolized. No unchanged parent molecule is found in urine, whereas minor amounts of unchanged cyprodinil are found in feces. Most of the administered cyprodinil is metabolized by sequential oxidation of the phenyl and pyrimidine ring. In urine and feces, there is no difference in the metabolite patterns of the phenyl or pyrimidyl labeled cyprodinil. Seven urinary, two biliary and two fecal metabolites have been identified, which in total accounts for 65–80% of the administered dose. Cyprodinil is absorbed in goats to a lesser extent and more slowly than in rats. The major route of excretion is in urine and feces, whereas excretion via the milk is minimal. In laying hens, cyprodinil is rapidly and completely eliminated. Residues in eggs and edible tissues are very low (Waechter *et al.*, 2001).

Carbamic acid derivative fungicides, such as EBDCs, are only partially absorbed, then rapidly metabolized and excreted with no evidence of long-term bioaccumulation. Absorption of oral doses is rapid and is excreted within 24 h with about half eliminated in the urine and half in the feces. Their common metabolite is ethylenethiourea (ETU). Only low-level residues are found in tissues particularly

in the thyroid. Another compound of this class, propamocarb is rapidly and nearly completely absorbed, distributed with concentration reaching peak levels within 1 h. Elimination from tissues is rapid; with half-lives ranging from 11 to 26 h, urine is the main route of excretion (about 75–91% within 24 h). Up to 6% of the administered dose is excreted in feces. Propamocarb is extensively metabolized and unchanged is found only in small quantities in urine. The metabolism involves aliphatic oxidation of the propyl chain (to form hydroxyl propamocarb) and by *N*-oxidation and *N*-demethylation of the tertiary amine resulting in propamocarb *N*-oxide and mono demethyl propamocarb, respectively (JMPR, 2005). Both benomyl and carbendazim are well absorbed after oral exposure (80–85%) but poorly absorbed after dermal exposure (1–2%) in rats, mice, dogs and hamsters. The major pathway of clearance is the urinary elimination in rats and mice but in dogs the majority of the dose (83.4%) is eliminated via feces with only 16.2% of the dose is eliminated in the urine after 72 h of dosing. In animals benomyl is converted into carbendazim through the loss of the *n*-butylcarbamyl side-chain prior to further metabolism (Gardiner *et al.*, 1974). In dogs and rats, carbendazim undergoes aryl hydroxylation–oxidation at the 5 and 6 positions of benzimidazole ring followed by sulfate or glucuronide conjugation before elimination. The urinary excretion half-life of carbendazim in both male and female rats is approximately 12 h. Benomyl, carbendazim and/or its metabolites are cleared rapidly from blood and exhibit minimal potential for bioaccumulation in rats exposed orally or intravenously (Mull and Hershberger, 2001).

Similarly, amide fungicides are rapidly absorbed and eliminated. Metalaxyl-M and metalaxyl can lead to stimulation of hepatic and renal cytochrome P450 and some other drug metabolizing enzymes. Tolylfluanid is rapidly and extensively absorbed followed by rapid metabolism and almost complete excretion, mainly in the urine and to a lesser extent in the bile, within 48 h. High tissue concentration has been seen soon after dosing in the kidney and liver, with lower concentrations in the peri-renal fat, brain, gonads and thyroid. In most of the species, the concentration of fluoride in the bone and teeth increases in a dose-related manner (JMPR, 2002, 2005).

Conazole fungicides such as triadimenol and triadimefon after oral dose are rapidly absorbed and widely distributed in liver and kidney. Excretion and metabolism is rapid and extensive; predominantly through oxidation of *t*-butyl methyl group. Propiconazole indicates rapid and extensive absorption (80% of the administered dose) and are widely distributed, having highest concentration in the liver and kidney. Excretion is more than 95% in the urine and feces in 48 h. There is extensive enterohepatic recirculation. The compound is extensively metabolized with oxidation of propyl side-chain, hydroxylation of phenyl and triazole rings, plus conjugation. The cleavage of dioxolane is significantly different according to species and sex (JMPR, 2004). The other compound, fludioxonil, is rapidly and extensively (80%) absorbed, widely distributed, extensively metabolized and rapidly excreted, primarily in feces (80%), with a small amount being excreted in the urine (20%). The maximum blood concentration is reached within 1 h after administration. Elimination is biphasic, with half-lives of between 2 and 5 h for the first phase and between 30 and 60 h for the second phase. The compound is extensively metabolized involving primarily oxidation of the pyrrole ring (57% of the administered dose) and a minor oxopyrrole metabolite (4% of the administered dose), followed by glucuronyl and sulfate conjugation. There is no potential of accumulation in the tissues. Trifloxystrobin is rapidly absorbed (66%) in 48 h and is widely distributed with highest concentration in blood, liver and kidney. Within 72 h, 72–96% of the administered dose is eliminated in the urine and feces. The metabolism is extensive and the compound undergoes hydroxylation, *O*-demethylation, oxidation, conjugation, chain shortening and cleavage between glyoxylphenyl and trifluoromethyl moieties (JMPR, 2004).

## MECHANISM OF ACTION

There are a series of biochemical changes or free radical-mediated processes; some may also be produced by other mechanisms that have been used to assess tissue injury. This is exemplified by the phenomenon of lipid peroxidation that has been invoked as a toxic mechanism in many situations, and also occurs subsequent to cell death and membrane lysis. However, in most of the situations it becomes difficult to pinpoint the exact mechanism of action. For example, in fungicides containing mercury, the mercury ions inhibit sulfhydryl group of enzymes involved in the transfer of amino acids across the blood brain barrier and then interfere with protein synthesis. Organomercurials can also release some mercury ions in the body, but their toxicity is not believed to be a primary action of mercury ions (Sandhu and Brar, 2000). There are several theories regarding the mechanism by which sulfur produces its toxic action. The oxidized sulfur theory attributes toxicity to its oxidation products, such as sulfur dioxide, sulfur trioxide, thiosulfuric acid or pentathionic acid. The reduced sulfur theory ascribes toxicity due to hydrogen sulfide. Direct action theory suggests toxicity due to cross linking of proteins, formation of other cellular components by free radicals of sulfur or polysulfides, or from extensive oxidation of thiol groups leading to loss of function or structural integrity of proteins. Pentachlorophenol (PCP), a halogenated substituted monocyclic aromatic, acts cellularly to uncouple oxidative phosphorylation, the target enzyme being $Na^+$, $K^+$-ATPase. Oxygen consumption is increased, while adenosine triphosphate (ATP) formation is decreased. The

energy is lost as heat instead of being stored as high-energy phosphate bonds. The electron transport chain responds by using up more and more available oxygen (increased oxygen demand) in an effort to produce ATP, but much of the free energy is lost as body heat. This leads to depletion of energy reserves (Eaton and Gallagher, 1997). Similarly organotin compounds, particularly triethyltin, uncouple oxidative phosphorylation, whereas other agents (such as sulfur) in the presence of sulfiting agents such as sulfur dioxide, thiamine is cleaved into its constituent pyrimidine and thiazole moieties, rendering it inactive.

Although the mechanism(s) by which captan and its analogs exert their cellular toxicity has not been established, captan is known to react with cellular thiols to produce thiophosgene, a potent and unstable chemical capable of reacting with sulfhydryl-, amino-, or hydroxyl-containing enzymes (Cremlyn, 1978). Thiols reduce the potency of captan. A volatile product of captan is responsible for mutagenic activity, the intermediate being short-lived and forming more quickly at higher levels at an alkaline pH. There are other several mechanisms by which these chemicals can induce cellular toxicity. For example, mouse tumors develop with oral administration above a threshold if maintained for at least 6 months. As shown in Figure 52.1, epithelial cells that comprise the villi are damaged first by exposure to captan and sloughed off into the intestinal lumen at an increased rate. Second, the basal cells in the crypt compartment that normally divide at a rate commensurate with the normal loss of villi cells from the tips of the villi increase resulting in high cell proliferation, which is not carcinogenic *per se*, but does play a role in tumor development (Gordon, 2001).

Chlorothalonil fungicide is a reactive molecule toward thio (—SH) groups. It is a soft electrophile with a preference for sulfur nucleophiles rather than nitrogen/oxygen nucleophiles. Such chemicals tend to show reactivity toward protein containing critical S electrophiles rather than toward DNA (containing critical O and N nucleophiles). A mechanistic interpretation for the carcinogenicity of chlorothalonil has been published by Wilkinson and Killeen (1996). Repeated administration of chlorothalonil causes hyperplasia in the forestomach of rats and mice. The data are consistent with a temporal sequence of events starting with increased cell proliferation, multifocal ulceration and erosion of the forestomach mucosa, regenerative hyperplasia and hyperkeratosis, ultimately progressing to the formation of gastric tumors within the forestomach. In dogs, there is neither any evidence of neoplastic development nor any evidence for the occurrence of pre-neoplastic lesions in the kidney or stomach. The absence of stomach lesions in the dog is attributable to the anatomical differences between rodents and dogs, that dogs do not possess a forestomach. Continued administration of chlorothalonil leads to the development of a regenerative hyperplasia within the renal proximal tubular epithelium. Continued regenerative hyperplasia ultimately results in progression of the kidney lesion to tubular adenoma and carcinoma. Initial cytotoxicity and regenerative hyperplasia within the proximal tubular epithelium are essential prerequisites for subsequent tumor development. The

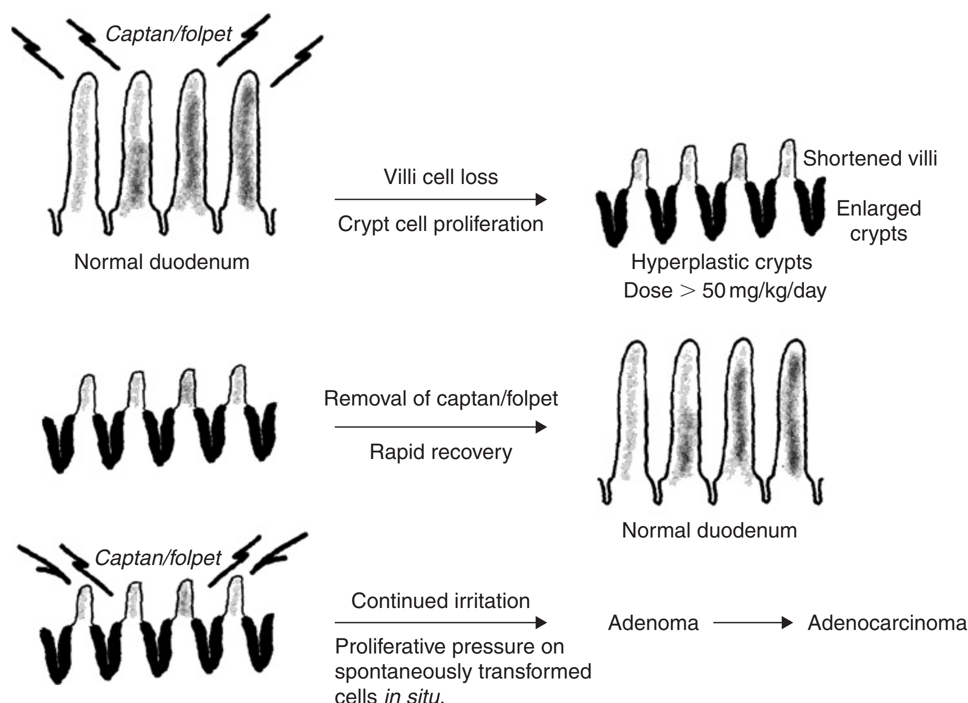



**FIGURE 52.1** Mode of action for captan and folpet in the mouse duodenum. From Gordon (2001) reproduced with permission.

proposed mode of action for the induction of renal toxicity in rodents is outlined in Figure 52.2.

The toxicology database supporting mechanism of action of carbamic acid derivatives such as EBDCs and their common metabolite ETU has been explained using modern studies with mancozeb, maneb and metiram because the principal target organ is the thyroid. These compounds inhibit the synthesis of thyroid hormone, thyroxine (T4) and triidothyronine (T3), leading to elevated levels of thyroid stimulating hormone (TSH) via feedback stimulation of the hypothalamus and pituitary (Atterwill and Aylard, 1995). Prolonged and continuous elevation of TSH levels results in hypertrophy and hyperplasia of the thyroid follicular cells in rats, mice, hamsters, monkeys and dogs, leading to development of follicular nodular hyperplasia, adenoma and/or carcinoma in rats and mice

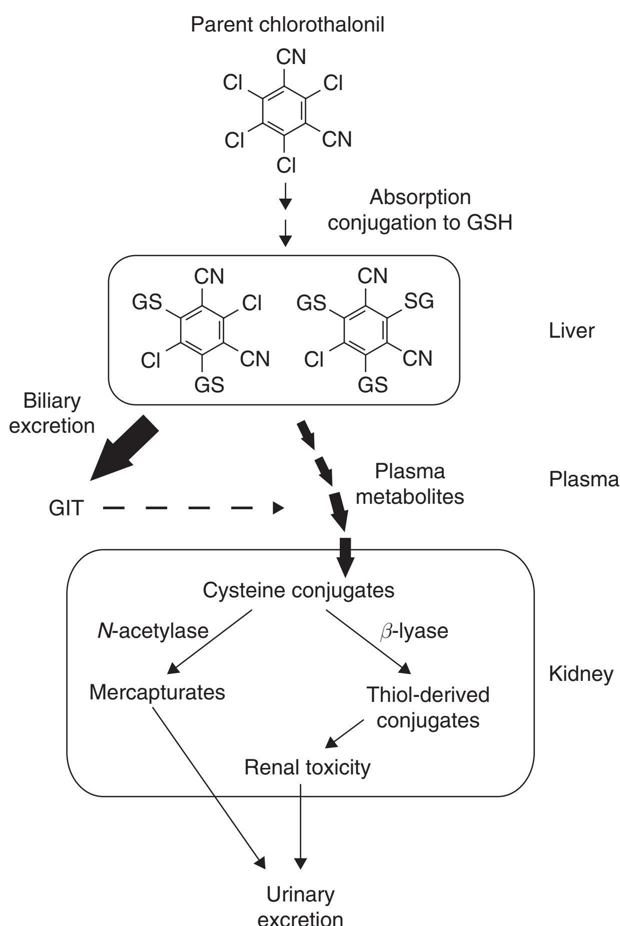



**FIGURE 52.2** Schematic outlining potential pathways of chlorothalonil metabolism in the rat that leads to formation of toxic metabolites within the kidney. Following absorption from the GIT, chlorothalonil is conjugated to glutathione in the liver. Further metabolic processing results in the formation of cysteine conjugates that may be detoxified via *N*-acetylase or activated to toxic thiol-derived species. GSH: glutathione; GIT: gastrointestinal tract. From Parsons (2001) reproduced with permission.

(Ollinger *et al.*, 2001). Conazoles such as propiconazole has phenobarbital-type mode of action leading to cell proliferation, increase in liver weight and microsomal enzyme induction (JMPR, 2004).

# TOXICITY

The spectrum of the chemical structure of fungicides is wide and diverse covering both inorganic and organic substances, thus leading to toxicity to livestock. Some of them are believed to be obsolete or discontinued for use. Till date several fungicides have been evaluated by WHO for their toxic potential and acceptable daily intake has been recommended by the Joint Meeting on Pesticide Residues (JMPR). A number of livestock poisoning cases from fungicides result from treated grains or potatoes, etc. Most of the available toxicity data are from laboratory animals; however, little information is available for farm animals and pets. In general, newer classes of fungicides have low-to-moderate toxicity. Chemical classes of fungicides by acute hazards along with their $LD_{50}$ values in rats as recommended by WHO have been summarized in Table 52.1.

These values are intended to be a guide only because the toxicity of fungicides may be due to the presence of other ingredients (such as surfactants, emulsifiers, etc.) present in the formulation. The details of guidelines for their hazard evaluation and their classification have already been summarized (IPCS, 2002; Gupta, 2006). Fungicides are believed to have a higher overall incidence than other pesticides in causing developmental abnormalities (Table 52.2) and oncogenesis. More than 80% of all oncogenic risk from the use of pesticides comes from a few fungicides, however, only a small proportion of pesticide-related deaths from fungicides have been reported (NAS, 1987; Costa, 1997).

## Inorganic fungicides

This class of fungicides includes potassium azide, potassium thiocyanate and titrated or sublimed sulfur. Sulfur had been in use in the 19th and early 20th century. Elemental sulfur and crude lime sulfur (calcium polysulfide and barium polysulfide) are commonly used as fungicides. Now, its use has declined due to availability of organic fungicides. The use of sulfur does not present a toxicological problem. Only micronized sulfur is responsible for sulfur poisoning. Lethal doses in cattle are 100–1000; sheep, 1000–1500; horses, 1000–1500 mg/kg b.w. The most affected animals are cattle, sheep, goats and dogs. Sulfur poisoning may lead to GI, neurological and pulmonary effects. On post-mortem congestion of the stomach and intestine, hemorrhagic suffusions and

**TABLE 52.1 WHO recommended category (hazardous nature) of major chemical classes of technical grade fungicides with representative examples of LD$_{50}$ values in rats**

| Chemical class | Category | LD$_{50}$ (mg/kg b.w.) |
|---|---|---|
| *Halogenated substituted monocyclic aromatics* | | |
| Chlorothalonil | U | >10,000 |
| Tecnazene | U | >10,000 |
| Dicloran | U | 4000 |
| HCB | Ia | [d]10,000 |
| Quintozene | U | >10,000 |
| Dinocap | III | 980 |
| Dichlorophen | III | 1250 |
| PCP | Ib | [c]80 |
| Chloroneb | O | – |
| *Chloroalkylthiodicarboximides* | | |
| Captan | U | 9000 |
| Captafol | Ia | 5000 |
| Folpet | U | >10,000 |
| *Anilinopyrimidines* | | |
| Mepanipyrim | U | >5000 |
| Pyrimethanil | U | 4150 |
| Cyprodinil | III | >2000 |
| *Carbamic acid derivatives* | | |
| Ferbam | U | >10,000 |
| Thiram | III | 560 |
| Ziram | III | 1400 |
| Propamocarb | U | 8600 |
| Maneb | U | 6750 |
| Mancozeb | U | >8000 |
| Zineb | U | >5000 |
| Nabam | II | 395 |
| Metiram | U | >10,000 |
| *Benzimidazoles* | | |
| Benomyl | U | >10,000 |
| Thiophanate-methyl | U | >6000 |
| Carbendazim | U | >10,000 |
| Fuberidazole | II | 336 |
| *Conazoles* | | |
| Cyproconazole | III | 1020 |
| Diniconazole | III | 639 |
| Etridiazole | III | 2000 |
| Hexaconazole | U | 2180 |
| Penconazole | U | 2120 |
| Triadimefon | III | 602 |
| Triadimenol | III | 900 |
| Azaconazole | II | 308 |
| Bromuconazole | II | 365 |
| Propiconazole | II | 1520 |
| Tetraconazole (oil) | II | 1031 |
| Imazalil | II | 320 |
| *Morpholines* | | |
| Dodemorph (liquid) | U | 4500 |
| Fenpropimorph (oil) | U | 3515 |
| Tridemorph | II | 650 |
| *Amides* | | |
| Fenhexamid | U | >5000 |
| Benalaxyl | U | [c]4200 |
| Metalaxyl | III | 670 |
| Flutolanil | U | >10,000 |
| Tolylfluanid | U | >5000 |
| Dichlofluanid | U | >5000 |
| *Others* | | |
| Thiabendazole | U | 3330 |
| Cycloheximide | O | – |
| Fludioxonil | U | >5000 |
| Dimethomorph | U | >5000 |
| Trifloxystrobin | U | >5000 |
| Fenpyroximate | II | 245 |

Ia: extremely hazardous; Ib: highly hazardous; II: moderately hazardous; III: slightly hazardous; U: unlike to present acute hazard in normal use; O: obsolete or discontinued; c: the variability is reflected in the prefix "c" and dermal in the prefix "d" before LD$_{50}$ values. Compiled from IPCS (2002) and JMPR (2005).

**TABLE 52.2 Selected list of fungicides having teratogenic potential in experimental animals**

| Name of the compound/chemical | Teratogenic effect |
|---|---|
| *Alkyldithiocarbamic acid* | |
| (Manganese, zinc and ammonium salts) | Multiple defects (rats) |
| *Halogenated substituted monocyclic aromatics* | |
| Dinocap[a] | Hydrocephaly (rabbit)/experimental teratogen |
| Hexachlorobenzene | Variety of defects (mice), rib variation and reduced weight (rats) |
| *Chloroalkylthiodicarboximides* | |
| Captafol, folpet | CNS, rib, tail and limb defects (hamsters) no teratogenic effects in other species |
| Captan | ??? |
| *Carbamic acid derivatives* | |
| Maneb and zineb metabolite ethylenethiuram monosulfide | ??? |
| Ferbam | Soft-tissue and skeletal (rats) |
| Mancozeb and maneb metabolites | Variety of defects |
| *Benzimidazoles* | |
| Benomyl | Skeletal malformations, increased mortality (rats), multiple anomalies (mice), small renal papillae but no malformations (rabbits) |

(*Continued*)

**TABLE 52.2 (Continued)**

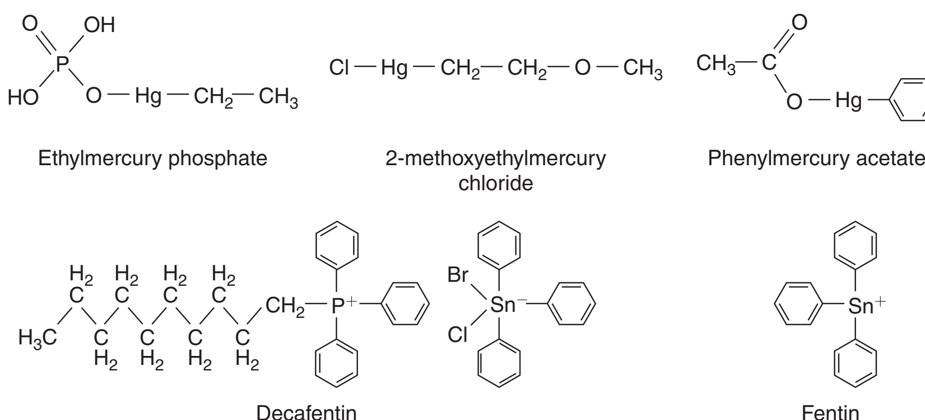
| | |
|---|---|
| Carbendazim | Limb malformations, postnatal behavior alterations, postural reflex, open field behavior (rat) |
| *Morpholine* | |
| Tridemorph | Cleft palate, other malformations and development toxicity (rats, mice) |
| *Others* | |
| Cycloheximide[b] | Skeletal defects, dactyly, hydrocephaly or other development defects |
| Bis(tri-*n*-butyltin)oxide | Cleft palate and development toxicity (rats, mice) |

a: withdrawn by manufacturers; b: obsolete; ???: variable results and needs further studies. Compiled from Schardein (2000).

petechiae along the GIT and occasionally on the surface of bladder have been observed. Barium polysulfide after reaction with gastric acid yields barium chloride which is super purgative (Lorgue *et al.*, 1996; Sandhu and Brar, 2000; Ballantyne, 2004).

## Metallic fungicides

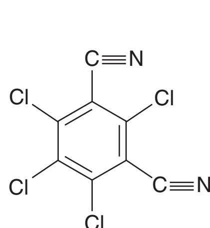
Inorganic metallic fungicides were first used in agriculture. They are protective and preventive. Mercuric and mercurous compounds have been withdrawn because of their toxicity. Ethylmercury phosphate, 2-methoxyethylmercury chloride, phenylmercury chloride and phenylmercury acetate are used as seed treatments for cereals and fodder beet (Lorgue *et al.*, 1996). Cattle, sheep and pigs are the most affected species. The oral $LD_{50}$ range from 10 to 500 mg/kg b.w. depending on the species and the particular compound involved. The organic metallic fungicides are both aliphatic and aromatic. Many are of moderate-to-high mammalian toxicity, several being immunotoxic and neurotoxic. Livestock exposed to mercurials may develop CNS and skin changes. The CNS signs include incoordination and ataxia, body swaying followed by prostration, convulsions and death. CNS stimulation is seen in calves, dogs, cats, rats and humans. However, other species (adult cattle, swine and fowls) manifest CNS depression. Other signs associated with organomercurials are bronchopneumonia, hyperpyrexia, epistasis, mucous membrane hemorrhages, hematuria and bloody feces. Skin lesions include eczema, pustules, ulceration, keratinization, dehydration, weakness and death (Sandhu and Brar, 2000). Typical organotins are di- and tri-alkyl and triphenyl tins. In general, they are severely irritant to the skin, eye and mucosae, and several are hepatorenotoxic and immunotoxic. They have been shown to increase susceptibility to infection, decrease lymphopoiesis and decrease T-lymphocyte production. Tri-alkyl tins cross the blood–brain barrier and are centrally neurotoxic (WHO, 1990).



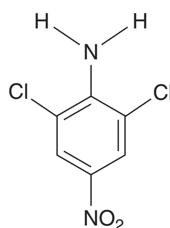

## Halogenated substituted monocyclic aromatics

This class of chemicals includes chlorothalonil, dicloran, HCB, quintozene, PCP, dichlorophen, dinocap, tecnazene and chloroneb. Chlorothalonil is a non-toxic halogenated benzonitrile fungicide (Table 52.1). Dermal irritation has been observed following repeated exposure in the rat and rabbit, indicating the potential for chlorothalonil to cause skin irritation. Chlorothalonil causes irreversible and severe ocular lesion in rabbits. Signs of toxicity include decreased body weight and decreased hematological parameters, increased absolute kidney weight, vacuolar degeneration in the proximal tubular epithelium and hyperplasia of forestomach. Chlorothalonil is not genotoxic *in vivo*.

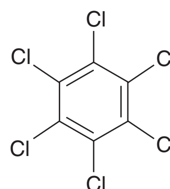
Treatment-related increase in renal tubular adenoma and carcinoma, squamous cell adenoma and carcinomas of the forestomach of both species have been reported. Chlorothalonil is not a developmental or reproductive toxicant when tested up to doses that cause significant maternal toxicity and maternal death; however, significant increase in the incidence of post-implantation loss due to early embryonic death has been observed (Parsons, 2001). Tecnazene has an oral rat $LD_{50}$ of 2047 mg/kg b.w. and is a mild irritant in the rabbit eye. The compound has the potential to produce pulmonary adenoma but it is neither embryotoxic nor teratogenic in mice (Ballantyne, 2004). Chloroneb, quintozene and dicloran have low toxicity (Table 52.1), and dinocap is a moderate eye irritant and has a human skin sensitizing potential. HCB, like other organochlorine compounds, possesses all the properties of chemical stability, slow degradation and biotransformation with potential to accumulate in adipose tissues and organs containing a high content of lipid membranes, and the ability to induce a range of tissue cytochrome-P450 as well as conjugate enzymes. Repeated exposure in animals results in hepatomegaly and porphyria as well as focal alopecia with itching and eruptions, followed by pigmented scars, anorexia and neurotoxicity expressed as irritability, ataxia and tremors. The compound can increase hepatic and thyroid tumors in hamsters and is teratogenic in mice (renal and palate malformations) and in rats (increased incidence of 14th rib). HCB is also toxic to developing perinatal animals (Table 52.2) and causes adverse effects on the immune system (Ecobichon, 2001). PCP has oral rat $LD_{50}$ of 150–210 mg/kg b.w. Common signs of toxicity are increased breathing rate, increased temperature, tremors, convulsions, loss in righting reflex and asphyxial spasms. Corneal injury may result from splashes or vapor over exposure. Toxicity is more due to contamination by commercial grade PCP. Technical grade PCP causes hepatic porphyria, increased microsomal monooxygenase activity and increased liver weight. Lethal dose in cattle and sheep is in the range of 120–140 mg/kg b.w. Chronic toxicity leads to emaciation, loss of weight, lowering of productivity (milk, eggs, meat, etc.) and increased mortality in poultry (ATSDR, 1994; Lorgue *et al.*, 1996).



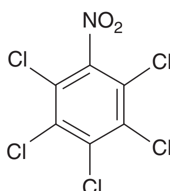
Chlorothalonil



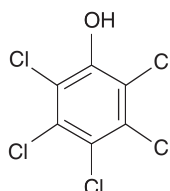
Dicloran



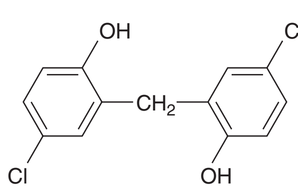
Hexachlorobenzene



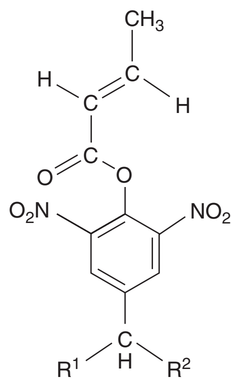
Quintozene



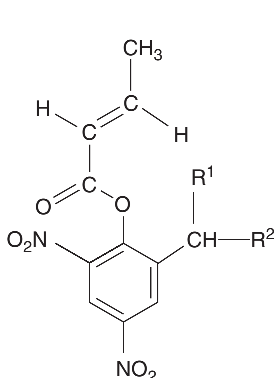
Pentachlorophenol



Dichlorophen



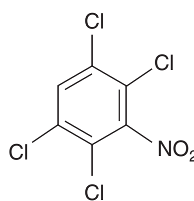
Dinocap-4



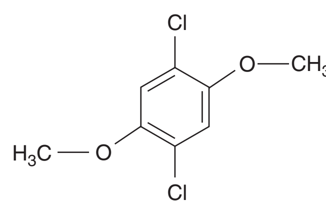
Dinocap-6

$R^1$ = methyl, ethyl or propyl $R^2$ = hexyl, pentyl or butyl

Dinocap



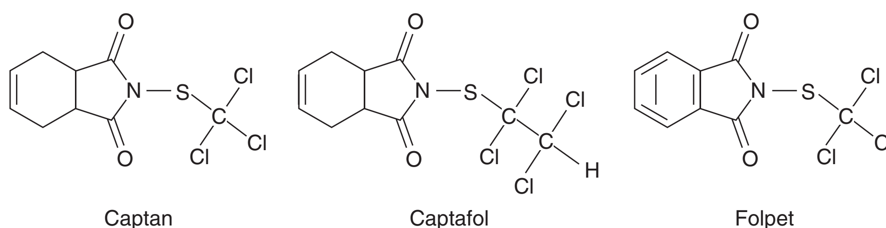
Tecnazene



Chloroneb

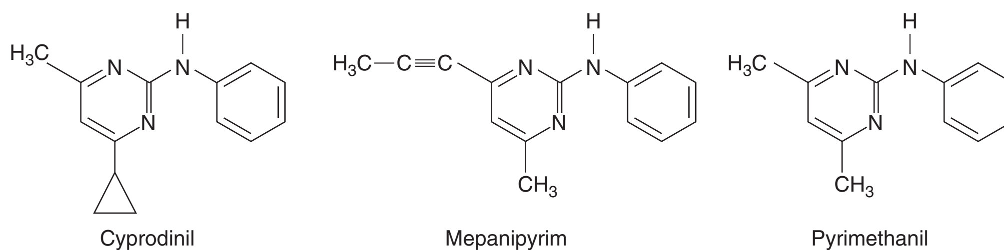
## Chloroalkylthiodicarboximides (phthalimides)

This class of chemicals contains broad-spectrum fungicides (captan, captafol and folpet) used as surface protectants on many crops. They are usually non-toxic to mammals. The oral $LD_{50}$ in rats is captan, >9000; folpet, >10000; and captafol, >5000 mg/kg b.w. (Table 52.1); however low-protein diet makes the animal more sensitive. Of this class of chemicals, folpet and captafol, true phthalimides have been deregistered and only captan, being structurally different (see structural formulae) with a cyclohexene ring, is being used. The compound is a severe eye irritant because of its high reactivity. Folpet induces incidences of diarrhea, vomiting, salivation, reduced food intake and reduced body weight gain. Testes weights are reduced in dogs. Single dose applied to the skin results in mild-to-low irritation. Long-term exposure to rats causes hyperkeratosis and acanthosis of the esophagus and stomach, particularly after folpet. Amongst ruminants, cattle is the most affected and captan produces toxicity (oral TD, 250–500 mg/kg b.w.) with labored respiration, anorexia, depression, hydrothorax, ascites and gastroenteritis (Sandhu and Brar, 2000). Mutagenicity may be associated with these agents at exceptionally high doses required to elicit biological effects. However, duodenal tumors in mice have been reported. Some compounds of this class cause teratogenicity (Table 52.2) whereas others are not proved because of, and/or masked by, maternal toxicity and possible nutritional deficits (Ecobichon, 2001). Captan induces hyperplasia of the crypt cells. Following treatment with folpet, the immune function is reduced, villi length is reduced and crypt compartments are expanded thereby reducing the villi-to-crypt ratio in mice (JMPR, 1990; Tinston, 1995; Waterson, 1995). The most characteristic pathologic finding consists of necrotizing and proliferative changes in the non-glandular portion of the stomach, dilation of the small intestine and focal epithelial hyperplasia in the proximal part of the small intestine in mice following treatment with captan. Captafol differs from captan and folpet in a number of areas including structure as well as chemical activity.



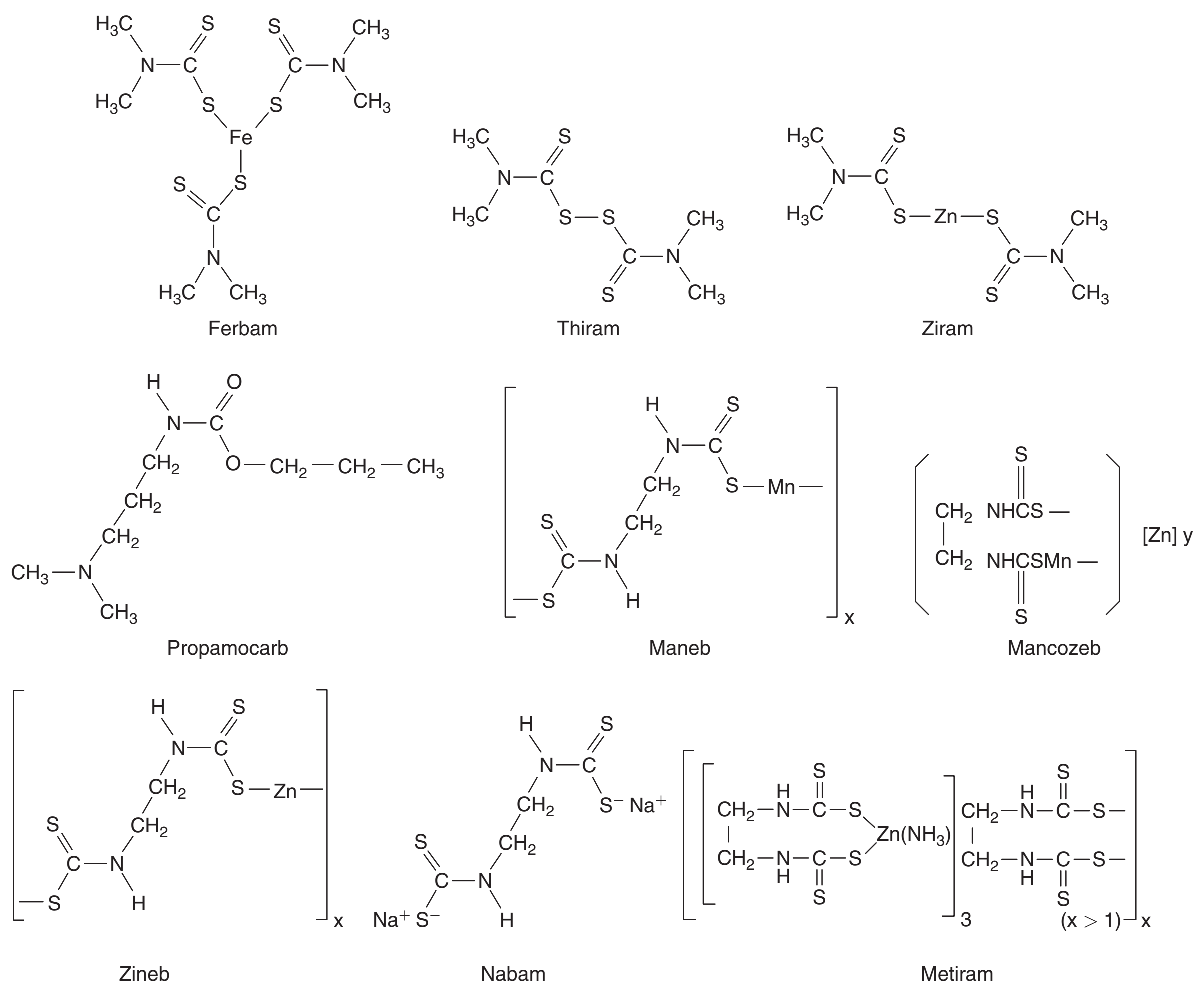

## Anilinopyrimidines

Anilinopyrimidine class of fungicides includes cyprodinil, mepanipyrim and pyrimethanil. The compounds have low toxicity and are unlikely to present acute hazards in normal use (Table 52.1). Cyprodinil produces hepatomagaly with hepatocellular hypertrophy and increased thyroid weights associated with follicular cell hypertrophy and hyprochromasia in rats. The compound also causes single cell necrosis in male and depletion of glycogen in female mice, while in dogs increased blood platelets have been observed at high doses. Mepanipyrim causes hepatocellular fatty vacuolation and lipofuscin deposition in Kupffer cells and hepatocytes of dogs, whereas such changes are not observed in cyprodinil-treated rats (Terada *et al.*, 1998). Pyrimethanil produces thyroid follicular cell tumors in rats and enhancement of hepatic thyroid hormone metabolism which may be responsible for thyroid tumorigenesis (Hurlety, 1998). In general, anilinopyrimidines do not have adverse effects on development toxicity. They are neither genotoxic nor have any carcinogenic potential (Waechter *et al.*, 2001).



## Carbamic acid derivatives

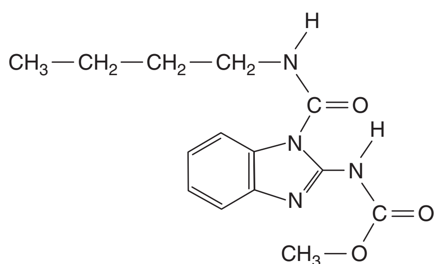
The carbamic acid class of fungicides includes dithiocarbamates (ferbam, thiram, ziram, propamocarb, etc.) and EDBCs (maneb, mancozeb, zineb, nabam, metiram, etc.). In general, carbamic acid derivatives have low or moderate acute toxicity by the oral, dermal and respiratory routes, except nabam (Table 52.1). The main features of toxicity include anorexia, diarrhea and flatulence followed by neurological effects, ataxia, muscular contractions and prostration. With repeated ingestion, there is a possibility of cutaneous effects, alopecia, risk of antithyroid effects specially with maneb. Certain compounds inhibit ovulation and egg laying (thiram, ziram). On histopathology, hepatic, renal and pulmonary congestion is common. Occasionally hepatic degeneration, ascites, enteritis and hydrothorax have been observed (Lorgue *et al.*, 1996). Propamocarb is non-irritating to the eye or skin. It induces sensitization in a Magnusson–Kligman maximization test. The signs of toxicity include hypokinesia, lethargy, hunched posture, body tremors, clonic convulsions, nasal hemorrhages, piloerection, staggering gait and ataxia. Vacuolar changes in various tissues including choroid plexus in the brain and reduction in organ weights have been observed in the rat and dog. The common development and reproductive abnormalities include reduction in copulation index (female rats) and body weight, retardation in ossification (rat) and increased post-implantation loss (rabbit) (JMPR, 2005). The principal target organ upon repeated exposure to EBDCs is thyroid. These fungicides alter thyroid hormone levels and/or weights. The developmental toxicity includes (Table 52.2) malformations and embryo-fetotoxic effects at maternally toxic dose levels with EBDCs in rats (Ollinger *et al.*, 2001).

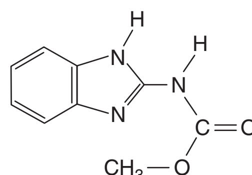


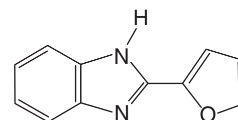

## Benzimidazoles

The major benzimidazole fungicides include benomyl, carbendazim and fuberidazole. Benomyl and carbendazim have low toxicity, whereas fuberidazole has moderate toxicity (Table 52.1). Both benomyl and carbendazim produce reproductive and developmental toxic effects in laboratory animals at high oral doses (Table 52.2). Reproductive toxic effects include decreased sperm count, decreased

testicular weights and histopathological changes, while developmental toxic effects include reduced fetal weight and anomalies of eyes (micropthalmia or anopthalmia), skull and head (hydrocephaly). The effects observed on the oocytes and uterine weight in female rats are direct and are not mediated by endocrine changes (Jeffay *et al.*, 1996; Spencer *et al.*, 1996). High dose of carbendazim causes increased incidence of diffuse proliferation of parafollicular cells of the thyroid in female rats. Both of these compounds are not carcinogenic in rats; however, lifetime exposure to mouse shows benign (not malignant), hepatocellular neoplasms and adenomas. Carbendazim is a developmental toxicant and teratogen (JMPR, 2005).

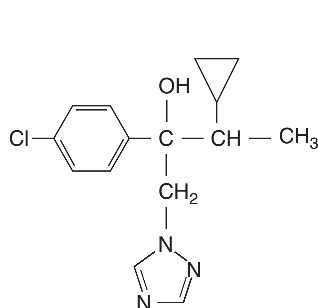

Benomyl

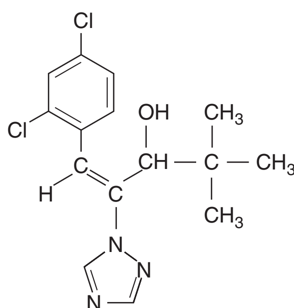

Carbendazim

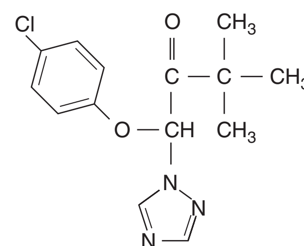

Fuberidazole

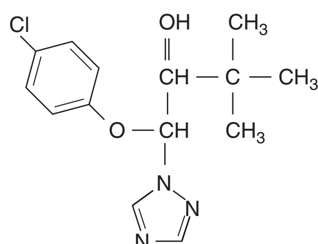
## Conazoles

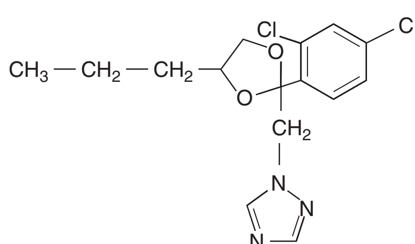
The conazole class of fungicides includes cyproconazole, diniconazole, triadimefon, triadimenol, propiconazole, imazalil, etc. and has low-to-moderate acute toxicity (Table 52.1). Triadimenol is triazole and triadimefon is closely chemically related to triadimenol with increasing toxicity for increasing isomer ratios of A (isomer B is less toxic). Triadimenol is non-irritating, whereas technical grade triadimefon is sensitizing. The other symptoms of toxicity include liver toxicity and central nervous system effects (general restlessness, alternating phases of increased and reduced motility and aggressive behavior). Liver adenomas have been observed in female mice. Developmental toxicity indicates increased ovary and testes weights, increased

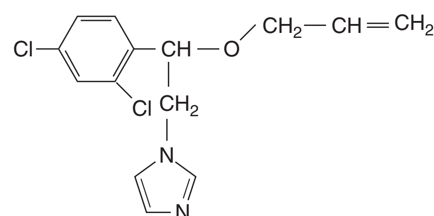

Cyproconazole


Diniconazole


Triadimefon


Triadimenol

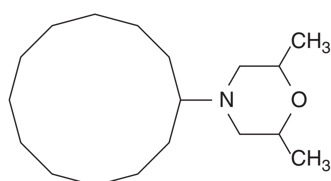

Propiconazole


Imazalil

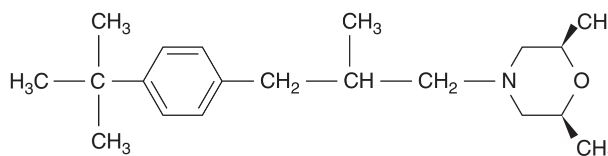
supernumerary lumber ribs with triadimenol, increased scapula malformations at maternal toxic doses in rabbits after triadimefon (JMPR, 2004). Propiconazole is not an eye irritant in rabbits, but is irritating to rabbit skin and is a skin sensitizer in guinea pigs. The compound causes reduction in body weights, liver toxicity and adverse changes in erythrocytes (rat) and in stomach (dog). On long-term exposure, liver hypertrophy and tumors (mice), uterine lumen dilation (rats), developmental toxicity indicative of reduced pup weight at parentally toxic dose and skeletal variations in laboratory animals have been observed (JMPR, 2004).

## Morpholines

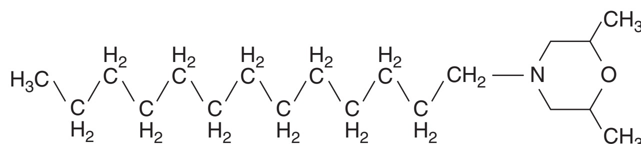
The class of morpholine fungicides includes dodemorph, fenpropimorph and tridemorph. These compounds are unlikely to cause acute hazards, except tridemorph which is moderately hazardous (Table 52.1). Dodemorph acetate is moderately irritating to rabbit skin and severely irritant to rabbit eye. Fenpropimorph is a mild irritant to rabbit skin, whereas tridemorph is non-irritant. Tridemorph and fenpropimorph lead to developmental toxicity (Table 52.2) with an increase in the total number of malformations (JMPR, 2004).



Dodemorph



Fenpropimorph



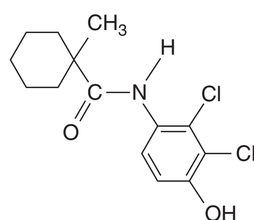
major component – 2,6-dimethyl-4-tridecylmorpholine

Tridemorph

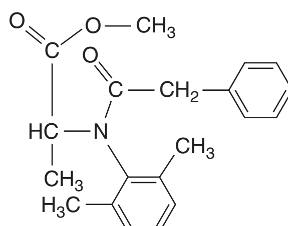
## Amides

Commonly used amide fungicides are fenhexamid, benalaxyl, metalaxyl, flutolanil tolylfluanid and dichlofluanid. The compounds are of low toxicity, except metalaxyl which is slightly hazardous (Table 52.1).

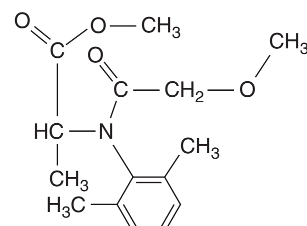
On long-term exposure, benalaxyl causes liver steatosis and hematological changes (rats) and atrophy of seminiferous tubules in dogs. In mice, increased mortality associated with amyloidosis has been observed. Reproductive abnormalities in rats include decreased body-weight gain,



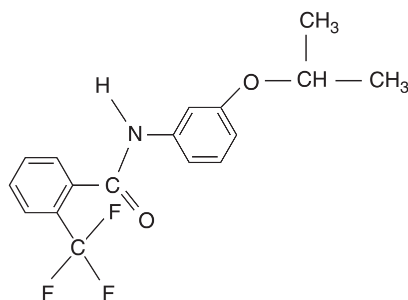
Fenhexamid



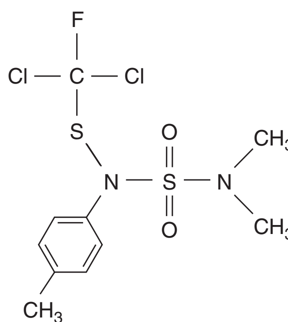
Benalaxyl



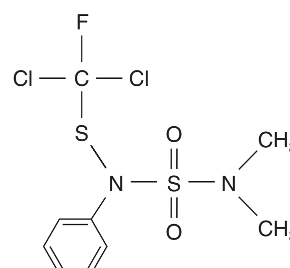
Metalaxyl



Flutolanil



Tolylfluanid

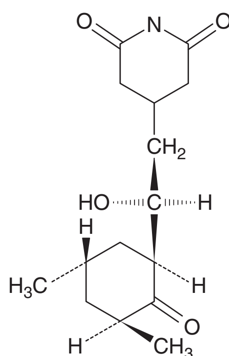


Dichlofluanid

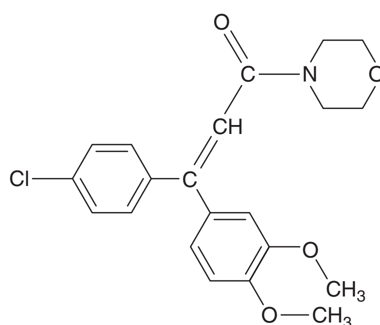
increased liver weight of pups and delayed ossification of cranial bones. Minor skeletal deviations at maternally toxic levels have been reported in rabbits (JMPR, 2005). Metalaxyl is a 1:1 mixture of R-enantiomer and S-enantiomer. Technical grade metalaxyl-M consists of a minimum of 97% of the R-enantiomer and 3% of the S-enantiomer. The two compounds are used as fungicides and are severe irritants to the rabbits. The dog is the most sensitive species with liver as the target organ. Both substances cause hepatocellular enlargement in rats, while dogs show changes in blood biochemical parameters indicative of hepatocellular damage (JMPR, 2002). Dichlofluanid and tolylfluanid have a fluorine atom substituted for one of the three chlorine atoms on the trichloromethylthio moiety of captan and folpet. Both compounds do not share a common mechanism of action with captan and folpet with regard to mouse duodenal tumors, principally because they do not induce these tumors. Flutolanil is slightly irritating to the eye. On long-term exposure, it leads to enlargement of liver, decreased body weights and mild hematological disturbances, with some evidence of increase in thyroid weight in rats and dogs. On long-term exposure to fenhexamid, the major target organ is the kidney in rats and mice, and the hematopoietic system (increase in Heinz bodies) and adrenal gland in dogs. At higher dose, delayed ossification has been observed in rabbits but is not teratogenic (JMPR, 2005). Tolylfluanid is a skin sensitizer and can lead to sedation, decreased motility, disturbed behavior and dyspnea. After intraperitoneal injection, signs consistent with local irritation, altered liver enzyme activity, increased liver weights and histopathological changes which are indicative of liver toxicity in mice, rats and dogs have been reported. At higher doses, the signs of renal toxicity and discoloration of bones and teeth, particularly the skull cap and incisors, have been observed. Alterations in thyroid hormones levels have also been observed in the number of studies in rats. The compound causes decreased pup viability at maternally toxic doses but is not teratogenic. These amides are neither genotoxic nor have any carcinogenic potential in animals (JMPR, 2002).

## Others

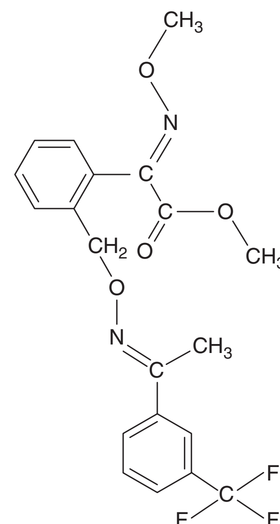
There are several other antibiotic substances, thiocarbonates and cinnamic acid derivatives which are used as fungicides. For example, sodium tetrathiocarbonate, a thiocarbonate fungicide, is moderately toxic. The rat oral $LD_{50}$ is 631 mg/kg b.w. It is a severe irritant to rabbit skin and a marked irritant to rabbit eye. Another compound cycloheximide is extremely toxic including development toxicity (Table 52.2) and has mutagenic potential. Dimethomorph has low oral toxicity in rats, is not an irritant to rabbit skin and is a minimal irritant to rabbit eye. Acute

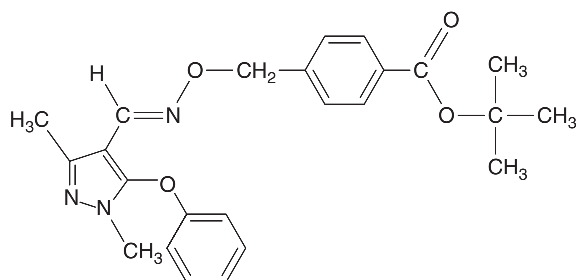


Cycloheximide

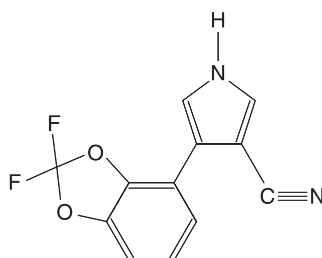


Dimethomorph





Fenpyroximate



Fludioxonil

Trifloxystrobin

oral $LD_{50}$ of fenpyroximate is 245 and 480 mg/kg b.w. in male and female rats, respectively. The compound may cause developmental toxicity in rats. Fludioxonil, a phenylpyrrole fungicide, and trifloxystrobin have low acute toxicity in rats. Fludioxonil is a slight eye irritant in rabbits, but is neither a skin irritant in rabbits nor a skin sensitizer in guinea-pigs. On long-term exposure it causes liver necrosis, kidney nephropathy and mild anemia, blue coloration of the urine and perineal fur. Reproductive toxicity indicates decreased pup weight gains in rats at parentally toxic doses. Trifloxystrobin is non-irritating but may be a skin sensitizer. Toxic symptoms are associated with liver toxicity, changes in kidney weight, atrophy of pancreas and spleen abnormalities. Development toxicity indicates decreased body-weight gain of pups accompanied by delayed eye opening at parentally toxic doses (JMPR, 2004).

## TREATMENT

In some cases there is no treatment, whereas in others supportive therapy as required by condition is indicated: (a) in the initial stages, use of emetics, gastric lavage or activated charcoal may be helpful for removal of residual material from GI tract; (b) oxygen should be provided immediately to suffice oxygen demand; (c) body temperature should be lowered (use of cool baths, cool sponging, placing the animal in the shade or in a cool, or in a quiet room); (d) phenothiazine tranquilizers by intramuscular route are advised to decrease exertion and stress and to facilitate handling of animal; however, these should not be used to sedate or comatose the animal; (e) balanced electrolyte solution should be administered to prevent dehydration and (f) animal should be removed immediately from the exposure site. In case of metallic poisoning, there is no satisfactory treatment of organomercurial toxicosis in farm or pet animals. Once developed, brain lesions are irreversible and treatment in such cases is meaningless. Traditional chelators like dimercaprol (BAL) or sodium thiosulfate have been recommended for many years, but have no or little value due to their poor affinity for organo-mercury compounds (Gupta, 1985b).

## CONCLUSION

Fungicides vary widely in chemical structure and thus in toxicity to livestock. The main hazard to livestock from fungicides is likely to arise from their use as dressings for the protection of stored grains, potatoes, etc. A number of livestock poisoning cases result from feeding of treated grains. More available toxicity data are from laboratory animals; however, little information is available for farm animals and pets. In general, fungicides have low-to-moderate toxicity. However, several fungicides such as alkyldithiocarbamic acid (manganese, zinc and ammonium salts), halogenated substituted monocyclic aromatics (dinocap), carbamic acid derivatives (maneb and zineb metabolite ethylenethiuram monosulfide), ferbam, mancozeb and maneb metabolites, HCB, benzimidazoles (benomyl and carbendazim), bis(tri-*n*-butyltin)oxide, chloroalkylthiodicarboximides (captafol and folpet), tridemorph, etc. are known to cause developmental toxicity and oncogenesis. More than 80% of all oncogenic risk from the use of pesticides come from a few fungicides, only a small proportion of pesticide-related deaths from fungicides have been reported. In view of teratogenicity several fungicides including cycloheximide have been deregistered or banned in many countries but still see some use in other, less regulated parts of the world. Other fungicides are undergoing re-evaluation because of suspected toxicity, particularly as teratogens or carcinogens and incomplete or outdated toxicity database.

## REFERENCES

ATSDR (1994) *Toxicological profile for pentachlorophenol*, US Department of Health and Human Services, Agency for Toxic Substances and Disease Registry. Atlanta, GA, USA.

Atterwill CP, Aylard SP (1995) Endocrine toxicology of the thyroid for industrial compounds. In *Toxicology of Industrial Compounds*, Thomas H, Hess R, Waechter (eds). Taylor & Francis, London, pp. 257–80.

Ballantyne B (2004) Toxicology of fungicides. In *Pesticide Toxicology and International Regulation*, Marrs TC, Bryan B (eds). John Wiley & Sons, Ltd. UK, pp. 194–303.

Costa LG (1997) Basic toxicology of pesticides. *Occup Med State Art Rev* **12:** 251–68.

Cremlyn R (1978) *Pesticides: Preparation and Mode of Action*, Wiley, New York.

Eaton DL, Gallagher EP (1997) Introduction to the principles of toxicology. In *Comprehensive Toxicology-General Principles,* Glenn S, Charlene I, McQueen A, Gandolfi J (eds). Vol. 1. Pergamon, Elsevier Science, Inc, USA, pp. 1–38.

Ecobichon DJ (2001) Toxic effects of pesticides. In *Casarett and Doull's Toxicology: The Basic Science of Poisons*, Klaassen CD (ed.). 6th edn, McGraw-Hill, New York, pp. 763–810.

Gardiner JA, Kirkland JJ, Klopping HL, Sherman H (1974) Fate of benomyl in animals. *J Agri Food Chem* **22**(3)**:** 419–27.

Gordon EB (2001) Captan and folpet. In *Hand Book of Pesticide Toxicology*, Krieger R (ed.). 2nd edn, Vol. 2. Academic Press, USA, pp. 1711–42.

Gupta PK (1985a) Pesticides. In *Modern Toxicology: Adverse Effects of Xenobiotics*, Gupta PK, Salunkhe DK (eds). Vol. 2, Metropolitan Book Co Pvt Ltd, New Delhi, pp. 1–60.

Gupta PK (1985b) Principles of non specific therapy. In *Modern Toxicology: Immuno and Clinical Toxicology*, Gupta PK, Salunkhe DK (eds). Vol. 3, Metropolitan Book Co Pvt Ltd, New Delhi, pp. 210–43.

Gupta PK (1986) *Pesticides in the Indian Environment*, Interprint, New Delhi.

Gupta PK (1988) *Veterinary Toxicology*, Cosmo Publications, New Delhi.

Gupta PK (2006) WHO/FAO guidelines for cholinesterase-inhibiting pesticide residues in food. In *Toxicology of Organophosphate and Carbamate Compounds*, Gupta RC (ed.). Elsevier, USA, pp. 643–54.

Hays Jr WJ (1982) *Pesticide Studies in Man*, Williams and Wilkins, Baltimore, MD, USA.

Hurlety PM (1998) Mode of carcinogenic action of pesticides inducing thyroid follicular cell tumors in rodents. *Environ Health Perspect* **106**: 437–5.

IPCS (2002) The WHO Recommended Classification of Pesticides by Hazard and Guidelines to Classification 2000–2002. WHO, Geneva.

Jeffay S, Libbus B, Barbee R, Perreault S (1996) Acute exposure of female hamsters to carbendazim (mbc) during meiosis results in aneuploid oocytes with subsequent arrest of embryonic cleavage and implantations. *Reprod Toxicol* **10**(3)**:** 183–9.

JMPR (1990) Pesticide residues in food. *Report of the Joint Meeting of the FAO. Panel of Experts on Pesticide Residues in Food and the Environment and a WHO Expert Group on Pesticide Residues*. FAO Plant Production and Protection Paper, 102, Rome.

JMPR (2002) Pesticide residues in food. *Report of the Joint Meeting of the FAO. Panel of Experts on Pesticide Residues in Food and the Environment and a WHO Expert Group on Pesticide Residues*. FAO Plant Production and Protection Paper, 176, Rome.

JMPR (2004) Pesticide residues in food. *Report of the Joint Meeting of the FAO. Panel of Experts on Pesticide Residues in Food and the Environment and a WHO Expert Group on Pesticide Residues*. FAO Plant Production and Protection Paper, 178, Rome.

JMPR (2005) Pesticide residues in food. *Report of the Joint Meeting of the FAO. Panel of Experts on Pesticide Residues in Food and the Environment and a WHO Expert Group on Pesticide Residues*. FAO Plant Production and Protection Paper, 179, Rome.

Krieger RI, Thongsinthusak T (1993) Captan metabolism in humans yields two biomarkers, tetrahydrophthalimide (THPI) and thiazolidine-2-thione-4-carboxylic acid (TTCA) in urine. *Drug Chem Toxicol* **16**(2)**:** 207–25.

Lorgue G, Lechenet J, Riviere A (1996) *Clinical Veterinary Toxicology*, English version by Chapman MJ Blackwell Science, UK.

Mull RL, Hershberger LW (2001) Inhibition of DNA biosynthesis-mitosis: benzimidazoles – the benzimidazole fungicides benomyl and carbendazim. In *Handbook of Pesticide Toxicology*, Krieger R(ed.). 2nd edn, Vol. 2. Academic Press, USA, pp. 1673–99.

NAS (1987) Regulating pesticides in food. The Delaney paradox. Report of Committee on Scientific and Regulatory Issues. Unlikely Pesticide Use Patterns. National Academy of Sciences, National Academic Press, Washington, DC.

Ollinger SJ, Arce G, Bui Q, Tobia AJ, Ravenswaay BV (2001) Dialkyldithiocarbamates (EBDCs). In *Handbook of Pesticide Toxicology*, KriegerR (ed.) 2nd edn, vol. 2. Academic Press, USA, pp. 1759–79.

Osteen CD, Padgitt M (2002) Economic issues of agricultural pesticide use and policy in the United States. In *Pesticides in Agriculture and the Environment,* Wheeler WB (ed.). Marcel Dekker, Inc, USA, pp. 59–95.

Parsons PP (2001) Mammalian toxicokinetics and toxicity of chlorothalonil. In *Handbook of Pesticide Toxicology*, Krieger R (ed.). vol. 2. Academic Press, USA, pp. 1743–57.

Phillips SD (2001) Fungicides and biocides. In *Clinical Environmental Health and Toxic Exposures*, Sullivan JB Krieger GR (eds). 2nd edn, vol. 2. Lippincott Williams and Wilkins, Philadelphia, PA, pp. 1109–25.

Sandhu HS, Brar RS (2000) *Text Book of Veterinary Toxicology*, Kalyani Publishers, Ludhiana, India.

Schardein JL (2000) *Chemically induced Birth Defects,* 3rd edn, Marcel Dekker Inc, New York.

Spencer F, Chi L, Zhu M (1996) Effect of benomyl and carbendazim on steroid and molecular mechanisms in uterine decidual growth in rats. *J Appl Toxicol* **16**: 211–14.

Terada M, Mizuhashi F, Tomita T, Inoue H, Murata K (1998) Mepanipyrim induced fatty liver in rats but not in mice and dogs. *J Toxicol Sci* **23:** 223–34.

Tinston DJ (1995) Captan: investigation of duodenal hyperplastic in mice. Report CTL/4532, Central Toxicology Laboratory, Alderley Park, Macclesfield, England, MRID 43982201.

Waechter F, Weber E, Herner T (2001) Cyprodinil: a fungicide of the anilinopyrimidine class. In *Hand Book of Pesticide Toxicology*, Krieger R (ed.). Vol. 2. Academic Press, USA, pp. 1701–10.

Waterson L (1995) Folpet: investigation of the effects on the duodenum of male mice after dietary administration for 28 days with recovery. Report MBS 45/943003, Huntingdon Research Centre Ltd, MRID 44286303.

WHO (1990) Methylmercury. Environmental Health Criteria No. 101, International Program on Chemical Safety. World Health Organization, Geneva.

Wilkinson CF Killeen JC (1996) A mechanistic interpretation of the oncogenicity of chlorothalonil in rodents and an assessment of human relevance. *Regulatory Toxicol Pharmacol* **24:** 69–84.

# Part 9

# Industrial Toxicants

C H A P T E R

# 53

# Alcohols and glycols

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## INTRODUCTION

Alcohols comprise a class of organic compounds composed of a hydrocarbon chain and a hydroxyl group. Alcohols that have one hydroxyl group are called monohydric, which include methanol, ethanol, and isopropanol, which are the three alcohols most commonly responsible for alcohol toxicosis. Alcohols are also classified as primary, secondary, or tertiary, according to the number of carbon atoms bonded to the carbon atom to which the hydroxyl group is bonded. Ethanol and methanol are primary alcohols, and isopropanol is a secondary alcohol. Glycols have two hydroxyl groups in their molecules and are dihydric. Ethylene glycol (EG) is the most common glycol responsible for poisonings, and EG poisoning is significantly more common in domestic animals than is alcohol toxicosis. Propylene glycol and butylene glycol are much less toxic than EG.

Alcohols and glycols are initially metabolized by hepatic alcohol dehydrogenase (ADH). Toxicosis from alcohols and glycols results in central nervous system (CNS) depression, ranging from decreased motor function to decreased consciousness, hypothermia, hypotension, coma and death from respiratory depression, and cardiovascular collapse. However, metabolites of the alcohols and glycols vary greatly in their toxicity. Metabolites of EG are nephrotoxic and result in acute renal failure. In primates, metabolites of methanol may result in blindness and permanent neurologic abnormalities.

## BACKGROUND

Reports of alcohol toxicosis are relatively quite rare in domestic animals when compared to the reported incidence in human beings. Ethanol, methanol, and isopropanol poisoning are all quite common in man. Methanol and isopropanol are usually ingested by adults as a substitute for ethanol, or in an attempt to commit suicide. In children, ingestion is usually accidental. Propylene glycol toxicosis is relatively rare in both human beings and domestic animals. Butylene glycol toxicosis is also rare, but the incidence in humans has increased recently as it is regarded as a neuromodulatory "recreational" drug (Irwin, 1996). EG toxicosis is relatively common, both in human beings and domestic animals (Barton and Oehme, 1981; Mueller, 1982; Rowland, 1987; Hornfeldt and Murphy, 1998). In humans, it is ingested either accidentally, as a substitute for ethanol, or to commit suicide; approximately 5000 episodes are reported in the United States each year (Litovitz *et al.*, 1997). The vast majority of these poisonings are unintentional, and approximately one-third of the cases occur in children (Litovitz *et al.*, 1997). EG is the most common cause of human poisoning in some countries such as Poland (Sienkiewicz and Kwiecinski, 1992).

Most incidents of EG toxicosis in domestic animals are also accidental, although malicious poisonings also occur. The mortality rate in dogs is reported to range from 50% to 70% (Barton and Oehme, 1981; Rowland, 1987; Connally, 1996) and is likely even higher in cats. EG intoxication is the second most common cause of fatal poisoning in animals, according to the American Association of Poison Control Centers (Hornfeldt *et al.*, 1998).

The first reported case of EG intoxication in a human being occurred in 1930 (Anonymous, 1930), but the toxicity of EG was not fully realized until 1938, when 76 people died after consuming an elixir of sulfanilamide containing 96% diethylene glycol (Geiling and Cannon, 1938). Since then, many reports of EG poisoning in humans and animals have been published.

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# ALCOHOL TOXICOSES

## Ethanol toxicosis

Ethanol (ethyl alcohol) has the structural formula $C_2H_5OH$, a molecular weight of 46 Da, and is the alcohol in alcoholic beverages. The percentage of ethanol in alcoholic beverages is one-half of the drink's proof value. It is also used in perfumes and mouthwashes. Ethanol toxicosis in dogs has been associated with ingestion of bread dough (Thrall, 1984a; Suter, 1992; Means, 2003). Uncooked bread dough contains *Saccharomyces cerevisiae* (common brewer's and baker's yeast), which metabolizes carbohydrate substrates to ethanol and carbon dioxide. Ethanol poisoning in dogs has also been associated with rotten apples (Kammerer *et al.*, 2001), and alcoholic beverages (van Wuijckhuise and Cremers, 2003). Ethanol toxicosis can also occur in dogs and cats when ethanol is given intravenously (IV) as a competitive substrate to treat EG toxicosis. In a study in which cats were experimentally poisoned with EG and then treated with intraperitoneal (IP) ethanol at a dose of 5 ml of 20% ethanol/kg body weight every 6 h, serum ethanol concentrations ranged from as low as 16 mg/dl at 6 h post-IP ethanol to as high as 240 mg/dl 30 min post-IP ethanol. Cats with serum ethanol concentrations of more than 200 mg/dl appeared to be near respiratory arrest and were hypothermic (Thrall *et al.*, 1988).

### Toxicokinetics

Ethanol is rapidly absorbed from the gastrointestinal (GI) tract; the rate of absorption can be slowed by the presence of food in the stomach or small intestine. Clinical findings in dogs and cats with ethanol intoxication can be correlated with blood ethanol concentration (BEC). Clinical signs include ataxia, lethargy, sedation, hypothermia, and metabolic acidosis. Time of onset of clinical signs is dependent on the dose ingested, and the amount of food present in the GI tract, but usually occurs within an hour of ingestion. Although respiratory depression usually develops when the concentration is between 400 and 500 mg/dl, death has been reported in a human being with BEC as low as 260 mg/dl (Maling, 1970).

### Mechanism of action

The mechanism of action of alcohol on the CNS is related in part to its interactions with biomembranes and its probable inhibition of gamma-amino butyric acid (GABA) receptors (Valentine, 1990).

### Diagnosis and treatment

A diagnosis can be made based on history, clinical signs, increased plasma osmolality and osmole gap, metabolic acidosis, and BEC, which can be measured by most laboratories. Hyperosmolality as a result of alcohol toxicosis must be determined using a freezing-point depression osmometer, as vapor pressure osmometers do not detect the osmotic effects of alcohols (Champion *et al.*, 1975). It is important to differentiate EG toxicosis from alcohol toxicosis. The clinical signs and early laboratory findings can be similar. However, patients with EG toxicosis must be given ADH inhibitors such as 4-methylpyrazole to prevent the formation of toxic metabolites, while patients with ethanol toxicosis can be treated supportively. Severe ethanol intoxication usually requires mechanical ventilation using a cuffed endotracheal tube. Hypothermia may develop, and alterations in hydration, electrolyte, and acid–base status should be corrected (Richardson, 2006).

## Methanol toxicosis

Methanol (methyl alcohol, wood alcohol) has the molecular structure $CH_3OH$, a molecular weight of 32 Da, and is widely used as a solvent, fuel (Sterno), gasoline additive, antifreeze, and windshield washer fluid (30–40% methanol). The minimum lethal dose in dogs is between 5.0 and 11.25 ml/kg, and in human beings is 1.25 ml/kg (Valentine, 1990). Methanol toxicosis is rare in dogs, but has been reported in a dog that chewed open a bottle of 98% methanol antifreeze (Hurd-Kuenzi, 1983).

### Toxicokinetics

Ingested methanol is absorbed quickly from the GI tract, and peak methanol concentrations occur within 30–60 min following ingestion (Barceloux *et al.*, 2002). Toxicosis has also been reported following inhalation or dermal absorption. Methanol is much more toxic to human beings and non-human primates than it is to other mammals. Methanol is metabolized by ADH to formaldehyde, which is oxidized to formic acid by formaldehyde dehydrogenase. In mammals other than primates, formic acid is metabolized relatively rapidly to carbon dioxide and water. Formic acid is metabolized less efficiently in primates.

### Mechanism of action

Formic acid is responsible for ocular and CNS lesions in primates as a result of inhibition of cytochrome oxidase (Roe, 1982). Blindness and permanent neurological abnormalities are common sequel in primates.

### Diagnosis and treatment

Clinical signs in animals other than primates are similar to those seen with ethanol toxicosis and are primarily related to CNS depression and metabolic acidosis. Vomiting and abdominal pain may be seen. In primates, following the

initial nausea and CNS depression, a latent period of approximately 12–24 h is followed by metabolic acidosis and impaired visual function. Laboratory findings in primates include hyperosmolality, increased anion gap, and severe metabolic acidosis (Bischoff, 2006a). Diagnosis can be made by history and measurement of blood methanol concentrations (or formic acid in primates).

Treatment in non-primates is symptomatic, and similar to treatment for ethanol toxicosis. Primates are treated with 4-methylpyrazole to compete with ADH and inhibit metabolism (Barceloux *et al*., 2002), or alternatively, with ethanol. Hemodialysis is also used to remove formic acid. Folic acid is given IV to enhance formic acid metabolism. It is probably inappropriate to treat methanol toxicosis in non-primates with ethanol because ethanol contributes to the sedation, and the metabolites of methanol do not cause blindness in dogs, as they do in primates.

## Isopropanol toxicosis

Isopropanol (isopropyl alcohol) has the structural formula $CH_3CH(OH)CH_3$, of a molecular weight of 60 Da, and is found in rubbing alcohol (70%), antifreeze, detergents, window cleaning products, and disinfectants. Ingestion is the usual cause of poisoning in human beings, although toxicity from inhalation and topical absorption has been reported. Isopropanol toxicosis is rare in domestic animals, possibly owing to its bitter taste. It has been reported in a horse that was mistakenly administered isopropanol via nasogastric intubation for colic; the isopropanol was mistaken for mineral oil (Somerville and Plumlee, 1996).

### *Toxicokinetics*

Isopropanol is approximately twofold more toxic than ethanol. It is rapidly absorbed from the GI tract, and approximately 80% is metabolized to acetone, which is also a CNS depressant, but acetone has a much longer half-life (16–20 h) than does alcohol.

### *Diagnosis and treatment*

Clinical signs associated with isopropanol toxicosis are similar to those for ethanol toxicosis, and include CNS depression, hypotension, vomiting, and abdominal pain due to severe gastritis, which is secondary to direct irritation. The breath of patients that have ingested isopropanol has a characteristic acetone-like odor (Somerville and Plumlee, 1996). Treatment is supportive and includes fluids, correction of acid–base abnormalities, and assisted respiration if necessary (Oehme and Kore, 2006). Hemodialysis is effective in removing isopropanol and acetone, and 4-methylpyrazole will prevent the metabolism of isopropanol to acetone.

# GLYCOL TOXICOSES

## Propylene glycol toxicosis

Propylene glycol (1,2-propanediol) has the structural formula $C_3H_8O_2$ and a molecular weight of 76 Da. It is one of the least toxic of the glycols, and is used as automotive antifreeze, an industrial and pharmaceutical solvent, in cosmetics, and a processed food additive for human and animal consumption. Although it is considered non-toxic when compared to EG, it causes CNS depression and lactic acidosis when ingested in large quantities. When used as an additive in semi-moist cat food, it historically caused Heinz body formation in erythrocytes, but did not cause an anemia when ingested in those small quantities (Christopher *et al*., 1989a, b; Weiss *et al*., 1990; Bauer *et al*., 1992a, b). However, cats eating such diets were more susceptible to other additional causes of oxidative injury, and even though overt anemia may not occur, red cells with Heinz bodies have a reduced life span. Consequently, it is no longer used as an additive in cat foods.

The oral median lethal dose for propylene glycol in dogs has been reported to be as low as 9 ml/kg (Bischoff, 2006b). Fatal cases of malicious propylene glycol toxicosis have been reported in dogs (Bischoff, 2006b). It is considered relatively unpalatable to dogs (Marshall and Doty, 1990). Fatal propylene glycol toxicosis was reported in a horse that was inadvertently given 7.6 ml/kg propylene glycol orally instead of mineral oil for potential grain overload (Dorman and Haschek, 1991). The horse had a serum concentration of 900 mg/dl propylene glycol at the time of death, 28 h following ingestion. Cause of death was presumably respiratory arrest. Propylene glycol toxicosis has been reported in at least two other horses with colic in which propylene glycol was mistaken for mineral oil (Myers and Usenik, 1969; McClanahan *et al*., 1998); both of these horses survived. In both cases, the horses were given approximately 6 ml/kg body weight via nasogastric tube. Heinz body formation also occurs in horses ingesting propylene glycol (McClanahan *et al*., 1998). Propylene glycol may be used to treat and prevent bovine ketosis, which may partially explain the availability and apparent ease with which it is confused with mineral oil.

### *Toxicokinetics*

It is rapidly absorbed in the GI tract and oxidized by ADH to D and L isomers of lactic acid, resulting in lactic acidosis. Almost all propylene glycol is metabolized within 24 h of ingestion.

### *Diagnosis and treatment*

Clinical signs depend on the quantity ingested and may include depression, ataxia, muscle fasciculations, hypotension, osmotic diuresis, respiratory arrest, and circulatory

collapse. Clinical signs in ruminants are similar to those seen in other species, and included ataxia, depression, and recumbency (Pintchuck *et al.*, 1993). Laboratory findings include metabolic acidosis, increased anion gap, and hyperosmolality of the plasma and the presence of Heinz bodies in cats and horses. Diagnosis is usually based on history of exposure, and can be confirmed by measuring propylene glycol concentrations in urine and serum by gas chromatography. Treatment for all species is supportive and includes correction of hydration and acid–base abnormalities.

## Butylene glycol toxicosis

Butylene glycol (1,2-, 1,3-, and 1,4-butanediol) has the structural formula $C_4H_{10}O_2$ and a molecular weight of 90 Da. Butylene glycol is used as antifreeze, an industrial cleaner, in cosmetics, is a component of polyurethane, and is used to make Spandex. Although no published reports of butylene glycol toxicosis in domestic animals were found in the literature, there are numerous reports of human intoxications from butylene glycol or the metabolite gamma-hydroxybutyrate (Dyer, 1991; Mack, 1993). Butylene glycol and the metabolite gamma-hydroxybutyrate are used as "recreational" drugs and were once marketed by health food stores as a food additive for bodybuilders, and to treat depression and insomnia.

1,3-butanediol has been used as an antidote for experimental EG toxicosis in dogs, since it a competitive substrate for ADH (Thrall *et al.*, 1982; Murphy *et al.*, 1984; Cox *et al.*, 1992). Although it was found to be a more effective antidote than ethanol, in that more unmetabolized EG was excreted in the urine in patients treated with 1,3-butanediol, CNS depression was as severe or more severe than that induced by ethanol therapy, and plasma hyperosmolality and metabolic acidosis were more severe than with ethanol therapy (Thrall *et al.*, 1982).

### *Toxicokinetics*

Butylene glycol is metabolized by ADH to acetoacetate and gamma-hydroxybutyrate, the so-called "date-rape" drug.

### *Mechanism of action*

Butylene glycol is a CNS depressant much like ethanol, due to the effect of the gamma-hydroxybutyrate on the CNS, and in large quantities can result in seizures and respiratory arrest.

### *Treatment*

Therapy for butylene glycol toxicosis in human beings is supportive, similar to therapy for ethanol toxicosis.

## EG toxicosis

EG has the structural formula $C_2H_6O_2$ and a molecular weight of 62 Da. EG is used primarily as an antifreeze and windshield de-icing agent. Its small molecular weight makes it very effective in lowering the freezing point of water. EG is also used as a cryoprotectant for embryo preservation, in the manufacture of polyester compounds, as a solvent in the paint and plastic industries, and as an ingredient in photographic developing solutions, hydraulic brake fluid and motor oil, and inks and wood stains (Davis *et al.*, 1997). The most readily available source of EG in the home is antifreeze solutions, which consist of approximately 95% EG.

### *Toxicokinetics*

Unlike the other alcohols (with the exception of methanol in primates) and glycols, the metabolites of EG are very toxic. EG is initially oxidized to glycoaldehyde by ADH, and glycoaldehyde is then oxidized to glycolic acid and then to glyoxylic acid. Glyoxylic acid is primarily converted to oxalic acid but may follow several metabolic pathways; end products may also include glycine, formic acid, hippuric acid, oxalomalic acid, and benzoic acid. Calcium is bound to oxalic acid, resulting in calcium oxalate crystal formation. Calcium oxalate crystal deposition is widespread but is most severe in the kidney, and crystalluria is a consistent finding in animals producing urine (Grauer *et al.*, 1984; Thrall *et al.*, 1984b).

### *Mechanism of action*

EG *per se* has no major effects other than GI irritation, and increased serum osmolality. Glycoaldehyde, the first metabolite, is thought to be primarily responsible for CNS dysfunction; respiration, glucose, and serotonin metabolism are depressed; and CNS amine concentrations are altered (Parry and Wallach, 1974; Gordon and Hunter, 1982). Hypocalcemia secondary to calcium oxalate deposition may contribute to CNS signs, although the concurrent metabolic acidosis shifts calcium to the ionized active state, reducing the chances of hypocalcemia-associated clinical signs. Acidosis is also thought to lead to altered levels of consciousness and cerebral damage. Most of the metabolites are very cytotoxic to renal tubular epithelium, and some renal epithelial and interstitial damage may be associated with calcium oxalate crystal formation within the renal tubules (de Water *et al.*, 1999). Renal epithelial cell death appears to be due primarily to destruction of cytoplasmic organelles, especially mitochondria (Bachman and Goldberg, 1971). Metabolic acidosis is often severe and has a deleterious effect on multiple organ systems. Glycolic acid accumulation is the primary cause of the metabolic acidosis associated with EG intoxication (Jacobsen *et al.*, 1984), although other acid metabolites

also contribute. Glycolic acid accumulates because the lactic dehydrogenase enzyme that metabolizes glycolic to glyoxylic acid becomes saturated.

### *Toxicity*

Before it is metabolized, EG is no more toxic than ethanol, although EG is a more potent CNS depressant than ethanol (Berger and Ayyar, 1981). However, EG is biotransformed to highly toxic metabolites that result in severe metabolic acidosis and acute renal failure, hallmarks of EG poisoning (Thrall *et al*., 1984b; Dial *et al*., 1994a, b; Davis *et al*., 1997). The minimum lethal dose of undiluted EG is 6.6 ml/kg in the dog (Kersting and Nielson, 1966) and 1.5 ml/kg in the cat (Milles, 1946).

### *Clinical signs*

Clinical signs are dose dependent and can be divided into those caused by unmetabolized EG and those caused by its toxic metabolites. The onset of clinical signs is almost always acute. Early clinical signs are usually observed 30 min after ingestion and often last until approximately 12 h after ingestion; they are primarily associated with EG-induced gastric irritation and high EG blood concentrations. These signs commonly include nausea and vomiting, CNS depression, ataxia and knuckling, muscle fasciculations, decreased withdrawal reflexes and righting ability, hypothermia, and osmotic diuresis with resultant polyuria and polydipsia (Grauer *et al*., 1984; Thrall *et al*., 1984b; Connally *et al*., 1996). As CNS depression increases in severity, dogs drink less, but osmotic diuresis persists, resulting in dehydration. In dogs, CNS signs abate after approximately 12 h, and patients may briefly appear to have recovered. Cats usually remain markedly depressed and do not exhibit polydipsia. Animals may be severely hypothermic, particularly if housed outside during the winter months. Clinical signs associated with the toxic metabolites are primarily related to oliguric renal failure, which is evident by 36–72 h following ingestion in dogs and by 12–24 h following ingestion in cats. Clinical signs may include severe lethargy or coma, seizures, anorexia, vomiting, oral ulcers and salivation, and oliguria with isosthenuria. Anuria often develops 72–96 h after ingestion. The kidneys are often swollen and painful, particularly in cats.

### *Early laboratory abnormalities*

Abnormal laboratory findings can also be divided into those associated with early EG intoxication, which may be related to the presence of EG per se or to its toxic metabolites, and those associated with late EG intoxication, most of which are related to renal failure. Early abnormalities are primarily due to the presence of acid metabolites of EG in the serum that result in metabolic acidosis and include decreased plasma bicarbonate concentration and increased anion gap. Additionally, hyperphosphatemia may occur owing to ingestion of a phosphate rust inhibitor present in some commercial antifreeze products (Grauer *et al*., 1984; Connally *et al*., 1996). The decreased plasma bicarbonate ($HCO_3$) concentration can be seen as early as 1 h following EG ingestion. Metabolites of EG significantly increase the pool of unmeasured anions and cause an increased anion gap. The anion gap is increased by 3 h after ingestion, peaks at 6 h after EG concentration, which peaks 1–6 h following ingestion, and EG is usually no longer detectable in the serum or urine 48–72 h after ingestion (Thrall *et al*., 1982; Grauer *et al*., 1984; Dial *et al*., 1994a, b). Kits[1] are available that accurately estimate blood EG concentrations with a minimum detection limit of 50 mg/dl, and the results correlate well with other established methods of measuring EG concentrations such as gas chromatography (Dasgupta *et al*., 1995), although the presence of propylene glycol or glycerol in the blood may cause a false-positive test reaction. Ethanol and methanol do not result in a false-positive test result. Cats may be intoxicated with a lethal dose of EG that is below the 50 mg/dl detectable level of the EG test kit. Therefore, if the test kit is negative and historical findings as well as clinical signs are compatible with EG ingestion, the recommendation is to initiate appropriate therapy for EG intoxication as well as submit a serum sample to a reference laboratory capable of determining a quantitative concentration.

Determination of serum osmolality is also useful for diagnosing early EG toxicosis, although other osmotically active, small molecular weight alcohols and glycols also increase serum osmolality (Ammar and Heckerling, 1996). Serum osmolality is increased by 1 h after ingestion of EG, increasing in parallel with serum EG concentrations (Dial *et al*., 1994a, b). When measured serum osmolality (by osmometry) is compared to calculated serum osmolality, the difference is referred to as the osmole or osmolal gap. If calculated osmolality is not provided on the biochemical profile printout, osmolality in mOsm/kg may be calculated using the following formula:

$$1.86\ (Na^+ + K^+) + \text{glucose}/18 + \text{BUN}/2.8 + 9$$

Normal serum osmolality is 280–310 mOsm/kg, and the normal osmole gap is less than 10 mOsm/kg. Serum osmolality as high as 450 mOsm/kg and an osmole gap as high as 150 mOsm/kg may be seen 3 h after ingestion, depending on the quantity of antifreeze ingested (Jacobsen *et al*., 1982b; Grauer *et al*., 1984). Both the gap and the measured osmolality may remain significantly high for

[1] Ethylene Glycol Test Kit, PRN Pharmacol, Inc., 5830 McAllister Avenue, Pensacola, FL 32504 (Tel.: +1 800 874 9764).

approximately 18 h after ingestion. Multiplication of the osmole gap by 5 yields an approximate serum EG concentration in mg/dl (Burkhart and Kulig, 1990). Each 100 mg/dl increment increase in EG concentration contributes approximately 16 mOsm/kg $H_2O$ to the serum osmolality (Eder *et al.*, 1998). Simultaneous or sequential increases in osmole and anion gaps are very suggestive of EG intoxication. As EG is metabolized, its contribution to the osmole gap diminishes because the accumulating negatively charged metabolites do not contribute to the osmole gap (Eder *et al.*, 1998). Two types of instruments are used to measure osmolality, freezing-point osmometers and vapor pressure osmometers. Because EG is nonvolatile (boiling point, 197°C), it is detected by either the freezing-point or vapor pressure methods. However, methanol, ethanol, and other volatile compounds, while contributing to serum osmolality, may go undetected if assayed by the vapor pressure method. Most clinical laboratories use the freezing-point method (Kruse and Cadnapaphornchai, 1994). Osmolality can be measured using serum or plasma; if the latter is used, heparin is the preferred anticoagulant. Other anticoagulants, such as EDTA, can markedly increase osmolality and can result in spurious increases in the osmole gap (Kruse and Cadnapaphornchai, 1994).

Dogs are isosthenuric (urine specific gravity of 1.008 to 1.012) by 3 h following ingestion of EG owing to osmotic diuresis and serum hyperosmolality-induced polydipsia (Grauer *et al.*, 1984; Dial *et al.*, 1994a). The urine specific gravity in cats is also decreased by 3 h after ingestion but may be above the isosthenuric range (Dial *et al.*, 1994b; Fogazzi, 1996). Calcium oxalate crystalluria is a common finding and may be observed as early as 3 and 6 h after ingestion in the cat and dog, respectively, as a result of oxalic acid combining with calcium (Dial *et al.*, 1994a, b). Calcium oxalate monohydrate crystals are variably sized, clear, six-sided prisms (Figure 53.1) (Scully *et al.*, 1979; Godolphin *et al.*, 1980; Terlinsky *et al.*, 1981; Jacobsen *et al.*, 1982a; Kramer *et al.*, 1984; Foit *et al.*, 1985; Thrall *et al.*, 1985; Steinhart, 1990). In animals and people poisoned with EG, the monohydrate form is observed more frequently than the dihydrate form, which appears as an envelope or Maltese cross (Connally *et al.*, 1996; Eder *et al.*, 1998). Dumbbell or sheaf-shaped crystals are observed infrequently. The detection of calcium oxalate crystalluria, particularly the monohydrate form, provides strong supporting evidence for the diagnosis of EG poisoning (Fogazzi, 1996). Urinary pH consistently decreases following EG ingestion.

Another diagnostic procedure that may be helpful in detecting early EG intoxication is examination of the oral cavity, face, paws, vomitus, and urine with a Wood's lamp to determine whether they appear fluorescent. Many antifreeze solutions manufactured today contain sodium fluorescein, a fluorescent dye that aids in the detection of

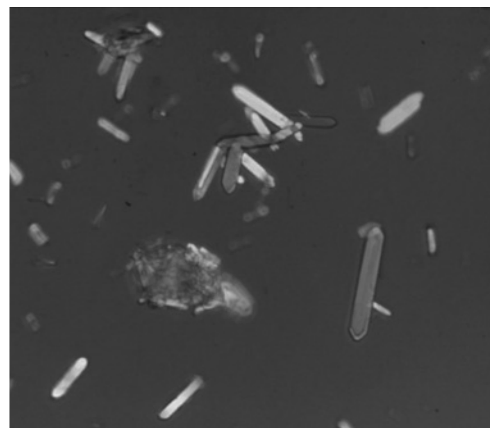

**FIGURE 53.1** Calcium oxalate monohydrate crystals (polarized light) from a dog with EG toxicosis. This figure is reproduced in color in the color plate section.

leaks in vehicle coolant systems. The dye is excreted in the urine for up to 6 h following ingestion of the antifreeze (Winter *et al.*, 1990). A negative test does not eliminate the possibility of EG ingestion, since not all antifreeze solutions contain the dye.

### *Late laboratory abnormalities*

With the onset of renal damage and subsequent decreased glomerular filtration, serum creatinine and blood urea nitrogen (BUN) concentrations increase. In the dog, these increases begin to occur between 24 and 48 h following EG ingestion. In the cat, BUN and creatinine begin to increase approximately 12 h after ingestion; however, since cats do not develop polydipsia, this may be in part due to dehydration. Serum phosphorus concentrations increase at this time owing to decreased glomerular filtration. Hyperkalemia develops with the onset of oliguria and anuria. Serum calcium concentration is decreased in approximately half of the patients (Thrall *et al.*, 1984b; Connally *et al.*, 1996), and is due to formation of insoluble calcium oxalate. Clinical signs of hypocalcemia are infrequently observed because acidosis results in a shift to the ionized, physiologically active form of calcium. Serum glucose concentration is increased in approximately 50% of dogs and cats (Thrall *et al.*, 1984b; Connally *et al.*, 1996) and is attributed to inhibition of glucose metabolism by aldehydes, increased epinephrine and endogenous corticosteroids, and uremia. Animals presenting with late EG poisoning are likely to have little to no osmole gap increase but will have an increased osmolality (whether calculated or measured) because of the azotemia and hyperglycemia. Animals remain isosthenuric in the later stages of toxicosis owing to renal dysfunction and impaired ability to concentrate urine. Calcium oxalate crystalluria persists for as long as animals are producing urine. Urine abnormalities

associated with renal damage may include hematuria, proteinuria, and glucosuria. Granular and cellular casts, white blood cells, red blood cells, and renal epithelial cells may be observed in the sediment of some patients (Thrall *et al.*, 1984b; Connally *et al.*, 1996).

### Treatment

Therapy for EG poisoning is aimed at preventing absorption, increasing excretion, and preventing metabolism of EG. Supportive care to correct fluid, acid–base, and electrolyte imbalances is also helpful. Although therapeutic recommendations have traditionally included induction of vomiting, gastric lavage, and administration of activated charcoal (Thrall *et al.*, 1995, 1998) it is likely that these procedures are not beneficial because of the rapidity with which EG is absorbed (Davis *et al.*, 1997). The most critical aspect of therapy is based on prevention of EG oxidation by ADH, the enzyme responsible for the initial reaction in the EG metabolic pathway (Parry and Wallach, 1974). Typically, dogs must be treated within 8 h following ingestion, and cats must be treated within 3 h for treatment to be successful (Dial *et al.*, 1994a, b). However, this is somewhat dependent on the amount of EG ingested. Historically, treating EG toxicosis has been directed toward inhibiting EG metabolism with ethanol, a competitive substrate that has a higher affinity for ADH than EG (Penumarthy and Oehme, 1975; Bostrom and Li, 1980). Ethanol has numerous disadvantages because it enhances many of the metabolic effects of EG. Both ethanol and EG are CNS depressants, and it is the compounded CNS depression that most limits the usefulness of ethanol as an antidote. Additional disadvantages of ethanol treatment include its metabolism to acetaldehyde, which impairs glucose metabolism and is a cerebral irritant. Ethanol also contributes to metabolic acidosis by enhancing the formation of lactic acid from pyruvate and may potentiate hypocalcemia (Money *et al.*, 1989). Moreover, ethanol compounds the effects of EG-induced osmotic diuresis and serum hyperosmolality (Kruse and Cadnapaphornchai, 1994).

4-Methylpyrazole (fomepizole) has become the preferred antidote in dogs (Grauer *et al.*, 1987; Dial *et al.*, 1989; Connally *et al.*, 1996), and more recently, cats (Connally *et al.*, 2002a, b; Thrall, 2006). Fomepizole is an ADH inhibitor, not a competitive substrate, and it does not induce CNS depression (in dogs), diuresis, or hyperosmolality at the recommended dosage. The recommended dose of fomepizole for dogs is 20 mg/kg body weight IV initially, followed by 15 mg/kg IV at 12 and 24 h, and 5 mg/kg IV at 36 h (Grauer *et al.*, 1987; Connally *et al.*, 1996; Thrall *et al.*, 2006). Cats must be given a much higher dose of fomepizole than dogs, because feline ADH is less effectively inhibited by fomepizole than is canine ADH (Connally *et al.*, 2000a, b). Cats are initially treated with 125 mg/kg fomepizole IV followed by 31.25 mg/kg IV fomepizole at 12, 24, and 36 h. The only adverse clinical sign that we have observed is CNS depression that appears to be fomepizole related (Connally *et al.*, 2002a, b). If ingestion of a large dose of EG is suspected, repeating serum quantification tests can be performed to determine whether continuation of therapy beyond 36 h is necessary. Alternatively, additional doses of fomepizole can be administered empirically. Fomepizole is commercially available as Antizol-Vet,[2] which can be conveniently reconstituted. Appropriate therapy also consists of IV fluids to correct dehydration, increase tissue perfusion, and promote diuresis. The fluid volume administered should be based on the maintenance, deficit, and continuing loss needs of the patient. Frequent measurement of urine production, serum urea nitrogen and creatinine, and blood pH, bicarbonate, ionized calcium, and electrolytes daily or twice daily will help guide fluid and electrolyte therapy (Grauer, 1998). Bicarbonate should be given slowly IV to correct the metabolic acidosis. Hypothermia can be controlled with blankets or the use of a pad with circulating warm water.

In animals that are azotemic and in oliguric renal failure on presentation, almost all of the EG has been metabolized, and treatment to inhibit ADH is likely to be of little benefit. However, ADH inhibitors should be given up to 36 h following ingestion to prevent the metabolism of any residual EG. Fluid, electrolyte, and acid–base disorders should be corrected and diuresis established, if possible. Diuretics, particularly mannitol, may be helpful. The tubular damage caused by EG may be reversible, but tubular repair can take weeks to months. Animals may take up to 1 year following EG toxicosis to regain concentrating ability, and some remain isosthenuric. Supportive care to maintain the patient during the period of renal tubular regeneration is necessary, and peritoneal dialysis may be useful (Shahar and Holmberg, 1985; Fox *et al.*, 1987; Crisp *et al.*, 1989). Hemodialysis has been attempted in dogs with EG-induced renal failure (DiBartola *et al.*, 1985) and has been shown to have a relatively good success rate in cats with acute renal failure (Langston *et al.*, 1997). Renal transplantation has also been used with variable success in cats with renal failure (Gregory *et al.*, 1992; Mathews and Gregory, 1997) and has been described in dogs (Nemeth *et al.*, 1997).

### Prognosis

EG has a very high potential for a lethal outcome, but with early recognition of the syndrome and timely institution of

[2] Antizol-Vet (fomepizole) for injection. Orphan Medical, Inc., 13911 Ridgedale Drive, Suite 475, Minnetonka, MN 55305. Available through The Butler Company (Tel.: +1 800 551 3861).

therapy, animals can be saved. The quantity of EG ingested, rate of absorption, and time interval prior to institution of therapy are variables that affect the prognosis. The prognosis is excellent in dogs treated with fomepizole within 5 h of ingesting EG. In a retrospective study of dogs with confirmed EG poisoning, all of the dogs that were azotemic when initially treated died. Of the dogs that did not have azotemia when initially treated, approximately 90% survived (Connally *et al*., 1996). The prognosis for cats is reasonably good if treatment is instituted within 3 h following ingestion (Dial *et al*., 1994b). In contrast, the prognosis in human beings who survive the initial syndrome of severe acidosis is very good. Terminal renal failure in human beings is rare, and most human patients regain renal function by 2 months following EG poisoning (Davis *et al*., 1997) likely owing to the effectiveness of hemodialysis therapy in humans (Christiansson *et al*., 1995).

## CONCLUSION

Ethanol, methanol, isopropanol, propylene glycol, butylene glycol, and marijuana toxicosis can produce ataxia and other CNS signs similar to those seen in acute EG poisoning but are much less common than EG toxicosis (Godbold *et al*., 1979; Hurd-Kuenzi, 1983; Thrall *et al*., 1984a; Suter, 1992). These disorders can be differentiated by the diagnostic laboratory tests discussed earlier. Other causes of an increased anion gap include diabetic ketoacidosis and lactic acidosis; these disorders can also be differentiated by appropriate laboratory tests. Other causes of increased osmolality include ethanol, isopropanol, methanol, and propylene glycol toxicosis. Ethanol, like EG, can also produce hypocalcemia (Money *et al*., 1989). Other differentials for acute renal failure include leptospirosis, ibuprofen and other non-steroidal anti-inflammatory drug toxicosis, aminoglycoside antibiotics, hemolytic–uremic syndrome, cholecalciferol toxicosis, grape and raisin toxicosis in dogs, and ingestion of oxalate-containing plants such as philodendron, and lily toxicosis in cats (Brown *et al*., 1985, 1996; Spyridakis *et al*., 1986; Gunther *et al*., 1988; Peterson *et al*., 1991; Holloway *et al*., 1993; Rivers *et al*., 1996; Vaden *et al*., 1997a, b; Poortinga and Hungerford, 1998; Forrester and Troy, 1999; Adin and Cowgill, 2000; Hovda, 2000; Rumbeiha *et al*., 2000; Singleton, 2001; Langston, 2002; Tefft, 2004). The majority of dogs with grape and raisin toxicosis are hypercalcemic, as are animals with cholecalciferol toxicosis (Fooshee and Forrester, 1990; Gwaltney-Brant *et al*., 2001); hypercalcemia is not associated with EG toxicosis (Thrall *et al*., 1984b; Connally *et al*., 1996). Acute renal failure must be differentiated from acutely decompensated chronic renal failure. Carbamylated hemoglobin concentration has been shown to be useful in making this differentiation (Vaden *et al*., 1997b; Heiene *et al*., 2001). Additionally, animals with chronic renal failure may be anemic and in poor body condition. A history of the duration of clinical signs is also helpful. Continuing to increase the awareness of the toxicity of EG, as well as other alcohols and glycols, will aid in preventing exposure and result in earlier presentation of animals.

## REFERENCES

Adin CA, Cowgill LD (2000) Treatment and outcome of dogs with leptospirosis: 36 cases (1990–1998). *J Am Vet Med Assoc* **216**: 371–5.

Ammar KA, Heckerling PS (1996) Ethylene glycol poisoning with a normal anion gap caused by concurrent ethanol ingestion: Importance of the osmolal gap. *Am J Kidney Dis* **27**: 130–3.

Anonymous (1930) Possible death from drinking ethylene glycol ("Prestone"). Queries and minor notes. *J Am Med Assoc* **94**: 1940.

Bachman E, Goldberg L (1971) Reappraisal of the toxicology of ethylene glycol. III. Mitochondrial effects. *Food Cosmet Toxicol* **9**: 39–55.

Barceloux DG, Bond GR, Krenzelok EP, Cooper H, Vale JA (2002) American Academy of Clinical Toxicology practice guidelines on the treatment of methanol poisoning. *Clin Toxicol* **40**: 415–46.

Barton J, Oehme FJ (1981) The incidence and characteristics of animal poisonings seen at Kansas State University from 1975 to 1980. *Vet Hum Toxicol* **23**: 101–2.

Bauer MC, Weiss DJ, Perman V (1992a) Hematologic alterations in adult cats fed 6 or 12% propylene glycol. *Am J Vet Res* **53**: 69–72.

Bauer MC, Weiss DJ, Perman V (1992b) Hematological alterations in kittens induced by 6 and 12% dietary propylene glycol. *Vet Hum Toxicol* **34**: 127–31.

Berger JR, Ayyar DR (1981) Neurological complications of ethylene glycol intoxication. Report of a case. *Arch Neurol* **38**: 724–6.

Bischoff K (2006a) Methanol. In *Small Animal Toxicology*, Peterson M, Talcott P (eds). Elsevier Saunders, St. Louis, MO, pp. 840–4.

Bischoff K (2006b) Propylene Glycol. In *Small Animal Toxicology*, Peterson M, Talcott P (eds). Elsevier Saunders, St. Louis, MO, pp. 996–1001.

Bostrom WF, Li T (1980) Alcohol dehydrogenase enzyme. In *Enzyme Basis of Detoxification*, vol. I, Jakoby WB (ed.). Academic Press, New York, pp. 231–48.

Brown SA, Barsanti JA, Crowell WA (1985) Gentamicin-associated acute renal failure in the dog. *J Am Vet Med Assoc* **186**: 686–90.

Brown CA, Roberts AW, Miller MA, Davis DA, Brown SA, Bolin CA, Jarecki-Black J, Greene CE, Miller-Liebl D (1996) *Leptospira interrogans* serovar grippotyphosa infection in dogs. *J Am Vet Med Assoc* **209**: 1265–7.

Burkhart KK, Kulig KW (1990) The other alcohols. *Emerg Med Clin North Am* **8**: 913–28.

Champion HR, Baker SP, Benner C (1975) Alcohol intoxication and serum osmolality. *Lancet* **1**: 1402–4.

Christiansson LK, Kaspersson KE, Kulling PE, Ovrebo S (1995) Treatment of severe ethylene glycol intoxication with continuous arteriovenous hemofiltration dialysis. *J Toxicol Clin Toxicol* **33**: 267–70.

Christopher MM, Perman V, Eaton JW (1989a) Contribution of propylene glycol-induced Heinz body formation to anemia in cats. *J Am Vet Med Assoc* **194**: 1045–56.

Christopher MM, Perman V, White JG (1989b) Propylene glycol-induced Heinz body formation and D-lactic acidosis in cats. *Prog Clin Biol Res* **319**: 69–87.

Connally HE, Thrall MA, Forney SD, Grauer GF, Hamar D (1996) Safety and efficacy of 4-methylpyrazole as treatment for suspected or confirmed ethylene glycol intoxication in dogs: 107 cases (1983–1995). *J Am Vet Med Assoc* **209**: 1880–3.

Connally HE, Hamar DW, Thrall MA (2000a) Inhibition of canine and feline alcohol dehydrogenase activity by fomepizole. *Am J Vet Res* **61**: 450–5.

Connally HE, Thrall MA, Hamar DW (2002b) Safety and efficacy of high dose fomepizole as therapy for ethylene glycol intoxication in cats. *Proceedings of the 8th International Veterinary Emergency and Critical Care Symposium*, p. 777.

Cox SK, Ferslew KE, Boelen LJ (1992) The toxicokinetics of 1,3 butylene glycol versus ethanol in the treatment of ethylene glycol poisoning. *Vet Hum Toxicol* **34**: 36–42.

Crisp MS, Chew DJ, DiBartola SP, Birchard SJ (1989) Peritoneal dialysis in dogs and cats: 27 cases (1976–1987). *J Am Vet Med Assoc* **195**: 1262–6.

Dasgupta A, Blackwell W, Griego J, Malik S (1995) Gas chromatographic-mass spectrometric identification and quantitation of ethylene glycol in serum after derivatization with perfluorooctanoyl chloride: A novel derivative. *J Chromatogr B Biomed Appl* **666**: 63–70.

Davis DP, Bramwell KJ, Hamilton RS, Williams SR (1997) Ethylene glycol poisoning: case report of a record-high level and a review. *J Emerg Med* **15**: 653–7.

de Water R, Noordermeer C, van der Kwast TH, Nizze H, Boeve ER, Kok DJ, Schroder FH (1999) Calcium oxalate nephrolithiasis: Effect of renal crystal deposition on the cellular composition of the renal interstitium. *Am J Kidney Dis* **33**: 761–71.

Dial SM, Thrall MA, Hamar DW (1989) 4-Methylpyrazole as treatment for naturally acquired ethylene glycol intoxication in dogs. *J Am Vet Med Assoc* **195**: 73–6.

Dial SM, Thrall MA, Hamar DW (1994a) Efficacy of 4-methylpyrazole for treatment of ethylene glycol intoxication in dogs. *Am J Vet Res* **55**: 1762–70.

Dial SM, Thrall MA, Hamar DW (1994b) Comparison of ethanol and 4-methylpyrazole as therapies for ethylene glycol intoxication in the cat. *Am J Vet Res* **55**: 1771–82.

DiBartola SP, Chew DJ, Tarr MJ, Sams RA (1985) Hemodialysis of a dog with acute renal failure. *J Am Vet Med Assoc* **186**: 1323–6.

Dorman DC, Hascheck WM (1991). Fatal propylene glycol toxicosis in a horse. *J Am Vet Med Assoc* **198**: 1643–4.

Dyer JE (1991) Gamma-hydroxybutyrate: a health-food product producing coma and seizure-like activity. *Am J Emerg Med* **9**: 321–4.

Eder AF, McGrath CM, Dowdy YG, Tomaszewski JE, Rosenberg FM, Wilson RB, Wolf BA, Shaw LM (1998) Ethylene glycol poisoning: Toxicokinetic and analytical factors affecting laboratory diagnosis. *Clin Chem* **44**: 168–77.

Fogazzi GB (1996) Crystalluria: a neglected aspect of urinary sediment analysis. *Nephrol Dial Transplant* **11**: 379–87.

Foit Jr FF, Cowell RL, Brobst DF, Moore MP, Tarr BD (1985) X-ray powder diffraction and microscopic analysis of crystalluria in dogs with ethylene glycol poisoning. *Am J Vet Res* **46**: 2404–8.

Fooshee SK, Forrester SD (1990) Hypercalcemia secondary to cholecalciferol rodenticide toxicosis in two dogs. *J Am Vet Med Assoc* **196**: 1265–8.

Forrester SD, Troy GC (1999) Renal effects of nonsteroidal antiinflammatory drugs. *Compend Contin Educ Pract Vet* **21**: 910–19.

Fox LE, Grauer GF, Dubielzig RR, Bjorling DE (1987) Reversal of ethylene glycol-induced nephrotoxicosis in a dog. *J Am Vet Med Assoc* **191**: 1433–5.

Geiling EM, Cannon PR (1938) Pathologic effects of elixer of sulfanilamide (diethylene glycol) poisoning. A clinical and experimental correlation: Final report. *J Am Med Assoc* **111**: 919–26.

Godbold Jr JC, Hawkins BJ, Woodward MG (1979) Acute oral marijuana poisoning in the dog. *J Am Vet Med Assoc* **175**: 1101–2.

Godolphin W, Meagher EP, Sanders HD (1980) Unusual calcium oxalate crystals in ethylene glycol poisoning. *Clin Toxicol* **16**: 479–86.

Gordon HL, Hunter JM (1982) Ethylene glycol poisoning. *Anaesthesia* **37**: 332–8.

Grauer GF (1998) Fluid therapy in acute and chronic renal failure. *Vet Clin North Am Small Anim Pract* **28**: 609–22.

Grauer GF, Thrall MA, Henre BA, Grauer RM, Hamar DW (1984) Early clinicopathologic findings in dogs ingesting ethylene glycol. *Am J Vet Res* **45**: 2299–309.

Grauer GF, Thrall MA, Henre BA, Hjelle JJ (1987) Comparison of the effects of ethanol and 4-methylpyrazole on the pharmacokinetics and toxicity of ethylene glycol in the dog. *Toxicol Lett* **35**: 307–14.

Gregory CR, Gourley IM, Kochin EJ, Broaddus TW (1992) Renal transplantation for treatment of end-stage renal failure in cats. *J Am Vet Med Assoc* **201**: 285–91.

Gunther R, Felice LJ, Nelson RK, Franson AM (1988) Toxicity of a vitamin D3 rodenticide to dogs. *J Am Vet Med Assoc* **193**: 211–14.

Gwaltney-Brant S, Holding JK, Donaldson CW, Eubig PA, Khan SA (2001) Renal failure associated with ingestion of grapes or raisins in dogs. *J Am Vet Med Assoc* **218**: 1555–6.

Heiene R, Vulliet PR, Williams RL, Cowgill LD (2001) Use of capillary electrophoresis to quantitate carbamylated hemoglobin concentrations in dogs with renal failure. *J Am Vet Res* **62**: 1302–6.

Holloway S, Senior D, Roth L, Tisher CC (1993) Hemolytic uremic syndrome in dogs. *J Vet Intern Med* **7**: 220–7.

Hornfeldt CA, Murphy M J (1998) American Association of Poison Control Centers Report on poisonings of animals, 1993–1994. *J Am Vet Med Assoc* **212**: 358–61.

Hovda L (2000) Common plant toxicities. In *Textbook of Veterinary Internal Medicine*, 5th edn, Ettinger SJ, Feldman EC (eds). W.B. Saunders Co, Philadelphia, PA.

Hurd-Kuenzi LA (1983) Methanol Intoxication in a dog. *J Am Vet Med Assoc* **183**: 882–3.

Irwin RD (1996) *1,4-Butanediol*. National Toxicology Program, Toxicity Report Series Number 54, NIH, US Department of Health and Human Services, NIH Publication 96–3932.

Jacobsen D, Akesson I, Shefter E (1982a) Urinary calcium oxalate monohydrate crystals in ethylene glycol poisoning. *Scand J Clin Lab Invest* **42**: 213–34.

Jacobsen D, Bredesen JE, Eide I (1982b) Anion and osmolal gaps in the diagnosis of methanol and ethylene glycol poisoning. *Acta Med Scand* **212**: 17–20.

Jacobsen D, Ovrebo S, Ostborg J, Sejersted OM (1984) Glycolate causes the acidosis in ethylene glycol poisoning and is effectively removed by hemodialysis. *Acta Med Scand* **216**: 409–16.

Kammerer M, Sachot E, Blanchot D (2001) Ethanol toxicosis from the ingestion of rotten apples by a dog. *Vet Hum Toxicol* **43**: 349–50.

Kersting EJ, Nielson SW (1966) Experimental ethylene glycol poisoning in the dog. *Am J Vet Res* **27**: 574–82.

Kramer JW, Bistline D, Sheridan P, Emerson C (1984) Identification of hippuric acid crystals in the urine of ethylene glycol-intoxicated dogs and cats. *J Am Vet Med Assoc* **184**: 584.

Kruse JA, Cadnapaphornchai P (1994) The serum osmole gap. *J Crit Care* **9**: 185–97.

Langston CE (2002) Acute renal failure caused by lily ingestion in six cats. *J Am Vet Med Assoc* **220**: 49–52.

Langston CE, Cowgill LD, Spano JA (1997) Applications and outcome of hemodialysis in cats: a review of 29 cases. *J Vet Intern Med* **11**: 348–55.

Litovitz TL, Smilkstein L, Felberg L (1997) 1996 annual report of the American Association of the Poison Control Centers Toxic Exposure Surveillance System. *Am J Emerg Med* **15**: 447–500.

Mack RB (1993) Love potion number 8 1/2. Gamma-hydroxybutyrate poisoning. *North Carol Med J* **54**: 232–3.

Maling HM (1970) Toxicology of single doses of ethyl alcohol. In *International Encyclopedia of Pharmacology and Therapeutics*, vol. II, Tremolieres J (ed.). Pergamon Press, New York, pp. 277–99.

Marshall DA, Doty RL (1990) Taste responses of dogs to ethylene glycol, propylene glycol, and ethylene glycol-based antifreeze. *J Am Vet Med Assoc* **197**: 1599–1602.

Mathews KG, Gregory CR (1997) Renal transplants in cats: 66 cases (1987–1996). *J Am Vet Med Assoc* **211**: 1432–6.

McClanahan S, Hunter J, Murphy M, Valberg S (1998) Propylene glycol toxicosis in a mare. *Vet Hum Toxicol* **40**: 294–6.

Means C (2003) Bread dough toxicosis in dogs. *J Vet Emerg Crit Care* **3**: 39–41.

Milles G (1946) Ethylene glycol poisoning with suggestions for its treatment as oxalate poisoning. *Am Med Assoc Arch Pathol* **41**: 631–8.

Money SR, Petroianu A, Kimura K, Jaffe BM (1989) Acute hypocalcemic effect of ethanol in dogs. Alcoholism. *Clin Exp Res* **13**: 453–6.

Mueller DH (1982) Epidemiologic considerations of ethylene glycol intoxication in small animals. *Vet Hum Toxicol* **24**: 21–4.

Murphy MJ, Ray AC, Jones LP, Reagor JC (1984) Butanedial treatment of ethylene glycol toxicosis in dogs. *Am J Vet Res* **45**: 2293–5.

Myers VS, Usenik EA (1969) Propylene glycol intoxication of horses. *J Am Vet Med Assoc* **155**: 1841.

Nemeth T, Toth J, Balogh L, Janoki G, Manczur F, Voros K, Dallos G (1997) Principles of renal transplantation in the dog: a review. *Acta Vet Hung* **45**: 213–26.

Oehme FW, Kore AM (2006) Miscellaneous indoor toxicants. In *Small Animal Toxicology*, Peterson M, Talcott P (eds). Elsevier Saunders, St. Louis, MO, pp. 223–43.

Parry MF, Wallach R (1974) Ethylene glycol poisoning. *Am J Med* **57**: 143–150.

Penumarthy R, Oehme FW (1975) Treatment of ethylene glycol toxicosis in cats. *Am J Vet Res* **36**: 209–212.

Peterson EN, Kirby R, Sommer M (1991) Cholecalciferol rodenticide intoxication in a cat. *J Am Vet Med Assoc* **199**: 904–6.

Pintchuck PA, Galey FD, George LW (1993) Propylene toxicity in adult dairy cows. *J Vet Intern Med* **7**: 150.

Poortinga EW, Hungerford LL (1998) A case–control study of acute ibuprofen toxicity in dogs. *Prevent Vet Med* **35**: 115–24.

Richardson JA (2006) Ethanol. In *Small Animal Toxicology*, Peterson M, Talcott P (eds). Elsevier Saunders, St. Louis, MO, pp. 698–701.

Rivers BJ, Walter PA, Letourneau J, Finlay DE, Ritenour ER, King VL, O'Brien TD, Polzin DJ (1996) Estimation of arcuate artery resistive index as a diagnostic tool for aminoglycoside-induced acute renal failure in dogs. *Am J Vet Res* **57**: 1536–44.

Roe O (1982) Species differences in methanol poisoning. *Crit Rev Toxicol* **10**: 275–86.

Rowland J (1987) Incidence of ethylene glycol intoxication in dogs and cats seen at Colorado State University Veterinary Teaching Hospital. *Vet Hum Toxicol* **29**: 41–4.

Rumbeiha WK, Braselton WE, Nachreiner RF, Refsal KR (2000) The postmortem diagnosis of cholecalciferol toxicosis: a novel approach and differentiation from ethylene glycol toxicosis. *J Vet Diagn Invest* **12**: 426–32.

Scully RE, Galbadine JJ, McNeely BV (1979) Case records of the Massachusetts General Hospital, Case 38-1979. *N Engl J Med* **30**: 650–7.

Shahar R, Holmberg DL (1985) Pleural dialysis in the management of acute renal failure in two dogs. *J Am Vet Med Assoc* **187**(9): 952–4.

Sienkiewicz J, Kwiecinski H (1992) Acute encephalopathy in ethylene glycol poisoning. *Wiadomosci Lekarskie* **45**: 536–9.

Singleton VL (2001) More information on grape or raisin toxicosis. *J Am Vet Med Assoc* **219**: 434–6.

Somerville BA, Plumlee KH (1996) Acute isopropyl alcohol intoxication in a horse. *Can Vet J* **37**: 359–60.

Spyridakis LK, Bacia JJ, Barsanti JA, Brown SA (1986) Ibuprofen toxicosis in a dog. *J Am Vet Med Assoc* **189**: 918–19.

Steinhart B (1990) Case report: severe ethylene glycol intoxication with normal osmolal gap – a chilling thought. *J Emerg Med* **8**: 583–5.

Suter RJ (1992) Presumed ethanol intoxication in sheep dogs fed uncooked pizza dough. *Aust Vet J* **69**: 20.

Tefft KM (2004) Lily nephrotoxicity in cats. *Compend Contin Educ Pract Vet* **26**: 149–56.

Terlinsky AS, Grochowski J, Geoly KL, Stauch BS, Hefter L (1981) Identification of atypical calcium oxalate crystalluria following ethylene glycol ingestion. *Am J Clin Pathol* **76**: 223–6.

Thrall MA, Grauer GF, Mero KN (1982) Ethanol, 1,3-butanediol, pyrazole, and 4-methylpyrazole therapy in dogs with experimental ethylene glycol intoxication (abstract). *Proc Am Soc Vet Clin Pathol*.

Thrall MA, Freemyer FG, Hamar DW, Jones RL (1984a) Ethanol toxicosis secondary to sourdough ingestion in a dog. *J Am Vet Med Assoc* **184**: 1513–14.

Thrall MA, Grauer GF, Mero KN (1984b) Clinicopathologic findings in dogs and cats with ethylene glycol intoxication. *J Am Vet Med Assoc* **184**: 37–41.

Thrall MA, Dial SM, Winder DR (1985) Identification of calcium oxalate monohydrate crystals by X-ray diffraction in urine of ethylene glycol-intoxicated dogs. *Vet Pathol* **22**: 625–8.

Thrall MA, Dial SM, Hamar DW (1988) Serum ethanol concentrations in ethylene glycol intoxicated cats treated with intraperitoneal ethanol. *Vet Clin Pathol* **17**: 14.

Thrall MA, Grauer GF, Dial SM (1995) Antifreeze poisoning. In *Kirk's Current Veterinary Therapy XII, Small Animal Practice*, Bonagura JD (ed.). W.B. Saunders, Philadelphia, PA, pp. 232–237.

Thrall MA, Connally HE, Grauer GF (1998) Don't freeze up! Quick response is key in ethylene glycol poisoning. *Vet Tech* **19**: 557–67.

Thrall MA, Connally HE, Grauer GF, Hamar D (2006) Ethylene Glycol. In *Small Animal Toxicology*, Peterson M, Talcott P (eds). Elsevier Saunders, St. Louis, MO, pp. 702–26.

Vaden SL, Gookin J, Trogdon M, Langston CE, Levine J, Cowgill LD (1997a) Use of carbamylated hemoglobin concentration to differentiate acute from chronic renal failure in dogs. *Am J Vet Res* **58**: 1193–6.

Vaden SL, Levine J, Breitschwerdt EB (1997b) A retrospective case–control of acute renal failure in 99 dogs. *J Vet Intern Med* **11**: 58–64.

Valentine WM (1990) Toxicology of selected pesticides, drugs, and chemicals. Short chain alcohols. *Vet Clin North Am* **20**: 515–23.

van Wuijckhuise L, Cremers GG (2003) Alcohol poisoning in dogs. *Tijdschr Diergeneeskd* **128**: 284–5.

Weiss DJ, McClay CB, Christopher MM (1990) Effects of propylene glycol-containing diets on acetaminophen-induced methemoglobinemia in cats. *J Am Vet Med Assoc* **196**: 1816–19.

Winter ML, Ellis MD, Snodgrass WR (1990) Urine fluorescence using a Wood's lamp to detect the antifreeze additive sodium fluorescein: a qualitative adjunctive test in suspected ethylene glycol ingestions. *Ann Emerg Med* **19**: 663–7.

C H A P T E R

# 54

# Petroleum

*Robert W. Coppock and Ralph G. Christian*

## INTRODUCTION

Intoxication of birds and animals with petroleum does occur. Land used for agriculture and land located in remote regions of the world are used for oil and gas production. Land uses include oil and gas well sites, tank battery sites, sweet and sour gas plants, compressor stations, and pipelines. Crude petroleum and chemicals used in the oilfields are spilled, and cattle and other animals do ingest spilled substances and can be subsequently poisoned. Birds and mammals have their feathers and fur soiled with oil. Intoxication occurs from dermal absorption and during preening. Emissions from oil and gas facilities occur. The production of sour gas (natural gas containing hydrogen sulfide, $H_2S$) and the removal of sulfur (sour gas processing plants) present toxicology issues. Animals are poisoned when petroleum products are used as medicaments, containers containing petroleum products are used for animal feedstuffs, and when petroleum containers are left open and unattended.

## OIL AND GAS WELL DRILLING AND COMPLETING

### Drilling

Oil and gas wells are drilled into oil and gas producing formations that exists at varying depths below the surface. During drilling operations, drilling fluids are used to cool and lubricate the drill bit, support the walls of the bore-hole, move the cuttings to surface. The weight of the drilling fluid (mud) controls subsurface pressure and aids in preventing blowouts. Different types of drilling fluids and additives are used for drilling the different geologic zones and penetrating the formation. There are individual preferences for different drilling fluids, cost considerations, and requirements of the drilling techniques employed. Wide variations in the chemical composition of drilling fluids are found within the same oilfield (Edwards and Gregory, 1991).

Two basic types of drilling fluids used are the water-based drilling fluid and the non-aqueous drilling fluids. Water-based drilling fluids generally consist of bentonite clay and barium sulfate for density and other chemicals are added to give desired properties such as lubrication and reduce foaming (Table 54.1). Brine or fresh water can be used in water-based drilling fluids. The non-aqueous drilling fluids (invert drilling fluids) have fresh or brine water emulsified in a hydrocarbon phase. Emulsifiers are used to disperse the water in the hydrocarbons. The water emulsion is generally less than 1 μm in diameter. Diesel fuel is often used as the hydrocarbon in land-based drilling operations. Hydrotreated mineral oil which has a low content of polyaromatic hydrocarbons can be used. Drilling mud can also contain heavy metals, especially aluminum, cadmium, chromium including chromate, copper, iron, lead, mercury, and zinc. If radioactive materials are encountered in the formations, radioactive materials and gas can be in drilling fluids and drill cuttings. Drilling and completion operations use a wide variety of chemicals (Table 54.1).

Regulations exist in most governmental jurisdictions to protect ground water reservoirs. During the drilling of the oil or gas well a surface casing is inserted and cemented in place to protect ground water. The regulations specify the depth the casing has to extend beyond freshwater aquifers.

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**TABLE 54.1 Examples of drilling mud additives, substances used to stimulate oil and gas wells, and substances used in production of oil and gas**

| Function of substance | Compound |
|---|---|
| Acids used for well stimulation and other uses | Acetic acid |
| | Acetic anhydride |
| | Benzoic acid |
| | Formic acid |
| | Hydrochloric acid |
| | Hydrofluoric acid |
| | Sulfuric acid |
| Add weight to drilling fluid | Attapulgite |
| | Barium sulfate (barite) |
| | Bentonite |
| | Galena (lead sulfate) |
| | Hematite (iron oxide) |
| | Siderite (iron carbonate) |
| Anti-hydrate (block formation of hydrates) | Alcohols |
| | Methanol |
| | Glycols and polyglycols |
| Biocides | Acrolein |
| | Anhydrous ammonia |
| | Amines |
| | Chlorinated phenols |
| | Formaldehyde |
| | Glutaraldehyde |
| | Isopropanol |
| | Methylene bisthiocyanate |
| | 2-(Thiocyanatomethylthio)-1,3-benzothiazole |
| | Thiazolin |
| | Quaternary ammonium |
| Breakers, emulsion/gels | Ammonium persulfate |
| | Benzoic acid |
| | Sodium acetate |
| | Sodium persulfate |
| Cement additives | Aluminum (coated) |
| | Calcium chloride |
| | Cellulose flakes |
| | Cellulose polymer |
| | Gilsonite |
| | Gypsum |
| | Latex |
| | Lignosulfonates |
| | Lime |
| | Long-chain alcohols |
| | Potassium chloride |
| | Sodium chloride |
| | Sodium metasilicates |
| Corrosion inhibitors | Acetylenic alcohols |
| | Amine formulations |
| | Ammonium bisulfite |
| | 2-Butoxyethanol |
| | Ironite sponge |
| | 4-4′-Methylene dianiline |
| | Paraformaldehyde |
| | Sodium chromate |
| | Sodium dichromate |
| | Sodium metasilicate |
| | Sodium polyacrylate |
| | Thiazolin |
| | Zinc carbonate |
| Corrosion inhibitor | Zinc oxide |
| | Zinc lignosulfonate |
| Deflocculants | Acrylic polymer |
| | Calcium lignosulfonate |
| | Chrome-free lignosulfonate |
| | Chromium lignosulfonate |
| | Iron lignosulfonate |
| | Quebracho |
| | Sodium acid pyrophosphate (SAPP) |
| | Sodium hexametaphosphate |
| | Sodium phosphate (oilfos) |
| | Sodium tetraphosphate |
| | Sodium tripolyphosphate (STP) |
| | Styrene, maleic anhydride co-polymer salt |
| | Sulfo-methylated tannin |
| Defoaming agents | Aluminum stearate |
| | Fatty acid salt formation |
| | Mixed alcohols |
| | Silicones |
| | Tributylphosphate |
| Dispersant – thinner | Causticized metal lignite |
| | Ferrochrome lignosulphate |
| | Sodium tetraphosphate |
| | Lignite |
| | Lignosulfonates |
| Fluoride generators | Ammonium bifluoride |
| | Ammonium fluoride |
| Lubricants/friction reducers | Acrylamide methacrylate co-polymers |
| | Graphite |
| | Lead grease |
| | Lithium grease |
| | Mineral oil formulations |
| | Molybdenum grease |
| | Motor oil |
| | Organo-fatty acid salt |
| | Petroleum grease |
| | Reprocessed motor oil |
| | Sulphonates |
| | Vegetable oil formulations |
| Multiple uses | Ammonium nitrate |
| | Potassium nitrate |
| Oil-based drilling fluids | Amid-polymer formulations |
| | Amine-treated lignite |
| | Asphalt |
| | Diesel |
| | Gilsonite |
| | Mineral oil |
| | Organophilic clay |
| | Organophilic hectorite |
| | Petroleum distillate |
| | polyethylene powder |
| | Polymerized organic acids |
| Oxygen remover | Ammonium bisulfite |

**TABLE 54.1 (Continued)**

| Function of substance | Compound | Function of substance | Compound |
|---|---|---|---|
| pH control | Calcium hydroxide | | Diacetone alcohol |
| | Potassium hydroxide | | Diesel oil |
| | Soda ash (sodium carbonate) | | Ethylene glycol monobutyl ether |
| | Sodium bicarbonate | | Kerosene |
| | Sodium hydroxide | | Isopropanol |
| Propping agents | Bauxite | | Methyl ethyl ketone (MEK) |
| | Resin-coated sand | | Methyl isobutyl ketone (MIBK) |
| | Sand | | Methylene chloride |
| | Zirconium compounds | | Methanol |
| Salt solutions | Aluminum chloride | | Naphtha |
| | Ammonium chloride | | 1,1,1-Trichloroethane |
| | Calcium bromide | | Toluene |
| | Calcium chloride | | Turpentine |
| | Calcium sulfate | | Xylene |
| | Ferrous sulfate | | |
| | Potassium chloride | | |
| | Sodium chloride | Surfactants – emulsifiers | General categories |
| | Sodium sulfate | | Cationic |
| | Zinc bromide | | Non-ionic |
| | Zinc chloride | | Anionic |
| | Zinc sulfate | | Amides |
| Scale inhibitors | Ethylenediaminetetra acetic acid (EDTA) | | Arylalkyl sulfonic acid |
| | | | Fatty alcohols |
| | | | Glycols |
| | Inorganic phosphates | | Isopropanol |
| | Nitrilotriacetic acid (NTA) | | Modified tall oils (from tree rosin byproduct of wood pulp industry) |
| | Organic phosphates | | |
| | Phosphonates | | |
| | Polyacrylate | | Nonylphenol ethoxylates |
| | Polyphosphates | | Oxyalkylated phenolic compounds |
| Solvents | Acetone | | Petroleum naphtha |
| | Aliphatic hydrocarbons | | Polyamines (diethylenetriamine and triethylenetetramine) |
| | t-butyl alcohol | | |
| | Carbon tetrachloride | | |
| | Chloroform | | |

During the cementing operation, cement is pushed up around the casing and the hardened cement is the seal between the borehole and the casing. A blowout preventer is attached to the casing. The production tubing is inserted into the borehole and extends from the surface to the oil or gas bearing formation. Production of oil and gas is from the tubing. Poor cement jobs and casing failure can result in contamination of the ground water with crude oil and natural gas.

## Completing

Oil and gas bearing formations are porous with the oil and gas contained within the pores. To stimulate production the formation is fractured. In the past, fracturing was done with explosives. Hydraulic fracturing is done by pumping chemicals down the hole and fracturing the formation. Acids can be pumped down the formation at very high prssure to form fractures by dissolving the formation. Propping agents such as resin-treated sand are placed in the fractures to prevent closure.

Oil and gas production can use large quantities of surface and ground water. The use of water generally increases with depletion of oil in the underground reservoir. Large quantities of water can also be used for *in situ* extraction of bitumen. In both of these operations water or steam is injected into the formation, sometimes at very high pressure. The water remains in the formation and oil or liquid bitumen is extracted. The loss of surface water to underground formations is an issue in the more arid regions of the world including western United States and Canada.

# COALBED METHANE

The production of coalbed methane can create water issues and the process is the *in situ* removal of methane from coal

deposits. Estimates of trillions of cubic meters of methane coalbed methane reserves exist making this an attractive source of natural gas. The procedure to produce coalbed methane is to drill a well into the coal deposit and inject liquids under high pressure to hydraulically fracture (fracking) the coal seam (Cobb, 2003; Keith *et al.*, 2003). After fracking, water is pumped from the coalbed and the decrease in pressure allows methane to escape into the natural and man-made cleats (cracks in the coalbed). In coalbed methane production, generally a larger number of wells are drilled than for conventional natural gas production. Coalbeds can occur in aquifers. In coalbed methane production, huge quantities of water can be removed from underground aquifers and aquifer drawdown can affect an area as large as a township. In some regions the produced water is high in salts (total dissolved solids, TDS) and surface water and soil pollution from this water are a significant problem. The salt water produced in coalbed methane production can be high enough to cause sodium ion intoxication in livestock (see Chapter 35).

## SOURCES OF TOXIC SUBSTANCES

Chemicals associated with petroleum intoxication in cattle are the gaseous, liquid and solid crude petroleum which are associated with the production of natural gas, crude oil and bitumen, respectively. Crude natural gas as it comes from the wellhead has volatile liquids and may contain $H_2S$ and other forms of sulfur including the polyaromatic thiophenes (Kropp and Fedorak, 1998). Natural gas that contains $H_2S$ is called sour gas. Additionally, a large number of chemicals are used in the production of crude petroleum. Sour gas can have the $H_2S$ removed and the sulfur gas disposed of by burning in flare stacks (vertical pipe with a flame at the end).

### Emissions

Gas wells are production tested after completion. Production testing is done to remove chemicals from the formation that would corrode pipelines and to estimate the economic value of the gas well. Products of value are the methane, liquids (propane, butane, etc.), and sulfur. The gas well is connected to a flare and the emissions from the well are burned. Toxic emissions can include a wide variety of combustion products such as chlorinated dibenzodioxins and furans if chlorine is present and favorable combustion conditions exist (Buckland *et al.*, 2000) (see Chapter 55). Polyaromatic hydrocarbons can also be formed. Sulfur dioxide ($SO_2$), reduced sulfur compounds (including carbon disulfide and carbonyl sulfide), polyaromatic hydrocarbons, and other compounds are produced by burning sour well emissions by flaring. In flaring sour gas, it is likely that the production of chlorinated dioxins and chlorinated dibenzofurans does not occur because of the quenching effect of sulfur on chlorine.

Process gas is often flared during the production of crude petroleum. In addition to sulfur compounds (e.g., carbonyl sulfide, carbon disulfide) a large number of polyaromatic compounds including thiophenes are formed. Flaring is generally the most inefficient method in terms of products of incomplete combustion to burn unwanted gases. Flare efficiency is remarkably reduced by wind and the presence of liquids in the gas. Strosher (1996) provided insight into the chemical complexity of flare emissions. Incinerators are more efficient than flare stacks.

Tank heaters are used in the production of heavy oil and bitumen. Heating the tank prevents heavy oil from congealing and bitumen from foaming. The tank heaters drive off a variety of hydrocarbons and water vapor. Issues exist because tank emissions are vented to atmosphere.

### Production water

Production water defines water that is brought to the surface during the production of oil and natural gas. Production water can be high in TDS and ingestion of production water can induce salt (sodium ion) poisoning. Production water generally has a unique ratio of ions and this ratio generally is often distinctly different from TDS in ground water. Livestock producers in the more arid regions use production water that is low in TDS, and sometimes produced water may be lower in TDS than water from shallow aquifers. Production water can be high in sulfates. Cattle consuming water high in sulfate have increased risk for thiamine-responsive polioencephalomalacia, and the risk increases with decreased dietary copper (Gould, 1998) (see Chapter 36).

Different organic chemicals and solvents are used in oil and gas production and pipeline transportation of petroleum. The interior of pipelines become coated with waxes and biofilms. These accumulations are removed with a scraper called a "pig"; the scraping procedure is called "pigging the pipeline". Substances used in pigging operations include solvents to remove wax and tar-like deposits, biocides to prevent the build up of biofilms inside pipelines, and anti-corrosives. These chemicals are caught in a tank located at an instillation called a "pig trap". Massive amounts of these substances can be spilled at a pig trap and contamination of ground water used for livestock can occur.

### Well rework

A variety of chemicals is used during rework (maintenance of down-hole equipment) operations. Spillage of these

chemicals occurs, and livestock poisonings have been reported (Monlux *et al.*, 1971). Detergents and other surfactants can be used during rework operations (Table 54.1). Detergents generally increase the gastrointestinal absorption of hydrocarbons. Acids, solvents, anti-corrosives, and chelating compounds can also be used. Some of these compounds are intrinsically toxic, and can enhance the toxicity of other substances. Chelating and descaling agents may increase the bioavailability of chemicals that otherwise would be considered to have a low order of toxicity. Downhole equipment can be laid out at unsecured sites over the weekend and cattle will lick the petroleum and other substances from the equipment.

## Sumps and contaminated soil

The chemical composition of drilling sumps is highly variable. The chemicals and substances found in sumps reflect chemicals used in drilling oil and gas wells, heavy metals in formations penetrated by the drilling operation, and other substances disposed of in the sumps (Heitman, 1986; Wascom, 1986). Sumps may not be adjacent to oil and gas well sites. Remote sumps miles from any oil or gas wells were used for waste disposal and until environmental issues arise, the current landowner may not be aware of the site exists. Although environmental practices have changed, old sumps can be a cause of concern (Murphy *et al.*, 1986). A sump can literally be minature abandoned hazardous waste site that has defaulted to the legal land owner. Issues arise regarding cleanup of these old sites, and many jurisdictions have laws that declare that the legal landowner is responsible. Some banks in Canada have given farmland 0 value for loans because of old oil and gas well sumps. Rough lands with buried sumps have become desirable building sites. Houses and barns have been built on or adjacent to old sumps and individuals have planted gardens over old sumps. Some jurisdictions have limited or eliminated oilfield sumps because of toxicology issues. Water can extrude drilling waste from sumps and the extruded buried waste substances to surface and the extruded substances are a toxicological hazard (Mostrom *et al.*, 1993).

Drill cuttings and drilling fluids eventually become wastes. For example, a well 20 cm in diameter and 1000 m deep generates about 30 $m^3$ drill cuttings. The volume of drilling fluids accumulating in the drilling sump varies from 789 to >160,000 $m^3$. The general rule is 0.5 $m^3$ of drilling waste/m of depth. Some of the drilling wastes are spread on agricultural lands because of perceived value for the content of nitrogen, potassium, and other plant nutrients. Issues exist from this practice including puddling of the drilling fluids and cattle drinking from the puddles and water repellency of soil. Waste drilling fluids and drill cuttings are disposed of in sumps on the oil lease or in lease sites called remote sumps. Some disposal sites used a series of backhoe trenches instead of one large pit.

Rain and snow runoff water can extrude contaminants from sumps and contaminated soil. Extrusions from a drilling sump containing invert drilling fluid have been associated with maladies in cattle (Mostrom *et al.*, 1993). Extrusions from an invert sump contaminated a pasture. Cows grazing this pasture had calves that were stunted. Necropsy findings were abnormal epiphyseal plates in the long bones and myeloid-like bodies in the proximal tubular cells of the kidney (Mostrom *et al.*, 1993). The activities of polysubstrate mixed function oxidase enzymes were also elevated. Sheep have been poisoned following extensive rains that caused extrusion of natural gas condensate from contaminated soil (Adler *et al.*, 1992). Valve failure on a condensate storage tank contaminated the soil, and subsequent rains extruded condensate from the soil outside the perimeter fence of the lease. Eight ewes were found dead the 1st day, and the extrusion area was fenced, but ewes continued to succumb during a 21-day period. Clinical signs observed were depression, fever, gas-like odor to the breath, dyspnea, anorexia, ruminal atony, bloody diarrhea, weight loss, nasal discharge, and recumbency. A gasoline-like odor was detected in ewes necropsied 10 days, but not 18 days, after the last exposure.

## Ions

Drilling sumps can also contain high levels of sodium and potassium ions (Darley and Gray, 1988). Heavy rainfall can extrude potassium salts from old sumps. The extrusions can be confused with naturally occurring alkali areas. When given by oral or intravenous routes, $K^+$ has an essentially equal order of toxicity (Ward, 1966a). The oral lethal dose of $K^+$ in a 475 kg cow has also been reported at 238 mg of $K^+$ (as KCl)/kg of body weight (Ward, 1966b). Because of decreased renal excretion of $K^+$, calves are more sensitive to $K^+$ poisoning than cows (Blaxter *et al.*, 1960; Ward, 1966a). In high doses, $K^+$ targets the cardiac and skeletal muscles. In calves given $K^+$ by intravenous infusion, changes in the electrocardiograph (ECG) were observed. With increasing plasma concentrations of $K^+$, the QRS interval increases, the P–R interval decreases, P waves decrease, and the Q–T interval becomes very prolonged. With increasing concentrations of $K^+$ in plasma, the QRS complex becomes disorganized, and atrial flutter, atrioventricular block, and occasional ventricular to complete ventricular arrest occur. With increasing concentrations of $K^+$ in plasma, respiration also increases in rate and amplitude (Bergman and Sellers, 1953, 1954). Altering the $K^+$:$Na^+$ ratio in the rumen has been associated with bloat (Turner, 1981).

Sodium ion poisoning can also occur (see Chapter 35). Clinical signs are muscle twitching, ataxia, bruxism,

aggression, circling, and head pressing. Ingestion of brine water causes dehydration (sunken eyes and loss of plasticity of the skin). Sodium ion in the blood increases and the electrophysiology of excitable tissues is disrupted. The pathology of $Na^+$ poisoning in cattle can be variable (Scott, 1924; McCoy and Edwards, 1980; Sullivan, 1985). Ingestion of concentrated $Na^+$ produces marked congestion of the omasal and abomasal mucosa. Gut contents are fluid, and hemorrhage may cause dark discoloration. Edema of the skeletal muscles and hydropericardium can occur. Edema of the brain can also occur and an eosinophilic infiltration of the Virchow–Robin spaces may or may not be observed in cattle. Lesions of polioencephalomalacia can also be present.

## Non-pesticide organophosphate esters

Natural gas compressor stations use non-pesticide organophosphate esters to prevent foaming of lubricating oils (Coppock *et al.*, 1995a). Phosphate esters have multiple uses in the petroleum industry, and have been used in high-performance lubricants. The trivial chemical names describing the industrial grade generally refer to complex chemical mixtures of these compounds (Dollahite and Pierce, 1969; Beck *et al.*, 1977; Sugden, 1981). The use of non-pesticide organophosphate esters has been reduced in North America, but these compounds maybe buried in disposal sumps.

For the phosphate esters used in industrial applications, the majority of the individual compounds have fragmented toxicological data (Coppock *et al.*, 1995a). Studies relating the chemical structure of individual tricresol phosphate (TCP) compounds to neurotoxicity have allowed structural grouping of many of the neurotoxic compounds (Johannsen *et al.*, 1977; Sprague and Castles, 1987; Abou-Donia and Gupta, 1994). The neurotoxicity of organophosphate esters is related to the inhibition of cholinesterases, and the malady produced by inadvertent exposure to triaryl phosphate group (TAP) is known as organophosphate-induced delayed neurotoxicity (Metcalf, 1984). There is wide variation in species sensitivity and the effect of age on sensitivity within species (Abou-Donia and Gupta, 1994). The neurotoxicology of the TAP group of compounds has been described in cattle (Dollahite and Pierce, 1969; Nicholson, 1974; Julian *et al.*, 1976; Beck *et al.*, 1977; Sugden, 1981; Prantner and Sosalla, 1993), other livestock species (Wilson *et al.*, 1954; Dollahite and Pierce, 1969), and chickens (Hixson, 1984). Humans are also susceptible to TAPs (Craig and Barth, 1999). The TAPs have also been found to target the seminiferous tubules in rats (Somkuti *et al.*, 1987).

The most common source of TAPs in livestock poisonings results from their use as additives in lubricating and hydraulic oils operating under high pressure and temperature. Cattle will voluntarily ingest water and feedstuffs adulterated with TAP (Dollahite and Pierce, 1969; Nicholson, 1974; Beck *et al.*, 1977; Prantner and Sosalla, 1993). Oilfield-related exposures of cattle to TAP have been primarily from compressor oils and hydraulic fluids (Dollahite and Pierce, 1969; Beck *et al.*, 1977; Coppock *et al.*, 1995a). Compressor lubricating oils in recycled barrels used to store wet molasses were the source of TAP is an incident in which cattle were poisoned (Nicholson, 1974). An undetermined source of waste oil used to lubricate a feed bunk chain was the source of TAP in a poisoning incident in heifers (Prantner and Sosalla, 1993). Waste oil used to treat ringworm was the source of TAP poisoning in a herd of cattle (Julian *et al.*, 1976).

Clinical signs of TAP poisoning generally starts with clinical signs of neurotoxicity (Table 54.2), expressed as some form of paralysis, observed initially in a few animals (Coppock *et al.*, 1995a). Clinical signs in animals inadvertently exposed to TAP are those resulting from cholinesterase inhibition and neurological deficiency. The number of affected animals depends on the mass of TAP available dose, duration of exposure, and age. In cases of inadvertent poisoning, there was a delay of 2–25 days between exposure and onset of clinical signs of intoxication. The morbidity depends on the dose, duration of exposure, and age. The morbidity pattern will continue until all animals that have consumed a toxic dose of TAP develop clinical evidence of neurotoxicity.

A herd of 50 animals, consisting of cows, 2 bulls and a few heifers, was exposed dermally to approximately 1–2 l of waste hydraulic oil that was poured over the anterior regions as a treatment for ringworm (Julian *et al.*, 1976). The cattle also ingested the oil by preening themselves and other animals. During the pathogenesis, the cattle were bright, alert, and had good appetites; normal body temperatures, and miosis and clinical evidence of visual impairment were not observed. Just before and during the time of intoxication, 10 calves were born. Cows that were not recumbent nursed their calves, and the calves were asymptomatic. Treatment with atropine and pantothenic acid was ineffective. Another group consisting of yearlings and 2-year-old steers and heifers was exposed to repeated applications of waste hydraulic oil that was dabbed on ringworm lesions. Animals in this herd did not develop clinical signs of intoxication. TAP poisoning occurred in four heifers exposed when oil was used to lubricate chains in a mechanical feeding system (Prantner and Sosalla, 1993). The relationship between exposure and the onset of clinical signs was not given. The onset of clinical signs occurred at differing intervals, i.e., 6 weeks between the 1st and 2nd heifers, 1 week between the 2nd and 3rd heifers, and 6 weeks between the 3rd and 4th heifers. The 3rd heifer vomited after being drenched with a solution of copper sulfate, and subsequently died. A herd of cattle was exposed to TAP when a seal failed in a natural gas compressor (Beck *et al.*, 1977). The interval between exposure and onset of clinical signs was not given. The owner observed that the cows were mute

**TABLE 54.2 Clinical Signs of delayed neurotoxicity in cattle**

| Source | Time sequence* | Clinical sign | Reference |
|---|---|---|---|
| Waste hydraulic oil | 12 days | Diarrhea | Julian *et al.* (1976) |
| | 20 days | Posterior weakness, knuckling at fetlocks, ataxia, difficulty in rising, dog-sitting while attempting to rise | |
| | 20+ days | Diarrhea, coughing, ataxia, difficulty turning, knuckling and buckling of rear limbs, unable to rise but could walk on knees, decreased sensory and motor response in rear limbs | |
| | 34+ days | Increased severity of clinical signs, incoordination progressing to front legs, standing in a crouched position, muscular wasting, dribbling urine, tail raised, coughing, dyspnea after attempting to rise and deaths (14/50) | |
| Waste oil | NS | Roaring sounds | Prantner and Sosalla (1993) |
| | 2 days | Ataxia of hind limbs and knuckling over at metatarsophalangeal joints | |
| | 42 days | 2nd heifer with signs same as first | |
| | 7 days | 3rd heifer, ataxia, dysuria, polyuria, dyspnea | |
| | 42 days | 4th heifer, clinical signs same as for heifer | |
| Compressor sump water | NS | Cows mute when separated from calves | Beck *et al.* (1977) |
| | 7 days | Knuckling of fetlocks, dyspnea, coughing | |
| | 7 days | Goose-stepping, diarrhea, mute, posterior paralysis, dog-sitting, recumbency and inability to rise | |
| Barrels previously used for hydraulic oils | NS | Standing in a crouched position, elevated tails, dribbling urine, posterior weakness | Nicholson (1974) |
| | 2 days | Posterior weakness, recumbency, death | |
| Lubricating–hydraulic oils | NS | Rough hair coats, loss of condition, muscular weakness and incoordination, tympanites, dyspnea and roaring | Dollahite and Pierce (1969) |

* After exposure. NS: Not specified.

when separated from the calves. The calves remained asymptomatic, and a calf born to an affected cow was normal. Calves had decreased growth, a finding that was attributed to some degree of agalactia in the cows. Cattle were poisoned with tri-*o*-tolyl phosphate, a TAP (Nicholson, 1974). In this incident of delayed neurotoxicity in cattle, molasses was contaminated with tri-*o*-tolyl phosphate when it was stored in barrels used previously for hydraulic oil supplied to a local pipeline pumping station. Initial clinical signs were urinary incontinence, elevated tails, crouched position, and posterior weakness. Two days later, 2/42 animals were dead, 5/42 were recumbent, and 25/42 had varying degrees of posterior weakness. The clinical sequence was posterior weakness progressing over a 2-day interval to posterior paralyses.

The mechanism of action for delayed neurotoxic effects of organophosphate esters has been partially elucidated (Abou-Donia and Gupta, 1994; Gupta and Abou-Donia, 1994). The majority of compounds in this group inhibit a neuropathy target enzyme, known as neurotoxic esterase (neurotoxic target esterase) (Gupta and Abou-Donia, 1994). Irreversible covalent binding or ageing of the neuropathy target esterase is essential for the induction of anatomical neurotoxicity. The anatomical neurotoxic effects have been associated with alterations in the cytoskeleton and the transport of nutrients from the cell body to the terminal parts of the axon.

## Anti-hydrates and antifreezes

Large quantities of methanol are used by the oil and gas industry, and cattle have been poisoned by these sources (Edwards *et al.*, 1979; Rousseaux *et al.*, 1982). Methanol is used to prevent freezing of water in pipes, and to prevent hydrate formation in gas wells and pipelines. Intoxication of cattle by methanol has been reported. A herd of 600 cows had access to methanol barrels stored on a lease and 2 cows were found dead. Manure accumulation behind the cows was taken as evidence that the cows had been recumbent for some time before death (Rousseaux *et al.*, 1982). Rumen contents from the 2 dead cows contained 370 mg of methanol/100 ml of rumen fluid. In another incident, 12/15 cows were found dead or in a moribund condition (Sesevicka *et al.*, 1979). Clinical signs observed were: ataxia, decreased rate and depth of respiration, frequent chewing motions, impaired vision, and hypoesthesia to nociceptive stimuli. These heifers had access to a pit that received waste water from a gas processing plant. Clinical signs of experimentally induced methanol poisoning in

cattle were similar to those reported for field exposures (Fritz and Coppock, 1992).

Large quantities of diethylene glycol are used in oil and gas operations (Edwards *et al.*, 1979). Research has shown that cattle are a species sensitive to diethylene glycol (Fritz and Coppock, 1992; Khan *et al.*, 1992). A dose of 1.51 ml/kg of body weight is fatal to a cow. Diethylene glycol is toxic to the eye, liver, kidney, and nervous system. Diethylene glycol-induced ocular changes that are similar in appearance to those of pinkeye. Diethylene glycol causes a unique lace-like hypertrophy of the perineuronal amphicytes in the Gasserian ganglia in cattle. This lesion has not been reported in laboratory animals. Other glycols are used in oil and gas production (see Chapter 53, Ethylene Glycol).

### Chromate

Cattle are exposed to environmental chromates from oilfield sources (Reagor and McDonald, 1980). Gross pathological findings in a calf (8 months old) were icterus, tracheal froth, excessive pleural fluid, and petechiae over the pleura. The intestinal contents were black and mucus covered. Edema and hemorrhage were observed in the mesentery and omentum. Hepatomegaly, yellowing of the liver, and edema of the gall bladder were observed. Histopathological observations included diffuse hepatocellular swelling, vacuolation of hepatocytes, inspissation of bile, and moderate biliary hyperplasia. The kidneys were pale and hemoglobinuric vacuolation was observed in the cortical tubular epithelium. Proteinaceous exudate was observed in the lung, and epicardial hemorrhages were observed. Chromium concentration in the liver was 14.8 ppm (see Chapter 38).

## GREASE AND MOTOR OIL

Lithium (Li) intoxication in cattle from Li grease has been reported. Wallace and Blodgett (1996) reported Li poisoning in 1 cow after the animal consumed Li grease, and Johnson *et al.* (1980) reported on Li poisoning in 19/90 animals. In both the incidents, the source of Li grease was discarded drums that had been used previously for transporting and storing grease. In the incident reported by Wallace and Blodgett (1996), the cow was euthanized and in the incident reported by Johnson *et al.* (1980), all 19 animals died. In the animals that consumed Li grease, clinical signs included muscular tremors, ataxia, stiffness, apparent disorientation, ptyalism, diarrhea, rapid respiration, and periodic seizure-like activity. The concentrations of Li in grease for the incidents reported were 2050 μg of Li/g of grease and 1250 μg of Li/g of grease, respectively (Johnson *et al.*, 1980; Wallace and Blodgett, 1996). Clinico-pathological findings (1 cow) included leukopenia, lymphopenia, and

**TABLE 54.3 Summary of clinical signs of Li intoxication in cattle (Johnson *et al.*, 1980)**

| Oral dose* | Time | Clinical signs |
|---|---|---|
| 250 mg/kg (4) | 2–6 h | Ptyalism |
| | 4–6 h | Depression, anorexia, diarrhea |
| | 72 h | Diarrhea |
| | 9 days | Recovered |
| 500 mg/kg (5) | 3–4 h | Ptyalism (lasted for 4–6 h) |
| | After 4–6 h | Depression, anorexia, hypodipsia, anuria and diarrhea |
| | 7 days | 4 animals died |
| | 11 days | 1 animal died |
| 700 mg/kg (4) | 2 h | Ptyalism (continued for 4–6 h) |
| | After 2 h | Depression, anorexia, hypodipsia, anuria, ataxia and severe diarrhea |
| | 8 hours to 7 days | Deaths occurred |

*Number of cows in trial is given in parentheses.

hyperfibrinogenemia (Wallace and Blodgett, 1996). The parameters of protein, cytology, glucose in cerebral spinal fluid were normal. An oral dosing study with Li has been done in cattle (Johnson *et al.*, 1980). Cattle were administered single oral doses *per gavage* of 250, 500, or 700 mg of LiCl/kg body weight. The LiCl was in water (qs to dissolve the Li) plus an equal volume of water to rinse the stomach tube. Clinical signs were reported (Table 54.3). The most consistent finding at necropsy was gastroenteritis of varying severity. Histopathological findings were congestion of the gastrointestinal tract. Lesions in the liver included cloudy swelling, edema, and cirrhosis in the portal triad areas. Renal lesions included cloudy swelling of the proximal tubular cells and a mild interstitial nephritis. Lithium can also be present in process water.

Grease can contain other metallic substances. Lead grease has excellent water repellence properties. Lead grease is also a hazard to livestock (see Chapter 31).

Motor oil contains additives to prevent foaming, keep carbon deposits is suspension and to reduce friction and wear of parts at high temperatures, viscosity improvers, pour-point improvers, and antioxidants. Synthetic oils can contain molybdenum and surfactants. Anti-wear additives such as zinc dialkyldithiophosphate compounds and derivatives are added to lubricants to reduce friction. Micronized metals can be added to lubricating oils. Many additives are mixtures of substances and most companies closely guard their intellectual property.

## TOXICOLOGY OF PETROLEUM

Cattle ingest crude, refined, and waste petroleum (Coppock *et al.*, 1995b). The toxicology of petroleum has been

**TABLE 54.4 Chemical characteristics of crude oils used in the Rowe and Bystrom studies**

| Fraction | Pembina crude[a,b] | Texas sweet[c] | Texas sour[c] |
|---|---|---|---|
| Light gasoline | NA | 14.2% | 9.6% |
| Total gasoline or total naphtha | 22.1% | 43.0% | 31.6% |
| Kerosene | 15.3% | 22.3% | 4.7% |
| Lubricating | NA | 8.8% | 16.8% |
| Sulfur | 0.24% | <0.1% | 1.5% |
| Nitrogen | 0.1% | 0.083% | 0.074% |
| Nickel | 2.53 ppm | NA | NA |
| Vanadium | 1.17 ppm | NA | NA |

[a] Pembina cardium crude from Drayton Valley, Alberta.
[b] Mass percentages.
[c] Units in terms of percent mass or percent volume were not given.
NA: Not available.

reported for experimental studies and field incidents. Rowe (1972, 1973) found that sweet crude oil was more toxic in cattle than was sour crude oil (oil that contains sulfur) (Table 54.4). Administration of 8 ml of sweet crude oil/kg body weight/day caused death within 7–14 days in 4/5 calves. For the same dose of sour crude oil, 5/5 calves died between days 16 and 24; for kerosene, 5/5 calves died between days 9 and 23. Administration of 37 ml of sweet crude oil/kg was fatal within a few minutes. The majority of the surviving animals had pneumonia.

## Experimental studies on crude oil

Bystrom (1989) studied the acute effects of crude oil in cattle. The dosage levels (administered *per gavage*) were 20, 40, 60, or 80 ml of sweet crude oil/kg of body weight, or 80 ml of potable water/kg of body weight. The analytical characterization of the oil is given in Table 54.4. Following exposure, daily mean rectal body temperatures were normal. Ruminal motility was decreased after animals were dosed with oil and slowly increased to pre-treatment values by day 8 (study day 0 was the day that cattle were dosed). Vomiting was not observed in the animals dosed with water. Vomiting occurred in 10/12 of the animals dosed with oil and, in some animals, vomiting was characterized by a projectile expulsion of oil. Emesis recurred after forage or water was ingested. Ruminal tympanites was observed in the majority of the animals, but did not require medical intervention. Neurological abnormalities were not observed in control animals. Central nervous signs expressed by nystagmus, muscular tremors, and "petit mal-like" seizures were observed in treated animals. Depression was the most common clinical sign observed. These effects were attributed to the "anesthetic" effects of the volatile hydrocarbons. The neurological effects contributed to aspiration pneumonia.

Gas chromatographic methods were used to determine hydrocarbons in blood and feces (Bystrom, 1989). Oil was found in feces at 23 h after dosing for the 20 and 40 ml/kg groups, at 7–31 h for the 60 ml/kg group, and at 5–19 h for the 80 ml/kg group. Oil was not detected in feces from the control animals. Using head-space (space between the liquid and stopper) analysis, it was demonstrated that volatile constituents from the oil were present in blood. *N*-heptane was used as a representative hydrocarbon. Semiquantitative values for total light naphtha were estimated to be as high as 10 mg/l of blood. Aspiration of oil into the lungs increased the levels of *N*-heptane in the blood.

Considerable variations have been reported for the dose–response of cattle to mineral petroleum. These effects can be due to presence of surfactants or emulsifiers, and additives in refined petroleum. A 200 ml single oral dose of odorless kerosene (Vacuum Oil Company No. EF471W, New Zealand) administered to a mature dairy cow caused a 90.3% reduction in dry matter intake, and the same dose of Stanvac Odorless Solvent caused a 42.5% reduction in dry matter intake (Reid, 1957). These effects were considered to be "eliminated" by day 3 of the study. Emulsification of kerosene with a non-ionic surfactant decreased the interval between exposure and the onset of effects. Similar findings were observed when kerosene was placed directly into the omasum. Fistulation of the rumen did not alter the response to kerosene or solvent.

Exposure to petroleum can reduce body stores of fat-soluble vitamins. McDowall (1957) administered 75 ml of heavy liquid paraffin/cow/day by drench for 26 days. A 40% reduction in blood carotene occurred over 16 days, and a 20% reduction in blood vitamin A ester, and a 40% reduction in tocopherol (vitamin E) was also observed. After cessation of treatment, 21 days were required for recovery to pre-exposure levels.

## Ingestion of petroleum

Numerous reports exist in the scientific literature that cattle and other animals have voluntarily ingested petroleum and other oilfield substances (Coppock *et al.*, 1986a, 1995b). Cattle are attracted to and will ingest several gallons of petroleum (Monlux *et al.*, 1971; Oehme, 1977). Deaths have occurred after cattle drank tractor paraffin and vaporizing oil (Eaton, 1943). Heifers drank gasoline (Albert and Ramey, 1964), and Messerli (1969) reported that cattle greedily ingested diesel oil flowing from a storage tank. Cattle have drunk from petroleum puddles near a tank battery, from slush pits, from puddles of volatile petroleum and petroleum distillate (Edwards *et al.*, 1979). Crude oil, spilled by a pipeline break, has been consumed by cattle, and cattle have drunk from puddles of road-oil after it was applied for dust suppression (Coale, 1947; Bumstead, 1949). Cattle will drink used motor oil (Gardner, 1977), and

will also ingest petroleum-contaminated forage (Stober, 1962; Beck *et al.*, 1977). An entire herd of heifers was irreversibly poisoned by ingesting water from a stream that was contaminated with aviation turbine fuel (Barber *et al.*, 1987). Monlux (1971) reported that the majority of animals avoid oil, but they also reported that, in some instances, the entire herd, especially feeder calves, will be attracted to oil. Cattle have ingested sump oil with adverse effects on health (Ballantyne, 1950, 1955).

Other species also ingest petroleum. Sheep have ingested Bunker "C" fuel oil (a residual fuel oil) following the sinking of a tanker ship (MacIntyre, 1970). Ingestion of surface water contaminated with extrusions by rain water of natural gas condensate has caused fatalities in sheep (Adler *et al.*, 1992). Voluntary ingestion of kerosene by 2 goats was reported by Pathan (1961). In Iran, goats ingested diesel fuel leaking from an overturned truck tanker (Toofanian *et al.*, 1979).

Experimental studies on the ingestion of crude oil by cattle have been done. Rowe (1972, 1973) found that calves will drink sweet and sour crude oils. After water had been withheld for 48 h, calves drank 4 l of either a sweet or sour crude oil, but would not repeat the ingestion of crude oil after water had been withheld for a total of 96 h. Two of the calves that drank oil had previously been administered *per gavage* kerosene in a previous study. Calves that were not water deprived did not drink oil. These findings suggest that cattle may acquire some aversion to drinking crude petroleum.

## Experimental study on ingestion of petroleum

An experimental study demonstrated that cows on a balanced diet and having water *ad libitum* are attracted to crude mineral petroleum, and will ingest it (Coppock *et al.*, 1992). In a study on voluntary ingestion of crude oil, a stall was designed to minimize competition at the oil source. All of the animals explored the oil and 5/10 animals ingested oil. For 3/4 animals that drank oil, ingestion occurred most frequently in the first few days during which oil was available. The most frequent method of ingesting oil was by licking; the cyclic pattern suggested that cattle will go on oil-licking "binges". The neurochemical reasons for the attraction of cattle to oil are not currently known.

## Clinical observations and findings

Varied clinical signs have been observed in field incidents of petroleum poisoning (Table 54.5). A herd of 58 yearling steers had access to crude petroleum distillate (condensate) on an oil-lease property (Edwards and Zinn, 1979).

**TABLE 54.5 Clinical signs of petroleum poisoning in cattle**

| Acute signs (within 24 h) | Subacute signs (After 24 h) | Chronic signs |
|---|---|---|
| Ruminal tympanites* | Depression | Lethargy |
| Emesis | Pneumonia | Anorexia |
| Postural weakness | Anorexia | Loss of condition |
| Ataxia and incoordination | Constipation | Impaired reproductive performance |
| Seizures | Coughing | Chronic cough |
| Hyperthermia | Ileus | Abortion |
| Dyspnea | Recumbency | Laminitis |
| Bloody diarrhea | Ruminal atony | Loss of body weight |
| Strong petroleum-like odor to breath/feces | Abortion | Pain – hardware-like signs |
| Ruminal atony | Loss of weight | |
| Depression | Sweet petroleum-like odor to breath and feces | |
| | Lethargy | |

* Ruminal tympanites is not a consistent finding.

Following oral exposure, 17 animals became sick and 9 died. The sick cattle had petroleum distillate dripping from the nostrils, and present in their feces; they also had petroleum on their tails and rear quarters. Surviving animals had varied signs including anorexia and weight loss. Some of the more severely affected animals died. Edwards and Zinn (1979) also described clinical signs in 18/135 animals that had access to a sump pit. The surviving animals lost weight and were unthrifty ("poor-doers"). These authors also reported that, of 200 steers with access to petroleum in puddles, 12 were found dead, and 13 died 24 h later. Oehme (1977) attributed abortions to ingestion of petroleum products. Loss of body condition can also result from petroleum ingestion-linked chronic pneumonia and pleural adhesions. Clinical evidence of pain can be associated with pleural adhesions (signs similar to hardware disease). Cattle attracted to an area saturated with condensate can have laminitis-like clinical signs.

A herd located in Alberta had access to sump oil (Ballantyne, 1950). A 545 kg cow, representative of a malady observed in 20 other cows from a large herd, was necropsied. After ingestion of oil this cow had a reduction in body weight. Necropsy findings were: visible oil in the rumen, the mucous membranes of the abomasum were stained black, and areas of inflammation were observed in the gut. There were 213 ml of oil/l of rumen contents. In another report (Bumstead, 1949), cattle drank from a pool of oil. Two animals died, and all of the animals that had wandered into the pool of oil showed clinical signs of intoxication. One heifer had severe enteritis, was dehydrated and had cachexia. All of the animals treated by the veterinarian made what was considered to be a complete recovery, which was attributed to treatment that rapidly removed oil from the digestive tract, and prophylactic treatment with an anti-microbial chemotherapeutic. In another report (Coale,

1947), 5 dairy cows drank crude oil that had escaped from a broken pipeline. All 5 of the animals were sold 90 days later because of lost productivity.

Gibson and Linzell (1948) reported that dairy cows drank petroleum after 5 gal (23 l) were mistakenly dumped in a water trough. Clinical signs in 6/8 cows were central nervous system (CNS) depression including coma, coughing, salivating, head shaking, hypothermia, and petroleum odor on respired air and milk. The diesel-like odor on the breath and in the milk persisted for 5 days. Three animals died at 12, 24, and 48 h, respectively, after exposure. During the interval between exposure and death, all of the animals would eat and ruminate, but were dull in temperament and were constipated. Forty-eight hours after ingestion, 2 cows had abnormal respiratory sounds, which persisted for 14 days in 1 cow. The pathological findings were chemical pneumonia, and evidence of inflammation of the mammary gland. Another author reported clinical signs of vomiting and death after a lactating shorthorn animal ingested approximately 1 gal (4.5 l) of tractor paraffin (Eaton, 1943). Twenty-four hours after ingesting tractor paraffin, a 3-year-old bullock had signs of marked excitement to wildness, blindness, and incoordination. Clinical signs of anorexia and constipation were reported after cattle ingested tractor-vaporizing oil (low grade of kerosene) (Parker and Williamson, 1951). Pathological findings were fatty changes in the liver, degeneration of the kidney, and multiple small hemorrhages in the lungs.

A herd of 24 dairy cattle ingested diesel oil after the valve on a storage tank was opened, possibly by the cattle (Messerli, 1969). Clinical signs of intoxication were observed in 9/24 cows 30 h later and 4/24 were considered to be seriously affected. Milk from all the cows had a diesel odor. From this herd, a Brown Swiss cow in critical condition was examined in detail. Clinical signs observed in the cow were: elevated body temperature, bradycardia, decreased appetite, decreased peristaltic ruminal movements, diarrhea, muscular weakness, and decreased milk production. Eight days after ingesting diesel, swelling of the rear fetlocks was observed. Eighteen days after ingesting oil, the herd milk production was diminished by 75%; 2 months later, unsteady movements were observed and after an unspecified interval, the cow regained her health. Three of the other seriously affected animals required 1 month for recovery, and had retarded growth for a substantially longer interval. The feces had a diesel odor for 5 days.

Peterson (1963) reported that after a herd of cattle had access to heating oil-contaminated water for 12 h, 2 cows showed remarkable clinical signs. One cow was pyrexic (41.1°C, 106°F), and had severe dyspnea. Body temperature of the second cow was 38.8°C (102°F), and there was clinical evidence of abdominal pain. The first cow was treated with intravenous antibiotics and laxatives; she passed fuel oil in her feces and made a complete recovery. The second cow received similar treatment, did not eat for 2 weeks, developed septic metritis, followed by pneumonia, and subsequently died. Poor health and an abnormal liver in a heifer were attributed to her drinking gasoline 15 months previously (Albert and Ramey, 1964).

Barber reported an environmental incident with aviation turbine fuel (Barber *et al.*, 1987). Fifty-one heifers 12–18-month olds were exposed to water in a stream that had been contaminate with aviation turbine fuel. The duration of exposure was not determined and, when discovered, the contaminated waterway was immediately fenced, and the heifers were provided an alternative source of drinking water. At the initial examination, 2 heifers were dead, ataxia was observed in 5 animals. Over a 3-week interval 8 heifers died or were euthanized *in extremis*. Animals that survived were dull, did not show interest in strangers walking about the field, and had marked weight loss, and increased respiratory rate was observed in some animals. Six weeks after the initial examination, 41 of the surviving animals appeared unhealthy. At 121 days after the initial examination, the heifers were compared to siblings, and were found to have lost 50–100 kg. Because of apparent chronic, poor health, 41 of the heifers were slaughtered approximately 124 days after the initial exposure. One heifer died during transport to slaughter. Approximately 124 days after the initial farm visit, the animals were slaughtered and submitted for pathological examinations. All of the heifers were poorly fleshed for their skeletal size. Some of the animals had liver flukes, but lung worms were not observed. Essentially all of the lungs were considered to be grossly abnormal, as exemplified by enlargement and grayish-blue areas of varying size, and had firm consistency. However, histopathological abnormalities were not observed in the lungs and kidneys.

## Dermal toxicity of petroleum

Pesticides applied to cattle using "cattle oilers" are often diluted with diesel oil and kerosene. Petroleum distillates can be used as vehicles for fly sprays. Diesel fuel was considered to be the cause of a severe skin reaction to fly spray (Edwards and Niles, 1981). The abdomens and legs of the cows were sprayed each time the cows entered the barn and, when the cows exited the barn, the same mixture was rubbed on their backs. The fly spray was a mixture of 3.8 l of proprietary fly spray mixed with 53 l of diesel fuel. Erythema of the skin, swelling of the carpus, and varying severity of edema were observed on the front legs between the elbow and fetlocks. Skin on the back, rear legs, and udder was not affected. The authors postulated that the udder and rear legs were protected by washing before milking. The fly spray–diesel mixture produced similar lesions on the backs of guinea pigs. Treatment consisted of washing the front legs, application of scarlet oil, and

discontinuing the use of the fly spray mixture. Using a different diesel fuel for diluting the fly spray was considered significant in causing the dermatitis. The causative agent in the diesel fuel was not identified. The mixture of pesticide and kerosene and diesel oil can increase the rate of absorption of pesticide. Diesel oil–pesticide mixture can be spill from overturned oilers.

Cattle that were attracted to soil that had been saturated with crude condensate. Puddles of condensate were observed in the cattle tracks. Clinical signs of laminitis were observed in many of the animals in this herd. It is assumed that the petroleum was absorbed through the hoof wall and caused inflammation.

# VETERINARY MEDICAL USE OF PETROLEUM

## White oils

Mineral oils are purified and used for medical purposes, added to human foodstuffs and animal feedstuffs (IRAC, 1984). The mineral oils are highly refined naphthenic or paraffinic distillates. The unsaturated compounds that add color and taste have been removed. Treatment methods include solvent extraction and steeping with strong sulfuric acid can be used as well as adsorption with clay minerals. Alternatively hydrogenation can be used. These oils have a very low sulfur and polyaromatic hydrocarbon content. Uses are the food industry, lubricants in manufacturing of human foodstuffs, direct addition to foodstuffs and as a pharmaceutic. Purified mineral oils are mixed with surfactants (pasture spray oil) and sprayed on alfalfa (lucern) forage before grazing as a prophylactic for bloat in cattle.

## Kerosene and diesel oils

There is historical record of various mineral petroleum fractions being used as veterinary medicaments; lamp paraffin (kerosene) and other kerosene-like products were used as a veterinary medicament for treating animals with intestinal helminths, frothy bloat and diarrhea, and crude oil was used as an aperient. The unpredictable adverse reactions of orally administered kerosene are anorexia, ptyalism, indigestion, irritation of the digestive tract, respiratory difficulties, and death. These effects were attributed to its variability in chemical composition (Reid, 1957; Stober, 1962). Clinical signs have been described for a cow that was drenched with lamp oil. In the first incident (Shenton, 1937), the owner administered a pint (0.57 l) of paraffin oil to control bloat. Twenty-four hours later, the cow was examined and a rumen cannula inserted. Forty-eight hours later, there was an absence of rumen motility, and the cow would vomit after drinking water. On the 3rd day, a stomach tube was passed and gas and water were removed from the rumen. On the 4th day, the cow drank gruel and water, and died on the 5th day. Necropsy findings were severe inflammation and necrosis of the esophagus, inflammation of the abomasum, and the smell of paraffin in the ingesta. Munch (1956) advised that, because of unpredictable adverse effects, crude oil should not be used as a laxative for cattle. Exposure to mineral oil can deplete fat-soluble vitamins. McDowall (1957) administered *per gavage* 75 ml of heavy paraffin/cow/day by drench for 26 days. A 40% reduction in blood carotene occurred over 16 days, and a 20% reduction in blood vitamin A ester, and a 40% reduction in tocopherol (vitamin E) was also observed. After cessation of treatment, 21 days were required for recovery to pre-exposure levels.

# SOUR GAS WELL BLOWOUTS

Sour gas is natural gas that contains $H_2S$ and other sulfur compounds. Sour gas well blowouts constitute a unique hazard to livestock. In the blowout incidents which have been reported in the literature, cattle were confined by fences, corrals, etc. and generally could not escape the airborne emissions. Ranchers and their families may have been forced to evacuate leaving cattle and other livestock unattended. These events can cause major disruptions in animal husbandry. Sour gas is heavier than air and settles into low areas especially during cool temperatures. Low areas tend to be sheltered from the wind and less mixing of the air occurs. The toxic effects of sour gas have been summarized in Table 54.6.

During rework operations a sour gas well in western Canada a blowout occurred (EPS, 1973). The rate of flow and $H_2S$ concentrations were estimated at 11.6 $ft^3$/s and 4–8%, respectively. Ideal conditions for the dispersal of gas were reported to have existed during the blowout. Cattle in one herd were reported to have a watery discharge from eyes and nostrils. Cattle from 6 farms were examined by the veterinary laboratory. The diagnosed was pneumonia.

Cattle were studied in western Canada after a sour gas well blowout (Lodgepole blowout) that occurred in 1982 (October 17 to December 23). The duration of the blowout was essentially 67 days and the estimated flow rate was $>4.2 \times 10^6\,m^3$/day giving an estimated release of elemental sulfur ranged from 900 to 1400 metric tons/day. The well was ignited at 14:07 h on November 1, 1982, and burned until November 17. The well was ignited again at 12:53 h on November 25, 1982, and burned until it was extinguished during the capping process. The well was not on fire for 27 days, and it was ignited for approximately 40 days.

**TABLE 54.6 Summary of observed effects of blowout emissions in cattle**

| Blowout | Observation | Reference |
|---|---|---|
| Canada | Watery discharge from eyes and nostrils. Pneumonia was observed. | EPS (1973) |
| Canada, Lodgepole blowout | Ocular and respiratory irritation, abortions, other reproductive problems. | Round (1992) |
| | Number of aborted fetuses and diagnostic trends did not change for the Provincial Laboratory (Edmonton) during and after the blowout. | Klavano and Christian (1992) |
| | Maladies in cattle increased with decreasing distance from the well and were increased in geographically low areas, problems associated with trace mineral deficiency were observed as were changes in hair color, *inertia uteri*, calves that were born with deformed feet, calves had failure to thrive, decreased growth of replacement heifers, possible decreased birth weights, and 20% reduction in milk production by dairy cows, calving interval increased, herd returned to normal. | Harris (1992) |
| | Significant reduction in weaning weights of calves exposed as cow–calf pairs. | Whitelock (1992) |
| | Possible association with exposure to well emissions were: physical examinations were within the expected normal variation, pathological findings were of varied diagnosis, and were not considered to be unique, 3 calves from these cows raised at the laboratory grew at average to above-average rates, parameters used may not have been sensitive enough to detect irreversible toxicological effects, and the owner may have been biased in his evaluation of long-term effects. | Church (1992) |
| Mississippi (USA) | Irritation of the eyes and respiratory tract, respiratory distress, intolerance to exercise. | Edwards (1992) |
| Canada | Clinical signs mimicked shipping fever, but infective agents or clinical evidence of infection were not detected, extended feeding time to finish exposed feedlot cattle, loss of stamina in cattle, exercise intolerance in horses, exposure to sour gas may have contributed to shipping fever in recently weaned calves, and reproductive failures were not established. | Coppock *et al.* (1986b) |
| Canada, multiphase pipeline leak | Ocular and nasal irritation, evidence of immune and nervous system dysfunction, aggressive behavior in cows, *in estrus* behavior of pregnant cows, above-average mortalities in cows and calves, calves lacked sucking instinct, cows failed to nurture new born calves, and lesions in lymph nodes and trachea. | Mostrom *et al.* (1995) |

There were differences of opinion, regarding the impact on cattle, of emissions from the Lodgepole well. Round (1992) reported clinical signs of ocular and respiratory irritation that was locally described as "red-eye syndrome". Improvement of cattle with the red-eye syndrome was observed immediately after the well was capped. The well emissions were considered to be suspect in causing abortions and infectious causes of abortions were not identified. The number of aborted fetuses and diagnostic trends were reviewed for the interval before, during, and after the Lodgepole blowout (Klavano and Christian, 1992). For the submissions sent to the Animal Health Laboratory, the Lodgepole blowout did not change the number of fetuses submitted to the laboratory or the diagnostic trends established for aborted fetuses. A study was done on the attitudes and opinions of livestock producers regarding the long-term effects in cattle of the emissions from the Lodgepole blowout (Harris, 1992). The producer-based observations were on 1700 beef and 40 dairy cows. Problems associated with trace mineral deficiencies, including change in hair color, were more prevalent among exposed cattle; complaints of blowout-associated maladies in cattle increased with increasing proximity to the well. Complaints of blowout-associated maladies also were more common for cattle located in low-lying areas. Concerns over low birth weights were more common for producers who had not recorded birth weights. Other problems included difficult calving due to *inertia uteri*, calves born with deformed feet, and calves that were "poor-doers". Farmers complained of reduced growth in replacement heifers. The study included one dairy producer. Lactating cows exposed to the blowout gas had a 20% reduction in milk production. There was no report of decreased milk production in cows which freshened after the blowout. The calving interval increased to 13.9 months during 1983, and then returned to 11.8 months in the subsequent year. The owner considered his herd to have been affected by the blowout emissions and then returned to normal.

Whitelock (1992) reported his observations in cattle attributed to the emissions from the Lodgepole blowout.

He observed a statistically significant reduction in weaning weights after exposure of cow–calf pairs and weaned calves to emissions from the Lodgepole well. Church (1992) interviewed farmers, and evaluated cattle for toxic effects. In a 118-cow herd of pure-bred Angus cattle exposed to the well emissions, eight parameters may have been related to exposure to blowout emissions. These were: (1) average decrease of 15 pounds (7 kg) in birth weight, (2) a 100 pound (45 kg) decrease in weaning weights, (3) a 4.6% increase in birth defects or stillborn calves, (4) a 9.5% increase in abortions, (5) loss of hair color (fading and graying), (6) decreased growth and milking ability of heifers born in 1983, (7) breeding problems such as abnormal cycling, and (8) a 23% increase in culling rate. Three of ten control herds had two of the problems listed for the exposed herd. One of the farmers requested that his cows be evaluated by laboratory procedures (Church, 1992). Six cows, considered by the owner to be the worst affected, were examined in 1986. Conclusions of the laboratory findings were: (1) three calves from these cows raised at the laboratory grew at average to above-average rates; (2) findings of physical examinations were within the expected normal variation, (3) pathological findings were of varied diagnosis, and were not considered to be unique, (4) parameters used may not have been sensitive enough to detect irreversible toxicological effects, and (5) the owner may have been biased in his evaluation of long-term effects.

Edwards (1992) described the impact of emissions in cattle from a sour gas well blowout that occurred in 1985 in the southern United States. The well blew for 72 days at a pressure of 1340 kg/cm$^2$ (19,000 lb/in.$^2$); the well produced 1.1 million m$^3$ (40 million ft$^3$) of emissions/day. The emissions were ignited because the gas was composed of 35% $H_2S$. A herd of red and gray Brahmans was examined 5 days after the blowout started. Clinical findings were irritation of the eyes and respiratory tract. Of the 55 animals with signs of respiratory distress in 25–30 days after the blowout started, 15 remained clinically affected ~9 months later.

A sour gas well blowout occurred in western Canada in 1984, and was out of control for 88 h (Coppock *et al.*, 1986b). Flow rates for the well were estimated at 239,000 m$^3$/day, and the approximate concentration of sulfur-containing compounds is given in Table 54.7. Cattle were exposed to the blowout gas, and the impacts of well gas in cattle were investigated. A total of 195 measurements of $H_2S$ were made in the blowout area; the concentrations ranged from 0.014 mg of $H_2S$/m$^3$ of air to 4.90 mg of $H_2S$/m$^3$. It was concluded that, within 4 km of the well, exposure to the gas caused irritation of the respiratory tract. Clinical signs of effects of the well gas in feedlot cattle mimicked those of shipping fever. Extensive laboratory testing did not identify infectious agents, clinicopathological parameters did not suggest infectious disease, and the pathogenesis was atypical of shipping fever. Reasonable evidence was established that cattle in a feedlot within 2 km of the well were affected adversely, and the time for finish was extended. Also observed in these cattle were deaths during a severe windstorm, and death of an exposed steer from polioencephalomalacia. The relationship between exposure to well emissions and lack of stamina or polioencephalomalacia was not established. Other farmers complained of loss of stamina in cattle, and exercise intolerance in horses. Lesser effects may have occurred at a greater distance from the well, and exposure to well gas may have contributed to respiratory disease in weaned calves. Evidence of reproductive failures associated with well gas exposure(s) was not established.

**TABLE 54.7 Chemical Composition of Well Gas Emitted from a sour gas well blowout**

| Compound | Concentration (ppm) |
|---|---|
| Hydrogen sulfide | 11,400 |
| Methylmercaptan | 57.0 |
| Carbonyl sulfide | 22.0 |
| Ethylmercaptan | 14.7 |
| Propylmercaptan | 14.3 |
| Carbon disulfide | 4.8 |
| Butylmercaptan | 3.3 |
| Dimethyl disulfide | $<0.5$ |

Mostrom (1995, 1996) investigated the effects of a leak from a sour multiphase pipeline in 2 herds of cattle. One of the herds was located immediately adjacent to a river where the pipeline break occurred, and was later moved to a farm approximately 4.5 km from the site. The other herd was located approximately 4 km from the site. The herds were exposed to emissions from the pipeline, and also to emissions from the cleanup operations. River ice, river bottom, and riparian lands were reclaimed by burning fugitive sour condensate and washing river gravel. Emissions from the washing operations were released into the atmosphere. Over 1400 tissue specimens were collected. This investigation found clinical evidence of exposure to an irritating gas, and evidence of immune and nervous system dysfunction in cattle. Some cows developed aggressive behavior. Pregnant cows were observed *in estrus*. Both herds had above-average mortalities in cows and calves. Many of the calves lacked the suckling instinct, and cows failed to nurture newborn calves. Stair *et al*. (1996) reported on the histopathological findings in these herds.

## EXPERIMENTAL STUDIES ON $H_2S$

Nordstrom (1975) exposed calves to ammonia ($NH_3$) and $H_2S$ gases individually or in combinations. Calves were housed and exposed in uniquely designed exposure

chambers. A manure pack was established in each chamber to stabilize a background production of $NH_3$ at approximately 12.95 ± 5 ppm (9.0 ± 3.5 mg/m$^3$). $H_2S$ was not identified in the manure gas. Calves were placed in the chambers for a 7-day acclimation period, and were exposed to the background level of $NH_3$ during this interval. The acclimation interval was immediately followed by 7-day exposure to $H_2S$. A subsequent 7-day observation period completed the experiment.

The predominant clinical sign in all treatment groups was ocular irritation as indicated by erythema and excessive lacrimation. Ocular irritation was observed at the background levels of $NH_3$. Nasal irritation, based on serous discharge from the nose, was observed at 65 ppm (45.2 mg/m$^3$) of $NH_3$, and the serous discharge was profuse at 150 ppm $NH_3$ (104.3 mg/m$^3$). Dry coughing was also observed, especially at 150 ppm $NH_3$. At 150 ppm exposure, the calves kept their eyes closed and had irregular shallow breathing. Based on diminishing clinical signs during the exposure periods, the author concluded that the calves appeared to develop some adaptation to $NH_3$. The calves were distressed and depressed by all exposure levels of $H_2S$. Signs of distress were restlessness, pawing, head shaking, tail switching, blowing through the nose, licking of the nose, and slobbering with the tongue protruding. Signs of depression were heads lowered and eyes closed. Diarrhea and vomiting were observed in some calves. Breath holding, panting, dyspnea, shallow breathing, and spasmodic coughing were signs of respiratory distress. Epistaxis was also observed. Neurological effects (nystagmus and depression) were reported. Ocular irritation appeared to be profound and, at 150 ppm (208.6 mg of $H_2S$/m$^3$) of exposure, the effect was described as degenerative. Photophobia and refusal to open the eyelids were observed at 20 ppm (27.8 mg of $H_2S$/m$^3$) level of exposure. At the high-exposure level, corneal opacity was severe, and the animals were diagnosed as blind. Keratoconjunctivitis and vesicular keratitis were observed. At the end of the exposure interval, rupture of the cornea seemed imminent. The eyes of white-faced calves appeared to be more susceptible to the irritating and inflammatory effects of $H_2S$ than calves with pigmented skin around the eyes.

Clinical signs of the effects on the upper respiratory tract were more severe in calves exposed to $NH_3$ plus $H_2S$ (Nordstrom, 1975). The severity of epistaxis increased at the exposures to high levels of $H_2S$ plus $NH_3$, and the author considered the effects to be additive or synergistic. The combination of 150 ppm $H_2S$ plus 150 ppm $NH_3$ decreased respiration from a pre-exposure rate of 87–63/min. Based on clinical evaluations of the ocular lesions at the 150 ppm of $H_2S$ level of exposure, the author concluded that $NH_3$ at 150 ppm decreased the effects of exposure to 150 ppm $H_2S$. Rumen motility was considered to be decreased in both rate and intensity. Exposure to $H_2S$ plus background $NH_3$ decreased feed consumption by 3.5% and 26% for 20 and 150 ppm levels of exposure, respectively. Animals exposed to 20 ppm $H_2S$ tended to recover appetite at the latter part of the exposure interval. During the 7-day post-exposure interval, feed consumption increased and was higher than that for the background $NH_3$ values. At levels of 20 ppm $H_2S$ plus 65 ppm $NH_3$, appetite depression in the calves was more or less comparable to that of calves exposed to 20 ppm $H_2S$ plus background $NH_3$. Feed consumption in calves exposed to 20 ppm $H_2S$ plus 150 ppm $NH_3$ was decreased 6.5%, or essentially double the appetite depression observed in calves exposed to 20 ppm $H_2S$ plus background $NH_3$. Exposure to 150 ppm $H_2S$ plus 150 ppm $NH_3$ caused a 32.5% decrease in feed consumption compared to that of the calves exposed to 20 ppm $H_2S$ plus background $NH_3$. These observations suggest that the ratio of $H_2S$ to $NH_3$ in the exposure mixture alters the interactive effect.

Clinicopathology observations were reported. Sulfhemoglobin was not detected in the blood from calves exposed to 20 ppm $H_2S$. Statistically significant changes in levels of blood ammonia, urea nitrogen and uric acid, serum concentration of bilirubin, glucose, phosphorus, calcium, total protein, albumin and cholesterol, or the activities of the enzymes glutamic oxaloacetic transaminase, lactic acid dehydrogenase, and alkaline phosphatase were not observed. Leukocytosis was observed in 2 calves exposed to $H_2S$ plus background concentrations of $NH_3$.

Goats were essentially continuously exposed to $H_2S$ during a 96-h interval in an exposure-hood delivery system (Hayes, 1972). The exposure-hood controlled the ambient environment around the head and neck of the animals, but permitted the animals to eat and drink. Exposure concentrations of $H_2S$ were 0.0 ppm, 4 goats; 10 ppm (13.9 mg/m$^3$), 4 goats; 50 ppm (69.5 mg/m$^3$), 4 goats; and 100 ppm (139.1 mg/m$^3$), 5 goats. Goats exposed to 50 or 100 ppm $H_2S$ trembled during activity. A decrease in urinary volume corresponded to a decrease in water consumption. At 50 ppm of exposure, epiphora occurred 24 h after exposure and persisted for the remainder of the exposure interval. Epiphora also occurred with exposure to 100 ppm $H_2S$, and ocular injury was visible following 24–48 h of exposure. Corneal opacities, that were considered to be reversible, caused partial loss of vision. There was a trend for exposure to $H_2S$ to decrease intake of feed. In animals exposed to 10 ppm, there was a trend to recovery during the exposure period, and the overall decrease in feed consumption was 20%. Exposure to 50 ppm $H_2S$ decreased feed and water intake, especially on the 1st day of exposure. Although a trend to recovery was observed, complete recovery did not occur. For the 50- and 100-ppm exposure groups, feed consumption decreased on exposure day 2 (37% decrease for the 100 ppm group) and remained depressed through exposure day 4. For the 10-ppm group, there was a decrease in water consumption on exposure day 1, and a rebound on days 2 through 4.

For the 50 ppm group, there was a sharp decrease in water consumption on exposure day 2. For the 100-ppm group, water consumption was also decreased. The author concluded that there was a dramatic decrease in the desire of the goats to consume food and water. Plasma concentrations of cortisol were increased 48% and 55% in the 50 and 100 ppm groups, respectively. Respiratory and heart rates, and blood pressure were recorded each day at 08:00, 10:00, 13:00, and 16:00 h. These data suggest that initial exposure to $H_2S$ at the 10 and 20 ppm levels caused a decrease in respiratory rate on first day of exposure. The 0.0 and 10 ppm treatment groups were not different for respiratory rate; exposure to 50 ppm significantly decreased respiration from 19.8 respirations/min on exposure day 2 to 16.5 respirations/min on day 3; goats exposed to 100 ppm had a significant decrease in respiratory rate on the last 2 days of exposure. Exposure to $H_2S$ did not significantly alter heart rate, blood pressure, or rectal temperature; however, there was a trend for rectal temperature to increase during the exposure period.

## EXPOSURE TO ENVIRONMENTAL SULFUR

The impact of elemental sulfur on cattle, especially to producers in close proximity to sulfur stockpiles, is a concern to cattle producers. A study was done on the impact in cattle of fugitive sulfur and other pollution from sulfur mines in Poland (Janowski and Chmielowiec, 1981). The principal pollutants were sulfur dust, sulfur gases, $SO_2$, and sulfur hydride. Fifty-five cattle within 1 km of the mine were evaluated during the summer months on pasture, and during the winter months when the cattle were predominantly stabled. The control group of 27 cattle was kept in an area that was free of sulfur pollution, 20 km from the mine. Analyses for sulfur dust and sulfur compounds are summarized in Table 54.8. Clinical findings in the polluted area were: (1) decreased occurrences of infestation with external parasites and mycotic skin infections (ringworm) (2) increased body temperature, heart and respiratory rates; and (3) decreased excitement response to pain. Other findings were abnormal eyes (90.4%), respiratory disease (94.2%), and digestive disorders (21.1%). Clinical findings in the eye were epiphora, erythema of the conjunctiva, and edema of the eyelids. Abnormal findings of the respiratory system included dyspnea, coughing, mucopurulent nasal discharge, and abnormal pulmonary sounds. Digestive disorders included abnormal ruminal motility and diarrhea. Cattle were healthier when kept indoors. Cattle in areas polluted with sulfur, as compared to controls, had a decreased burden of parasites in the digestive tract. Pathological findings in the sulfur-exposed cattle were: the respiratory tract contained gray-yellow tinged mucus, inflammation of the trachea, thinning of cellular layers in the bronchi, almost to the point of squamous metaplasia, and interstitial inflammation. The authors concluded that sulfur pollution produces intoxication in the form of a chronic disease that they called sulfurosis (Janowski and Chmielowiec, 1981).

**TABLE 54.8 Concentrations of sulfur and sulfur compounds in sulfurosis study**

| Parameter | Study area* | Control area |
|---|---|---|
| Urinary sulfur | Barn 3.01 g/l<br>Outdoors 5.33 g/l | 0.52 g/l |
| Milk sulfur | 1.87 g/l | 0.30 g/l |
| Serum sulfur | 2.58 g/l | 0.46 g/l |
| Sulfur dust | Barn 1.02 g/$m^2$<br>Outdoors 1.31 g/$m^2$ | ND |
| Sulfur dioxide | Barn 0.089 mg/$m^3$ (0.034) | ND |
| Hydrogen sulfide | Barn 0.178 mg/$m^3$ (0.128)<br>Outdoors 0.089 mg/$m^3$ (0.064) | Barn ND<br>Outdoors 0.002 mg/$m^3$ |

* Value in parentheses is given in ppm.
ND: Not detectable.

## TOXICOLOGY OF SULFUR DIOXIDE IN CATTLE

Inhalation studies (head only exposure) in cattle showed that sulfur dioxide had an effect on the immune system and an effect on metabolism and if cold temperature had an effect on $SO_2$ toxicity (Komarnisky, 2003). A brief summary of this study is given. Eight steers were progressively exposed to room air containing 1 ppm $SO_2$ for 10 days, 5 ppm $SO_2$ for 7 days, and 20 ppm for 7 days. Four steers were exposed to $SO_2$ at room temperature ~18.5ºC and 4 steers were exposed to $SO_2$ at –16ºC. In the cold environment, metabolic rate was increased by 33%, 39%, and 44% at 1, 5, and 20 ppm of $SO_2$, respectively. Exposure to $SO_2$ in the warm environment did not significantly alter metabolic rate. For a 500 kg steer intermittently exposed to 1 ppm of $SO_2$ the extra feed required for maintenance and growth would be equivalent to an additional 1.5 kg/day of grain or 2.1 kg/day of hay. Sulfur dioxide was shown to have immunotoxic effects in cattle. Exposure of steers to 5 and 20 ppm in the cold environment decreased the respiratory bursts in neutrophils. Exposure to $SO_2$ in the cold environment decreased the threshold dose for shedding of respiratory epithelial cells into broncho-alveolar lavage (BAL) fluid. Neutrophil numbers in BAL were increased by exposure to $SO_2$ and exposure to cold temperature further increased neutrophil numbers. Exposure to $SO_2$ decreased the number of pulmonary macrophages in BAL fluid. At the 20 ppm level, lactate dehydrogenase was increased in the BAL fluid.

# PATHOLOGY OF PETROLEUM AND OILFIELD CHEMICALS

## Clinical pathology

Barber (1987) reported on the toxicology of aviation turbine fuel in heifers. Ten animals were considered to have elevated serum activity of aspartate aminotransferase, interpreted as a reflection of acute hepatic dysfunction, activity of $\gamma$-glutamyl transpeptidase was considered to be within the normal range. Elevated concentrations of non-esterified fatty acids were observed in 11 of the animals, 2 had elevated concentrations of blood urea nitrogen and 5 had a leukocytosis. Six weeks after the initial farm visit, all the 41 animals had elevated serum activity of $\gamma$-glutamyl transpeptidase.

Bystrom (1989) found that sweet crude oil did not alter hematological parameters. There was a correlation between the severity of clinical signs and increased concentrations of plasma fibrinogen. The activity of hepatocellular enzymes in serum and serum glucose were not consistently increased after exposure to sweet crude oil, but serum calcium and potassium values consistently decreased and bilirubin values consistently increased. Rowe (1972) found that hematological parameters, especially leukocyte numbers were elevated as chemical pneumonia advanced. A single dose of sour crude oil caused a constant decline in plasma glucose. A transient decline in plasma glucose was observed in calves given sweet crude oil or kerosene.

Exposure to grease has been reported to cause changes in hematology and clinic chemical parameters (Wallace and Blodgett, 1996). A cow exposed to Li grease had leukopenia ($3.7 \times 10^9$ WBC/l), lymphopenia ($1.8 \times 10^9$ lymphocytes/l), and hyperfibrinogenemia (6 g/l and normal given as $<5$ g/l).

Clinicopathological observations are available for cattle. TAP have greater affinity for pseudocholinesterase, also known as plasma cholinesterase, and butyrylcholinesterase, than for acetylcholinesterase (Abou-Donia and Gupta, 1994). Erythrocytic acetylcholinesterase was inhibited for a short duration after a calf was initially dosed with 10 g of TAP/kg of body weight, and the dose was repeated as 5 g/kg on day 30, and as 10 g/kg on day 56 (Dollahite and Pierce, 1969). In four animals poisoned with tri-*o*-tolyl phosphate, changes in the routine automated clinicochemical and hematological parameters were not observed (Nicholson, 1974). In an experimental study, 3 calves were given oral doses at 5, 10, or 20 g of TAP/kg of body weight, with the total dose divided equally over 10 days (Beck *et al.*, 1977). On day 8 or 9, there was a 70–77% reduction in the activity of whole blood acetylcholinesterase. In 1 calf given 5 g/kg as a single oral dose, activity of whole blood acetylcholinesterase was reduced by 68% on day 2 and by 78% on day 9. In cows dosed with 0.5 or 1.0 g of TAP/kg, activity of whole blood cholinesterase was reduced by 50% and 63%, respectively on day 2, and by 56% and 61%, respectively, on day 7. The consensus is that a relationship between the inhibition of blood cholinesterases and organophosphate ester-induced delayed neurotoxicity does not exist (Abou-Donia and Gupta, 1994).

Exposure to nitrates from drilling fluids can cause methemoglobinemia. The concentration of nitrates can be determined in rumen fluid, aqueous humor (anterior chamber of the eye), urine, plasma, and serum. Exposure to $H_2S$ does not produce sulfhemoglobin.

## Pulmonary pathology of petroleum

The lung is a target for unweathered (unchanged by exposure to atmosphere) petroleum. Hydrocarbon-induced chemical (inhalation) pneumonia occurs when oil is inhaled. Aspiration of oil during emesis is the most common mechanism of inhalation exposure (Coppock *et al.*, 1986a, 1996).

Necropsy findings of oil-induced chemical pneumonia have been described (Dungworth, 1985, 1993). Generally, the cranioventral lobes of the lungs are affected first. During necropsy, oil may be identified in the lungs by oil discoloration and a petroleum odor, visible oil in the lung, oil floating to the surface when a piece of lung is immersed in water or formalin, or oil layering on the surface of the supernatant when a piece of lung is homogenized.

The pulmonary histopathology for unweathered petroleum is similar for the various forms of petroleum. Histopathological changes are proliferative, and macrophages with a foamy-appearing cytoplasm are found in and tend to fill the alveoli (Dungworth, 1985). In the pathogenesis, oil-containing macrophages are found in the lymphatics, especially those adjacent to blood vessels around bronchi. Fibrosis and proliferation of type II macrophages are prominent features. Foamy macrophages may be incorporated into the alveolar septa by fibroblastic proliferation. As determined by histochemistry and special stains, lipids are found in both intracellular and extracellular sites, and oil can be identified in the alveolar spaces. In the early stages, the lesion may be an acute necrotizing fibrinous bronchopneumonia. Generally, neutrophils, lymphocytes, and plasma cells surround the necrotic areas. Fibrinous exudate, inflammatory cells and amorphous eosinophilic and oily material can fill the alveolar spaces. Empty spaces in the tissues suggest that liquid foreign material may have been absorbed. Plant material from aspirated ingesta may also be seen in alveoli. Fibrin hemorrhages and a mixed population of mononuclear cells may be observed on the pleural surface. Extensive coagulative necrosis and suppuration may be observed in the consolidated areas. Bacteria may be present in the necrotic areas and alveoli. Hematogenous exposure of the lungs to

petroleum hydrocarbons also occurs. Volatile components of petroleum are absorbed by the gastrointestinal tract and are volatilized from the blood in the lungs. Oil emboli can occur in the lungs through absorption of oil from the gastrointestinal tract, and form oil emboli that block the small blood vessels in the lungs and brain. Oil emboli in blood vessels of the lungs and brain have not been reported in cattle intoxicated with crude petroleum. The pathogenesis and observed lesions may be altered by concurrent bacterial infection and cytotoxicity of the oil.

There are reports of field and experimental-induced petroleum intoxication. Ulceration of the ventral aspect of the trachea can occur in kerosene poisoning and the ulcers may be covered with a pseudomembrane (Rowe, 1972, 1973),. Areas of the lung can be consolidated and have a dark purple, reddened or tan-gray, mottled discoloration. The areas of consolidation often have a nodular appearance. Fibrinous pleural adhesions and serofibrinous pleural exudate can occur. Pulmonary abscesses can also be observed (Rowe, 1972, 1973). Multiple small hemorrhages were observed in the lungs of cattle poisoned with tractor-vaporizing oil (Parker and Williamson, 1951). Chemical pneumonia was reported in cattle dying from diesel fuel poisoning (Gibson and Linzell, 1948). Pneumonia and pulmonary abscesses-containing Gram-negative organisms were found in cattle that died acutely from drinking water contaminated with aviation turbine engine fuel (Barber *et al.*, 1987). The rumen contents smelled of oil, and the odor matched the source of the petroleum. The carcasses appeared to repel scavengers and flies, and appeared to have retarded putrefaction. Microscopic examination of tissues from the animals that died in the first 3 weeks showed fatty degeneration of hepatocytes and focal areas of hepatitis, and 1 of 4 had tubulonephrosis, renal vascular thrombi, and interstitial nephritis. A diagnosis of pneumonia, and abscesses-containing gram-negative organisms was made in 2/4 of the animals.

Approximately 124 days after the ingestion of aviation turbine fuel-contaminated water, the remaining cattle were necropsied. At slaughter, the lungs of the cattle were considered abnormal, and were characterized by enlargement and gray-blue areas of varying and abnormal firmness. However, histopathological abnormalities were not observed. Infection in the lungs can occur rapidly following chemical injury. Cattle that survive for 6 weeks or longer with chemical pneumonia usually have marked loss of weight and chronic ill health (Rowe *et al.*, 1973). Pulmonary lesions have been observed in sheep following a 1-day exposure to natural gas condensate-contaminated water (Adler *et al.*, 1992). Gross pathological observations included severe bilateral consolidations and tan to gray mottling of the ventral lobes. Histological diagnosis of the lesions was a necrotizing bronchopneumonia. There were extensive areas of coagulation necrosis bordered by a dense zone of neutrophils and lesser numbers of lymphocytes, plasmacytes, and macrophages. The alveoli were filled with fibrinous exudate and amorphous eosinophilic material. Plant material was also observed in the pulmonary parenchyma. The pleural surface was coated with fibrin, hemorrhages, and mononuclear cells. Pathogenic and potentially pathogenic bacteria (*Pasteurella haemolytica*, *Klebsiella pneumoniae*, *Fusobacterium necrophorum*, *Actinomyces* (*Corynebacterium*) *pyogenes*, *Escherichia coli* and *Pseudomonas* spp.) were isolated from the lungs of some of the sheep.

Experimental studies have been done in crude oil (Bystrom, 1989). Cattle were administrated a sweet crude oil *per gavage*. Many of the animals, especially those that received larger doses of oil, had black discoloration of the lungs. Visible oil was present in the cranioventral lobes. In one animal, it was estimated that 80% of the lungs were affected by black discoloration, and visible oil was observed. Pathological diagnoses included pulmonary consolidation, fibronecrotizing pneumonia, pleuritis, emphysema, atelectasis, multifocal interstitial pneumonia, proliferative alveolitis, bronchopneumonia, and alveolitis. In animals that survived 8 days, hyperplasia of type II epithelial cells was observed, and the alveoli were filled with foamy, alveolar macrophages.

Lung lesions can occur when animals are exposed to gaseous petroleum and sour gas. Stair *et al*. (1996) reported lesions of hyperplasia of the submucosal glands in the trachea of cattle exposed to emissions from a multiphase sour gas leak (Mostrom and Campbell, 1996). Other lesions observed included hyperplasia of tracheal, bronchial and bronchiolar glands, metaplasia of the tracheal mucosa, and loss of cilia from the respiratory epithelium. Lymphoid hypoplasia was also observed.

A study was done in feral cats that were exposed to emissions from fires in the Kuwait (Moeller *et al.*, 1994). The cats were collected in Kuwait approximately 8 months following ignition of the oil wells. To ensure that the cats had been exposed for the entire duration of the oilfield fires, only cats with permanent teeth were examined. Twelve cats were collected in Kuwait City, an area that was relatively smoke-free, and 14 cats were collected in Ahmadi, a city that was partially evacuated because of intense smoke from the fires in the adjacent Bergan oilfield. These findings have been summarized in Table 54.9. The authors concluded that these lesions were probably reversible, and were most likely caused by exposure to pollution from the oilfield fires. However, other causes such as infectious diseases and parasitism could not be completely eliminated.

## Hepatic pathology of petroleum

Hepatic pathology can be observed following exposure to oil. Parker and Williamson (1951) reported fatty changes in the liver from a cow that had ingested tractor-vaporizing

**TABLE 54.9 Respiratory tract lesions observed in feral cats exposed to emissions from the Kuwait oilfield fires**

| Lesion | Kuwait city | Ahmadi |
|---|---|---|
| Mild accumulation of anthracotic pigment | 5/12 | 11/14 |
| Hyperplasia of bronchial and bronchiolar glands | 3/12 | 5/14 |
| Scattered occurrence of bronchi that were dilated and filled with cellular debris and a few neutrophils, and connective tissue around bronchiolar glands contained lymphocytes and plasma cells | 1/12 | 1/14 |
| Hyperplasia of smooth muscle around terminal and secondary bronchioli | 4/12 | 10/14 |
| Tracheal gland hyperplasia, and segmental thickening of the submucosa | 3/12 | 4/14 |
| Multifocal squamous metaplasia of tracheal mucosa | 7/12 | 10/14 |
| Minimal hyperplasia of the submucosal glands of the larynx, some thickening of the laryngeal submucosa and slight goblet cell hyperplasia | 2/12 | 0/14 |
| Multifocal squamous metaplasia of the laryngeal epithelium | 2/12 | 3/14 |

oil. Fatty degenerative changes and focal areas of hepatitis were observed in cattle following ingestion of water contaminated with aviation turbine engine fuel (Barber *et al.*, 1987). An abnormal-appearing liver in a heifer was attributed to the ingestion of gasoline 15 months previously (Albert and Ramey, 1964). A sweet crude oil was found to be hepatotoxic in cattle. Pathological changes included swelling of the liver, increased friability, and centrilobular congestion (Bystrom, 1989). Cows dosed with crude oil had an increase in liver weights. Histologically, the hepatocytes contained numerous vacuoles, and these vacuoles were thought to have previously contained oil. Wallace and Blodgett (1996) reported the histopathological changes in the liver of a cow that consumed Li grease containing 2050 μg Li/g of grease. The observations were vacuolated and atrophied hepatocytes. Experimental oral exposure of cattle to Li produced similar hepatic lesions (Johnson *et al.*, 1980). Hepatic changes observed in sheep poisoned by natural gas condensate were fatty degeneration, and periportal infiltration of lymphocytes and plasma cells. Biliary hyperplasia and periportal fibrosis were also found (Adler *et al.*, 1992).

## Gastrointestinal pathology of petroleum

The gastrointestinal tract in oral exposure has primary contact with the oil. The chemical composition of the oil will cause variation in the pathology observed. Petroleum can be observed and the odor of petroleum can be detected in the rumen and intestinal tract (Wallace and Blodgett, 1996). Kerosene-induced ulcers of the esophagus have been reported (Rowe *et al.*, 1973). Bystrom (1989) reported pyloric ulcers of the abomasum of cattle exposed to unweathered sweet crude oil; also rectal lesions of mild congestion to mild hemorrhages and focal areas of inflammation were observed. Other gastrointestinal lesions were not reported. Bumstead (1949) reported oil to be very irritating to the intestinal tract of cattle. Ingestion of natural gas condensate was observed to produce reddening of the serosal surface of the gastrointestinal tract of sheep (Adler *et al.*, 1992). Hemorrhage into lumen of the gut was also observed. Histological observations were submucosal and mucosal congestion, and mucosal and serosal hemorrhage. An inflammatory response was observed in all areas of the gastrointestinal tract. Superficial erosions, mild acute cryptitis and neutrophilic and lymphocytic exudates were reported. Isolated necrosis of enterocytes was also observed. Lithium grease has been reported to cause gastroenteritis in cattle (Johnson *et al.*, 1980).

## Renal pathology of petroleum

The kidneys are also target organs for petroleum. Some animal species and gender within a species are more sensitive to the nephrotoxicity of petroleum than others. The sensitivity of cattle to the nephrotoxicity of petroleum has not been well defined. A cow that consumed Li grease had mild swelling and vacuolation of the proximal tubular cells (Wallace and Blodgett, 1996). Experimental oral exposure of cattle to Li grease containing 1250 μg Li/g of grease produced similar renal lesions (Johnson *et al.*, 1980). Parker and Williamson (1951) reported degeneration of the kidney in cattle after they consumed tractor-vaporizing oil. Tubulonephrosis, renal vascular thrombi, and interstitial nephritis were observed in cattle following environmental exposure to aviation turbine engine fuel (Barber *et al.*, 1987). Renal lesions were not observed in surviving animals slaughtered 124 days later. Renal lesions have been observed in cattle dosed with a light crude oil (Bystrom, 1989). These lesions were shrunken or collapsed glomeruli (2–5 in a 4 times field), mild focal necrosis of epithelial cells in the collecting ducts, and inflammatory cells that were observed in the renal cortex. In sheep poisoned with natural gas condensate, tubular epithelial necrosis was observed (Adler *et al.*, 1992). Granular eosinophilic casts (negative for hemoglobin) and protein droplets filled Bowman's space and many renal tubules. Inflammatory cells were seen around tubular casts.

## Nervous system pathology of petroleum

Cattle exposed orally to Li grease had slight hemorrhage into the cerebellar peduncles (Johnson *et al.*, 1980; Wallace

and Blodgett, 1996). Lesions in the CNS were observed in sheep poisoned by natural gas condensate (Adler *et al.*, 1992). Mild perivascular hemorrhage was seen in the pia mater and in the white mater of the cerebellum and cerebrum. Increased separation between the pia and arachnoid membranes was prominent, and edema was observed in the stroma of the choroid plexus. Lesions of the CNS were observed in harbor seals (*Phoca vitulina*) exposed to crude petroleum (Prudhoe Bay Crude Oil, PBCO) from the T/V *Exxon Valdez*, which grounded in Prince William Sound (Spraker *et al.*, 1994). Lesions were observed in neurons and axons of the midbrain. Histopathological observations included intramyelinic edema of the large myelinated axons, neuronal swelling, neuronal necrosis, and axonal swelling and degeneration of the midbrain. These lesions were most prominent in the thalamic nuclei. Lesions of the CNS also were observed in polar bears. The bears were dermally exposed to Midale crude oil (Oritsland *et al.*, 1981). The lesion consisted of degenerated and necrotic cells in the small cell pyramidal layer of the hippocampus. Other lesions observed were perineuronal vacuolation in the cerebral cortex. Enlargement of the Virchow–Robin spaces and extravasation of blood around small blood vessels occurred.

## Cardiac pathology of petroleum

Cardiac lesions associated with petroleum poisoning in cattle do not appear to have been reported, but have been observed in sheep exposed to petroleum condensate (Adler *et al.*, 1992). Gross pathological changes included epicardial hemorrhages, serosanguineous pericardial fluid, pale-appearing myocardium, and reddened endocardium. Microscopic observations were segmental myocardial necrosis and calcification. The myofibrils had loss of cross striations and a beaded appearance. There were multifocal areas infiltrated with lymphocytes, macrophages, and occasional neutrophils. The blood vessels were congested.

## Pathological effects of petroleum in embryos

Lesions in the bovine fetus linked to exposure to crude oil have not been reported. However, studies on the embryotoxicity of PBCO have been done. Couillard and Leighton (1989, 1990a, b) reported that PBCO was embryotoxic and that the toxicity was different from hypoxia induced by sealing the eggs with a sealing wax. Mineral oil (USP), used as a control, was not toxic to chicken embryos. Dosages ranged from 2 to 20 μl PBCO/egg; the oil was applied on the surface of the egg below the airspace. Hepatic lesions were hepatocellular necrosis, mineralization, and perivascular and multifocal accumulations of heterophils. Hepatocellular necrosis was dose dependent. The liver was observed to have superficial yellowish zones that were multifocal to diffuse. These yellowish zones and mineralization corresponded to areas of hepatocellular necrosis. Mineralization occurred in the necrotic areas. Vacuolation of the hepatocytes was observed. The number of mitotic figures in the liver increased with the dose. Renal lesions were limited to the mesonephrous. The capillaries of the glomeruli were distended and cellular casts and mineralization were observed in the renal tubules. Splenic lesions were limited to increased granulopoietic cells arranged in distinct cords. Other pathological findings were ascites and subcutaneous edema. Hepatic lesions were observed 2 days after treatment; renal and splenic lesions on the 3rd day. Edema fluid was more pronounced after the 3rd day.

In another study, the toxicity of PBCO was evaluated in chicken embryos (Lusimbo and Leighton, 1996). The chicken embryos were exposed to oil on day 9 of incubation. The oil was placed on the eggshell over a prominent blood vessel of the chorio-allantoic membrane. Dosages ranged from 0 to 16 μl PBCO/egg, and embryonal mortality was dose dependent. Exposure to PBCO at 4 μl decreased weight gains during a 3-week post-hatching observation period. Lesions were observed in the liver, subcutaneous tissues, bursa of Fabricius, and pipping muscle (musculus complexus). Hepatic lesions were multifocal to locally extensive areas of hepatic necrosis and mineralization. Large fluid-filled vesicles were observed on the dorsocaudal aspect of 5% and 16% of embryos dosed with 1 and 2 μl of PBCO respectively. The bursa of Fabricius had depleted lymphoid tissue and the interstitium was infiltrated with heterophils. Lesions in the pipping muscle were edema and hemorrhage, sparse multifocal fragmentation and occasional vacuolation of the myofibers.

## Pathology of $H_2S$ and Sulfur

Detailed reports on the pathology of $H_2S$ and sour gas in cattle do not appear to exist in the scientific literature. There are reports on the toxicopathology of $H_2S$ in other species. Massive pulmonary edema was the pathological finding in humans killed by accidental exposure to $H_2S$ and in rats experimentally exposed to high concentrations of $H_2S$ (Burnett *et al.*, 1977; Lopez *et al.*, 1986, 1987, 1988, 1989). Ocular lesions in rats included corneal edema and necrosis, and detachment of corneal epithelial cells has been reported for experimental $H_2S$ exposures of 56 mg/m$^3$ (40.3 ppm) (Beasly, 1963; Lopez *et al.*, 1989). Calves continually exposed to $H_2S$ by inhalation developed corneal opacity (Nordstrom, 1975; Beauchamp *et al.*, 1984). Unfortunately, the corneal pathology was not described in these reports.

Lopez *et al.* (1986) exposed rats to 0, 56 and 420 mg of $H_2S$/m$^3$ (0, 40.3 and 302 ppm) for 6 h. Mild pulmonary

edema was observed in rats immediately after exposure to atmospheres containing 56 mg $H_2S/m^3$ and in rats that were killed by exposure to 420 mg $H_2S/m^3$. Pulmonary edema was described as primarily perivascular, and edema fluid was rarely observed in the alveoli. Pulmonary edema was not observed in rats exposed to 56 mg/$m^3$ at 18 and 42 h after exposure. Lymphorhexia of the lymph nodes or thymus appeared to increase in severity and frequency in the high-dose group. Since nasal ulcers were observed in all of the rats, activation of the immune system could increase the sensitivity of lymphoid tissue to $H_2S$.

Lopez *et al.* (1987) reported that exposure of rats to $H_2S$ concentrations of 0, 10, 200, and 400 ppm (0, 13.9, 278.1 and 556.2 mg/$m^3$) for 4 h increased the cellularity of nasal saline washes by 0%, 139%, 483% and 817%, respectively. The cells present in the nasal washes were exfoliated epithelial cells and neutrophils. A 3000% increase in the protein concentrations in BAL fluids was observed 1-h post-exposure in rats exposed to 400 ppm $H_2S$. The increase in protein and leukocytes was considered to be the result of alterations of vascular permeability induced by $H_2S$.

Lopez *et al.* (1988) exposed rats to 0, 14, 280 or 560 mg of $H_2S/m^3$ (0, 10.1, 201.3, and 402.7 ppm), respectively. Squamous epithelium appears to be resistant to the corrosive effects of $H_2S$, and lesions were not observed in these regions of the nasal epithelium. Lesions of the respiratory and olfactory epithelium were observed in all of the rats exposed to 560 mg $H_2S/m^3$. Cellular necrosis, erosions, and ulcers of the respiratory epithelium were observed. Twenty hours after exposure, eroded or ulcerated areas were covered by a thin layer of basophilic cells, and a neutrophilic exudate was observed in the lamina propria and submucosa. At 44 h after exposure, the respiratory epithelium was covered with pre-ciliated secretory cells, and mitotic figures were observed. Specific lesions were observed in the olfactory epithelium. There were multifocal areas of cellular necrosis, and the lesions were more pronounced at 20 h after exposure. Evidence of healing was not observed at 44 h after exposure. The olfactory epithelium is considered a sensitive target in the nasal passages for $H_2S$. No report was found for cattle. However, lesions of the olfactory epithelium could alter maternal behavior by altering offspring recognition.

Ciliated cells in the nasal passages, trachea, and bronchi are sensitive targets for airborne irritants including $H_2S$, $SO_2$, and $NO_2$ (Lopez *et al.*, 1988). Ciliated cells transport mucus or foreign particulate matter from the lower respiratory tract toward the pharynx where it is swallowed, and from the nasal passages to exit at the nares. The cilia of the columnar epithelium are lost following exposure to irritating airborne substances. Loss of cilia or ciliated cells can increase the impact of particulate substances on the pulmonary tract. Rats exposed to 2317.6 mg of $H_2S/m^3$ or rats given intraperitoneal injections of sodium bisulfide (NaHS) died within 3 min (Lopez *et al.*, 1989). The rats that were exposed to $H_2S$ had massive extravasation of eosinophilic fluid into the broncho-alveolar space.

Pathological findings in environmental sulfur poisoning of cattle were gray-yellow tinged mucus in the respiratory tract, inflammation of the trachea, thinning of cells in the bronchus almost to the point of squamous metaplasia, and interstitial inflammation (Janowski and Chmielowiec, 1981). Sulfur dioxide produced histopathological evidence of severe irritation of the respiratory tract and eyes. Manure-gas poisoning produced congestion and edema of the lungs, gastroenteritis, and peritoneal effusions.

## Sour gas and sour condensate

Stair *et al.* (1996) reported on cattle that were exposed to sour gas/condensate from a break in a multiphase pipeline. Clinical signs in the cattle were estrus in cows in advanced pregnancy and failure of cows to bond with calves. Some cows had neurological signs of proprioceptive-locomotor deficiencies. Many of the calves failed to thrive, and a high occurrence of mortality was observed. Pathological findings in the respiratory tract included hyperplasia of the submucosal tracheal glands, discharged goblet cells, squamous metaplasia of the tracheal mucosa, and submucosal perivascular lymphoid infiltration. Also observed was hypoplasia of the lymph nodes. These effects were associated with exposure to sour gas/condensate in an area of intensive sour gas and crude oil production and processing.

## Non-pesticide organophosphorus esters

Non-pesticide phosphate esters are used as additives for lubricating oils operating under extreme conditions. Coppock (1995a) published a review of the neurotoxicity of these esters.

The pathology of the TAP esters has been described in several species. Specific pathological changes may not be observed during necropsy (Dollahite and Pierce, 1969; Nicholson, 1974; Beck *et al.*, 1977). Irritation and hemorrhage in the gut have been associated with TAP poisoning (Julian *et al.*, 1976). Histopathological changes of axonopathy may progress in severity toward the cell body (Maydew *et al.*, 1983; Jubb and Huxtable, 1992). The peripheral axonal lesion was described as demyelination and swelling (3–4 times) of the axonal cylinders (Dollahite and Pierce, 1969). Vacuolation and degeneration of axons were observed in the sciatic and femoral nerves (Julian *et al.*, 1976). Dollahite and Pierce (1969) observed demyelination in the spinal nerve roots, of which the ventral nerve roots were the most severely affected. Cytoplasmic vacuolation of the large motor neurons was also observed. Lesions of the spinal cord were described by Nicholson (1974) as axonal degeneration in the gray mater of the ventral horn and in the

ascending nerve tracts. Swelling of the axonal cylinder (20 times), with a transition from basophilic to acidophilic staining properties, were observed in the spinal cord of cattle inadvertently or experimentally exposed to TAP (Beck *et al.*, 1977). From lumbar to cervical regions, the lesions appeared to change in pattern and severity. Histological evidence of axonal degeneration was not observed in the L5 vertebral region, however, a few swollen axons were observed at the level of L2 and posterior thoracic (T12) vertebrae. A marked increase in degenerated axons appeared at the level of T5 and the seventh cervical vertebrae (C7). At the level of C3, the lesions were more severe at the tips of the dorsal and lateral horns. Axonal degeneration was not observed in the brain. Lesions of demyelination and degeneration were observed bilaterally in the ascending tracts of the cervical regions of the spinal cord (Julian *et al.*, 1976). The lesions were immediately ventral to the dorsolateral sulcus in the area of the spinocerebellar tract and in the fasciculus gracilis. The degenerating areas had large vacuoles containing eosinophilic globules. Prantner and Sosalla (1993) reported multifocal areas of spongiosis in the medial and lateral funiculi at the levels of vertebrae L2 through L5. Focal perivascular hemorrhages were also observed in the basal nuclei of the brain. The chemical composition of the TAP, age of the animal, and the ascending progression of lesions when they were observed may explain these discrepancies (Gupta and Abou-Donia, 1994).

## ANALYTICAL TOXICOLOGY

The best procedure is to phone the laboratory and request instructions for sampling containers and methods of preservation. The sampling procedure must be written out, signed, and dated. Labels must correspond with the description of the sample. The majority of samples can be stored by freezing. Care must be taken to ensure that samples do not break during freezing and the frost on the container during thawing does not alter the label. Glass containers should be used and the containers must be chemically clean. Plastic containers can have substances migrate into the sample and present expensive cleanup problems for the analytical laboratory. If aluminum foil is used to seal containers, the dull side has contact with the sample. Pictures should be taken and a description of the pictures written.

The tissues that should be collected at necropsy are rumen and stomach contents, contents from various areas of the gastrointestinal tract, liver, kidney, lung (including description of the lobe), perirenal or bone marrow fat, brain, soiled hair or feathers. Samples should be taken of the suspect petroleum. Environmental samples include soil, water with floating substances, and water plus sediment. The suspect water and oil should be sampled.

Petroleum and the compounds associated with petroleum and oil–gas field operations is difficult because of the complex mixture of chemicals. The most common approach used is to match chromatographic fingerprints. The chromatographs are extracts from tissues such as lung and tissues and chromatographs of the suspect petroleum. Chemicals in petroleum that are resistant to biodegradation are called biomarker chemicals (e.g., phytane and pristine). Many of the biomarkers in crude petroleum are sensitive to petroleum refining and up-grading processes. Some biomarkers accumulate in fat. The percent, of fat found in tissue extracts must be recorded along with the concentration of the biomarker of interest. If possible, multiple biomarkers should be used and the ratio of the biomarkers calculated for the suspect petroleum and the biomarkers found in tissue. For flare emission issues, analyses of tissues especially fat may show the presence of a number of polycyclic aromatic hydrocarbons (PAHs). The problem is that there are generally multiple sources of these PAHs.

Ions and heavy metal pollutants can be assayed. In addition to the concentration of the ions or metals, the ratio of the ions and metals can be helpful in identification. For example if production water has contaminated domestic water, the ratio of the ions in the domestic water should reflect the ion ratio found in the produced water. The same applies to gas from a gas well that have contaminated a domestic water supply.

## DIFFERENTIAL DIAGNOSIS

The diagnosis of petroleum toxicity can be straightforward or very difficult depending on the circumstances. If the exposure is current or recent and there is direct evidence of toxic substances in or on the animals and obvious detrimental effects, the diagnosis may still require careful assessment and testing, but should be accomplished relatively easily.

However, if the exposure has not been recent and clinical signs are not specific or are ones that may have several possible causes, the diagnosis can be very difficult. Pneumonic lesions associated with crude oil toxicity are easier to interpret if oil is still present in the gastrointestinal tract. The presence of vacuolated macrophages is helpful in differentiation from infectious causes of pneumonia. Pneumonia initiated by toxic gases is difficult to diagnose because the initial site of injury is at the broncho-alveolar junction, the same location as the initial site of infection for several viral and bacterial pathogens. The bronchiolitis and alveolar reactions of necrosis, edema, and cellular infiltration can be indistinguishable between toxic gas responses and infectious agent responses. As well toxic gases can interfere with the normal functions of cells lining

the trachea and bronchi leading to reduced resistance to infection (Dungworth, 1983). Multiple agents may also be present at the same time, making the determination of the initial cause impossible.

Acute neurological signs may result from sodium ion toxicity. The differential includes thiamine deficiency-linked polioencephalomalacia, which results in brain lesions that are indistinguishable from lead poisoning. The analysis for sodium levels in rumen content is essential.

The occurrence of delayed neurotoxicity signs requires an in-depth investigation of potential causes, including TAP's and organophosphorus pesticides, to determine possible exposure.

The occurrence of ocular and respiratory irritation in the presence of toxic gases is a typical sign. However, similar irritation may result from dry, dusty, and windy conditions and form irritating particles in feed. The measurement of toxic gas levels in the vicinity of the livestock is seldom possible and assumptions of exposure based on data from the emission source and nearby monitoring sites may be the only data available. Differentiation from infectious pinkeye may be made by isolating the infectious agent and ruling out toxic gas exposure.

Poor growth and weight loss may have many possible causes including nutritional deficiency, parasitism, chronic pneumonia, chronic intestinal disease, mycotoxicosis, and others. Temporal connection to petroleum sources such as crude oil and previous acute cases is important in the differentiation. The possible role of adverse effects of petroleum compounds on the immune system increasing susceptibility to infectious disease further complicates the situation.

Reproductive losses may have multiple causes. The time delay often present between exposure and clinical signs makes diagnosis very difficult, if not impossible. Abortions that result during an acute toxic episode are the easiest to associate with the toxin. The potential hormonal disruption from chemicals that disturb the endocrine hormone system relating to conception and pregnancy may not be possible to determine days or weeks after the exposure.

## CONCLUDING REMARKS AND/OR FUTURE DIRECTIONS

Domestic and wild animals ingest petroleum and chemicals used in exploration and production of oil and gas. Many of the chemicals used have limited toxicology information and the toxicology of chemical mixtures is unknown. The toxicology in aquatic organisms may be known because of the requirement for toxicology assessments before use on offshore platforms.

Areas that require future research are analytical methods and toxicological assessment in laboratory exposure systems. Definitive toxicology data is required before epidemiological studies can be accurately interpreted.

## REFERENCES

Abou-Donia MB, Gupta RP (1994) Involvement of cytoskeletal proteins in chemically induced neuropathies. In *Principles of Neurotoxicology*, Chang LW (ed.). New Marcel Dekker, New York, pp. 153–210.

Adler R, Boermans HJ, Moulton JE, Moore DA (1992) Toxicosis in sheep following ingestion of natural gas condensate. *Vet Pathol* **29**: 11–20.

Albert TF, Ramey DB (1964) Abomasal displacement associated with ingestion of gasoline. *J Am Vet Med Assoc* **145**: 460–1.

Ballantyne EE (1950) Crude petroleum oil poisoning. *Annual Report: Veterinary Services Branch*. Alberta Agriculture Edmonton, AB, **16**.

Ballantyne EE (1955) Oilwell poisoning. *Annual Report: Veterinary Services Branch*. Alberta Agriculture, Edmonton, AB, **13**.

Barber DL M, Cousin DAH, Seawright D (1987) An episode of kerosine poisoning in dairy heifers. *Vet Rec* **120**: 462–3.

Beasly RWS (1963) The eye and hydrogen sulfide. *Br J Ind Med* **20**: 32–4.

Beauchamp RO, Bus JS, Popp JA, Boreiko CJ, Andjelkovich DA (1984) A critical review of the literature on hydrogen sulfide toxicity. *CRC Crit Rev Toxicol* **13**: 25–97.

Beck BE, Wood CD, Whenham GR (1977) Triaryl phosphate poisoning in cattle. *Vet Pathol* **14**: 128–37.

Bergman EN, Sellers AF (1953) Studies on intravenous administration of calcium, potassium, and magnesium to dairy calves. I. Some biochemical and general toxic effects. *Am J Vet Res* **14**: 520–9.

Bergman EN, Sellers AF (1954) Studies on intravenous administration of calcium, potassium, and magnesium to dairy calves. II. Some cardiac and respiratory effects. *Am J Vet Res* 15, 25–35.

Blaxter KL, Cowlishaw B, Rook JAF (1960) Potassium and hypomagnesemic tetany in calves. *Anim Prod* **2**: (Part 1), 1–10.

Buckland SJ, Ellis HK, Dyke P (2000) *New Zealand Inventory of Dioxin Emissions to Air, Land and Water, and Reservoir Sources*. Ministry for The Environment, Wellington, New Zealand, p. 164.

Bumstead WA (1949) Unusual case of crude oil poisoning of cattle. *North Am Vet* **30**: 712.

Burnett WW, King EG, Grace M, Hall WF (1977) Hydrogen sulfide poisoning. A review of 5 years experience. *Can Med Assoc J* **117**: 1277–80.

Bystrom JM (1989) Study of the acute toxicity of ingested crude petroleum oil to cattle. MSc Thesis. University of Saskatchewan Faculty of Graduate Studies, Saskatoon, Saskatchewan, p. 279.

Church TL (1992) Field investigation findings of the long-term effects in Alberta livestock exposed to acid forming emissions: a case study report. In *Proceedings, International Workshop on Effects of Acid Forming Emissions in Livestock*, Coppock RW, Lillie LE, (eds). Publication AECV92-P2, Vegreville, AB.

Coale AJ (1947) Drinking of crude oil by cattle. *North Am Vet* **28**: 221.

Cobb JC (2003) Coalbed Methane. University of Kentucky, Lexington, KY.

Coppock RW, Mostrom MS, Smetzer DL (1986a) Volatile hydrocarbons (solvents, fuels) and petrochemicals. In *Current Veterinary Therapy IX: Small Animal Practice*, Kirk RW (ed.). Saunders, Toronto, Ontario, pp. 197–202.

Coppock RW, Lillie LE, Beck BE, Christian RG (1986b) A report on the field investigation into livestock health complaints subsequent to the Drummond 6-30 sour gaswell blowout: September 24–8, 1984. Publication AECV86-R3, Vegreville, AB, p. 238.

Coppock RW, Florence LZ, Miller C.G, Khan AA, Fritz DL (1992) Study on the ethology of crude oil ingestion by cattle. *Toxicologist* **12**: 336.

Coppock RW, Mostrom MS, Khan AA Stair EL (1995a) A review of nonpesticide phosphate ester-induced neurotoxicity in cattle. *Vet Hum Toxicol* **37**: 576–9.

Coppock RW, Mostrom MS, Khan AA, Semalulu SS (1995b) Toxicology of oilfield pollutants in cattle: a review. *Vet Hum Toxicol* **37**: 569–76.

Coppock RW, Mostrom MS, Stair EL, Semalulu SS (1996) Toxicopathology of oilfield poisoning in cattle: a review. *Vet Hum Toxicol* **38**: 36–42.

Couillard CM, Leighton FA (1989) Comparative pathology of Prudhoe Bay crude oil and inert shell sealants in chicken embryos. *Fundam Appl Toxicol* **13**: 165–73.

Couillard CM, Leighton FA (1990a) The toxicopathology of Prudhoe Bay crude oil in chicken embryos. *Fundam Appl Toxicol* **14**: 30–9.

Couillard CM, Leighton FA (1990b) Sequential study of the pathology of Prudhoe Bay crude oil in chicken embryos. *Ecotoxicol Environ Safety* **19**: 17–23.

Craig PH, Barth ML (1999) Evaluation of the hazards of industrial exposure to tricresyl phosphate: a review and interpretation of the literature. *J Toxicol Environ Health B Crit Rev* **2**: 281–300.

Darley HCH, Gray GR (1988) Composition and Properties of Drilling and Completion Fluids. 5th ed. Gulf Publishing Company, Houston, TX, pp. 58–9.

Dollahite JW, Pierce KR (1969) Neurologic disturbances due to triaryl phosphate toxicity. *Am J Vet Res* **30**: 1461–4.

Dungworth DL (1985) The respiratory system. In *Pathology of Domestic Animals*, 3rd edn. Jubb KVF, Kennedy PC, Palmer N (eds.). Vol. **2**: Academic Press, Toronto, Ontario, pp. 413–556.

Dungworth DL (1993) The respiratory system. In *Pathology of Domestic Animals*, 4th edn. Jubb KVF, Kennedy PC, Palmer N, (eds.). Vol. **2**: Academic Press, Toronto, Ontario, pp. 507–9.

Eaton G (1943) Paraffin poisoning in cattle. *Vet Rec* **55**: 19.

Edwards WC (1992) Investigation of animal health effects of sour gas acid forming emissions. In *Proceedings, International Workshop on Effects of Acid Forming Emissions in Livestock*, Coppock RW, Lillie LE (eds). Publication AECV92-P2, Vegreville, AB.

Edwards WC, Gregory DG (1991). Livestock poisoning from oilfield drilling fluids, muds and additives. *Vet Hum Toxicol* **33**: 502–4.

Edwards WC, Niles GA (1981) Dermatitis induced by diesel fuel on dairy cows. *Vet Med Small Anim Clin* **76**: 873–4.

Edwards WC, Zinn LL (1979) Petroleum hydrocarbon poisoning. *Vet Med Small Anim Clin* **74**: 1516–8.

Edwards WC, Coppock RW, Zinn LL (1979) Toxicosis related to the petroleum industry. *Vet Hum Toxicol* **21**: 328–37.

Environmental Protection Services (EPS) (1973). *Report of New Norway Scientific Committee Regarding A Gaswell Blowout October 2, 1973 Near Camrose*. Alberta Environmental Protection Services, Edmonton, AB, p. 36.

Fritz DL, Coppock RW (1992) Toxicopathy of diethylene glycol in cattle. *Toxicologist* **12**: 119.

Gardner DL (1977) Toxicology of waste petroleum products in cattle. *Vet Med Small Anim Clin* **72**: 1874–5.

Gibson EA Linzell JL (1948) Diesel oil poisoning in cattle. *Vet Rec* **60**: 60–1.

Gould DH (1998) Polioencephalomalacia. *J Anim Sci* **76**: 309–14.

Gupta RP, Abou-Donia MB (1994) Axonopathy. In *Principles of Neurotoxicology*, Chang LW (ed.). Marcel Dekker, Inc, New York, pp. 135–51.

Harris B (1992) Field investigation findings of the long-term effects in Alberta livestock exposed to acid forming emissions: survey following the Lodgepole blowout. In *Proceedings, International Workshop on Effects of Acid Forming Emissions in Livestock*, Coppock RW, Lillie LE (eds). Publication AECV92-P2, Vegreville, AB.

Hayes FL (1972) Studies on the effects of atmospheric hydrogen sulfide in animals. PhD Thesis. University of Missouri Graduate School, Columbia, MO.

Heitman JF (1986) Chemical stratification and environmental concerns of Oklahoma off site disposal pits. In *Proceedings of A National Conference on Drilling Muds*, Kamat RE (ed.). Environmental Groundwater Institute, Norman, OK.

Hixson EJ (1984) Consideration of dose for delayed neurotoxicity testing in hens: the relationship of neurotoxic dosage to acute $LD_{50}$ values. In *Delayed Neurotoxicity*, Cranmer JM, Hixson EJ (eds). Intox Press, Little Rock, AR, pp. 104–10.

International Agency for Research on Cancer (IRAC) (1984). *Polynuclear Aromatic Hydrocarbons*, Part 2, Carbon Blacks, Mineral Oils (Lubricant Base Oils and Derived Products) and Some Nitroarenes. IRAC Monographs on The Evaluation of Carcinogenic Risk of Chemicals to Humans **33**: 87–254.

Janowski TM, Chmielowiec J (1981) Sulfuroza bydla. *Med Weter* **137**: 265–8.

Johannsen FR Wright PL, Gordon DE, Levinskas GJ, Radue RW, Graham PR (1977) Evaluation of delayed neurotoxicity and dose–response relationships of phosphate esters in the adult hen. *Toxicol Appl Pharm* **41**: 291–304.

Johnson JH, Crookshank HR, Smalley HE (1980) Lithium toxicity in cattle. *Vet Hum Toxicol* **22**: 248–51.

Jubb KVF, Huxtable CR (1992) The nervous system. In *Pathology of Domestic Animals*, 4th edn. vol. 1. Jubb KVF Kennedy PC, Palmer N (eds)., Academic Press, Toronto, Ontario, pp. 267–439.

Julian RJ, Gal, DE, Butler D (1976) Diagnosis of triorthocresyl phosphate poisoning in cattle. *Proc Am Assoc Vet Laborat Diagn* **1**: 407–18.

Keith K, Bauder J, Wheaton J (2003) Coal Bed Methane (CBM). Frequently Asked Questions. Montana State University, Bozeman, MT.

Klavano GG, Christian RG (1992) Findings of a retrospective survey conducted after the Lodgepole sour gaswell blowout to determine if the natural occurrence of bovine abortions and fetal abnormalities increased. In *Proceedings, International Workshop on Effects of Acid Forming Emissions in Livestock* Coppock RW, Lillie LE (eds). Publication AECV92-P2, Vegreville, AB.

Khan AA, Coppock RW, Schuler MM (1992) Hepatic biochemical effects in cattle exposed to methanol and diethylene glycol. *Toxicologist* **12**: 338.

Komarnisky L (2003) The Effect of Sulfur Dioxide and Cold Stress on Cattle. PhD Thesis. University of Alberta, Edmonton, AB.

Kropp KG, Fedorak PM (1998) A review of the occurrence, toxicity, and biodegradation of condensed thiophenes found in petroleum. *Can J Microbiol* **44**: 605–22.

Lopez A, Prior MG, Leblanc D, Yong S, Albassam M, Lillie LE (1986) *Morphological Observations in Rats Exposed for Six Hours to An Atmosphere of 0, 56, or 420 mg $m^{-3}$ Hydrogen Sulfide*. Alberta: Alberta Environmental Centre Publication AECV86-A1, Vegreville, AB, p. 28.

Lopez A, Prior MG, Yong S, Albassam M, Lillie LE (1987) Biochemical and cytological alterations in the respiratory tract of rats exposed for 4 hours to hydrogen sulfide. *Fundam Appl Toxicol* **9**: 753–62.

Lopez A, Prior M, Yong S, Lillie LE, Lefebvre M (1988) Nasal lesions in rats exposed to hydrogen sulfide for four hours. *Am J Vet Res* **49**: 1107–11.

Lopez A, Prior MG, Reiffenstein RJ, Goodwin L (1989) Peracute toxic effects of inhaled hydrogen sulfide and injected sodium hydrogen sulfide in lungs of rats. *Fundam Appl Toxicol* **12:** 367–73.

Lusimbo WS, Leighton FA (1996) Effects of Prudhoe Bay crude oil on hatching success and associated changes in pipping muscles in embryos of domestic chicken (*Gallus gallus*). *J Wildl Dis* **32**: 209–15.

MacIntyre TM (1970) Effect of Bunker "C" oil on sheep. *Can J Anim Sci* **50**: 748–9.

Maydew MS, Kruckenberg SM, Schoneweis DA, Cook JE, Dennis SM (1983) Clinical signs and histopathological changes of the spinal cord in pigs treated with tri-*o*-cresyl phosphate. *Neurotoxicology* **4**: 163–72.

McCoy EP, Edwards WC (1980) Sodium ion poisoning in livestock from oilfield wastes. *Bovine Pract* **15**: 152–4.

McDowall FH, McGillivray WA, Reid CS (1957) Effects of ingestion of paraffins by ruminants II. Ingestion of heavy liquid paraffin by milking cows in relation to yield and composition of milk to properties and fat-soluble vitamins of butterfat. *New Zeal J Sci Tech* **38**: 839–51.

Metcalf RL (1984) Historical perspective of organophosphorus ester-induced delayed neurotoxicity. In *Delayed Neurotoxicity*, Cranmer JM, Hixson EL (eds). Intox Press, Little Rock, AR, pp. 7–22.

Messerli VW (1969) Vergiftungen in einer rinderherde durch dieselol. *Schweizer Archiv Fur Tierheilkunde* **111**: 642–4.

Moeller Jr RB , Kalasinsky VF, Razzaque M, Centeno JA, Dick EJ, Abdal M, Petrov II, DeWitt TW, Al-Attar M, Pletcher JM, Briskey EJ (1994) Assessment of the histopathological lesions and chemical analysis of feral cats to the smoke from the Kuwait oil fires. *J Environ Pathol Toxicol Oncol* **13**: 137–49.

Monlux AW, Schoeppel RJ, Pearson CC, Waller GR (1971) The effect of oilfield pollutants on vegetation and farm animals. *Am J Vet Res* **158**: 1379–90.

Mostrom MS, Khan AA, Fritz DL, Coppock RW (1993) Alterations in xenobiotic metabolizing enzymes and tissue ultrastructure in a calf with exposure to oil field chemicals. *Toxicologist.* **13:** 267.

Mostrom MS, Campbell CAJ, Coppock RW (1995) Use of livestock as monitors of environmental health following a petroleum pipeline break. *Pacific Basin Conference on Hazardous Waste*. Edmonton, AB.

Mostrom MS, Campbell CAJ (1996) 1994 *Livestock Field Investigation of Two Ranches Associated with a Pipeline Break. Publications Office, Communications Division*. Alberta Agriculture Food and Rural Development, Edmonton, AB, p. 351.

Munch JC (1956) Poisoning from oilwell wastes. *North Am Vet* **88**: 474.

Murphy EC, Beal WA, Kehew AE (1986) The effect of buried drilling fluid on shallow ground water in North Dakota. In *Proceedings of A National Conference on Drilling Muds*, Kamat RE (ed.). Environmental Groundwater Institute, Norman, OK.

Nicholson SS (1974) Bovine posterior paralysis due to organophosphate poisoning. *J Am Vet Med Assoc* **165**: 280–1.

Nordstrom GA (1975) A Study Of Calf Response To Ammonia And Hydrogen Sulfide Gases. MSc Thesis. University of Alberta Faculty of Graduate Studies, Edmonton, AB.

Oehme FW (1977) Veterinary toxicology: the epidemiology of poisonings in domestic animals. *Clin Toxicol* **10**: 1–21.

Oritsland NA, Engelhardt FR, Juck FA, Hurst RJ, Watts PD (1981) *Environmental Studies No 24: Effect of Crude Oil on Polar Bears*. Northern Affairs Publication No QS-8283-020-EE-A1, Ottawa, p. 268.

Parker WH, Williamson TF (1951) Paraffin poisoning in cattle. *Vet Rec* **63**: 430–2.

Pathan MH (1961) A case record of kerosene poisoning in two goats. *Ind Vet J* **38**: 559–61.

Peterson DD (1963) Fuel oil poisoning. *Vet Med* **58**: 748.

Prantner MM, Sosalla MJ (1993) Delayed organophosphate neurotoxicosis in four heifers. *J Am Vet Med Assoc* **203**: 1453–5.

Reagor JC, McDonald D (1980) Chromate poisoning in cattle: a case report. *Southwest Vet* **33**: 10–11.

Reid CSW (1957) Effects of ingestion of paraffins by ruminants. 1. The effect of different liquid paraffins on the feed intake of nonlactating monozygotic twin cows. *New Zeal J Sci Technol* **38**: 825–38.

Rousseaux CR, Audette RJ, Ellefson G (1982) Methyl alcohol toxicity in cattle. *Can Vet J* **23**: 252.

Round J (1992) Clinical syndromes in livestock associated with acid forming emissions. In *Proceedings, International Workshop on Effects of Acid Forming Emissions in Livestock*, Coppock RW, Lillie LE, (eds). Publication AECV92-P2, Vegreville, AB.

Rowe LD (1972) The Toxicity of Two Crude Oils and Kerosene to Cattle. MSc Thesis. Texas A & M University, College Station, TX, p. 55.

Rowe LD, Dollahite JW, Camp BJ (1973) Toxicity of two crude oils and of kerosene to cattle. *J Am Vet Med Assoc* **162**: 61–6.

Scott W (1924) Salt poisoning in cattle. *Vet J* **80**: 19–26.

Sesevicka L, Guoth J, Verner O, Hazlinsky M, Trebisov M (1979) A case of methanol poisoning in heifers. *Statni Zemedelske Nakladatelstivi Veterinaistvi* **29**: 414–5.

Shenton A (1937) Death following the administration of paraffin (lamp) oil. *Vet Rec* **49**: 454.

Somkuti SG, Lapadula DM, Chapin RE, Lamb JC, IV, Abou-Donia MB (1987) Reproductive tract lesions resulting from subchronic administration (63 days) of tri-*o*-cresyl phosphate in male rats. *Toxicol Appl Pharmacol* **89**: 49–63.

Stair EL, Mostrom MS, Coppock RW, Kosanke SD, Campbell CAJ (1996) Histopathologic lesions in cattle after exposure to multiphase sour condensate/gas pipeline leak. *Proceedings of Acidifying Emissions Symposium* (April 1996). Alberta Clean Air Strategic Alliance, Red Deer, AB.

Stober VM (1962) Vetraglichkeitsprufungen mit roh- und heizol an rindern. *Deutsche Tierarztliche Wochenschrift* **69**: 386–90.

Strosher M (1996) Investigations of Flare Gas Emissions in Alberta. Alberta Research Council, Edmonton, AB, p. 30.

Sprague GL, Castles TR (1987) Estimation of the delayed neurotoxic potential and potency for a series of triaryl phosphates using an *in vitro* test with metabolic activation. *Neurotoxicol* **6**: 79–86.

Spraker TR, Lowry LF, Frost KJ (1994) Gross necropsy and histopathological lesions in harbor seals. In *Marine Mammals and The Exxon Valdez* Loughlin TR (ed.). Academic Press, San Diego, CA, pp. 281–310.

Sugden EA (1981) Delayed neurotoxic effects caused by triaryl phosphate or triorthocresyl phosphate: a problem of definition. *Can Vet J* **22**: 210.

Sullivan ND (1985) The nervous system. In *Pathology of Domestic Animals*, 3rd edn. Jubb KVF,Kennedy PC, Palmer N (eds). Vol. 2, Academic Press, Toronto, Ontario, pp. 201–338.

Toofanian F, Aliakbari S, Ivoghli B (1979) Acute diesel fuel poisoning in goats. *Trop Anim Health Prod* **11**: 98–101.

Turner MA (1981) Dietary potassium–sodium imbalance as a factor in aetiology of primary ruminal tympany in dairy cows. *Vet Res Comun* **5**: 159–64.

Wallace MA, Blodgett DJ (1996) Lithium toxicosis in a cow. *Vet Hum Toxicol* **138**: 99–100.

Ward GM (1966a) Potassium metabolism of domestic ruminants: a review. *J Dairy Sci* **19**: 268–76.

Ward GM (1966b) Oral potassium chloride fatal to a cow. *J Am Vet Med Assoc* **148:** 543–4.

Wascom CD (1986) Oilfield pit regulations. A first for the Louisiana oil and gas industry. In *Proceedings of A National Conference on Drilling Muds* Kamat RW (ed.). Environmental Groundwater Institute, Norman, OK, pp. 434–50.

Whitelock C (1992) Producer's observations of the short-term effects of acid forming emissions in cattle. In *Proceedings, International Workshop on Effects of Acid Forming Emissions in Livestock*, Coppock RW, Lillie LE (eds). Publication AECV92-P2, Vegreville, AB.

Wilson RD, Rowe LD, Lovering SL, Witzel DA (1954) Acute toxicity of tri-*ortho*-cresyl phosphate in sheep and swine. *Am J Vet Res* **43**: 1954–7.

CHAPTER

# 55

# Polychlorinated biphenyls, polybrominated biphenyls, polychlorinated dibenzo-*p*-dioxins and polychlorinated dibenzofurans

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## INTRODUCTION

Polychlorinated biphenyls (PCBs), polybrominated biphenyls (PBBs), polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) form a large group of compounds, the polyhalogenated aromatic hydrocarbons (PHAHs), that are structurally related and are environmentally and biologically persistent (Safe, 1990, 1998; Van den Berg *et al.*, 1994; Fries, 1995; Huwe, 2002; Mandal, 2005; Schecter *et al.*, 2006).

PCBs and PBBs were produced commercially for a variety of applications while the PCDDs and PCDFs occur as by-products of industrial and natural processes. The structurally similar PCBs and PBBs are formed by substituting chlorine or bromine, respectively, for hydrogen on the biphenyl molecule that consists of two benzene rings (Figure 55.1). Theoretically, there are 209 possible PCB and PBB congeners considering the five chlorine or bromine binding sites on each ring. Each congener has been assigned a unique number from 1 to 209 in accordance with the rules of the International Union of Pure and Applied Chemistry (IUPAC). Commercial PCB and PBB products were mixtures of congeners that differed with respect to the extent and positions of chlorination or bromination. PCDDs are composed of two benzene rings connected by two oxygen atoms and contain four to eight chlorines, for a total of 75 congeners (Figure 55.1). PCDFs are also composed of two benzene rings. The rings have a single oxygen between them and have four chlorine binding sites available on each ring (Figure 55.1). There are 135 different PCDF congeners (DiCarlo *et al.*, 1978; Safe, 1990, 1998; Fries, 1995; Headrick *et al.*, 1999; Huwe, 2002; Mandal, 2005; Schecter *et al.*, 2006).

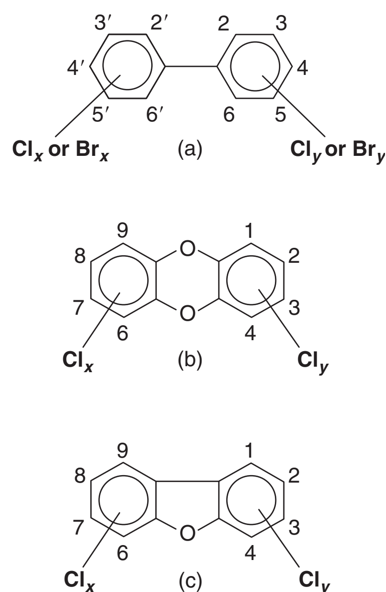


**FIGURE 55.1** Structures and numbering of generic (a) PCB/PBB, (b) PCDD and (c) PCDF molecules.

Certain approximate stereoisomers in this group, often referred to collectively as dioxins and dioxin-like compounds, induce a common suite of effects and have a common mechanism of action mediated by binding of the PHAH ligand to a specific high-affinity cellular protein.

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This group of dioxins and dioxin-like chemicals includes 7 PCDD congeners, 10 PCDF congeners and 12 PCB congeners. While the PBB congeners analogous to the 12 PCB congeners could also be considered dioxin-like chemicals, the relatively short commercial lifespan and restricted environmental distribution of PBBs generally precludes them from consideration. The prototype for the dioxins is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Toxicity and persistence of the PHAHs are determined by structure, with lateral substitutions on the ring resulting in the highest degree of toxicity. For the PCDDs and PCDFs, congeners with chlorines in the 2, 3, 7 and 8 positions fall into this category. The dioxin-like PCB congeners are the non-*ortho*- and mono-*ortho*-substituted compounds with none or no more than one chlorine (or bromine) on the 2, 2′ , 6 or 6′ position (Safe, 1990; Fries, 1995; Headrick *et al.*, 1999; Huwe, 2002; Mandal, 2005; Schecter *et al.*, 2006).

Mechanistic studies indicate that the toxic and biochemical effects associated with exposure to TCDD and its approximate stereoisomers are mediated by initial binding of the chemical to the cytosolic aryl hydrocarbon receptor (AhR) present in target tissues and organs. There is a correlation between the AhR binding affinity of these chemicals and their structure–toxicity relationships, which supports the idea that the AhR is involved in the mediation of responses induced by the TCCD-like PCDD, PCDF and PCB congeners (Okey *et al.*, 1994; Hahn, 1998, 2002; Safe, 1998; Denison *et al.*, 2002; Denison and Nagy, 2003; Mandal, 2005).

The common mechanism of action of TCDD and related compounds allows for use of the toxic equivalency factor (TEF) approach to estimate the TCDD-like toxicity of complex mixtures containing chemicals that resemble TCDD. The TEF value for a TCDD-like congener is defined as the potency of the individual congener relative to TCDD. Using the TEF concept, TCDD toxic equivalents (TEQs), which is the sum of the product of the concentration of each congener and its respective TEF, can be calculated for any complex mixture containing TCDD-like chemicals to provide an estimation of the total TCDD-like toxicity (Safe, 1998; Huwe, 2002).

The PCDDs, PCDFs and PCBs are widely distributed into the global environment and, due to long-range transport, can be very resistant to environmental degradation and metabolism. As a result, they readily accumulate in the food chain with the greatest tissue concentrations being found in species at the higher trophic levels. Residues have been detected in a variety of animal species, including humans (Van den Berg *et al.*, 1994; Safe, 1998). In some situations, the environmental concentrations of these contaminants are such that there is a health risk to animals and humans. Because of this risk, there continues to be an effort on the part of state, federal and international regulatory agencies to minimize exposure to this significant class of environmental contaminants.

# BACKGROUND

## Sources of PCBs, PCDDs, PCDFs and PBBs

### *PCBs*

PCBs were first synthesized by Schmidt and Schultz in 1881. Commercial production of PCBs for a variety of uses began in the United States in 1929 until 1977, primarily by the Monsanto Corporation (Kimbrough, 1987, 1995; Tanabe, 1988; Headrick *et al.*, 1999). They were used in closed use systems such as electrical transformers, capacitors, and heat transfer and hydraulic systems. For a period of time, PCBs also had a large number of open-ended applications. They were used in paints, polymers and adhesives, as lubricants, plasticizers, fire retardants, immersion oils, vehicles for pesticide application and as agents for the suspension of pigments in carbonless copy paper (Safe, 1990; Headrick *et al.*, 1999). The PCB products that were manufactured by Monsanto in the United States had the trade name Aroclor followed by four digits that identified the particular mixture. The first two digits referred to the 12 carbon atoms of the biphenyl molecule and the last two digits referred to the percent of chlorine in the mixture, by weight. Aroclors 1221, 1232, 1242, 1254 and 1260 were the commercial PCB products that were produced by Monsanto, containing 21%, 32%, 42%, 54% and 60% chlorine by weight, respectively. Similar commercial PCB mixtures were produced by other manufacturers worldwide including the Clophens (Bayer, Germany), Pheoclors and Pyralenes (Prodelec, France), Fenclors (Caffro, Italy) and Kanechlors (Kanegafuchi, Japan) (Kimbrough, 1987, 1995; Safe, 1994).

The physical and chemical properties of PCBs, such as high stability, inertness and dielectric properties, that were advantageous for many industrial purposes, led to the international use of PCBs in large quantities (Tanabe, 1988). For example, the estimated cumulative production of PCBs in the United States between 1930 and 1975 was 700,000 tons and 1.2 million tons were estimated to have been produced worldwide. Domestic sales of PCBs in the United States during this time period totaled 627,000 tons (Kimbrough, 1987, 1995; Tanabe, 1988). As a result of widespread use, PCBs were identified in environmental media and biota as early as the 1960s. After the discovery of their widespread environmental contamination in the 1970s, PCB production decreased and eventually ceased (Tanabe, 1988). In 1971, Monsanto voluntarily stopped production of PCBs for open-ended uses and subsequently only the lower chlorinated biphenyls were produced (Aroclor 1242 and 1016). In 1977, Monsanto ceased production entirely (Kimbrough, 1987, 1995).

Although PCBs are no longer used commercially because of their persistence, they are still present in the environment. About 31% (370,000 tons) of all the PCBs produced is

present in the global environment. It is estimated that 780,000 tons are still in use in older electric equipment and other products, deposited in landfills and dumps or in storage (Tanabe, 1988).

### *PCDDs and PCDFs*

PCDDs and PCDFs are by-products that are formed during the synthesis of certain industrial halogenated aromatics chemicals, by-products from other commercial processes and by-products of combustion (Safe, 1990). Some of the important industrial sources of PCDDs and PCDFs have included their formation as by-products in the production of PCBs, chlorinated phenols and chlorinated phenol-derived chemicals, hexachlorobenzene, technical hexachlorocyclohexanes, and chlorides of iron, aluminum and copper. PCDDs and PCDFs have also been identified in effluents, wastes, and pulp samples from the pulp and paper industry and in finished paper products. Emissions from municipal and hazardous waste incinerators as well as home heating systems that use wood and coal, diesel engines, forest and grass fires and agricultural and backyard burning contain PCDDs and PCDFs. Another contribution might come from naturally formed PCDDs and PCDFs, which have been detected in deep soils and clays from the southern United States and Germany (Safe, 1990; Huwe, 2002).

The United States Environmental Protection Agency (EPA) estimated that annual emissions of PCDDs and PCDFs decreased from 13.5 to 2.8 kg TEQ/year between 1987 and 1995. This was due primarily to improvements in incinerator performance and removal of incinerators that could not meet emission standards. Other regulations, including bans or restrictions on the production and use of chemicals such as the wood preservative pentachlorophenol (PCP), the phase-out of leaded gasoline that contained halogenated additives and the elimination of chlorine bleaching in the pulp industry also contributed to reducing concentrations of PCDDs and PCDFs (Huwe, 2002).

### *PBBs*

PBBs were manufactured for use as flame retardants in industrial and consumer products (Damstra *et al.*, 1982). It is estimated that approximately 13 million pounds were produced in the United States from 1970 to 1976 and used by more than 130 companies for incorporation into plastic products that included business machine housings, radios, televisions, thermostats, electric shavers, hand tools and various automotive parts (DiCarlo *et al.*, 1978; Headrick *et al.*, 1999). Three commercial PBB products were manufactured in the United States: hexabromobiphenyl, octabromobiphenyl and decabromobiphenyl (DiCarlo *et al.*, 1978; Hardy, 2000). Hexabromobiphenyl was the predominant product with approximately 11.8 million pounds being produced solely by Michigan Chemical Company (DiCarlo *et al.*, 1978). Over 98% of the hexabromobiphenyl was produced as FireMaster BP-6 with the remainder being produced as FireMaster FF-1 (Hesse and Powers, 1978) after addition of an anti-caking agent to FireMaster BP-6. Michigan Chemical Company stopped production of their PBB products in 1974 (DiCarlo *et al.*, 1978). White Chemical Company and Hexcel Corporation manufactured octa- and decabromobiphenyl in the United States until 1979 (IARC, 1986). Production of decabromobiphenyl was discontinued in Great Britain in 1977 and Germany stopped production of brominated biphenyls in 1985. In 2000, France discontinued the remaining commercial production of PBBs (Hardy, 2000).

While FireMaster BP-6 was sold as a hexabromobiphenyl, it consisted of 18 different congeners, some of which were tetra-, penta- hepta- and octabromobiphenyls. The predominant hexabromobiphenyl was 2,4,5,2′,4′,5′-hexabromobiphenyl. Commercial octabromobiphenyl contained at least four congeners; a heptabromobiphenyl, isomeric octabromobiphenyls and a nonabromobiphenyl, which constituted the majority of the mixture. Commercial decabromobiphenyl was over 95% decabromobiphenyl with the remainder being a nonabromobiphenyl and an octabromobiphenyl (DiCarlo *et al.*, 1978).

## Environmental fate of PCBs, PCDDs, PCDFs and PBBs

The release of PCBs into the environment primarily has been the result of leaks, spills and improper disposal. As stated previously in the section PCBs, it is estimated that approximately 370,000 tons of PCBs are present in the global environment (Tanabe, 1988). The volatility of PCBs allows their evaporation from water surfaces and movement through the atmosphere, resulting in widespread environmental dispersal (Headrick *et al.*, 1999). PCDDs and PCDFs are released into the atmosphere primarily by combustion sources and by evaporation from PCDD/PCDF-containing soils and water. Similar to PCBs, the PCDDs and PCDFs can be transported long distances by winds, contributing both to general background concentrations and to contamination of remote areas far from the original source. PCBs, PCDDs and PCDFs are removed from the atmosphere by physical processes such as wet and dry deposition and vapor uptake and are deposited on soils, surface waters and plant surfaces. Most of the PCBs, PCDDs and PCDFs that are deposited on surface waters sorb onto suspended sediments. Once bound to soil and sediment, these chemicals generally remain fixed except for bulk transport due to soil erosion, flooding and dredging (Dickson and Buzik, 1993). Ingestion of these compounds by animals results in their preferential bioaccumulation and biomagnification in higher trophic levels of the food chain (Safe, 1994).

Because PBBs were manufactured for a relatively short time and because of their restricted use, they are not considered to be a significant environmental contaminant with the exception of specific locations in Michigan related to production and disposal. Environmental losses of PBBs from manufacturing sites were estimated to be 0.11% into the atmosphere as particulate matter, negligible losses to storm sewers and 5% as solid waste to landfills. Soil samples collected from the loading and bagging area of the Michigan Chemical Company plant manufacturing FireMaster BP-6 and FF-1 contained 3500 and 2500 ppm (mg/kg) PBBs, respectively (DiCarlo *et al*., 19787). Like PCBs, PCDDs and PCDFs, PBBs are very stable compounds and persist in the environment. Studies have indicated that PBBs have a high affinity for soil and undergo very little degradation and translocation into vegetation (Fries, 1985). Like PCBs, PCDDs and PCDFs, the PBBs are very lipophilic and have the potential to bioaccumulate and biomagnify in the food chain (Damstra *et al*., 1982).

It is important to remember that the commercial PCB products and PCBs, PCDDs and PCDFs in environmental extracts are complex mixtures of congeners. Because of various physical and biological processes, the composition of the commercial PCB mixture and an environmental PCB/PCDD/PCDF mixture may vary significantly from one another. Thus, the impacts of PCBs, PCDDs and PCDFs on a environment and biota are due to the individual components of these mixtures and their additive and/or non-additive (synergistic/antagonistic) interactions with themselves and other classes of pollutants (Safe, 1994).

## Exposure to PCBs, PCDDs, PCDFs and PBBs

There are a number of ways by which animals can be and have been exposed to PCBs, PCDDs/PCDFs and PBBs. Some of the scenarios described involve ingestion of low concentrations of these chemicals through consumption of environmentally contaminated feed or feed components while other scenarios involve accidental incorporation of the chemical into the feed resulting in exposure to relatively high concentrations of the contaminant. Usually, food animal exposures to PCBs, PCDDs and PCDFs occur below concentrations resulting in acute toxicity. Clinical signs are not evident and there often is not a noticeable economic impact on the health of the animal, although there may be detectable contamination of food products such as milk, meat and eggs (Headrick *et al*., 1999).

### *PCBs*

During the 1940s and 1950s, silos constructed with concrete were sealed with a PCB-containing paint, which eventually peeled off from the walls resulting in contaminated silage. Dairy and beef cattle were exposed to the paint in the feed resulting in accumulation of PCBs in adipose tissue. As a result, food products such as milk and meat contained detectable concentrations of PCBs. Examples of other exposure incidents resulting in PCB residues in food animals were summarized by Headrick *et al*. (1999). These include consumption of tar paper by veal calves, consumption of fish viscera by swine, pullet consumption of feed containing PCB-contaminated fat added during processing, exposure of chickens to ceiling insulation and fiberglass insulation that contained PCBs and treatment of boars with a topical pesticide containing PCB-contaminated oil. In 1979, a spare electrical transformer in a Montana hog slaughter plant was damaged allowing PCBs to leak into the plant' s drainage system. The PCBs and animal wastes were processed into grease and animal feed, which was distributed and subsequently fed to hogs, beef cattle, dairy cattle, chickens and mink in 19 states in the United States and in Canada and Japan before the contamination was detected. Ultimately, 800,000 chickens, nearly 4,000,000 eggs, 4000 hogs, 74,000 bakery items, 800,000 lb of animal feeds and 1,200,000 lb of grease were destroyed with an estimated cost of recalls and destroyed products amounting to over $2 million (Headrick *et al*., 1999).

### *Michigan PBB Incident*

The most extensive exposure of food animals and humans to PBBs occurred in Michigan in the mid-1970s. In 1970, the Michigan Chemical Company began to manufacture PBBs under the trade name of FireMaster BP-6 in St. Louis, MI (Fries, 1985). FireMaster BP-6 was a mixture of PBB congeners containing two to eight bromines. The major constituents of FireMaster BP-6 were 2,2′,4,4′,5,5′-hexabromobiphenyl (56%) and 2,2′,3,4,4′,5,5′-heptabromobiphenyl (27%) (Damstra *et al*., 1982). In 1972, the company changed the formulation of the retardant by grinding BP-6 and adding 2% calcium silicate as an anti-caking agent. This new formulation, now called FireMaster FF-1, was a white powder as opposed to brown flakes, which was the appearance of BP-6 (Fries, 1985).

In May of 1973, 650 lb of FF-1 were mistakenly included in a shipment of feed-grade magnesium oxide to a feed mill in Climax, MI. Michigan Chemical Company, in addition to producing FireMaster FF-1, also produced the magnesium oxide product, which had an appearance identical to FireMaster FF-1 and was sold under the trade name NutriMaster. Normally the two products were packaged in paper bags with unique color codes. However, during a paper shortage, both FireMaster and NutriMaster were packaged in plain brown bags differentiated only by the product names stenciled on the bags. Both products were stored in the same warehouse (Dunckel, 1975; Fries, 1985).

A portion of the magnesium oxide that was shipped to the Climax feed mill was used to mix feeds primarily for

dairy cattle at that location, which were subsequently shipped to area farms or other retail units. The remaining magnesium oxide was shipped to other mills within the state and used in feeds mixed at those locations. Feeds that were not formulated to contain magnesium oxide also became contaminated because of carry-over from the contaminated feed-mixing equipment (Fries, 1985). In total, 101 feed mills were found to be affected (Dunckel, 1975).

Most of the high-level exposures occurred during the fall of 1973 before sale of the initial batch of feed was stopped in December 1973 because of dairy producer complaints of animal health problems. Three initial feed preparations containing different concentrations of PBBs were Feed No. 405, which had 2.4 ppm PBBs, No. 410 having 1790 ppm PBBs and No. 407, which contained 4300 ppm PBBs. The highest feed concentration reported was 13,500 ppm PBBs (DiCarlo *et al*., 1978; Damstra *et al*., 1982). Low-level contamination of feed continued beyond the chance identification of PBBs as the contaminant in April 1974 because of their persistence. In 1974, 68% of 1770 feed samples collected in Michigan contained PBB residues. Resampling in 1975 indicated that 6% of 1208 feed samples were contaminated and in 1976, only 0.3% of 663 samples contained PBBs (DiCarlo *et al*., 1978). Shortly after PBBs were identified as the feed contaminant, the US Food and Drug Administration (FDA) set a temporary guideline of 1 ppm PBBs in milk fat, meat and poultry, 0.1 ppm in whole eggs and 0.3 ppm in animal feeds (DiCarlo *et al*., 1978). The Michigan Department of Agriculture started to identify and quarantine all dairy herds with PBB concentrations in excess of 5 ppm. Because of the long half-life of PBBs, the decision was made to depopulate affected farms and to dispose of the animals at a burial site in northern Michigan. Initially, 9400 head of cattle, 2000 swine, 400 sheep and 2,000,000 chickens were buried in addition to 865 tons of feed and animal by-products such as cheese, butter, dry milk products and eggs (DiCarlo *et al*., 1978; Damstra *et al*., 1982; Fries, 1985; Headrick *et al*., 1999). In October of 1974, FDA lowered the guidelines for PBBs in milk and meat from 1 ppm to 0.3 ppm, which resulted in disposal of 20,000 additional head of cattle as well as 3900 swine and 1100 sheep (Damstra *et al*., 1982). In total, 507 farms were affected (Dunckel, 1975). In response to increasing concerns about the effects of PBBs on human and animal health, the Michigan legislature lowered the PBB tolerance to 0.02 ppm in body fat of all dairy cattle offered for slaughter in 1977. A small number of dairy producers who had repopulated after the initial quarantine in 1974 continued to have violative cattle because of residual contamination on their facilities, although this number was less than 2% of all culled cows. Over the next 5 years, the number of violative cattle diminished rapidly as they approached the end of production.

A potential environmental source of exposure to animals on contaminated farms was the soil, if animals had direct access to the contaminated soil. Four years after the initial PBB contamination incident, there were several farms in Michigan that exceeded the 20 ppb tolerance for PBBs in fat of cull cows even though farmers had depopulated, cleaned up the premises and repopulated with clean animals. The major route of animal exposure was through soil consumption because crops grown on contaminated soils did not accumulate PBBs (Fries, 1985). Major sources of PBBs on the farms were soils of fields on which manure had been spread and soil of unpaved exercise lots where feces were deposited and feed was spilled. It was shown that all of the problem farms were using unpaved lots that had been used in 1974 for some part of their production system and that ingestion of soil from unpaved lots was the major route of PBB exposure causing animal contamination on problem farms (Fries, 1995).

### *PCDDs and PCDFs*

For livestock, atmospheric deposition of PCDDs and PCDFs onto forage and soils is assumed to be the major source of exposure (Huwe, 2002). Extensive field studies in contaminated areas have demonstrated a positive correlation between PCDD and PCDF concentrations in animals and their soil contact (Van den Berg *et al*., 1994). The relative importance of soil depends upon the species of animal and the management system. Ruminants are expected to be more vulnerable to PCDD and PCDF exposure than poultry and swine because of their grazing activities. The use of pasture is of particular importance because consumption of contaminated plants is additive to soil ingestion of PCDDs and PCDFs. Volatilization of PCDDs and PCDFs from soil and deposition on plants is an important pathway of animal exposure when forage is abundant. However, that soil may be more important when grazing is sparse. The soil ingestion pathway is not limited to grazing animals. Cattle confined to unpaved lots consume small amounts of soil that can lead to product residues. Although most poultry and pork production is conducted in confined operations, the soil ingestion pathway of exposure may be important when these species have access to contaminated soil (Fries, 1995).

In addition to exposure to PCDDs and PCDFs through consumption of contaminated soil and/or forage, a number of other incidents of animal exposure to these compounds have been reported. The first known exposure to PCDDs occurred in the late 1950s. "Chick edema disease", as the condition was initially called, resulted from consumption of feed containing fat contaminated with a number of PCDD congeners originating from the production of PCP. In 1975, several horses died in Missouri as a result of exposure to TCDD-contaminated waste oil that was used for dust control on horse tracks. The oil contained waste from a hexachlorophene manufacturing plant and it had also been used for dust control on the unpaved streets of Times

Beach. The high concentrations of TCDD in the residential soils led to purchase and evacuation of the town by the US government (Fries, 1995). In a geographical survey, high concentrations of TEQs in cattle were strongly correlated to PCP-treated wood used for fence posts and feed bunks at several beef cattle operations across 13 states in the United States. Although the use of PCP was restricted in the 1980s, PCP was used heavily on farms as a wood preservative in the late 1970s (Huwe, 2002). In an EPA survey of poultry, two chicken samples were found with PCDD concentrations considerably above background. The origin of this contamination was ball clay, which had been added as an anti-caking agent to soy meal in the feed. This same contaminated feed was also used by the catfish industry and resulted in high TEQ concentrations in catfish from Arkansas (Huwe, 2002).

Several dioxin contamination incidents have occurred in Europe. In 1998, during routine monitoring, dairy products were identified that had dioxin concentrations that were 2–4 times higher than background concentrations. The source of the contamination was traced to citrus pulp used as a cattle feed component. The citrus pulp and contaminated feeds were immediately removed from the market. In another incident, PCB/PCDD/PCDF-contaminated oil was added to recycled fat used as an additive in animal feeds. The affected feeds contaminated Belgian poultry, dairy and meat, and were discovered only after toxic effects characteristic of "chick edema disease" were seen in chickens. Animals and products were quarantined, recalled and eventually destroyed. The incident led to international recalls and bans against Belgian products (van Larebeke *et al*., 2001; Bernard *et al*., 2002; Huwe, 2002).

## Differential toxicity

Comparison of the relative toxicities of PCDDs, PCDFs and PCBs as three separate classes suggests that the dioxins are more toxic than the furans, which in turn are more toxic than the PCBs. It was concluded that PBBs are slightly more toxic than their chlorinated counterparts (McConnell, 1985). Studies also suggest that the toxicity of commercial PCB formulations increases with increasing chlorine content (Aroclor 1221 $<$ 1232 $<$ 1242 $<$ 1248 $<$ 1254), but highly chlorinated Aroclors 1260, 1262, and 1268 are less toxic than Aroclor 1254 (Tanabe, 1988).

2,3,7,8-TCDD binds with the greatest affinity to the AhR and is the most potent isomer in terms of toxicity. PCDDs and PCDFs substituted with chlorines in at least three of the four lateral positions (2,3,7, 8; Figure 55.1) bind most strongly to the AhR. If chlorines are removed from these lateral positions or if chlorines are added to the non-lateral positions (1,4,6,9; Figure 55.1), binding affinities decrease markedly, as do toxicities. There are seven 2,3,7,8-substituted PCDDs (2,3,7,8-TCDD; 1,2,3,7,8-penta CDD; 1,2,3,4,7,8-hexaCDD; 1,2,3,6,7,8-hexaCDD; 1,2,3,7,8,9-hexaCDD; 1,2,3,4,6,7,8-heptaCDD and octaCDD) and ten 2,3,7,8-substituted PCDFs (2,3,7,8-TCDF; 1,2,3,7,8-pentaCDF; 2,3,4,7,8-pentaCDF; 1,2,3,4,7,8-hexaCDF; 1,2,3,6,7,8-hexaCDF; 1,2,3,7,8,9-hexaCDF; 2,3,4,6,7,8-hexaCDF; 1,2,3,4,6,7,8-heptaCDF; 1,2,3,4,6,7,8-heptaCDF and octaCDF) that induce TCDD-like toxicity. There are 209 theoretically possible PCB congeners having different toxic and biologic responses. The most toxic PCB congeners have four or more chlorine atoms at both the para (4,4′) and meta positions (3,3′,5,5′; Figure 55.1) in the biphenyl rings, but no chlorine (bromine) atoms in the ortho positions (2,2′,6,6′; Figure 55.1). Of the 209 PCB congeners, four PCB congeners (3,3′,4,4′-tetraCB (IUPAC 77), 3,4,4′,5-tetraCB (IUPAC 81), 3,3′,4,4′,5- pentaCB (IUPAC 126) and 3,3′,4,4′,5,5′-hexaCB (IUPAC 169)) are approximate stereoisomers of the highly toxic 2,3,7,8-TCDD and thus bind to the AhR and elicit toxic and biologic responses typical of TCDD, although at higher doses. These four congeners are considered to be coplanar because both rings of the biphenyl molecule lie in the same plane, which enables binding to the AhR (Tanabe, 1988). There are eight PCB congeners with chlorine substitution in one of the ortho positions (2,2′,6,6′). These congeners may have partial coplanarity and thus exhibit lower competitive binding affinities for the AhR and lower toxic potency. The mono-*ortho* PCB congeners are 2,3,3′,4,4′-pentaCB (IUPAC 105); 2,3,4,4′,5-pentaCB (IUPAC 114); 2,3′,4,4′,5-pentaCB (IUPAC 118); 2′,3,4,4′,5-pentaCB (IUPAC 123); 2,3,3′,4,4′,5-hexaCB (IUPAC 156); 2,3,3′,4,4′,5-hexaCB (IUPAC 157); 2,3′,4,4′,5,5′-hexaCB (IUPAC 167) and 2,3,3′,4,4′,5,5′-heptaCB (IUPAC 189) (Poland and Knutson, 1982; Tanabe, 1988; Safe, 1990, 1998; Giesy and Kannan, 1998; Huwe, 2002; Whyte *et al*., 2004).

## TEFs

The relationship between the structure of individual PCDD, PCDF and PCB congeners and their toxicity is the basis of TEFs and the TEQ approach. The TEQ approach is used to determine the toxic potency of complex mixtures of PCDDs, PCDFs and PCBs found in the environment. Assuming a similar mechanism of action (binding to the AhR), the potency of each chemical in a mixture to cause a particular toxic or biological effect can be expressed as a fraction of the potency of 2,3,7,8-TCDD to cause the same effect. Thus, the TEF is a ratio of EC50 (TCDD-like chemical)/EC50 (TCDD). 2,3,7,8-TCDD has been assigned a TEF value of 1.0. Based on a variety of biological endpoints, relative potency factors (RPFs) are assigned to the different TCDD-like congeners. All RPFs for an individual congener are evaluated to derive a consensus value (the TEF) that describes an order-of-magnitude potency for that

**TABLE 55.1 Summary of World Health Organization (WHO) 2005 Toxic Equivalency Factor (TEF) values***

| Compound | WHO 2005 TEF |
|---|---|
| *Chlorinated dibenzo-p-dioxins* | |
| 2,3,7,8-TCDD | 1 |
| 1,2,3,7,8-PeCDD | 1 |
| 1,2,3,4,7,8-HxCDD | 0.1 |
| 1,2,3,6,7,8-HxCDD | 0.1 |
| 1,2,3,7,8,9-HxCDD | 0.1 |
| 1,2,3,4,6,7,8-HpCDD | 0.01 |
| OCDD | 0.0003 |
| *Chlorinated dibenzofurans* | |
| 2,3,7,8-TCDF | 0.1 |
| 1,2,3,7,8-PeCDF | 0.03 |
| 2,3,4,7,8-PeCDF | 0.3 |
| 1,2,3,4,7,8-HxCDF | 0.1 |
| 1,2,3,6,7,8-HxCDF | 0.1 |
| 1,2,3,7,8,9-HxCDF | 0.1 |
| 2,3,4,6,7,8-HxCDF | 0.1 |
| 1,2,3,4,6,7,8-HpCDF | 0.01 |
| 1,2,3,6,7,8,9-HpCDF | 0.01 |
| OCDF | 0.0003 |
| *Non-ortho substituted PCBs* | |
| 3,3′,4,4′-tetraCB (PCB 77) | 0.0001 |
| 3,4,4′,5-tetraCB (PCB 81) | 0.0003 |
| 3,3′,4,4′,5-pentaCB (PCB 126) | 0.1 |
| 3,3′,4,4′,5,5′-hexaCB (PCB 169) | 0.03 |
| *Mono-ortho substituted PCBs* | |
| 2,3,3′,4,4′-pentaCB (PCB 105) | 0.00003 |
| 2,3,4,4′,5-pentaCB (PCB 114) | 0.00003 |
| 2,3′,4,4′,5-pentaCB (PCB 118) | 0.00003 |
| 2′,3,4,4′,5-pentaCB (PCB 123) | 0.00003 |
| 2,3,3′,4,4′,5-hexaCB (PCB 156) | 0.00003 |
| 2,3,3′,4,4′,5′-hexaCB (PCB 157) | 0.00003 |
| 2,3′,4,4′,5,5′-hexaCB (PCB 167) | 0.00003 |
| 2,3,3′,4,4′,5,5′-heptaCB (PCB 189) | 0.00003 |

*Van den Berg *et al*. (2006).

**TABLE 55.2 Concentrations of PCDD PCDF and PCB congeners and TEQs in fish collected downstream from a contaminated industrial site**

| Compound | TEF | Congener and TEQs concentration (pg/g) |
|---|---|---|
| *PCDDs* | | |
| 2,3,7,8-TCDD | 1.00000 | 0.5 (0.5) |
| 1,2,3,7,8-PeCDD | 1.00000 | 0.3 (0.3) |
| TEQs from PCDDs | | 0.8 |
| % total TEQs | | 1.8 |
| *PCDFs* | | |
| 2,3,7,8-TCDF | 0.10000 | 3.0 (0.3) |
| 2,3,4,7,8-pentaCDF | 0.30000 | 4.6 (1.4) |
| TEQs from PCDFs | | 1.7 |
| % total TEQs | | 3.8 |
| *Non-ortho PCBs* | | |
| #126 | 0.10000 | 410 (41.0) |
| TEQs from non-*ortho* PCBs | | 41.0 |
| % total TEQs | | 90.5 |
| *Mono-ortho PCBs* | | |
| #123 | 0.00003 | 38000 (1.1) |
| #156 | 0.00003 | 23000 (0.7) |
| TEQs from mono-*ortho* PCBs | | 1.8 |
| % total TEQs | | 4.0 |
| *Grand total TEQs* (pg/g wet weight) | | 45.3 |

congener. The toxic potency of a mixture of PCDDs, PCDFs and/or PCBs is estimated by multiplying the concentrations of individual congeners by their respective TEFs and summing the products to yield total TEQs. The total TEQs express the toxicity as if the mixture were pure TCDD (Safe, 1990, 1998; Dickson and Buzik, 1993; Van den Berg *et al*., 1994, 2006; Fries, 1995; Whyte *et al*., 2004; Schecter *et al*., 2006). Several assumptions are made when using the TEF approach: (1) the effects of individual PCDDs, PCDFs and/or PCBs in a mixture are additive; (2) only tissue and environmentally persistent organochlorine compounds have been assigned TEFs and (3) all of these compounds bind to the AhR and elicit receptor-mediated biochemical and toxic responses (Safe, 1998). The TEQ approach and current values (Table 55.1) have been adopted internationally as the most appropriate way to estimate the potential health risk of mixtures of TCDD-like chemicals (Schecter *et al*., 2006).

Table 55.2 illustrates how the TEF approach can be used. The table presents the concentrations of various PCDD, PCDF and PCB congeners found in fish collected downstream from the abandoned site of a manufacturing facility that used PCBs. By multiplying the congener concentration detected in the fish by the appropriate TEF value, the quantity of the TCDD-like toxicity contributed by each congener can be determined. In the example given, the PCDDs contribute 0.8 pg TEQ/g, the PCDFs contribute 3.8 pg TEQs/g and the non-*ortho* PCBs contribute 41 pg TEQs/g or over 90% of the TCDD-like activity in the fish sample. While the relative toxicities of the mono-*ortho* PCB congeners are relatively low, as indicated by their TEF values, their high concentrations in the fish allow them to contribute a measurable quantity of TCDD-like activity.

# TOXICOKINETICS

## PCBs and PBBs

Because commercial PCB and PBB products are mixtures of individual congeners that differ in the number and position of chlorine or bromine atoms and thus differ in terms of their biological activities, it is difficult to accurately assess their absorption, distribution, metabolism and elimination. A number of experiments have been conducted with a variety of species including cows, pigs, rats and birds on the absorption, distribution, metabolism and elimination of the commercial PBB mixture, FireMaster

BP-6, which have been summarized in an extensive review by Fries (1985). Because of the similarities between PCBs and PBBs, information pertaining to one can generally be applied to the other.

In general, PBBs are rapidly and extensively absorbed, with absorption being inversely dependent on the number of bromine atoms (Damstra *et al.*, 1982; Fries, 1985). For example, less than 10% of a dose of $C^{14}$-labeled 2,2′,4,4′,5,5′-hexabromobiphenyl was eliminated in rats (Matthews *et al.*, 1977; Fries, 1985) indicating almost complete absorption compared to 62% fecal elimination of $C^{14}$-labeled octobromobiphenyl 24 h after dosing suggesting incomplete absorption (Norris *et al.*, 1975; Fries, 1985).

PBBs are widely distributed throughout the body of all species studied. Initial concentrations are generally greatest in the liver and adipose tissue (Damstra *et al.*, 1982; Fries, 1985) with highest equilibrium concentrations on a wet tissue basis being in adipose tissue (Miceli and Marks, 1981; Damstra *et al.*, 1982; Fries, 1985). Concentrations of PBBs in muscle and organ tissues are usually an order of magnitude lower (Fries, 1985) compared to adipose tissue. Generally, differences in concentration between tissues can be attributed, at least in part, to variations in fat content of the tissues (Fries, 1985).

Individual PBB congeners in FireMaster BP-6 undergo hydroxylation by metabolic routes that are similar for the related PCBs with the rate of metabolism being determined primarily by the position of bromine atoms on the ring and secondarily by the bromine content of the molecule (Damstra *et al.*, 1982). *In vivo* studies suggest that, like PCBs, metabolism can occur if there are two adjacent unbrominated positions (Matthews *et al.*, 1978; Fries, 1985), although no studies indicated significant metabolism of congeners comprising the commercial PBB mixture (Fries, 1985).

Elimination of individual PBB congeners occurs at different rates with those congeners that are more slowly removed becoming more concentrated in tissues relative to those congeners that are more rapidly eliminated. The predominant congener in FireMaster BP-6, 2,2′4,4′,5,5′-hexabromobiphenyl, is the most persistent congener in the various species studied (Damstra *et al.*, 1982). PBBs are eliminated primarily by biliary excretion into the feces, but fecal concentrations are low compared to whole-body concentrations (Damstra *et al.*, 1982; Fries, 1985). For example, less than 7% of an intravenous dose of 2,2′, 4,4′,5,5′-hexabromobiphenyl was eliminated by rats over a 42-day period (Matthews *et al.*, 1977; Fries, 1985) and rhesus monkeys excreted on a daily basis approximately 0.5% of a single dose of the same congener from 10 to 42 days post-dosing (Rozman *et al.*, 1982; Fries, 1985).

Placental transfer of PBBs occurs to some extent, but the concentrations in fetal or offspring tissues are relatively small compared to concentrations in maternal tissues. Transfer of PBBs to the offspring during nursing results in much greater whole-body concentrations compared to PBB transfer during gestation. For example, pigs from sows that were fed PBBs during gestation and lactation had a 5-fold increase in body burden during the 4-week lactation period with residues accumulated during lactation accounting for 95% of the total body burden (Werner and Sleight, 1981; Fries, 1985).

As suggested above, milk is the major route of elimination of PBBs for lactating mammals, although in the case of females nursing their young, the contaminant is simply transferred from one animal to another. Concentrations of PBBs in milk fat generally exceed dietary concentrations, with bovine milk fat concentrations exceeding dietary concentrations by 3–4-fold (Fries and Marrow, 1975; Willett and Irving, 1976; Damstra *et al.*, 1982; Fries, 1985).

The egg is a major route of elimination for egg-laying birds with concentrations in chicken or Japanese quail eggs being 1–1.5 times that of dietary concentrations (Babish *et al.*, 1975; Fries *et al.*, 1976; Polin and Ringer, 1978a; Damstra *et al.*, 1982; Fries, 1985). Elimination of PBBs via the egg can account for as much as 50% of the daily dose if there is no deleterious effect on egg production (Fries, 1985).

Dairy cows at different stages of production were administered daily doses of FireMaster BP-6 ranging from 0.25 mg to 25 g for periods ranging from 1 to 202 days. Penta-, hexa- and heptabromobiphenyls in the commercial mixture were absorbed through the gastrointestinal tract and were detected in the plasma after 4 h and reached steady state concentrations after 15 days. The concentration of PBBs in tissues was, in general, correlated with the lipid content of the particular tissue with exception of the liver which contained relatively high concentrations of PBBs in relation to its lipid content. Fecal elimination was the predominant route of excretion in non-lactating cows, while milk fat was the major route of elimination in those animals that were lactating. Concentrations of PBBs in milk were approximately 3 times those in feces. The half-life of PBBs being excreted via lactation was 28 days (Willett and Durst, 1978; Damstra *et al.*, 1982).

Chickens fed FireMaster FF-1 for 5 weeks accumulated PBB residues at average ratios of tissue PBBs:diet PBBs of 3:1 for adipose tissue, 1.5:1 for whole egg, 0.8:1 for liver and 0.008:1 for muscle (Polin and Ringer, 1978a, b). The distribution of a chicken′ s daily intake of PBBs on reaching a steady state was estimated to be: excreta, 11%; eggs, 58%; adipose, 10%; muscle, 12%; liver, 2%; unknown, 7% (Ringer and Polin, 1977). A second chicken study demonstrated that after 63 days of consuming a diet containing PBBs, body fat concentrations were approximately 4 times greater than dietary concentrations (Fries *et al.*, 1976; Damstra *et al.*, 1982).

The concentrations of PBBs in adipose tissue of mink fed various concentrations of FireMaster FF-1 were about 60 times higher than dietary concentrations (Aulerich and Ringer, 1979).

The kinetics of some individual PBB congeners has been studied. Rats orally administered $^{14}C$-labeled 2,2′,4,

4′,5,5′-hexabromobiphenyl at doses up to 30 mg/kg over 4 days absorbed at least 90% of the congener. Twenty-four hours after the last dose, 40% of the radioactivity was measured in the muscle, 10% in the liver and 25% in the adipose tissue. After 7 days, adipose tissue contained 60% of the radioactivity while the liver and muscle contained 2% and 7%, respectively. Very little of the congener was metabolized and eliminated. It was estimated that less than 10% of the total dose would undergo fecal excretion (Matthews *et al*., 1977; DiCarlo *et al*., 1978). An octabromobiphenyl mixture consisting of hepta-, octa-, nona- and decabromobiphenyls that comprised approximately 11% of FireMaster BP-6 was fed to rats at a dietary concentration of 1000 ppm for 4 weeks. Bromine concentrations in the adipose tissue of these animals were approximately 600 times greater than bromine concentrations in the adipose tissue of untreated rats. Adipose tissue concentrations of bromine increased to 800 times greater than concentrations in untreated animals 18 weeks after the treated animals had been placed on clean feed. These results indicated that the octabromobiphenyl mixture was resistant to metabolism and elimination (Lee *et al*., 1975; Damstra *et al*., 1982).

PBBs can have a relatively long biological half-life in animals. Data suggested that only 10% of the total dose of 2,2′4,4′5,5′-hexabromobiphenyl would be eliminated during the lifetime of a rat (Matthews *et al*., 1977; DiCarlo *et al*., 1978). Rats receiving a single dose of $C^{14}$-octabromobiphenyl had biphasic fecal excretion with the initial half-life being less than 24 h and second phase half-life being greater than 16 days (Norris *et al*., 1975; DiCarlo *et al*., 1978). Studies with cows suggested biphasic elimination of PBBs via the milk with an initial half-life of 11 days and a second half-life of 58 days (Fries and Marrow, 1975; Gutenmann and Lisk, 1975; Fries *et al*., 1976; DiCarlo *et al*., 1978). In cases where observation periods were long, a biological half-life of 180 days was estimated for lactating cows (Fries, 1985). It was estimated that the concentration of FireMaster BP-6 in bovine milk fat would decrease from approximately 300 ppm to 0.3 ppm in 120 weeks (Detering *et al*., 1975; DiCarlo *et al*., 1978). A half-life of 17 days was calculated for FireMaster FF-1 in the eggs of chickens fed a diet containing the commercial mixture. Half-lives of 17 days and 31 days were calculated for muscle and liver, respectively and the concentration of PBBs in adipose tissue was essentially unchanged after 56 days (Ringer and Polin, 1977; Polin and Ringer, 1978b). It was estimated that a chicken exposed to 1 ppm PBB in the feed for at least 10 days (the minimum time required to attain a steady state concentration in the contents of a whole egg) would require 87 days of feeding uncontaminated feed to reach a concentration of 0.05 ppm in the whole egg (Ringer and Polin, 1977). The half-life of hexabromobiphenyl was 28 days and that of heptabromobiphenyl was 20 days in chicken eggs (Fries *et al*., 1976; DiCarlo *et al*., 1978).

## PCDDs and PCDFs

The absorption, distribution, metabolism and elimination of PCDDs and PCDFs have been extensively reviewed by Van den Berg *et al*. (1994). Absorption from the gastrointestinal tract of mammals is effective and can exceed 75% of the dose for the lower chlorinated congeners. With increasing molecular size, absorption from the intestines is greatly reduced, which is most apparent for the hepta- and octachlorinated congeners. The liver and adipose tissue are the major storage sites of PCDDs and PCDFs for most mammalian and avian species. The biotransformation of PCDDs and PCDFs depends on the number and position of the chlorine atoms on the molecule. Metabolic reactions include oxidation and reductive dechlorination as well as breakage of the oxygen bonds. Sulfur-containing metabolites have also been identified. In general, the urinary and biliary elimination of 2,3,7,8-substituted congeners depends on the metabolism of these compounds. Whole-body half-lives of the group of 2,3,7,8-substituted congeners in rodents range from a few to more than 100 days.

The absorption of PCDDs and PCDFs from the gastrointestinal tract has been studied for a number of individual congeners. The extent of absorption of 2,3,7,8-TCDD or related compounds is variable, depending on the vehicle and the substitution pattern of the congener. There appear to be no differences between species in terms of absorption of these compounds through the gastrointestinal tract. There are indications that passage across the intestinal wall is predominately limited by molecular size and solubility with the influence of these two parameters being most significant for hepta- and octachlorinated congeners, which exhibit decreased absorption. Studies with rats, mice, hamsters, guinea pigs, cows and chickens, in general, indicate that 2,3,7,8-tetra- and pentachlorinated congeners are well absorbed from the gastrointestinal tract (50–90%), and octachlorodibenzs P-dioxin (OCCD) is absorbed only to a limited extent (2–15%) (Pohjanvirta and Tuomisto, 1994; Van den Berg *et al*., 1994).

The tissue distribution of PCDDs and PCDFs has been extensively studied in laboratory experiments using rodents and non-human primates. Upon absorption, 2,3,7,8-substituted PCDDs and PCDFs are bound to chylomicrons, lipoproteins and other serum proteins and transported throughout the circulatory system. The liver and adipose tissue are the major storage sites of PCDDs and PCDFs for most mammalian and avian species, whereas, depending on species, the skin and adrenals can also act as primary sites for deposition. Several studies with rats, mice, hamsters, guinea pigs and monkeys indicated that the 2,3,7,8-substituted PCDDs and PCDFs are the predominant congeners retained in tissues and body fluids. In rats and mice, most of the 2,3,7,8-substituted penta- to octachlorinated congeners are retained in the liver at higher

concentrations than 2,3,7,8-TCDD and TCDF. In the hamster, the adrenal glands also appear to be a major storage site in addition to the liver and adipose tissue. Recent studies suggest that the tissue distribution of 2,3,7,8-TCDD and related compounds is dose dependent in that as the dose increases, so does the liver:adipose distribution ratio. In the liver, 2,3,7,8-TCDD induces both cytochromes CYP1A1 and CYP1A2. Induced CYP1A2, in turn, appears to be a crucial binding species for 2,3,7,8-TCDD and related compounds in rodents. The hepatocellular binding of 2,3,7,8-TCDD to CYP1A1 is so strong that only a very limited amount will be released back into the circulation. Placental transfer of 2,3,7,8-substituted PCDDs and PCDFs was found to be strongly dependent on molecular size with 2,3,7,8-TCDD being retained to the greatest extent in the fetus. In a number of mammalian species, the transfer of PCDDs and PCDFs from the mother to the offspring via lactation is quantitatively more important than transport to the fetus across the placenta. Excretion via lactation generally decreases as chlorine content increases, being most pronounced for the hepta- and octachlorinated congeners (Pohjanvirta and Tuomisto, 1994; Van den Berg *et al.*, 1994).

As in mammals, the liver and adipose tissue of avian species are the major sites for storage and accumulation of 2,3,7,8-substituted PCDDs and PCDFs. Hepatic deposition of 2,3,7,8-substituted PCDDs and PCDFs appeared to increase with increasing chlorination, resulting in a limited transfer of the more highly chlorinated congeners to the egg (Van den Berg *et al.*, 1994).

Metabolism of 2,3,7,8-TCDD and related compounds is necessary for urinary and biliary elimination, thus playing a major role in regulating the rate of excretion of these compounds. In rats, metabolic reactions include oxidation, preferably in the lateral positions and reductive dechlorination as well as oxygen bridge cleavage of the diphenyl ether in 2,3,7,8-substituted PCDDs. As with lower chlorinated PCDDs, oxidation of PCDFs preferably occurs on the 2 and 3 positions. Metabolism of 2,3,7,8-TCDF results in a number of metabolites containing one and two hydroxyl groups. However, in contrast to 2,3,7,8-TCDD, there was no cleavage of the oxygen bridge. In the 2,3,7,8-substituted PCDF molecule, the 4 and 6 positions are more susceptible to metabolic attack than the 1 and 9 positions. As a result, PCDFs with chlorines in the 4 and 6 positions are highly persistent in biota (Van den Berg *et al.*, 1994). Metabolism of PCDDs and PCDFs is generally thought to be a detoxification process, thus toxicity is attributable to the unchanged parent compound (Pohjanvirta and Tuomisto, 1994).

The induction of CYP1A1 and CYP1A2 enzyme activities by 2,3,7,8-substituted PCDDs and PCDFs has been shown to be one of the most sensitive parameters for biological activity of these compounds. The enzymes most studied are the CYP1A1-dependent ethoxy resorufin-*ortho*-deethylase (EROD) and aryl hydrocarbon hydroxylase (AHH). In addition, 2,3,7,8-substituted PCDDs and PCDFs also induce phase II enzymes (Van den Berg *et al.*, 1994).

In mammals, the liver and adipose tissue are the major compartments for the deposition of PCDDs and PCDFs. The elimination of polar metabolites of 2,3,7,8-substituted PCDDs and PCDFs occurs predominantly via the bile and feces, with urinary excretion playing a minor role. However, urinary elimination plays an important role in the hamster. In rats, the whole-body half-lives of PCDDs and PCDFs range from 17 to 31 days, depending on the dose and strain of rat used, while in hamsters and mice, the whole-body half-lives range from 11 to 15 days and 11 to 24 days, respectively. In rats, it was shown that lactation can reduce the half-life of these compounds while egg-laying can reduce the half-life in avian species. In lactating cows, mean half-lives ranged from 40 to 50 days for tetra- to heptaCDDs and CDFs . 2,3,7,8-Tetrachlorodibenzofuran is eliminated more rapidly than 2,3,7,8-TCDD, having a whole-body half-life of 2 days. The rapid elimination of 2,3,7,8-TCDF is thought to be due to its rapid metabolism. In contrast, 2,3,4,7,8-pentaCDF is eliminated very slowly in the rat, with a whole-body half-life of 64 days. The slow elimination rate is probably due to tight binding of this congener by CYP1A2, in addition to limited metabolism. As chlorine content increases, the rate of elimination of PCDDs and PCDFs decreases (Pohjanvirta and Tuomisto, 1994; Van den Berg *et al.*, 1994).

Interspecies differences in toxicity can only be partly explained by differences in toxicokinetics. The hamster is the species most resistant to the acute toxicity of 2,3,7,8-TCDD. Although the elimination rate of 2,3,7,8-TCDD is 2–3-fold greater in the hamster than the rat and mouse, this does not explain entirely the 10–100-fold difference in acute toxicity between the hamster and other rodent species. The guinea pig is most sensitive to the acute effects of 2,3,7,8-TCDD and it is the species with the slowest metabolism and elimination of 2,3,7,8-TCDD, suggesting that toxicokinetics in part explains the unique sensitivity of the guinea pig to the acute toxicity of 2,3,7,8-TCDD and 2,3,7,8-TCDF (Van den Berg *et al.*, 1994).

## MECHANISM OF ACTION

The AhR is a ligand-activated transcription factor that is involved in the regulation of a number of genes, including those for enzymes that play a role in the metabolism of xenobiotics as well as genes involved in cell growth regulation and differentiation (Okey *et al.*, 1994; Safe, 1994; Hahn, 1998, 2002; Denison *et al.*, 2002; Denison and Nagy, 2003; Mandal, 2005). The AhR plays an important role in the altered gene expression and species- and tissue-specific toxicity resulting from exposure to specific PCB, PCDD

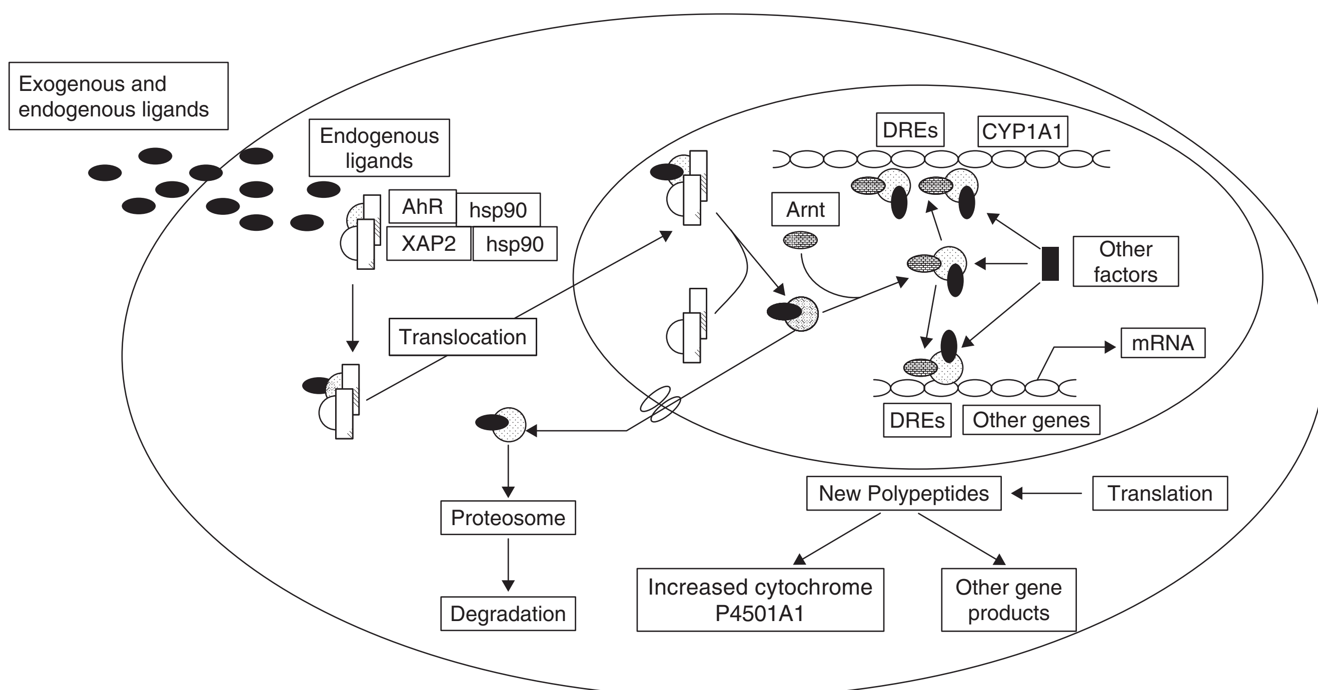


**FIGURE 55.2** The proposed molecular mechanism of action of 2,3,7,8-TCDD and TCDD-like chemicals. See text for details.

and PCDF congeners. It is now well established that the majority of the toxic effects attributed to 2,3,7,8-TCDD and TCDD-like chemicals require activation of the AhR. The toxicity of individual congeners is closely related to the affinity with which these compounds bind to the AhR with the most toxic compounds being those that bind with the greatest affinity (Okey *et al.*, 1994). There are large species and strain differences in sensitivity to TCDD and related chemicals. Mouse and rat strain differences in sensitivity to TCDD can be partially explained by differences in the ligand-binding affinity of their polymorphic AhR variants. A recent study in birds indicated that species differences in sensitivity to TCDD and related chemicals could be due, at least in part, to differences in amino acid composition of the ligand-binding domain of the AhR (Karchner *et al.*, 2006). However, differences in AhR concentration or binding affinity do not fully explain the differences in species susceptibility to TCDD and TCDD-like chemicals (Denison *et al.*, 1986).

The AhR is a basic helix–loop–helix (bHLH) and Per-Arnt-Sim (PAS) containing transcription factor (Denison *et al.*, 2002; Denison and Nagy, 2003). In the absence of a ligand, AhR occurs as a soluble multiprotein complex in the cytosol of the cell. The chaperone proteins are two molecules of hsp90 (a heat shock protein of 90 kDa), the X-associated protein 2 (XAP2) and p23 (a co-chaperone protein of 23 kDa). When TCDD or another ligand diffuses across the plasma membrane and binds to the AhR, the ligand AhR complex undergoes a conformational change that exposes a nuclear localization sequence (NLS) (Figure 55.2). The complex translocates into the nucleus of the cell and the chaperone proteins dissociate from the complex. The AhR–ligand then binds to the bHLH–PAS nuclear protein, AhR nuclear translocator or Arnt. The formation of this heterodimer initiates conversion of the complex into a form that binds to DNA with high affinity on a specific recognition site called the dioxin responsive element (DRE). Binding of the ligand–AhR–Arnt complex to the DRE stimulates transcription of genes encoding ctyochrome P450 enzymes in the CYP1A1 subfamily and other AhR-responsive genes that are located upstream of the DRE (Denison *et al.*, 2002; Denison and Nagy, 2003). Continuous and inappropriate modulation of gene expression is thought to be responsible for a series of biochemical, cellular and tissue changes that result in toxicity characteristic of TCDD and related compounds (Denison and Heath-Pagliuso, 1998; Mandal, 2005).

# TOXICITY

## PBBs

### *Laboratory studies*

Toxicological assessment of FireMaster FF-1 is complicated by the fact that the commercial product is a mixture of individual congeners of differing toxicities. In addition, the commercial mixture also contained traces of brominated

naphthalenes as impurities with their own toxicity. As with many chemicals, the type and severity of effects are influenced by species, age, duration of exposure and dose. None of the effects caused by PBBs are unique to this particular group of brominated congeners, but they are characteristic of polyhalogenated hydrocarbons including PBBs, PCBs, PCDDs and PCDFs. In general, the concentrations required to produce deleterious effects in animals are quite high.

The acute oral LD50s of FireMaster BP-6 as well as octabromobiphenyl and decabromobiphenyl are in the range of 2–22 g/kg body weight for a number of species (DiCarlo *et al.*, 1978; Fries, 1985). Death resulting from acute exposures can occur up to 2 weeks after dosing. Thus, the LD50 value is not a good indicator of PBB toxicity because it gives no consideration to subacute effects and acute effects that develop slowly. When observation periods are longer, the LD50 value tends to decrease (Fries, 1985). The PBB dose required to cause lethality is consistent among most mammalian and avian species. Generally, total doses exceeding 1000 mg/kg body weight or dietary concentrations in excess of 500 ppm are required. Notable exceptions include the guinea pig, which is one order of magnitude more sensitive and the mink, which is two orders of magnitude more sensitive to PBBs (Fries, 1985). However, as with other halogenated hydrocarbons, PBBs accumulate in adipose tissue upon chronic exposure and are toxic in animals at concentrations much lower than indicated from LD50 values (Ringer and Polin, 1977).

Generally speaking, prolonged exposure to low concentrations of PBBs causes more deleterious effects than exposure to a large, single dose (Damstra *et al.*, 1982). The appearance of clinical signs after a single dose may take a number of days. This delay in appearance of clinical signs in conjunction with the relatively long half-life of PBBs, which results in long-term exposure from a single dose, makes the distinction between acute and chronic toxicity less clear. The development of clinical signs is similar across species (Fries, 1985).

In most toxicity studies conducted with PBBs, the characteristic effects are a decrease in feed consumption with a concomitant decrease in body weight loss or body weight gain, which may not be entirely related to the decrease in feed consumption. Milk production and egg production may also decrease as a result, at least in part, of reduced feed consumption. Changes in hematology and serum clinical chemistry values generally were unremarkable in all species examined and with a few exceptions occurred only in conjunction with clinical signs indicative of severe toxicity. The most prominent gross morphological and histological changes occurred in the liver of most species examined, with the exception of cattle in which the kidney appears to be the target organ (Damstra *et al.*, 1982; Fries, 1985). In rodents, changes in the liver can include a dose-related increase in weight, which is associated with induction of microsomal enzymes (Sleight and Sanger, 1976; Fries, 1985). Microscopic lesions are characterized by swelling and vacuolation of hepatocytes with fatty infiltration (Corbett *et al.*, 1978; Kimbrough *et al.*, 1978; Gupta and Moore, 1979; Gupta *et al.*, 1981; Fries, 1985). Mitochondria increase in size and degenerate over time (Sleight and Sanger, 1976; Corbett *et al.*, 1978; Kasza *et al.*, 1978; Fries, 1985) and there is an increase in smooth endoplasmic reticulum (Sleight and Sanger, 1976; Corbett *et al.*, 1978; Kasza *et al.*, 1978; Gupta and Moore, 1979; Gupta *et al.*, 1981; Fries, 1985). At higher doses, livers become friable and the external surface is mottled (Gupta *et al.*, 1981; Fries, 1985). In birds, liver weights increased in addition to vacuolation and mitochondrial swelling within the hepatocytes (Dharma *et al.*, 1982; Fries, 1985). The livers of cattle exposed to PBBs were enlarged, but had minor histological changes (Moorehead *et al.*, 1977; Fries, 1985). In most species exposed to PBBs, there is minimal renal involvement with the exception of cattle. In cattle, the kidneys are the site of the most severe damage, which is characterized by enlargement and distension due to accumulation of fluid. Lesions included dilatation of collecting ducts and convoluted tubules as well as degeneration of tubular epithelium (Moorehead *et al.*, 1977; Fries, 1985). Both the thymus and the avian bursa of Fabricius decreased in size in some species at relatively low doses. There is a loss of cells specific to the respective tissue and a disruption of the normal architecture (Gupta and Moore, 1979; Gupta *et al.*, 1981; Dharma *et al.*, 1982; Fries, 1985).

Male rats fed FireMaster BP-6 at concentrations as low as 50 ppm for 10 weeks had increased liver weights (Harris *et al.*, 1978; Damstra *et al.*, 1982). Rats fed a diet containing 10 ppm PBBs for 30 days developed slight hepatotoxic effects and pups exposed to PBBs through lactation had microscopic and ultrastructural hepatic lesions (Sleight and Sanger, 1976; Gupta *et al.*, 1981). Rats administered 22 doses of FireMaster FF-1 ranging from 30 to 100 mg/kg body weight/day had decreased body weight gain accompanied by a decrease in feed intake, anemia and increased liver weights by the end of the 180-day trial. Histological assessment of tissues from these animals indicated lesions in the liver, kidneys, prostate gland and thyroid glands in males and hepatic involvement in the females. Animals receiving higher doses (300 and 1000 mg/kg body weight/day) died within 73 days and had atrophied thymuses and spleens (Gupta and Moore, 1979; Damstra *et al.*, 1982). A study involving administration of 22 oral doses up to 30 mg/kg body weight of FireMaster FF-1 or up to 16.8 mg/kg body weight 2,2′,4,4′,5,5′-hexachlorobiphenyl to rats and mice over a 30-day period with an additional 90 days holding period demonstrated hepatotoxicity in the females of both species with effects being more severe in the FireMaster FF-1 groups than the 2,2′,4,4′,5,5′ -hexabromobiphenyl groups. The liver was enlarged due to swelling of hepatocytes, fatty infiltration and proliferation of the endoplasmic reticulum (Gupta *et al.*, 1981). Mice that were fed diets containing 1000 ppm

FireMaster BP-6 for up to 14 days lost body weight and had enlarged livers at necropsy. Examination of hepatic ultrastructure indicated increased smooth endoplasmic reticulum and lysosomes, degeneration of the mitochondria and decrease glycogen content (Corbett *et al.*, 1978; Damstra *et al.*, 1982). Mink were fed diets containing FireMaster FF-1 at concentrations ranging from 1.0 to 15.6 ppm, a diet containing contaminated chicken that provided a PBB concentration of 1.5 ppm or a diet containing contaminated beef that provided a PBB concentration of 12.0 ppm for up to 10 months. The animals had decreased feed consumption accompanied by a decrease in body weights and unthrifty appearance. Kidneys and livers tended to be enlarged with the latter being characterized by fatty infiltration (Aulerich and Ringer, 1979). Chickens fed diets containing 125 ppm FireMaster FF-1 had reduced feed consumption (Polin and Ringer, 1978a). Japanese quail also had reduced feed consumption at similar PBB concentrations (Babish *et al.*, 1975; Ringer and Polin, 1977). Chickens fed diets containing 50–250 ppm FireMaster FF-1 had decreased body weights, which were due in part to a decrease in feed consumption. Additionally, weights of the comb, testes, spleen and bursa were decreased and liver and thyroid weights were increased. Hydropericardium and ascites were also observed. In a second experiment, chicks fed diets containing 75 or 150 ppm FireMaster FF-1 for 9 weeks developed an anemic condition characterized by decreased heart rate, cardiac output, packed cell volume and hemoglobin concentration (Ringer and Polin, 1977; Ringer, 1978). Rhesus monkeys fed 0.3 ppm FireMaster FF-1 for approximately 2 years had reduced body weights without a concomitant decrease in feed intake, prolonged menstrual cycles and decreased progesterone concentrations (Allen *et al.*, 1978; Damstra *et al.*, 1982). Monkeys fed greater concentrations of the commercial fire retardant had a decrease in serum cholesterol, enlarged livers with fatty infiltration, hyperplastic gastroenteritis, dry, scaly skin, alopecia, generalized subcutaneous edema and edema of the eyelids (Allen *et al.*, 1978; Lambrecht *et al.*, 1978; Damstra *et al.*, 1982).

There is some evidence, in addition to reported atrophy of lymphoid tissues in a variety of species, that exposure to PBBs causes suppression of the immune system (Moorehead *et al.*, 1977; Kimbrough *et al.*, 1978; Gupta and Moore, 1979; Gupta *et al.*, 1981, 1983; Fries, 1985). However, doses sufficient to cause other toxic effects are usually required to induce immunosuppression and there appears to be differences between species in terms of sensitivity (Damstra *et al.*, 1982; Fries, 1985). Dogs receiving FireMaster FF-1 for 61 days had depletion of lymphocytes in lymph nodes but only at doses that resulted in other toxic effects (Farber *et al.*, 1978; Damstra *et al.*, 1982). Alterations in B-cell and T-cell function occurred as a result of feeding rhesus monkeys 1.5 ppm PBBs for 5 months and 25 ppm for 10 weeks (Allen and Lambrecht, 1978; Damstra *et al.*, 1982). Decreased immunoresponsiveness was reported for rats receiving 3.0–30 mg PBBs/kg body weight/day for 30 days (Luster *et al.*, 1978; Damstra *et al.*, 1982) and for rats and mice receiving up to 10 mg/kg body weight/day (5 days/week) for 6 months (Luster *et al.*, 1980; Damstra *et al.*, 1982). Mice fed diets containing 1, 10 or 100 ppm PBBs had antibody responses to sheep red blood cells that were 80%, 30% or 12%, respectively, of control responses, although cell-mediated immunity was not affected (Fraker, 1980; Damstra *et al.*, 1982).

A number of studies have demonstrated PBB-induced reproductive effects in a variety of species. Exposure of poultry to deleterious concentrations of PBBs results in an initial decrease in feed consumption accompanied by a decrease in egg production. If concentrations are high enough, there is then a dose-related decrease in egg hatchability with embryo mortality occurring late in incubation (Fries, 1985). Dietary concentrations of FireMaster FF-1 greater than 30 ppm resulted in a significant decrease in egg production of chickens, which returned to control values from 2 to 6 weeks after withdrawal of the contaminated feed. Concentrations above 30 ppm also had an adverse effect on hatchability and subsequent survivability of chicks (Ringer and Polin, 1977; Polin and Ringer, 1978a). Edema of the abdominal and cervical regions of the chicken embryos and hatchlings was the prevalent pathological condition observed and was the only effect that had an increased incidence compared to the incidence of abnormalities observed in controls (Ringer and Polin, 1977; Polin and Ringer, 1978b). A dietary PBB concentration of 20 ppm had no effect on egg production and hatchability in Japanese quail, but 100 ppm resulted in a significant decrease in both parameters (Babish *et al.*, 1975). Rats administered FireMaster BP-6 at doses of 5 or 25 mg/kg body weight every other day for 14 days had a reduced number of implantation sites compared to controls at both doses and increased resorptions and fetal deaths at the higher dose (Beaudoin, 1977; Damstra *et al.*, 1982). Single doses of 400 and 800 mg/kg body weight caused a decrease in rat fetal weights at term as well as an increased incidence of cleft palate and diaphragmatic hernia (Beaudoin, 1977; Damstra *et al.*, 1982). Pregnant rats fed diets containing 100 and 1000 ppm FireMaster BP-6 experienced increased fetal mortality and fetal weights were reduced at term (Corbett *et al.*, 1975; Damstra *et al.*, 1982). Mice exposed to 200 ppm dietary PBBs on gestation days 4 through 16 had an increase in fetal deaths and resorptions as well as reduced fetal weights at term (Preache *et al.*, 1976; Damstra *et al.*, 1982). Exencephaly and cleft palate were observed in offspring of mice fed FireMaster BP-6 at concentrations up to 1000 ppm (Corbett *et al.*, 1975; DiCarlo *et al.*, 1978). The predominant congener in FireMaster BP-6, 2,2′,4,4′,5,5′-hexabromobiphenyl, did not cause any effects in offspring when administered to pregnant mice at a dose of 40 mg/kg body weight on gestation days 10 through 16 (Damstra *et al.*, 1982). Pigs that

were fed diets containing up to 200 ppm PBBs during gestation had normal young but as the offspring nursed, there was 50% mortality in those receiving 200 ppm via lactation and significant growth depression in offspring consuming 100 ppm in the milk (Werner and Sleight, 1981; Fries, 1985). Rhesus monkeys fed diets containing 0.3 ppm FireMaster FF-1 for 7 months prior to breeding had prolonged menstrual cycles and decreased progesterone concentrations. Offspring had depressed birth weights and growth rates through 16 weeks of age (Allen and Lambrecht, 1978; Damstra *et al.*, 1982). Mink were fed diets containing FireMaster FF-1 at concentrations ranging from 1.0 to 15.6 ppm, a diet containing contaminated chicken that provided a PBB concentration of 1.5 ppm or a diet containing contaminated beef that provided a PBB concentration of 12.0 ppm for up to 10 months. Adults experienced increased mortality at dietary concentrations of 6.25 ppm or greater. There was also a significant decrease in litter size, birth weights and kit survivability through 4 weeks of age at dietary concentrations from 1.0 to 2.5 ppm and greater. Results indicated that the PBB-contaminated poultry and beef were more toxic than the commercial mixture. A concentration lethal to 50% of the population (LC50) for FireMaster FF-1 was estimated to be 4 ppm when fed for over 300 days. This study indicated that PBBs were not as fetotoxic as two commercial PCB mixtures (Aroclors 1242 and 1254) but were lethal to adults at a lower dietary concentration (Aulerich and Ringer, 1979).

Commercial PBB mixtures have been shown to cause liver cancer in rats and mice (Damstra *et al.*, 1982; Fries, 1985). Sherman rats were administered a single dose of 1000 mg/kg FireMaster BP-6 and observed for 14 months. There was pronounced liver pathology in both sexes that included neoplastic lesions that were more prominent in the females (Kimbrough *et al.*, 1978). Subsequently, groups of female Sherman strain COBS rats given a single dose of 1000 mg/kg body weight of FireMaster FF-1, 12 doses of 100 mg/kg body weight or a single dose of 200 mg/kg body weight single or multiple doses of FireMaster FF-1 of 1000 mg/kg body weight or higher had an incidence of liver tumors. There was an incidence of hepatocellular carcinoma ranging from 41% to 68% at the two higher doses while animals in the lowest dose group had a 31% incidence of neoplastic nodules (Kimbrough *et al.*, 1981; Damstra *et al.*, 1982). Fischer 344 rats of both sexes had a significant incidence of neoplastic nodules, hepatocellular carcinomas and cholangiocarcinomas 23 months after receiving daily doses of FireMaster FF-1 at 10.0 mg/kg body weight/day for 6 months. Atypical foci in the liver of male rats occurred at doses as low as 0.3 mg/kg body weight. Hepatocellular carcinomas were noted in $D6C3F_1$ mice receiving the 10 mg/kg body weight dose (Gupta *et al.*, 1983; Fries, 1985). Studies have indicated no evidence of tumors in tissues other than the liver and that the commercial PBB mixture acts as a promoter rather than an initiator of carcinogenesis (Fries, 1985).

### *Michigan PBB Incident*

One dairy producer who received an early shipment of PBB-containing feed soon suspected that there was something wrong with the feed. The sequence of events that occurred on this particular farm as result of feeding the commercial PBB mixture was reported by Jackson and Halbert (1974) and provides one of the earliest accounts of PBB toxicity in a domestic animal. The producer had a 400-cow herd with a 305-day average of 15,000 lb of milk. Because soil concentrations of magnesium were low in the area, the herd was fed magnesium oxide that was added to pelleted feed at a rate of 8 lb/ton. Cows that produced more than 40 lb of milk/day had free access to the pelleted ration twice a day while being milked, which resulted in an estimated consumption of at least 15 lb/day. Cows that produced less milk consumed approximately 8 lb of the feed on a daily basis.

Early in September of 1973, the herd was provided the pelleted ration containing PBBs. By late September, milk production of the herd decreased from 13,000 lb/day to 7600 lb/day over a 20-day period and feed intake decreased by 50%. During this first phase of clinical signs, some cows had increased urination and lacrimation. Lameness also was apparent in some of the animals. Approximately 16 days after feeding of the suspect concentrate began the ration was changed by switching to alfalfa hay and eliminating the pelleted feed. Although feed consumption rapidly increased, milk production continued to be about 40% lower than the previous average. Also during this time period, it was observed that cows bred 4–6 weeks earlier came back into estrus, suggesting early fetal resorptions.

The second phase of clinical signs began about 30 days after the drop in milk production was observed. Hematomas developed in about 10% of the cows and animals continued to lose weight despite resumption of normal feed consumption. Approximately 60 days after initial observations, it was noted that the hooves of approximately 25% of the cows were abnormally long. Some cows had alopecia with thickened skin on the thorax, neck and shoulders. Cows consuming contaminated feed during the last trimester of pregnancy experienced dystocia and often the calf was born dead or died soon after parturition. Thirty freshening cows developed metritis in January of 1974 and in March, 20 non-lactating cows went off feed several weeks prepartum. Half of the latter cows died without calving.

From mid-October 1973 to mid-April 1974, 24 cows died. Gross findings at necropsy included hematomas and abscesses in the peritoneal and thoracic cavities. Pathology reports indicated liver lesions in seven of 10 cows assessed, including hemorrhagic necrotic hepatitis, fatty metamorphosis, large fat vacuoles and amyloidosis. Four cows also had kidney involvement characterized by interstitial nephritis (Jackson and Halbert, 1974).

## PCBs, PCDDs and PCDFs

The toxic and biochemical effects of various commercial PCB mixtures have been studied in various laboratory animals, fish and wildlife species and have been summarized by Safe (1994). Commercial PCBs cause a broad spectrum of toxic responses that are dependent on several factors including the chlorine content and purity of the commercial mixture, the route and duration of exposure as well as on the age, sex, species and strain of animal. The majority of effects caused by commercial PCB mixtures are the same as those induced by 2,3,7,8-TCDD because the responses caused by commercial PCB mixtures are due, in part, to the individual non-*ortho* coplanar and mono-*ortho* coplanar PCBs present in these mixtures that act as AhR agonists. Because some of the mono-*ortho* coplanar PCBs are present in relatively high concentrations in commercial mixtures and environmental extracts, this class of PCBs may contribute significantly to the TCDD-like activity of PCB mixtures (Safe, 1994).

There are a number of reports that describe the common effects induced by 2,3,7,8-TCDD and related PCDDs, PCDFs and PCBs. The only major difference in these compounds is their relative toxic potencies. These effects are thought to be mediated through initial binding to the AhR and are dependent on dose, age, sex, species and strain of animal. In most cases, if a given species is more sensitive to a given class of compound (e.g. PCBs), this species will also be sensitive to the other classes (PCDDs, PCDFs, PBBs). It has also been observed that in most instances young animals are more sensitive than adults and females more sensitive than males. Generally speaking, chickens, guinea pigs and mink are the most sensitive species of animals to the toxic effects induced by 2,3,7,8-TCDD and TCDD-like chemicals. In contrast, hamsters and amphibians appear to be fairly resistant to the toxic effects. The effects include acute lethality, wasting syndrome, thymic and splenic atrophy, impairment of immune responses, hepatotoxicity and porphyria, chloracne and related dermal lesions, tissue-specific hypo- and hyperplastic responses, disruption of multiple endocrine pathways, carcinogenesis, teratogenicity and reproductive toxicity (Poland and Knutson, 1982; McConnell, 1985; Safe, 1986, 1990, 1998; Dickson and Buzik, 1993; Fries, 1995; Schecter *et al.*, 2006).

TCDD causes lethality at very low doses in specific species. The oral LD50 values for various species are: 0.6-2.0 μg/kg body weight for the guinea pig, 22–45 μg/kg for the rat, 25–50 μg/kg for the chicken, 70 μg/kg for the monkey, 115 μg/kg for the rabbit, 100–200 μg/kg for the dog, 114–284 μg/kg for the mouse and 1157–5000 μg/kg for the hamster (Safe, 1990). There is a delayed appearance of lethality, irrespective of dose. Death typically does not occur earlier than 1 week after exposure and it may not occur until 6 weeks of exposure. Before death, the exposed animal generally undergoes a rapid and substantial weight loss called "wasting syndrome" (McConnell, 1985; Pohjanvirta and Tuomisto, 1994). This is accompanied by decreased food and water intake, which accounts for some, but not all of the weight loss. These may be the only signs observed in rats, mice, guinea pigs, mink and poultry after oral administration. However, skin lesions have been reported in hairless mice and acne-like lesions have been described in the ears of rabbits after local application. At times, poultry may show an increase in body weight prior to death due to accumulation of body fluids (subcutaneous edema, ascites, hydrothorax and hydropericardium). In addition to body weight loss, the clinical syndrome in monkeys and cattle is characterized by skin and eyelid lesions and abnormal growth of finger or toe nails or hooves. The lesions in monkeys are follicular dermatitis (acne) of the face, neck and forearms, enlarged tortuous Meibomian glands in the eyelid, and overgrowth and loss of nails of the hands and feet. Alopecia may be present, particularly in the areas of the body showing dermatitis. The skin of cattle is thickened and dry, particularly over the neck, shoulders and back (McConnell, 1985).

TCDD is a highly immunosuppressive chemical in laboratory animals. Effects include decreased host resistance to infectious disease and suppressed humoral and cell-mediated immune responses. Both pre- and post-natal exposure of mice and guinea pigs reduced both the delayed hypersensitivity and lymphoproliferative response. TCDD appears to selectively interfere with antigen-specific activation of T-cells. In addition to immune suppression, TCDD promotes inflammatory responses (Dickson and Buzik, 1993; Pohjanvirta and Tuomisto, 1994; Mandal, 2005).

Thymic atrophy is one of the most uniform and consistent findings in TCDD-exposed mammals. It consists of depletion of small immature cortical thymocytes, making a distinction between cortex and medulla difficult. Although both pyknotic and scattered necrotic lymphocytes have been reported, frank necrosis is not a typical feature of the lesion. At less toxic doses, the thymus might look normal histopathologically while being one half normal size (McConnell, 1985; Pohjanvirta and Tuomisto, 1994).

TCDD exposure results in hepatomegaly in all species investigated, but there is extensive variation among species in the severity of this lesion. The changes in the liver are accompanied by altered liver function characterized by enzyme induction, porphyria, impaired plasma membrane function and hyperlipidemia. Liver lesions are most severe in the rabbit, which displays extensive necrosis. In other species, such as the rat, mouse, mink and, to a lesser extent, the guinea pig, hamster, bovine and monkey, the predominant features are hepatocellular hypertrophy, multinuclear hepatocytes, steatosis and inflammatory cell infiltration, often accompanied by scattered focal necrosis with a preferentially centrilobular location. Female rodents are more susceptible to the hepatic effects of TCDD compared to males

(McConnell, 1985; Dickson and Buzik, 1993; Pohjanvirta and Tuomisto, 1994; Safe, 1994).

Intrahepatic bile duct hyperplasia has been described in rodents and monkeys, but is a more prominent feature in chronically exposed animals. Marked epithelial proliferation of the extrahepatic bile duct and gall bladder has been described in monkeys and cattle. Epithelial erosions, ulcers and inflammation are characteristic components of the lesion. Hyperplasia of the epithelium lining of the urinary tract has been described in guinea pigs, cattle and monkeys. The lesion extends from the renal pelvis to the urinary bladder stopping at the level of the urethra. The histological appearance of the epithelium appears normal with the exception of an increase in the number of cell layers. Monkeys exposed to TCDD-like compounds develop a lesion referred to as "simian gastropathy". In acute lethal exposures, the chief (acid-producing) cells are replaced by hyperplastic mucous-producing cells. In more chronic exposures, the hyperplastic change becomes more pronounced and at times appears to invade adjacent tissues. A similar, but much less severe lesion has been described in rats exposed to PBBs. The large intestine also shows hyperplastic changes in monkeys chronically exposed to these chemicals (McConnell, 1985).

The skin and associated structures in monkeys, rabbits and certain strains of mice show characteristic lesions as a result of exposure to TCDD and related chemicals. In monkeys, the lesion is characterized microscopically by mild epithelial hyperkeratosis and severe atrophy of sebaceous glands and hyperkeratosis of their ducts. The ducts become occluded with keratinaceous debris and grossly the lesion mimics an acne-like lesion. A similar lesion is observed in the Meibomian glands of the eyelid and ceruminous glands of the external auditory canal. Alopecia, dry scaly skin and fingernail loss are also features of exposure in monkeys. While most strains of mice do not show skin lesions, certain "hairless" strains show a similar skin lesion to that in monkeys. The inner surface of a rabbit' s ear also develops acne-like lesions if the compound is applied directly to the skin. Cattle also show a characteristic skin disease when exposed to these compounds (McConnell, 1985; Safe, 1994).

A relatively consistent hematological feature of exposure to TCDD and similar compounds across species, except for the guinea pig, is a shift in the proportional number of leukocytes with a trend toward an increase in neutrophils at the expense of lymphocytes. This is a classical manifestation of stress. Another common alteration is thrombocytopenia. There is an increase in hemoglobin concentration, hematocrit and red blood cell numbers that may result from hemoconcentration in the early phases of intoxication. In chronic studies, the most consistent hematologic finding is a mild to moderate degree of anemia. Porphyria and jaundice are typically seen as a result of prolonged exposure to TCDD. The porphyrinogenic effect of TCDD leads to an accumulation of hepatic porphyrins as a result of inhibition of uroporphyrinogen decarboxylase activity (McConnell, 1985; Pohjanvirta and Tuomisto, 1994).

The study of blood serum changes is one of the more complex features of intoxication by TCDD and related chemicals because the pathology varies between species. The liver damage induced by TCDD is reflected in increases in the serum activities of the liver-specific transaminases (alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase) (McConnell, 1985; Pohjanvirta and Tuomisto, 1994).

TCDD reduces serum thyroxin ($T_4$) concentrations rapidly in rats. TCDD-induced changes in $T_4$ concentrations in other species are less consistent. The decrease in serum $T_4$ concentrations is thought to be due to accelerated clearance of the hormone. In addition to altering thyroid hormone concentrations, TCDD appears to modulate the concentration of thyroid hormone receptors. Other endocrine changes include alterations in corticosteroid concentrations and in the concentrations of hormones associated with the reproductive system in rodents, monkeys and birds (Pohjanvirta and Tuomisto, 1994; Safe, 1994; Ulbrich and Stahlmann, 2004).

It is assumed that the interaction of TCDD with the AhR regulates a number of genes, although CYP1A1 has been studied most thoroughly. Thus, in addition to monooxygenases dependent on cytochrome P4501A1, a number of other enzymes are induced (Pohjanvirta and Tuomisto, 1994; Huwe, 2002).

Effects on the nervous system are manifested as hyperactivity, impaired learning and changes in brain neurochemical concentrations in rats and monkeys (Safe, 1994).

PCBs appear to have a detrimental as well as enhancing effect on learning performance (Ulbrich and Stahlmann, 2004).

TCDD and approximate stereoisomers have been shown to affect female reproductive endpoints in a variety of animal studies. Among the effects reported are a decrease in the number of females mated in rats, mink and monkeys, fewer completed pregnancies in rats, mink and monkeys, lower maternal weight gain during pregnancy in rats, rabbits and monkeys, decreased litter size in rats, rabbits, mink and swine, effects on female gonads in guinea pigs and mice and altered estrous and menstrual cycles in mice, rats and monkeys. Decreased egg production and hatchability occur in a number of avian species. Numerous studies have indicated that TCDD and related chemicals are anti-estrogenic presumably due to increased metabolism of estrogen and a decreased number of estrogen receptors. One manifestation of the anti-estrogenic effect is the TCDD-induced decrease in uterine weight in rats. Occasionally, some signs of ovarian dysfunction such as anovulation and suppression of the estrous cycle have been reported (Golub *et al.*, 1991; Peterson *et al.*, 1993; Safe, 1994).

TCDD and related compounds decrease testis and accessory sex organ weight, cause abnormal testicular morphology, decrease spermatogenesis and reduce fertility when given to adult animals in doses sufficient to reduce feed intake and/or body weight. Some of these effects have been reported in chickens, rhesus monkeys, rats, guinea pigs and mice treated with overtly toxic doses of TCDD or TCDD-like chemicals. TCDD effects on spermatogenesis are characterized by loss of germ cells, the appearance of degenerating spermatocytes and mature spermatozoa within the lumens of seminiferous tubules and a reduction in the number of tubules containing mature spermatozoa. Effects of TCDD on the male reproductive system are thought to be due in part to an androgen deficiency. The deficiency in rats is caused by decreased plasma testosterone and dihydroxytestor-terone concentrations, and unchanged plasma clearance of androgens and luteinizing hormone (LH) (Dickson and Buzik, 1993; Peterson *et al.*, 1993; Safe, 1994).

Avian embryos are more sensitive to TCDD toxicity compared to mammals based on LD50 values. Among bird species, most of the developmental toxicity research has been done on the chicken, which is considered to be the most sensitive avian species to TCDD-like chemicals. Injection of TCDD or its approximate stereoisomers into fertilized chicken eggs causes a toxicity syndrome in the embryo characterized by pericardial and subcutaneous edema, liver lesions, inhibition of lymphoid development in the thymus and bursa of Fabricius, microophthalmia, beak deformities, cardiovascular malformations and mortality. Clinical signs in turkey embryos include microophthalmia, beak deformities and embryo mortality, but not liver lesions, edema or thymic hypoplasia while ring-necked pheasant embryos experienced only mortality. Thus, the clinical signs of toxicity of TCDD and its approximate stereoisomers are species dependent with embryo mortality being the only common effect (Peterson *et al.*, 1993).

Exposure to TCDD during pregnancy causes prenatal mortality in the mouse, rat, guinea pig, hamster, rabbit, mink and monkey. The time period during which exposure of the embryo/fetus to TCDD occurs is just as important as the dose of TCDD administered in terms of prenatal mortality. In most laboratory mammals, gestational exposure to TCDD produces a characteristic pattern of fetotoxic responses that consist of thymic hypoplasia, subcutaneous edema, decreased fetal growth and prenatal mortality. In addition to these common fetotoxic effects, there are other effects of TCDD that are highly species specific. Such effects include cleft palate formation in the mouse and intestinal hemorrhage in the rat. In the mouse, hydronephrosis is the sensitive sign of prenatal toxicity, followed by cleft palate formation and atrophy of the thymus at higher doses, and by subcutaneous edema and mortality at maternally toxic doses. In the rat, TCDD prenatal toxicity is characterized by intestinal hemorrhage, subcutaneous edema, decreased fetal growth and mortality. Structural abnormalities occur in the rat only at relatively large doses. In the hamster fetus, hydronephrosis and renal congestion are the most sensitive effects, followed by subcutaneous edema and mortality. In the rabbit, an increased incidence of extra ribs and prenatal mortality is found, whereas in the guinea pig and rhesus monkey, prenatal mortality is seen (Dickson and Buzik, 1993; Peterson *et al.*, 1993).

Normal development of male reproductive organs and imprinting of typical adult sexual behavior patterns require sufficient testosterone be secreted by the fetal and neonatal testes at critical times in early development before and shortly after birth. Perinatal exposure of male rats to TCDD can produce both prenatal and postnatal androgenic deficiencies that are manifested as a decrease in anogenital distance, a delay in testis descent, decreased plasma concentrations of testosterone, 5$\alpha$-dihydrotestosterone and LH and a decrease in the weights of the seminal vesicles and the ventral prostate. Perinatal exposure of the rat to TCDD also results in decreased spermatogenesis as well as decreased testis and epididymis weights. Additionally, demasculinization and feminization of sexual behavior has been reported (Golub *et al.*, 1991; Peterson *et al.*, 1993).

There have been several long-term bioassays for carcinogenicity of TCDD in several species with all studies producing positive results. Studies in Sprague Dawley rats indicated an increased incidence of hepatocellular hyperplastic nodules and hepatocellular carcinomas in female rats but not male rats. Additional lesions noted were squamous cell carcinoma of the tongue and nasal turbinates/hard palate in both sexes. In addition to the liver, tongue, nasal turbinates and hard palate, increased incidences of lung tumors were observed in the females (Kociba *et al.*, 1978; Lucier *et al.*, 1993). In a study with Osborne–Mendel rats and B6C3F1 mice, TCDD induced malignant liver tumors in female rats and male and female mice. The incidence of thyroid gland tumors increased in male rats while TCDD-induced neoplasms of the adrenal gland occurred in both male and female rats. Fibrosarcomas of the subcutaneous tissue occurred in both female rats and mice. Lymphomas and lung tumors were also observed in female mice (NTP, 1982; Lucier *et al.*, 1993). In the male Syrian Golden hamster, exposure to TCDD resulted in squamous cell carcinomas of the skin in the facial region with some of the lesion metastasizing to the lung (Rao *et al.*, 1988; Lucier *et al.*, 1993). B6C3 and B6C mice of both sexes administered TCDD developed thymic lymphomas and hepatic neoplasms (Della Porta *et al.*, 1987; Lucier *et al.*, 1993).

The studies summarized above by Lucier *et al.* (1993) indicate that TCDD is a multisite carcinogen in both sexes and in several species, it is a carcinogen in sites remote from the site of treatment and it increases cancer incidence at doses below the maximum tolerated dose (MTD). In

two-stage models for liver and skin cancer, it is clear that TCDD is a potent promoting agent with weak or no initiating activity. The consensus is that TCDD is an example of a receptor-mediated carcinogenesis in that; (1) interaction with the AhR appears to be a necessary early step; (2) TCDD modifies a number of receptor and hormone systems involved in cell growth and differentiation, such as epidermal growth factor receptor and the estrogen receptor and (3) hormones exert a profound influence on the carcinogenic actions of TCDD. Although tumor promotion data are limited for PCDFs and co-planar PCBs, it appears that these chemicals are liver tumor promoters with potencies dependent on their binding affinity to the AhR (Lucier *et al.*, 1993; Schwarz and Appel, 2005).

## TREATMENT

The PBB incident in Michigan that resulted in the contamination and subsequent disposal of thousands of animals and millions of pounds of contaminated food products because of the long biological half-lives of these compounds prompted investigation of ways to enhance elimination of persistent PHAHs. Strategies reported in Fries (1985) included activated charcoal in rats and cows (Cook *et al.*, 1978; McConnell *et al.*, 1980), mineral oil in rats and monkeys (Kimbrough *et al.*, 1980; Rozman *et al.*, 1982), high fiber diets in rats (Kimbrough *et al.*, 1980), phenobarbital in cows (Cook *et al.*, 1978) and colestipol, mineral oil, propylene glycol or petroleum jelly with and without restricted feeding in chickens (Polin *et al.*, 1985, 1989). In general, none of these elimination enhancement strategies proved to be effective despite the fact that they were employed for periods of 3–6 months (Fries, 1985). More recently, studies have been conducted that have examined different strategies to increase clearance of dioxins from animals. Rats fed clenbuterol-supplemented feed for 10 days after exposure to dioxin, had 30% less fat than control rats and a 30% decrease in the dioxin body burden. In other studies, rats and mice fed dietary fiber, chlorophyll or an insoluble evacuation substance (chlorophyllin-chitosan) significantly increased excretion of PCDDs and PCDFs and reduced the TEQ body burden (Huwe, 2002).

As suggested above, once animals have been contaminated with the persistent PHAHs, there are currently no practical methods available to quickly reduce body burdens. Therefore, in a contamination incident, products are removed from the market and animals may have to be destroyed. A common strategy to reduce concentrations of contaminants in exposed animals is to provide the animal with uncontaminated feed and withhold products from the market until concentrations of the contaminant have decreased to an acceptable level. In the case of the persistent PCBs, PBBs, PCDDs, long half-lives in the animals require long withdrawal periods. Estimates of the half-lives of PCBs/PCDDs/PCDFs in milk range from 40 to190 days. In beef cattle adipose tissue, half-lives are in the range of 100–200 days. The half-lives of dioxins in adipose tissue and eggs of chickens range from 25 to 60 days. Depuration is the only way to reduce body burdens, but may be uneconomical in many situations because of the length of time required (Huwe, 2002).

At present, the best way to reduce dioxin concentrations in livestock is to minimize exposure. In general, the substitution of plant meals for animal and fish meals may prove to be an effective to lower dioxin intake in livestock and aquaculture (Huwe, 2002).

## CONCLUSION

The PHAHs, which include PCBs, PBBs, PCDDs and PCDFs are environmentally persistent, lipophilic compounds that have a tendency to bioaccumulate and biomagnify at the higher levels of the food chain. Concentrations of these chemicals have been detected in remote areas of the world and in a variety of animal species, including humans. While certain of these chemicals can pose a very serious threat to the health of animals and humans, exposure situations are generally such that risks of health effects are generally low. The most significant problem for those involved in producing a safe food supply is contamination of food products beginning at the animal. Additionally, there are areas of the environment that are heavily contaminated by these chemicals because of past industrial activities. Animals and humans residing in or near contaminated locations certainly are at risk for serious health effects. In those situations, efforts must continue to eliminate or reduce exposure to these very persistent and toxic chemicals.

## REFERENCES

Allen JR, Lambrecht L (1978) Response of rhesus monkeys to polybrominated biphenyls. *Toxicol Appl Pharmacol* **45**: 340–1.

Allen JR, Lambrecht LK, Barsotti DA (1978) Effects of polybrominated biphenyls in non-human primates. *J Am Vet Med Assoc* **173**: 1485–9.

Aulerich RJ, Ringer RK (1979) Toxic effects of dietary polybrominated biphenyls on mink. *Arch Environ Contam Toxicol* **8**: 487–98.

Babish JG, Gutenmann WH, Stoewsand GS (1975) Polybrominated biphenyls: tissue distribution and effect on hepatic microsomal enzymes in Japanese quail. *J Agric Food Chem* **23**: 879–82.

Beaudoin AR (1977) Teratogenicity of polybrominated biphenyls in rats. *Environ Res* **14**: 81–6.

Bernard A, Broeckaert F, De Poorter G, De Cock A, Hermans C, Saegerman C, Houins G (2002) The Belgian PCB/dioxin incident: analysis of the food chain contamination and health risk evaluation. *Environ Res* **88A**: 1–18.

Cook RM, Prewitt LR, Fries GF (1978) Effects of activated carbon, phenobarbital, and vitamins A, D, and E on polybrominated biphenyl excretion in cows. *J Dairy Sci* **61**: 414–19.

Corbett TH, Beaudoin AR, Cornell RG, Anver MR, Schumacher R, Endres J, Szwambowska M (1975) Toxicity of polybrominated biphenyls (Firemaster BP-6) in rodents. *Environ Res* **10**: 390–6.

Corbett TH, Simmons JL, Kawanishi H, Endres JL (1978) EM changes and other toxic effects of FireMaster BP-6® (polybrominated biphenyls) in the mouse. *Environ Health Perspect* **23**: 275–81.

Damstra T, Jurgelski Jr W, Posner HS, Vouk VB, Bernheim NJ, Guthrie J, Luster M, Falk HL (1982) Toxicity of polybrominated biphenyls in domestic and laboratory animals. *Environ Health Perspect* **44**: 175–188.

Della Porta G, Dragani TA, Sozzi G (1987) Carcinogenic effects of infantile and long-term 2,3,7,8-tetrachlorodibenzo-*p*-dioxin treatment in the mouse. *Tumorigenesis* **73**: 99–107.

Denison MS, Heath-Pagliuso S (1998) The Ah receptor: a regulator of the biochemical and toxicological actions of structurally diverse chemicals. *Bull Environ Contam Toxicol* **61**: 557–68.

Denison MS, Nagy SR (2003) Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals. *Ann Rev Pharmacol Toxicol* **43**: 309–34.

Denison MS, Vella LM, Okey AB (1986) Structure and function of the Ah receptor for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin: species differences in molecular properties of the receptor from mouse and rat hepatic cytosol. *J Biol Chem* **261**: 3987–95.

Denison MS, Pandini A, Nagy SR, Baldwin EP, Bonati L (2002) Ligand binding and activation of the Ah receptor. *Chem Biol Interact* **141**: 3–24.

Detering CN, Prewitt LR, Cook RM, Fries GF (1975) Placental transfer of polybrominated biphenyls by Holstein cows. *J Dairy Sci* **58**: 764–5.

Dharma DN, Sleight SD, Ringer RK, Aust SD (1982) Pathologic effects of 2,2′,4,4′,5,5′- and 2,3′,4,4′5,5′-hexabromobiphenyl in white leghorn cockerels. *Avian Dis* **26**: 542–52.

DiCarlo FJ, Seifter J, DeCarlo VJ (1978) Assessment of the hazards of polybrominated biphenyls. *Environ Health Perspect* **23**: 351–65.

Dickson LC, Buzik SC (1993) Health risks of "dioxins": a review of environmental and toxicological considerations. *Vet Hum Toxicol* **35**: 68–77.

Dunckel, A.E. (1975). An updating on the polybrominated biphenyl disaster in Michigan. *J Am Vet Med Assoc* **167**: 838–41.

Farber T, Kasza L, Giovetti A, Carter C, Earl F, Balzas T (1978) Effect of polybrominated biphenyls (Firemaster BP-6) on the immunological system of the beagle dog. *Toxicol Appl Pharmacol* **45**: 343.

Fraker PJ (1980) The antibody-mediated and delayed type hypersensitivity response of mice exposed to polybrominated biphenyls. *Toxicol Appl Pharmacol* **53**: 1–7.

Fries GF (1985) The PBB episode in Michigan: an overall appraisal. *Crit Rev Toxicol* **16**: 105–56.

Fries G (1995) A review of the significance of animal food products as potential pathways of human exposure to dioxins. *J Anim Sci* **73**: 1639–50.

Fries GF, Marrow GS (1975) Excretion of polybrominated biphenyls into the milk of cows. *J Dairy Sci* **58:** 947–75.

Fries GF, Cecil HC, Bitman J, Lillie RJ (1976) Retention and excretion of polybrominated biphenyls by hens. *Bull Environ Contam Toxicol* **15**: 278–82.

Giesy JP, Kannan K (1998) Dioxin-like and non-dioxin-like toxic effects of polychlorinated biphenyls (PCBs): implications for risk assessment. *Crit Rev Toxicol* **28**: 511–69.

Golub MS, Donald JM, Reyes JA (1991) Reproductive toxicity of commercial PCB mixtures: LOAELs and NOAELs from animal studies. *Environ Health Perspect* **94**: 245–53.

Gupta BN, Moore JA (1979) Toxicologic assessments of a commercial polybrominated biphenyl mixture in the rat. *Am J Vet Res* **40**: 1458–68.

Gupta BN, McConnell EE, Harris MW, Moore JA (1981) Polybrominated biphenyl toxicosis in the rat and mouse. *Toxicol Appl Pharmacol* **57**: 99–118.

Gupta BN, McConnell EE, Goldstein JA, Harris MW, Moore JA (1983) Effects of a polybrominated biphenyl mixture in the rat and mouse. I. Six-month exposure. *Toxicol Appl Pharmacol* **68**: 1–18.

Gutenmann WH, Lisk DJ (1975) Tissue storage and excretion in milk of polybrominated biphenyls in ruminants. *J Agric Food Chem* **23**: 1005–1007.

Hahn ME (1998) The aryl hydrocarbon receptor: a comparative perspective. *Comp Biochem Physiol* **121C**: 23–53.

Hahn ME (2002) Aryl hydrocarbon receptors: diversity and evolution. *Chem Biol Interact* **141**: 131–60.

Hardy ML (2000) The toxicity of the commercial polybrominated diphenyl oxide flame retardants: DBDPO, OBDPO, PeBDPO. *Organohalogen Comp* **47**: 41–4.

Harris SJ, Cecil HC, Bitman J (1978) Effects of feeding a polybrominated biphenyl flame retardant (Firemaster BP-6) to male rats. *Bull Environ Contam Toxicol* **19**: 692–6.

Headrick ML, Hollinger K, Lovell RA, Matheson JC (1999) PBBs, PCBs, and dioxins in food animals, their public health implications. *Vet Clin North Am Food Anim Pract* **15**: 109–31.

Hesse JL, Powers RA (1978) Polybrominated biphenyl (PBB) contamination of the Pine River, Gratiot and Midland Counties, Michigan. *Environ Health Perspect* **23**: 19–25.

Huwe JK (2002) Dioxins in food: A modern agricultural perspective. *J Agric Food Chem* **50**: 1739–50.

International Agency for Research on Cancer (IARC) (1986) *IARC Monographs of the Evaluation of Carcinogenic Risks to Humans*, Vol. 41. *Some Halogenated Hydrocarbons and Pesticide Exposures*. World Health Organization, Lyon, France, pp. 261–92.

Jackson TF, Halbert FL (1974) A toxic syndrome associated with the feeding of polybrominated-contaminated protein concentrate to dairy cattle. *J Am Vet Med Assoc* **165**: 437–9.

Karchner SI, Franks DG, Kennedy SW, Hahn ME (2006) The molecular basis for differential dioxin sensitivity in birds: role of the aryl hydrocarbon receptor. *Proc Natl Acad Sci USA* **103**: 6252–7.

Kasza L, Weinberger MA, Hinton DE, Trump BF, Patel C, Friedman L, Gartoff LH (1978) Comparative toxicity of polychlorinated biphenyl and polybrominated biphenyl in the rat liver: light and electron microscopic alterations after subacute dietary exposure. *J Environ Pathol Toxicol* **1**: 241–57.

Kimbrough RD (1987) Human health effects of polychlorinated biphenyls (PCBs) and polybrominated biphenyls (PBBs). *Ann Rev Pharmacol Toxicol* **27**: 87–111.

Kimbrough RD (1995) Polychlorinated biphenyls (PCBs) and human health: an update. *Crit Rev Toxicol* **25**: 133–63.

Kimbrough RD, Burse VW, Liddle JA (1978) Persistent liver lesions in rats after a single oral dose of polybrominated biphenyls (FireMaster FF-1®) and concomitant PBB tissue levels. *Environ Health Perspect* **23**: 265–73.

Kimbrough RD, Korver MP, Burse VW, Groce DF (1980) The effect of different diets or mineral oil on liver pathology and polybrominated biphenyl concentration in tissues. *Toxicol Appl Pharmacol* **52**: 442–53.

Kimbrough RD, Groce DF, Lorver MP, Burse VM (1981) Induction of liver tumors in female Sherman strain rats by polybrominated biphenyls. *J Natl Cancer Inst* **66**: 535–42.

Kociba RJ, Keyes DG, Beyer JE, Carreon RM, Wade CE, Dittenber DA, Kalnins RP, Frauson LE, Parks CN, Barnard SD, Hummel RA, Humiston CG (1978) Results of a two-year chronic toxicity and oncogenicity study of 2,3,7,8-tetrachlorodibenzo-*p*-dioxins in rats. *Toxicol Appl Pharmacol* **46**: 279–303.

Lambrecht LK, Barsotti DA, Allen JR (1978) Responses of nonhuman primates to a polybrominated biphenyl mixture. *Environ Health Perspect* **23**: 139–45.

Lee KP, Herbert RR, Sherman H, Aftosmis JG, Waritz RS (1975) Bromine tissue residue and hepatotoxic effects of octabromobiphenyl in rats. *Toxicol Appl Pharmacol* **34**: 115–27.

Lucier G, Clark G, Hiermath C, Tritscher A, Sewall C, Huff J (1993) Carcinogenicity of TCDD in laboratory animals: implications for risk assessment. *Toxicol Ind Health* **9**: 631–68.

Luster MI, Faith RE, Moore JA (1978) Effects of polybrominated biphenyls (PBB) on immune response in rodents. *Environ Health Perspect* **23**: 227–32.

Luster MI, Boorman GA, Harris MW, Moore JA (1980) Laboratory studies on polybrominated biphenyl induced immune alterations following low-level chronic or pre/postnatal exposure. *Int J Immunopharmacol* **2**: 69–80.

Mandal PK (2005) Dioxin: a review of its environmental effects and its aryl hydrocarbon receptor biology. *J Comp Physiol B* **175**: 221–30.

Matthews HB, Kato S, Morales NM, Tuey DB (1977) Distribution and excretion of 2,4,5,2′,4′,5′-hexabromobiphenyl, the major component of Firemaster BP-6®. *J Toxicol Environ Health* **3**: 599–605.

Matthews HB, Fries G, Gardner A, Garthoff L, Goldstein J, Ku Y, Moore J (1978) Metabolism and biochemical toxicity of PCBs and PBBs. *Environ Health Perspect* **24**: 147–55.

McConnell EE (1985) Comparative toxicity of PCBs and related compounds in various species of animals. *Environ Health Perspect* **60**: 29–33.

McConnell EE, Harris MW, Moore JA, (1980) Studies on the use of activated charcoal and cholestyramine for reducing the body burden of polybrominated biphenyls. *Drug Chem Toxicol* **3**: 277–92.

Miceli JN, Marks BH (1981) Tissue distribution and elimination kinetics of polybrominated biphenyls (PBB) from rat tissue. *Toxicol Lett* **9**: 315–20.

Moorehead PD, Willett LB, Brumm CJ, Mercer HD (1977) Pathology of experimentally induced polybrominated biphenyl toxicosis in pregnant heifers. *J Am Vet Med Assoc* **170**: 307–13.

National Toxicology Program (NTP) (1982) Bioassay of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin for possible carcinogenicity (gavage study). Research Triangle Park, NC. Technical Report Number 201.

Norris JM, Kociba RJ, Schwetz BA, Rose JQ, Humiston CG, Jewett GL, Gehring PJ, Mailhes JB (1975) Toxicology of octabromobiphenyl and decabromobiphenyl oxide. *Environ Health Perspect* **11**: 153–61.

Okey AB, Riddick DS, Harper PA (1994) The Ah receptor: mediator of the toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and related compounds. *Toxicol Lett* **70**: 1–22.

Peterson RE, Theobald HM, Kimmel GL (1993) Developmental and reproductive toxicity of dioxins and related compounds: cross-species comparisons. *Crit Rev Toxicol* **23**: 283–335.

Pohjanvirta R, Tuomisto J (1994) Short-term toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in laboratory animals: effects, mechanisms, and animal models. *Pharmacol Rev* **46**: 483–549.

Poland A, Knutson JC (1982) 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and related halogenated aromatic hydrocarbons: examination of the mechanism of toxicity. *Ann Rev Pharmacol Toxicol* **22**: 517–54.

Polin D, Ringer RK (1978a) PBB fed to adult female chickens: its effect on egg production, viability of offspring, and residues in tissues and eggs. *Environ Health Perspect* **23**: 283–90.

Polin D, Ringer RK (1978b) Polybrominated biphenyls in chicken eggs vs. hatchability. *Proc Soc Exp Biol Med* **159**: 131–5.

Polin D, Lehning E, Pullen D, Bursian S, Leavitt R (1985) Procedures to enhance withdrawals of xenbiotics from chickens. *J Toxicol Environ Health* **16**: 243–254

Polin D, Underwood M, Lehning E, Olsan B, Bursian S (1989). Enhanced withdrawal of polychlorinated biphenyls: a comparison of colestipol, mineral oil, propylene glycol, and petroleum jelly with or without restricted feeding. *Poultry Sci.* **68**: 885–90

Preache MM, Cagan SJ, Gibson JE (1976) Perinatal toxicity in mice following dietary exposure to polybrominated biphenyls. *Toxicol Appl Pharmacol* **37**: 171.

Rao MS, Subbarao V, Prasad JD, Scarpelli DC (1988) Carcinogenicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in the Syrian golden hamster. *Carcinogenesis* **9**: 1677–9.

Ringer RK (1978) PBB fed to immature chickens: its effects on organ weights and function and on the cardiovascular system. *Environ Health Perspect* **23**: 247–55.

Ringer RK, Polin D (1977) The biological effects of polybrominated biphenyls in avian species. *Fed Proc* **36**: 1894–8.

Rozman KK, Rozman TA, Williams J, Greim HA (1982) Effects of mineral oil and/or cholestryamine in the diet on biliary and intestinal elimination of 2,2′,4,4′,5,5′-hexachlorobiphenyl in the rhesus monkey. *J Toxicol Environ Health* **9**: 611–18.

Safe S (1986) Comparative toxicology and mechanism of action of polychlorinated dibenzo-*p*-dioxins and dibenzofurans. *Ann Rev Pharmacol Toxicol* **26**: 371–99.

Safe S (1990) Polychlorinated biphenyls (PCBs), dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs), and related compounds: environmental and mechanistic considerations which support the development of toxic equivalency factors (TEFs). *Crit Rev Toxicol* **21**: 51–88.

Safe S (1994) Polychlorinated biphenyls (PCBs): environmental impact, biochemical and toxic responses, and implications for risk assessment. *Crit Rev Toxicol* **24**: 87–149.

Safe S (1998) Development validation and problems with the toxic equivalency factor approach for risk assessment of dioxins and related compounds. *J Anim Sci* **76**: 134–41.

Schecter A, Birnbaum L, Ryan JJ, Constable JD (2006) Dioxins: an overview. *Environ Res* **101**: 419–28.

Schwarz M, Appel KE (2005) Carcinogenic risks of dioxin: mechanistic considerations. *Reg Toxicol Pharmacol* **43**: 19–34.

Sleight SD, Sanger VL (1976) Pathologic features of polybrominated biphenyls toxicosis in the rat and guinea pig. *J Am Vet Med Assoc* **169**: 1231–5.

Tanabe S (1988) PCB problems in the future: foresight from current knowledge. *Environ Pollut* **50**: 5–28.

Ulbrich B, Stahlmann R (2004) Developmental toxicity of polychlorinated biphenyls: a systematic review of experimental data. *Arch Toxicol* **78**: 252–68.

Van den Berg M, De Jongh J, Poiger H, Olson JR (1994) The toxicokinetics and metabolism of polychlorinated dibenzo-*p*-dioxins (PCDDs) and dibenzofuran (PCDFs) and their relevance for toxicity. *Crit Rev Toxicol* **24**: 1–74.

Van den Berg M, Birnbaum LS, Denison M, DeVito M, Farland W, Feeley M, Fiedler H, Hakansson H, Hanberg A, Haws L, Rose M, Safe S, Schrenk D, Tohyama C, Tritscher A, Tuomisto J, Tysklind M, Walker N, Peterson RE (2006) The 2005 World Health Organization re-evaluation of human and mammalian toxic equivalency factors for dioxins and dioxin-like compounds. *Tox Sci* **93**: 223–241.

Van Larebeke N, Hens L, Schepens P, Covaci A, Baeyens J, Everaert K, Bernheim JL (2001) The Belgian PCB and dioxin incident of January–June 1999: exposure data and potential impact on health. *Environ Health Perspect* **109**: 265–73.

Werner PR, Sleight SD (1981) Toxicosis in sows and their pigs caused by feeding rations containing polybrominated biphenyls to sows during pregnancy and lactation. *Am J Vet Res* **42**: 183–9.

Willett LB, Durst HI (1978) Effects of PBBs on cattle. IV. Distribution and clearance of components of FireMaster BP-6®. *Environ Health Perspect* **23**: 67–74.

Willett LB, Irving HA (1976) Distribution and clearance of polybrominated biphenyls in cows and calves. *J Dairy Sci* **59**: 1429–39.

Whyte JJ, Schmitt CJ, Tillitt DE (2004) The H4IIE cell bioassay as an indicator of dioxin-like chemicals in wildlife and the environment. *Crit Rev Toxicol* **34**: 1–83.

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# Part 10

# Environmental Toxicology

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C H A P T E R

# 56

# Avian toxicology

*Robert H. Poppenga*

## INTRODUCTION

There are about 30 orders of birds in the Aves class with approximately 2000 genera and 10,000 species. Obviously, such a large class of animals possesses a bewildering array of unique physiologic adaptations, behaviors, and ecologic niches. Thus, a discussion of a broad topic such as avian toxicology presents a daunting task. This chapter is intended to provide an overview of toxicologic hazards to three subsets of birds: pet and aviary birds, poultry and other farm-raised birds, and wild birds. Given the breadth of the topic, this chapter is not an exhaustive treatise, but will hopefully provide an overview of major avian toxicologic hazards.

### Pet and aviary birds

The popularity of birds as pets has increased substantially over the past several years. The increased popularity is due to a variety of factors including the availability of species with a diversity of sizes, colors, and temperaments, the ability to house birds in a small area and, in some cases, a long lifespan. The fact that most pet birds are confined to the home environment exposes them to toxicants that poultry and wild birds are unlikely to come into contact with such as the pyrolysis products from Teflon®-coated cookware. Alternatively, pet birds can be exposed to toxicants to which poultry and wild birds are also exposed, but which are in different forms or from different sources. For example, pet birds can be intoxicated by lead, most often in the form of lead-based paint or lead objects such as drapery weights or toys, whereas wild birds are frequently intoxicated following ingestion of lead ammunition or lead-containing fishing gear.

### Wild birds

Obviously, wild birds exist in a much less well-controlled environment than pet birds or poultry and are therefore exposed to a greater variety of potential toxicants. It is safe to say that avian toxicology had its origins in the 1950s and 1960s when the effects of organochlorine insecticides (OCs) such as dichlorodiphenyltrichloroethane (DDT) on a variety of bird species were first recognized. The rapid decline of a variety of high-profile avian species such as bald eagles, peregrine falcons, and pelicans during those years and, more recently, the declines of many songbird and waterfowl populations, has resulted in significant government funding to more fully characterize the effects of toxicants on avian wildlife. In addition, regulatory requirements to obtain premarket toxicology information for pesticides using laboratory surrogates, such as quail and mallard ducks, has generated a substantial database of toxicologic information.

One unique aspect of wild bird toxicology is the potential exposure of wild birds to toxicants via their food. Raptors and scavengers such as turkey vultures or condors are often poisoned as a result of feeding on animal carcasses contaminated with pesticides or lead. Thus, secondary or rely toxicity is a more common occurrence in wild birds than in pet birds or poultry. In addition, bioaccumulation and biomagnification of persistent environmental toxicants are much more likely to be of concern in wild birds compared to pet birds or poultry. Bioaccumulation is defined as the net accumulation of a contaminant in or on an organism from all sources including water, air, and diet (Newman and Unger, 2003). Biomagnification is defined as the increase in contaminant concentrations from one trophic level to the next due to accumulation from food (Newman and Unger, 2003). For example, fish-eating birds

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are exposed to methyl mercury and polychlorinated biphenyls (PCBs) through ingestion of contaminated prey. Concentrations of such toxicants increase (bioaccumulate) with increasing age of the individual due to their long biologic half-lives.

Wild birds are much more likely to be maliciously poisoned than pet birds or poultry. Some avian species such as grackles, blackbirds, and pigeons are considered to be pests and are intentionally poisoned to reduce their numbers. Avicides, such as 4-aminopyridine, are specifically marketed to control pest birds.

## Poultry

Most commercial poultry are raised in well-controlled environments and are provided quality feed and water. Thus, their potential for exposure to toxicants is more limited. However, poultry can become intoxicated following exposure to common feed additives such as organic arsenicals or ionophores if feed misformulations occur. Obviously, free-ranging poultry are potentially exposed to a greater variety of hazardous chemicals.

## Physiologic differences of birds relevant to toxicology

### *Respiratory system*

The unique physiology of bird respiration makes them more susceptible to some inhaled toxicants such as carbon monoxide (CO) and pyrolysis products from overheated Teflon®. The sensitivity of birds to inhaled toxicants brings to mind the image of the canary in the coal mine. Several physiologic differences of birds increase their sensitivity to inhaled toxicants such as a higher mass specific minute ventilation, a higher mass specific ventilation of gas-exchange tissues, cross-current and counter-current gas exchange mechanisms, and a gas diffusion barrier one-half the thickness of that of mammals (Brown *et al*., 1997). Some of these physiologic adaptations are as a result of high metabolic rates of birds and the concomitant need for a high ventilatory capacity.

### *Metabolic system*

There have been a number of studies examining the metabolism of xenobiotics in a variety of avian species and comparing metabolic capabilities of birds and mammals (Pan, 1978; Watkins and Klaassen, 1986; Dalvi *et al*., 1987; Short *et al*., 1988; Coulet *et al*., 1996; Schlinger, 1997; Bailey *et al*., 1998; Gupta and Abou-Donia, 1998). Xenobiotic metabolism has been studied most extensively in chickens, pigeons, Japanese quail, and domestic ducks (Pan, 1978). As expected, there are substantial differences in metabolic pathways and capacities between birds and mammals that influence susceptibility to intoxication (Watkins and Klaassen, 1986; Short *et al*., 1988). Organophosphate (OP) insecticides are more toxic to birds than mammals and the basis for this difference is primarily due to differences in OP metabolism. Dimethoate is 20 times more toxic for pheasants than rats. Dimethoate is metabolized by pheasants to a toxic metabolite, while the toxic metabolite is detoxified by rats (Pan 1978). In addition, there are differences between bird species and between sexes and ages (Pan, 1978; Dalvi *et al*., 1987). Geese, chickens, and turkeys have similar liver aniline hydoxylase activity compared with rats, while quail and ducks had lower activities (Dalvi *et al*., 1987). Conjugating capabilities also differ. For example, glucuronidation of *p*-nitrophenol was greater in ducks than in chickens or turkeys (Short *et al*., 1988).

### *Excretion*

Birds have several unique excretory pathways for xenobiotics. Many metals are incorporated into feathers which are subsequently molted. In addition, incorporation of lipophilic toxicants into eggs provides a significant excretory pathway for female birds.

# GENERAL COMMENTS ABOUT DIAGNOSING AVIAN INTOXICATIONS

Although most diagnostic approaches for documenting intoxication in mammals are applicable to birds, there are several unique aspects that bear mentioning. Given the small size of many birds, obtaining a sufficient amount of sample for testing can sometimes present a challenge. For example, it is not unusual to be able to obtain only 100 μl (sometimes less) of whole blood or serum antemortem for lead and zinc testing, respectively. Quantities of gastrointestinal contents or tissues such as liver are sometimes limiting, especially if multiple tests are desired. The availability of less than optimal sample sizes oftentimes decreases the sensitivity of toxicologic testing, so it is possible that low toxicant concentrations are not detected. In the case of wild bird die-offs, pooling of samples from several dead birds is often required.

Investigation of wild bird die-offs is often a challenge from the standpoint that frequently birds are not discovered until significant postmortem autolysis and/or predation has occurred. Postmortem autolysis often precludes thorough pathologic, microbiologic, and virologic testing. However, for the vast majority of toxicologic tests, the condition of the tissue or fluid sample is of less concern and severely autolyzed samples may be perfectly suitable for toxicologic testing.

Antemortem, whole blood is most often requested from mammals for cholinesterase activity determinations. However, in birds, there is little cholinesterase activity that is associated with the red blood cell. Therefore, for avian species, plasma is preferred. Feathers and egg samples are sometimes useful for diagnosing either exposure to or intoxication from toxicants.

As is the case for any suspected intoxication related to feed, it is critical to obtain a representative feed sample. For example, feed-related mycotoxicoses are common concerns in poultry. However, the distribution and concentration of a mycotoxin in a feed can be quite variable. Therefore, it is important to obtain a number of samples that can then be pooled and subsampled. Other toxicants for which obtaining representative feed samples are critical include drugs, growth promotants such as ionophores, sodium chloride, and nutritionally important minerals such as copper, zinc, and selenium.

Since malicious poisonings of wild birds are common and some poisoned birds are protected by state or federal laws, the potential for litigation should always be of concern. Therefore, it may be critical to obtain and submit samples following chain-of-custody procedures.

# SPECIFIC TOXICANTS

# NATURAL TOXICANTS

## Algal toxins

### Cyanobacteria

Cyanobacteria (blue-green algae) are prokaryotic, plantlike organisms that are widely distributed in warm, nutrient-enriched waters such as eutrophic temperate lakes and reservoirs during warm weather months or in eutrophic subtropical lakes throughout the year (Zurawell *et al.*, 2005). A variety of factors contribute to the occurrence of an algal bloom such as a stable water column, warm water, high nutrient content (nitrogen, phosphorus, and organics), low N:P ratio, low $CO_2$ availability, and low grazing rates by large zooplankton (Zurawell *et al.*, 2005). One consequence of cyanobacterial blooms is the production of both neurotoxins and hepatotoxins, which are believed to be produced to reduce grazing by zooplankton and protozoa. The hepatotoxic microcystins, produced primarily by *Microcystis* spp. as well as some *Anabaena*, *Anabaenopsis*, *Aphanizomenon*, *Nostoc*, and *Planktothrix* spp., are the most commonly occurring toxins. Other hepatotoxins include nodularins and cylindrospermopsin. Cyanobacterial neurotoxins include anatoxin-a, anatoxin-a(s), and paralytic shellfish poison (PSP). Anatoxin-a is produced by *Anabaena flos-aquae*, *Aphanizomenon flos-aquae*, and *Planktothrix* spp. Anatoxin-a(s) is produced by *Anabaena flos-aquae* and *A. lemmermannii*. In fresh waters, PSPs are produced by *Aphanizomenon issatschenkoi*, *Cylindrospermopsis raciborskii*, and *Planktothrix* spp.

#### Microcystins

A variety of microcystins have been isolated and chemically characterized. They have variable toxicity based upon intraperitoneal (i.p.) injection $LD_{50}$s in mice (from ~50 to >1200 μg/kg) (Zurawell *et al.*, 2005). Microcystins are preferentially taken up by hepatocytes via bile acid transport mechanisms. They are inhibitors of protein phosphatases 1 and 2A and are potent liver tumor initiators and promotors. Protein phosphatases regulate the phosphorylation and dephosphorylation of regulatory intracellular proteins. *In vitro*, microcystins act on intermediate filaments, microtubules, and microfilaments causing altered structural integrity of the hepatocyte cytoskeleton. Apoptosis is also induced by microcystins. They cause rapid blebbing of cell membranes, cell contraction, organelle disruption, chromatin condensation, DNA fragmentation, and ladder formation (McDermott *et al.*, 1998). Following cytoskeletal disruption and subsequent separation of hepatocytes, sinusoidal endothelial cell breakdown allows severe intrahepatic hemorrhage to occur. Death results from hypovolemic shock. In an experimental study in quail, the i.p. $LD_{50}$ of microcystin RR was 256 μg/kg. Interestingly, no liver lesions were noted on necropsy, although spleens of intoxicated birds were approximately twice the size of control birds (Takahashi and Kaya, 1993).

#### Anatoxins

Anatoxin-a is a low molecular weight secondary amine that mimics acetylcholine (ACh). It binds to nicotinic ACh receptors with higher affinity than ACh and it is not susceptible to hydrolysis by acetylcholinesterase (AChE). Anatoxin-a(s) is an OP that binds to AChE preventing ACh hydrolysis.

Wild bird species are more likely to be intoxicated by cyanobacterial toxins than pet birds or poultry given their most common water sources. However, it is possible that zoo birds or production birds such as poultry, gamebirds, or waterfowl can be exposed via their water depending on its source and if conditions conducive to algal blooms are present. There are few documented instances of intoxication of birds, although cyanobacterial toxins are often found in lakes, reservoirs, and rivers used by wild birds. A recent survey of the saline Salton Sea in California detected microcystins in 85% of the samples tested (Carmichael and Li, 2006). Analyses of liver samples of eared grebe (*Podiceps nigricollis*) found microcystins at concentrations believed to be clinically significant. However, the role that microcystins play, if any, in documented large eared grebe die-offs remains to be determined. A bloom of *Microcystis*

*aeurginosa* in a pond in Japan was believed to have played a role in the death of approximately 20 spot-billed ducks (Matsunaga *et al.*, 1999). Although analysis for microcystins was not done, hepatic necrosis was noted in the one bird necropsied.

Anatoxin-a(s) was believed to have been the cause of bird mortality in several Danish lakes. *Anabaena lemmermannii* dominated the blooms and anatoxin-a(s) was detected and bloom material was shown to be toxic to mice (Henriksen *et al.*, 1997; Onodera *et al.*, 1997). Both anatoxin-a and microcystins (LR and RR) were implicated in the mass mortalities of flamingos in Kenya (Krienitz *et al.*, 2003).

#### *Avian vacuolar myelinopathy*

Avian vacuolar myelinopathy (AVM) is a disease of birds that has sporadically caused mortality of bald eagles (*Haliaeetus leucocephalus*), American coots (*Fulica americana*), and other waterbirds in several reservoirs in the southeastern Unites States. AVM is characterized by widespread, bilaterally symmetrical vacuolation of the white matter of the brain and spinal cord. Recent work has demonstrated an association between bird mortality and the presence of the invasive plant hydrilla (*Hydrilla verticillata*) (Wilde *et al.*, 2005). An unknown cyanobacterial species belonging to *Stigonematales* has been found associated with the plant. AVM was reproduced using mallard ducks exposed to *Stigonematales* blooms. It is believed that a currently uncharacterized algal neurotoxin is the cause of AVM.

## Plants

A number of plants are recognized for their toxicity. Plants contain a large variety of biologically active constituents including volatile oils, resins, alkaloids, polysaccharides, phenols, glycosides, and fixed oils. Free-flying companion birds are likely to encounter and eat household plants. Free-ranging production birds encounter plants in their outdoor environment. Both companion birds and production birds can be exposed to potentially toxic plants via their feed. Generally, wild birds are adapted to their environments and are unlikely to inadvertently ingest toxic plants.

The susceptibility of different bird species to specific toxic plants is variable. In addition, birds may be unaffected by plants that are toxic to other animals such as mammals. For example, cedar waxwings and house finches can consume fruit from the pepper tree (*Capsicum annuum*) that is toxic to mammals (Navarro, 1992).

Feeding behaviors also influence the susceptibility to intoxication. For example, it has been suggested that parrots can consume otherwise toxic plants because they remover the outer covering of fruits and seeds, which can contain high concentrations of toxins, before consumption.

There are few documented cases of bird intoxication following plant ingestion. However, there are several experimental studies of the susceptibility of small companion birds such as budgerigars and canaries along with numerous feeding studies using chickens and turkeys. Table 56.1 summarizes published information on plants poisonous to birds. There are published and anecdotal reports of fruit-eating birds being intoxicated by ethanol as a result of eating fermented fruit (Fitzgerald *et al.*, 1990).

## Zootoxins

There are few reports of bird intoxication resulting from exposure to zootoxins. Snakebite was believed to have been responsible for the deaths of two red-tailed hawks (*Buteo jamaicensis*) and a Cooper's hawk (*Accipeter cooperii*) based upon the carcasses being found in the vicinity of venomous snakes and gross findings of hemorrhages, muscle degeneration, and gangrenous necrosis of a limb (Heckel *et al.*, 1994). Chickens, turkeys, and ducks have also reportedly been killed by snakes based upon bite sites being identified on postmortem examination along with the occurrence of compatible gross organ lesions (Lawal *et al.*, 1992).

### *Bacterial toxins*

#### *Botulinum toxin*

Avian botulism results from the ingestion of botulinum toxin produced by *Clostridium botulinum*. There are eight antigenically distinct toxin types designated A, B, C alpha, C beta, D, E, F, and G); the type designations reflect their order of discovery. Type C alpha cultures produce three toxins designated C1, C2, C3, and small amounts of type *D. C. botulinum* is an anaerobic bacterium that persists in the form of dormant spores when environmental conditions are not conducive for bacterial growth. The spores are quite resistant to destruction and can remain viable for years. Spores of type C toxin are widely distributed in wetland sediments and in tissue of wetland organisms. A number of factors play a role in the occurrence of an outbreak of botulism. These include optimal environmental conditions for spore germination and bacterial growth, availability of suitable material to support bacterial replication, and a means for toxin transfer to birds (Friend and Franson, 1999). Most outbreaks occur during summer and fall months when ambient temperatures are relatively high. Bacterial replication requires substrates that are high in protein; decomposing carcasses are good bacterial substrates. Types C1 and D are produced by clostridia containing pseudolysogenic tox+ phages encoding for the respective toxin genes (Zechmeister *et al.*, 2004; Sakaguchi *et al.*, 2005). Genes encoding for C1 toxin are ubiquitous in wetlands inhabited by bird populations (Zechmeister *et al.*, 2004).

Botulinum toxin is one of the most toxic chemicals known, although for any given species toxicity varies

**TABLE 56.1 Plants reported to be toxic to birds**

| Common name | Scientific name | Toxin | Plant part demonstrated to be toxic | Mechanism of toxic action | Primary organ system affected | Species/bird type reported to be affected | References |
|---|---|---|---|---|---|---|---|
| Avocado | *Persea americana* | Persin | Fruit, leaves | Unknown | Cardiovascular system | Budgerigars (*Melopsittacus undulates*); canaries (*Serinus canaria*); cockatiels, ostriches (*Struthio camelus*) | Hargis *et al*., (1989), Shropshire *et al*. (1992), Burger *et al*. (1994), Burrows and Tyrl (2001) |
| Black locust | *Robinia pseudoacacia* | Unknown: possibly glycoprotein lectins, proteins | Leaves, bark | Unknown | Cardiovascular system and gastrointestinal tract | Budgerigars (*Melopsittacus undulates*) | Shropshire *et al*. (1992), Burrows and Tyrl (2001) |
| Bladder pod, rattlebox, coffeeweed, daubentonia, others | *Sesbania* spp. | Saponins, others | Seeds | Smooth muscle inhibition, vasodilation | Nervous system, gastrointestinal tract, kidneys | Chickens | Flory and Hebert (1984), Burrows and Tyrl (2001) |
| Cacao | *Theobroma cacao* | Theobromine, caffeine | Seeds | Adenosine receptor antagonism, increased intracellular $Ca^{++}$ concentrations | Nervous system, cardiovascular system | Chickens | Day and Dilworth (1984) |
| Canola (also referred to as rapeseed), mustards | *Brassica* spp. | Glucosinolates, other | Seeds | Accumulation of trimethylamine affects eggs (odor and taste), liver, and muscle damage due to unknown mechanism. | Eggs, liver, muscle, cardiovascular system | Chickens, Turkeys, ducks | Ratanasethkul *et al*. (1976), Wight *et al*. (1987), Burrows and Tyrl (2001) |
| Cassava | *Manihot* spp. | Cyanide | Root | See section on cyanide | | | |
| Castor bean | *Ricinus communis* | Ricin | Seeds | Protein synthesis inhibition | Gastrointestinal tract, liver | Ducks | Jensen and Allen (1981) |
| Coffee senna, sickle pod, coffeeweed, others | *Senna* spp. (previously, *Cassia* spp.) | Anthraquinones | Fruit and seeds | Irritation, mitochondrial myopathy | Gastrointestinal tract, skeletal muscle | Chickens | Cavaliere *et al*. (1997), Burrows and Tyrl (2001), Haraguchi *et al*. (2003) |
| Corn cockle | *Agrostemma githago* | Saponins | Seeds | Irritation | Gastrointestinal tract | Chickens | Heuser and Schumacher (1942), Burrows and Tyrl (2001) |

*(Continued)*

TABLE 56.1 (Continued)

| Common name | Scientific name | Toxin | Plant part demonstrated to be toxic | Mechanism of toxic action | Primary organ system affected | Species/bird type reported to be affected | References |
|---|---|---|---|---|---|---|---|
| Cottonseed | *Gossypium* spp. | Gossypol | Seeds | Unknown | Gastrointestinal, kidneys, liver, egg production | Poultry | Brown and Julian (2003) |
| Coyotillo | *Karwinskia humboldtiana* | Anthracenones | Seeds | Uncoupling of oxidative phosphorylation, inhibition of axoplasmal transport | Nervous system | Poultry | Burrows and Tyrl (2001) |
| Day jessamine, day cestrum, wild jasmine, Chinese inkberry | *Cestrum diurnum* | Analog of 1,25-dihydroxy-cholecalciferol | Leaves | Hypercalcemia and dystrophic tissue calcification | Cardiovascular system, kidneys | Chickens | Sarkar *et al*. (1981) |
| Death camas, others | *Zygadenus* spp. | Alkaloids | Seeds, stems, roots | Increase reflex activity, stimulate afferent pathway receptors, increase and extend negative afterpotentials | Nervous system | Poultry | Burrows and Tyrl (2001) |
| Dieffenbachia | *Dieffenbachia* spp. | Insoluble oxalates | Leaves | Physical irritation | Gastrointestinal tract | Canaries (*Serinus canaria*) | Arai *et al*. (1992) |
| Digitalis | *Digitalis purpurea* | Cardiac glycosides | Leaves | Inhibit $Na^{+}$–$K^{+}$ ATPase | Cardiovascular system | Canaries (*Serinus canaria*) | Arai *et al*. (1992) |
| Eucalyptus | *Eucalyptus cladocalyx* | Cyanide | Leaves | Inhibit cytochrome oxidase c | Multiorgan | Poultry | Brown and Julian (2003) |
| Jimsonweed, thornapple | *Datura* spp. | Tropane alkaloids | Seeds | Cholinergic (muscarinic) receptor antagonism | Nervous system | Chickens | Day and Dilworth (1984), Kovatsis *et al*. (1994), Burrows and Tyrl (2001) |
| Jojoba | *Simmondsia chinensis* | Simmondsin | Seeds | Inhibition of appetite via unknown mechanism | Overall growth rate | Chickens | Arnouts *et al*. (1993) |
| White popinac, lead tree, guacis, jumby bean | *Leucaena leucocephala* | Mimosine | Seeds | Inhibit reactions requiring pyridoxine, inhibit DNA and RNA synthesis | Bone, depressed growth | Poultry | Kamada *et al*. (1998), Burrows and Tyrl (2001) |
| Lily-of-the-valley | *Convallaria majalis* | Cardenolides | Leaves, rhizomes, and roots | Inhibit $Na^{+}$–$K^{+}$ ATPase | Cardiovascular system | Poultry | Burrows and Tyrl (2001) |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Lupine | *Lupinus* spp. | Quinolizidine alkaloids | Leaves | Cholinergic receptor agonists | Nervous system | Canaries (*Serinus canaria*) | Arai *et al*. (1992), Burrows and Tyrl (2001) |
| Milkweed | *Asclepias* spp. | Cardenolides, uncharacterized neurotoxin | Entire plant, latex sap | Inhibit $Na^{+}$–$K^{+}$ ATPase, uncertain for neurotoxic effect | Cardiovascular system, nervous system | | Burrows and Tyrl (2001) |
| Nightshades, potato | *Solanum* spp. | Steroidal glycosides (chaconine, solanine, others) | Immature seeds, foliage | Saponin-induced irritation, AChE inhibition | Gastrointestinal tract, nervous system | Chickens | Temperton (1944), Burrows and Tyrl (2001) |
| Oak | *Quercus* spp. | Tannins | Leaves, early buds | Astringent effect (protein precipitation), direct cell damage | Gastrointestinal tract, kidneys | Double-wattled cassowary (*Casuarius casuarius*) | Kinde, 1988; Burrows and Tyrl, 2001. |
| Oleander | *Nerium oleander* | Oleandrin | Leaves | Inhibit $Na^{+}$–$K^{+}$ ATPase | Cardiovascular system | Budgerigars (*Melopsittacus undulates*); Canaries (*Serinus canaria*); geese (species not identified) | Arai *et al*., 1992; Shropshire *et al*., 1992; Alfonso *et al*., (1994) |
| Oxalates, soluble | Many plants (*Halogeton* spp., *Sarcobatus* spp., others) | Calcium or potassium oxalates | Leaves, stems | Hypocalcemia, calcium oxalate precipitation in renal tubules | Cardiovascular system, kidneys | Poultry | Burrows and Tyrl, (2001) |
| Parsley | *Ammi majus* | Furanocoumarins | Seeds, leaves | Primary photosensitization, other (calcium channel inhibition) | Skin, liver, and kidneys | Ducks, geese; chickens, and turkeys are less sensitive | Shlosberg *et al*., 1974; Burrows and Tyrl, 2001 |
| Poison hemlock | *Conium maculatum* | Pyridine alkaloids including coniine, *N*-methylconiine, and $\gamma$-coniceine | Seeds | Nicotinic receptor agonist | Nervous system | Range turkeys (species not identified) | Frank and Reed (1987); Burrows and Tyrl, (2001) |
| Pokeberry | *Phytolacca americana* | Saponins, oxalates | Berries | Irritation | Gastrointestinal tract | Turkeys | Cattley and Barnett (1977); Burrows and Tyrl, 2001 |
| Rattlebox, others | *Crotalaria* spp. | Pyrrolizidine alkaloids | Seeds, leaves, and stems | Inhibition of protein and RNA synthesis, alkylate DNA, inhibit mitosis | Liver | Chickens, geese | Gopinath and Ford (1977); Alfonso and Sanchez, (1993), Burrows and Tyrl (2001) |

*(Continued)*

TABLE 56.1 (Continued)

| Common name | Scientific name | Toxin | Plant part demonstrated to be toxic | Mechanism of toxic action | Primary organ system affected | Species/bird type reported to be affected | References |
|---|---|---|---|---|---|---|---|
| Ragwort, groundsel | *Senecio* spp. | | | | | | |
| Sweet pea | *Lathyrus* spp. | Amino acids, nitriles, β-amino-proprionitrile | Seeds | Glutamate receptor agonism, inhibition of lysyl oxidase | Nervous system, reproduction (decrease ovarian activity), eggs | Chickens, turkeys | Chowdhury (1988), Burrows and Tyrl (2001) |
| Tobacco | *Nicotiana* spp. | Nicotine, nornicotine, anabasine | Leaves and stems | Nicotinic receptor stimulation, teratogen | Nervous system, embryo development | Poultry | Burrows and Tyrl (2001) |
| Velvetweed | Abutilon theophrasti | | | Affects egg yolks due to unknown mechanism | Eggs | Poultry | Brown and Julian (2003) |
| Vetch | *Vicia* spp. | Cyanogenic glycosides (probably not toxic); β -cyano-L-alanine | Seeds | Cyanide inhibits cytochrome oxidase c; signs similar to pyridoxine deficiency | Nervous system | Chickens | Harper and Arscott (1962), Burrows and Tyrl (2001) |
| Virginia creeper | *Parthenocissus quinquefolia* | | Leaves | Unknown | Gastroinestinal system | Budgerigars (*Melopsittacus undulates*) | Shropshire *et al*. (1992), Burrows and Tyrl (2001) |
| Yew | *Taxus* spp. | Taxine | Leaves, seeds | Alter atrioventricular (AV) conduction via inhibition of $Na^{+}$–$Ca^{++}$ fluxes and possible $K^{+}$ channel effects | Cardiovascular system | Budgerigars (*Melopsittacus undulates*); Canaries (*Serinus canaria*); emus (*Dromaius novaehollandiae*) | Shropshire *et al*. (1992), Fiedler and Perron (1994), Burrows and Tyrl (2001) |

between toxin type and age (Barnes, 2003). For example, chickens, turkeys, pheasants, and peafowl are susceptible to types A, B, C, and E, but not to D or F (Gross and Smith, 1971). As broiler chickens age, they become less susceptible to type C toxin (Barnes, 2003). Most outbreaks of botulism in birds are due to exposure to type C1 toxin. Less frequently, exposure to type E has caused mortality of fish-eating birds (Friend and Franson, 1999). Domestic chickens have been intoxicated by type A. Recently, a mosaic neurotoxin (2/3 type C and 1/3 type D) was characterized that was believed to have been responsible for some forms of avian botulism (Takeda *et al*., 2005).

Avian botulism is one of the most important diseases of migratory birds (Friend and Franson, 1999). Filter feeding and dabbling waterfowl are among the species most at risk. Raptors have been intoxicated by feeding on poultry carcasses that have not been disposed of properly. Waterfowl are exposed to botulinum toxin via ingestion of contaminated substrates or via contaminated invertebrates such as maggots that feed on decaying carcasses or other organisms such as zooplankton that are inadvertently contaminated. As few as two maggots can be lethal if ingested. Outbreaks of botulism in avian wildlife result in losses that are quite variable, ranging from a few hundred birds to over one million.

Poultry and other production birds can be affected. Game farm pheasants have been intoxicated by feeding on maggots (Foreyt and Abinanti, 1980). Wound contamination by spores of *C. botulinum* was hypothesized to have contributed to the deaths of caponized chickens (Trampel *et al*., 2005).

Vultures and other carrion eaters are resistant to botulinum toxin, perhaps, in part, due to the production of antibodies to botulinum toxin which does not occur in sensitive species such as chickens and ducks (Gross and Smith, 1971; Ohishi *et al*., 1979; Friend and Franson, 1999).

Botulinum toxin blocks the release of ACh at neuromuscular junctions which results in paralysis. Blockage occurs as a result of the failure of synaptic vesicles containing ACh to fuse with the plasma membrane (Davletov *et al*., 2005). This occurs as a result of the ability of botulinum toxin to cleave and thereby inactivate cellular proteins necessary for fusion.

Clinical signs are consistent with neuromuscular paralysis. Early, affected waterfowl are unable to sustain flight (Friend and Franson, 1999). Leg paralysis in waterfowl causes birds to propel themselves using their wings. Paralysis of the nictitating membrane and neck muscles follows, resulting in an inability to hold the head erect (thus the term limberneck). In chickens, an ascending flaccid paralysis is noted (Barnes, 2003). Initially, affected birds are reluctant to walk and, when forced, appear lame. Similar to waterfowl, the wings droop and the neck becomes weak followed by eyelid paralysis. Waterfowl often drown; affected terrestrial birds die from respiratory and cardiac failure. The time between the onset of clinical signs and death can be as short as few hours. Postmortem lesions are generally not observed; in affected waterfowl, lesions may be consistent with drowning.

A presumptive diagnosis is based upon characteristic clinical signs and environmental conditions conducive to bacterial growth and toxin production. Confirmation of botulism is most often made by using the mouse bioassay to detect the presence of the toxin. Toxin specificity is determined by protecting inoculated mice with specific antitoxins. More recently, enzyme-linked immunosorbent assay (ELISA) and liquid chromatography–mass spectrometry methods have been developed to confirm the presence of toxin (Zechmesiter *et al*., 2004; Barr *et al*., 2005). The mouse bioassay is still considered to be the "gold standard" assay due to the sensitivity of the mouse to botulinum toxin. However, newer methods will likely replace the mouse bioassay in the future.

Treatment of individual affected birds with antitoxin can result in recovery, especially in waterfowl. Other affected species such as coots, shorebirds, gulls, and grebes rarely recover (Friend and Franson, 1999). Difficult logistics, limited availability of antitoxin, and cost of treatment preclude effective intervention in large outbreaks.

Prevention and control of outbreaks are critical to minimize losses. In wetlands, the amount of organic and decaying material should be limited. Prompt removal and disposal via burial or burning of animal carcasses is important during outbreaks to interrupt bacterial growth and toxin production. Immunization with inactivated bacterin-toxoids is protective but vaccination of large poultry flocks or waterfowl is expensive and, in the case of waterfowl, logistically difficult.

### *Biogenic amines*

Biogenic amines, such as gizzerosine, histamine, histidine, cadaverine, spermine, and putrescine result from the heating or bacterial spoilage of fish and animal by-products (Barnes *et al*., 2001; Brown and Julian, 2003). Poultry are potentially exposed to the amines when fish or meat meal is incorporated into their diets. Toxic concentrations vary depending on the specific amine. Dietary histamine at 0.1% is associated with reduced feed conversion and body weights in broiler chickens (Barnes *et al*., 2001). Dietary spermine is toxic to 120-week-old chicks at 0.2% (Sousadias and Smith, 1995). Putrescine is toxic at concentrations >0.05% (Chowdhury, 2001). Interestingly, a dietary concentration of putrescine of 0.05% has been associated with improved eggshell quality (Chowdhury, 2001). The improvement was hypothesized to be due to increased calcium transport.

Biogenic amines at toxic concentrations cause digestive disturbances. Gizzerosine causes gizzard erosion and hemorrhage. A dietary histamine concentration of 4 ppm results

in localized gizzard lesions and reduced growth rates in chicks (Harry and Tucker, 1976). In addition to gastrointestinal effects, toxic biogenic amine concentrations cause a malabsorption syndrome in chickens characterized by reduced feed efficiency and enlargement of the proventriculus (Barnes *et al.*, 2001).

## Feed additives

### *Arsenicals*

Arsenic exists in a variety of inorganic and organic forms and valences. Arsenic trioxide was used as an herbicide. Pentavalent and trivalent sodium, potassium, and calcium salts of arsenic have been used as insecticides, especially for ants. Trivalent organic forms of arsenic include the herbicides monosodium methanarsonate (MSMA) and disodium methanarsonate (DSMA) and thiacetarsamide, previously used for treating dogs with heartworm. Pentavalent arsenicals used as feed additives include arsanilic acid, sodium arsanilate, 3-nitro-4-hydroxyphenylarsonic acid (3-nitro, roxarsone), and 4-nitrophenylarsonic acid. Arsanilic acid and 3-nitro are the most commonly used organic arsenicals in poultry production (Feed Additive Compendium, 2005). They are used to increase feed efficiency, promote growth, improve pigmentation, and prevent coccidiosis. While exposure of birds to inorganic arsenic salts can occur, most avian arsenic intoxications involve exposure of poultry to pentavalent organic arsenicals.

Pentavalent organic arsenicals are believed to substitute for phosphate in oxidative phosphorylation. Uncoupling of oxidative phosphorylation results in cellular energy deficits (Ensley, 2004). Intoxication occurs most commonly due to feed mixing errors. Clinical signs have occurred in turkeys exposed to dietary concentrations of 3-nitro approximately twice the recommended dose. Clinical signs in affected birds include stunting, depression, apparent lameness, and ataxia. Gross lesions are typically absent, but histopathologically, Wallerian degeneration of the optic and peripheral nerves occurs. Wallerian degeneration can also occur in the spinal cord (Ensley, 2004). Ulcerative cholecystitis has been reported in turkey poults intoxicated by 3-nitro (Brown *et al.*, 1991).

Diagnosis of organic arsenical intoxication in poultry depends on detection of potentially toxic concentrations in representative feed samples and the occurrence of compatible clinical signs and postmortem lesions. Treatment consists of removing the offending feed and providing accessible feed and water.

Arsenic has been detected at relatively high concentrations in some seabirds including albatrosses and gulls (Kubota *et al.*, 2003). It is present in tissues primarily as the organic arsenical, arsenobetaine, which is relatively non-toxic.

A significant percentage of the human tolerable daily intake of arsenic can come from arsenic residues in poultry meat (Taylor, 2004). Measurable increases in soil arsenic concentrations can occur as a result of using litter from arsenic-treated poultry as fertilizer (Garbarino *et al.*, 2003; Rutherford *et al.*, 2003).

### *Sodium*

Sodium ion intoxication is a significant problem in poultry production. Intoxication can result from excessive sodium in the feed, water deprivation, or ingestion of saline waters (Brown and Julian, 2003). The most common form of sodium associated with intoxication is sodium chloride. Sodium sesquicarbonate, used as a buffering agent in ruminant feeds containing high concentrations of urea, was responsible for high mortality in broiler chickens as a result of a feed misformulation (Sander *et al.*, 1998). High dietary sodium can be well tolerated if access to water is not restricted. For example, 15-day-old turkey poults tolerated up to 10% sodium chloride in their mash for 14–16 days when drinking water was not restricted (Gitter *et al.*, 1979). However, young chicks and poults can be intoxicated from sodium in feed despite adequate water intake. Sodium concentrations in both the feed and water need to be considered in assessing sodium exposure.

A number of factors influence the toxicity of the sodium ion including bird age, bird species, dietary factors, and water quality and accessibility. Young birds are more sensitive to sodium ion toxicity, most likely due to less developed renal function. Turkeys are more susceptible to intoxication than chickens (Berger, 1993). In addition, birds have less ability to excrete salts in excess of water, so their ability to reduce high-plasma osmolality is limited (Barnes, 2003). Some waterfowl have nasal salt glands that provide an additional excretory route. In general, the addition of 1% salt (as sodium chloride) to poultry rations is safe, even for very young birds. A level of 0.25% salt in drinking water is considered to be safe (Berger, 1993).

At high sodium intakes, birds develop acute, severe diarrhea and dehydration, lose weight, and die. Renal function is often impaired, especially if sodium bicarbonate is the source for exposure (Barnes, 2003). Lower, but still toxic, sodium intakes primarily cause cardiac overload, ascites, dyspnea, and edema. Loose droppings, decreased feed intake, and poor growth are often observed. Thirteen-week-old tom turkeys affected by high dietary sodium chloride concentrations (~8%) exhibited polydipsia, diarrhea, ataxia, tremors, depression, sternal and lateral recumbency, torticollis, and death (Wages *et al.*, 1995).

Ascites, edema, fluid in the lungs, hydropericardium, cardiac hypertrophy (right-sided in chickens and bilateral in poults), and dilatory cardiomyopathy (poults) are found on postmortem examination. Most microscopic lesions are secondary to cardiac failure, although bilaterally symmetrical

areas of cerebral necrosis, vascular congestion, and edema were noted in intoxicated tom turkeys (Wages *et al*., 1995).

Sodium intoxication in waterfowl has been associated with the ingestion of hypersaline water or salt that has precipitated on feathers (Windingstand *et al*., 1987; Meteyer *et al*., 1997; Gordus *et al*., 2002; Stolley and Meteyer, 2004). Significant gross and microscopic lesions in ruddy ducks intoxicated by sodium include conjunctivitis, lens opacity, cataract formation, vascular congestion in multiple organs especially in the meninges, and myocardial and skeletal muscle degeneration (Gordus *et al*., 2002).

The use of sodium chloride as a deicing agent on roads is believed to intoxicate passerine birds. Experimentally sodium chloride was lethal to house sparrows dosed with a concentrated sodium chloride solution providing 8000 mg/kg (0.8%) (Bollinger *et al*., 2005).

A diagnosis of sodium ion intoxication relies on detection of high dietary and/or water, serum and/or brain sodium concentrations. The significance of detected dietary and water sodium concentrations needs to be assessed in conjunction with other historical, clinical, and postmortem findings. Serum sodium concentrations >150 mEq/l are elevated and brain sodium concentrations >2000 ppm wet weight are highly suggestive of intoxication (Puls, 1994).

### *Vitamin A*

Vitamin A is an essential micronutrient that plays a role in normal vision, reproduction, immunity, membrane integrity, growth, and embryogenesis. Vitamin A intoxication has been produced experimentally in poultry and cockatiels (Tang *et al*., 1985; Koutsos *et al*., 2003). Osteodystrophy occurred in broiler and leghorn chicks exposed to 330–660 IU vitamin A per day for 21 days (Tang *et al*., 1985). Skeletal development was affected in growing chickens given 200 mg retinyl acetate per kg (Baker *et al*., 1967). Anorexia, conjunctivitis, eyelid adhesions, and encrustations around the mouth were also noted by Tang. Cockatiels fed approximately 30,000 μg per kg of vitamin A (as retinol) for 269 days exhibited intensified vocalization patterns, pancreatitis, multifocal accumulation of lymphocytes in the lamina propria of the duodenum, and reduced body condition (Koutsos *et al*., 2003). Intakes of 3000 μg per kg of vitamin A also caused clinical signs, but they were less severe. A diagnosis of vitamin A intoxication can be made based upon measurement of high vitamin A concentrations in representative feed samples and in serum or liver tissue along with the occurrence of compatible clinical signs.

### *Vitamin D*

Vitamin D is required for normal calcium and phosphorus homeostasis which, in turn, is important for normal bone, beak, claw, and eggshell formation. Poultry diets are commonly supplemented with vitamin D in the form of cholecalciferol ($D_3$) (Barnes, 2003). Vitamin D stimulates the absorption of calcium from the gastrointestinal tract, influences osteoblast and osteoclast activity, and increases renal tubular reabsorption of calcium. Exposure to excessive vitamin D results in increases in calcium absorption from the gastrointestinal tract and renal tubules, and calcium mobilization from bone. High blood and tissue calcium concentrations cause metastatic tissue calcification. In broiler chicks, dietary concentrations of 30,000 IU/kg are toxic when fed during growth. Much higher doses of vitamin D can rapidly cause renal calcification and damage. Aortic and arterial calcification can also occur. Postmortem lesions include parathyroid gland atrophy, renal tubular and aortic calcification, and calcification of blood vessel walls in the brain. Older birds are less sensitive to vitamin D than younger, actively growing birds. Other forms of vitamin D are also potentially toxic. Relative toxicity is vitamin $D_2$ > vitamin $D_3$ > 25-monohydroxycholecalciferol > 1,25-dihydroxycholecalciferol (Barnes, 2003). Nephrocalcinosis was experimentally produced in chicks by feeding powdered leaves from *Cestrum diurnum* which contains an analog of 1,25-dihydroxycholecalciferol (Sarkar *et al*., 1981).

### *Drugs*

A number of chemotherapeutic agents have caused intoxications in birds, primarily in poultry and pet birds. In most cases, intoxication results from inappropriate or overuse of a drug. Table 56.2 lists chemotherapeutic agents associated with adverse effects in birds. Two drugs have been documented to intoxicate birds of prey as a result of feeding on carcasses from animals administered the drugs prior to death. Carcass residues of the non-steroidal anti-inflammatory, diclofenac, have been shown to cause renal failure, visceral gout, and death in oriental white-backed vultures (*Gyps bengalensis*) and long-billed vultures (*G. indicus*) in the Indian subcontinent (Oaks *et al*., 2004; Shultz *et al*., 2004). Diclofenac has been a widely used veterinary drug in regions with affected birds. In the United States, use of pentobarbital for euthanasia has resulted in secondary intoxication of raptors feeding on carcasses from euthanized animals (O'Rourke, 2002). Intoxication has occurred from scavenging carcasses not disposed of properly on farms or landfills.

## Pesticides

### *Insecticides*

#### *Organochlorine insecticides*

Historically, OCs were widely used from the 1940s to the 1970s in agriculture and forestry and for mosquito control. The most widely used organochlorines included the dichlorodiphenylethanes (DDT, methoxychlor, and dicofol), cyclodienes (aldrin, dieldrin, heptachlor, chlordane, and endosulfan), and hexachlorocyclohexanes (lindane

**TABLE 56.2 Drug intoxications in birds**

| Drug | Effects | Clinical signs | Pathologic lesions | References |
|---|---|---|---|---|
| Sulfonamides | Blood dyscrasias, bone marrow depression, anemia, thrombocytopenia, lymphoid depression, impaired immune function. | Depression, pallor, weight loss, decreased egg production and quality, icterus, depigmentation of brown eggs | Widespread hemorrhage, pale bone marrow, swollen, pale red or icteric liver, splenic enlargement, ulcers at proventricular-gizzard junction | Reece *et al*. (1985), Daft *et al*. (1989), Brown and Julian (2003) |
| Nitrofurans | Furazolidone causes a dose-related biventricular cardiomyopathy<br>Chronic exposure can delay sexual maturity in male broiler breeder chickens | Depression, incoordination, ruffled feathers, growth retardation, hyperexcitability, tremors, loud vocalization, opisthotonus, aimless running, seizures | Dilation of ventricles of the heart, thinning of right or left ventricular wall, passive congestion with lung edema, liver congestion, ascites | Reece *et al*. (1985), Brown and Julian (2003) |
| Aminoglycosides | Lysosomal dysfunction in renal tubular epithelial cells leading to necrosis; impaired synthesis of protective vasodilatory renal prostaglandins<br>Apoptosis in cochlear sensory hair cells | Gentamicin causes depression, edema, injection site hemorrhages, large pale nephritic kidneys; weakness, and apnea<br>Ototoxicity<br>Egg inoculation associated with embryo deaths<br>Streptomycin and dihydrostreptomycin sulfate associated with respiratory distress, paresis, and mild convulsions | Increased number of lysosomes containing myelin figures in renal tubule cells<br>Renal tubular cell necrosis<br>Degeneration of the apical portion of cochlear hair cells | Bird *et al*. (1983), Boothe (2001), Woolley *et al*. , (2001), Brown and Julian (2003) |
| Nicarbazin | Increases metabolic rate and heat production<br>Increases lipoprotein lipase activity<br>Acts as a $Ca^{++}$ ionophore | Poor eggshell pigmentation, decreased egg weight, decreased egg hatchability, growth depression, increased susceptibility to heat stress | No diagnostic lesions | Reece *et al*. (1985), Hughes *et al*. (1991) , Brown and Julian (2003), Yoder *et al*. (2006), |
| Dimetridazole (other nitroimidazoles) | Possible free radical damage to DNA and other molecules | Growth depression, decreased egg production, incoordination, inability to fly, aimless running, vocalization, tremors, seizures | No diagnostic lesions | Riddell (1984), Brown and Julian (2003) |
| 3,5-dinitro-*o*-toluamide | Purkinje cell dysfunction | Ataxia, torticollis, reduced growth | Necrosis and depletion of Purkinje cells of the cerebellar cortex | Reece and Hooper (1984), Brown *et al*. (2003) |
| Ivermectin (and other macrolide endectocides) | GABA agonist | Somnolence, listlessness, ataxia, bradypnea, mydriasis | No diagnostic lesions | Kim and Crichlow (1995), Brown and Julian (2003) |

and benzene hexachloride). While these insecticides are no longer used in the United States, Canada, and Europe, they continue to be used in developing countries. These compounds are highly lipophilic and, in general, have long environmental and body half-lives (Blus *et al.*, 1996). Because of their lipophilic and persistent nature, they biomagnify within food webs. They were banned due to their environmental persistence, concern about their impact on human health, and widespread insect resistance.

OCs can be acutely toxic and a large number of avian mortality events following exposure have been documented (Friend and Franson, 1999). Acutely, the OCs are neurotoxic as a result of their ability to prolong the opening of sodium channels (DDT) or act at presynaptic terminals (cyclodienes and lindane) (Blus *et al.*, 1996). Signs of neurotoxicity include hypersensitivity, muscle twitching and tremors, and intermittent to continuous tonic–clonic seizures (Blus *et al.*, 1996). Death is most often due to respiratory failure and hypoxia. OCs can accumulate within fat reserves to high concentrations; storage in fat prevents adverse effects from occurring within the nervous system. However, rapid mobilization of residues from fat can precipitate signs of intoxication.

Dichlorodiphenyldichloroethylene (DDE, a metabolite of DDT) was responsible for perhaps the most widely known sublethal effect of the OCs on several avian wildlife populations. Sublethal effects included failure of adults to breed, eggshell thinning, and a decrease in hatchability of eggs and survival of young birds (Blus *et al.*, 1996). DDE impairs the translocation of calcium from the mucosa cells of the shell gland to the shell gland cavity and alters the ratio of calcium bound per mole of ATP hydrolyzed during shell formation. DDE can also inhibit calmodulin. The sublethal, reproductive effects of DDE were species specific. Widespread population declines were noted in peregrine falcons, double-crested and great cormorants, ospreys, bald eagles, merlins, and brown pelicans (Blus, 2003). Interestingly, some bird species such as American kestrels, Great blue herons, and domestic chickens are resistant to the reproductive effects of DDE. OCs such as DDT, DDE, methoxychlor, and chlordecone exhibit estrogenic activity. Also, OCs induce liver microsomal enzymes which, in turn, alter sex hormone metabolism (Blus, 2003).

Although acute intoxication is rare, a diagnosis is based upon the occurrence of characteristic signs and detection and quantification of OCs in brain samples. Gross or histopathologic findings are generally absent (Friend and Franson, 1999). Occasionally, residues of OCs are a concern in poultry intended for human consumption following exposure to sublethal concentrations.

*Cholinesterase inhibitors (organophosphorus and carbamate insectisides)*

Due to their more rapid breakdown in the environment, OP and carbamate insecticides replaced the organochlorine pesticides as the later were banned for use in North America and Europe in the 1960s and 1970s. A number of different formulations are available for use either in the environment (e.g. agricultural or residential use) or on animals (e.g., livestock dips or sprays). OPs and carbamates are formulated as liquids, granules, and powders. The more toxic insecticides of each group are generally restricted to agricultural uses, whereas less toxic members are approved for use on animals or in residential environments. Although chemically distinct, the OPs and carbamates have a common mechanism of toxic action, namely, the inhibition of cholinesterase enzymes.

Possible exposure scenarios are numerous. Pet birds can be exposed via their diets, via home or premise use, or via direct application. Inhalation exposure is also possible from the use of dichlorvos-impregnated pest strips or premise spraying or fogging. Poultry and other production birds can be exposed in similar ways. There are relatively few documented reports of acute pet or production bird intoxication from the use of OPs or carbamates. However, acute OP and carbamate intoxications are common in wild birds and, while the numbers of affected birds is difficult to assess, losses are significant. For example, at the height of use of granular formulations of the carbamate insecticide, carbofuran, 17 to 91 million birds were estimated to have died annually (Mineau, 2005).

Exposure of wild birds to these insecticides can occur via ingestion of treated seeds or vegetation (accidental, intentional, or misuse of a product), poisoned insects or animals (impaired live animals or carcasses), product (especially granular formulations), or contaminated water (Friend and Franson, 1999). Inhalation or dermal exposure is also possible from spraying or spills. With avian wildlife there can be some degree of seasonality to OP or carbamate intoxications as a result of season patterns of insecticide use.

The acute oral toxicity of individual OPs and carbamates varies considerably within each of their classes. For example, within the carbamate insecticide class, the acute oral $LD_{50}$s for carbofuran and carbaryl in mallard ducks are 0.5 mg/kg b.w. (body weight) and $>2000$ mg/kg b.w., respectively (Friend and Franson, 1999). Also, acute oral toxicities of specific OP or carbamate insecticides vary considerably between species. The acute oral $LD_{50}$s of ethion range from 45 mg/kg for blackbirds to $>2000$ mg/kg for mallard ducks (Friend and Franson, 1999). A number of other factors can influence toxicity including age, sex, diet, body condition, and product formulation.

Some OPs, including insecticides such as leptophos, mipafox, and cyanofenphos and industrial chemicals such as tri-*ortho*-cresyl-phosphate (TOCP) cause a delayed neurotoxic effect referred to as OP-induced delayed neuropathy (OPIDN). OPIDN occurs as a result of inhibition of neurotoxic esterase, an enzyme found in peripheral nerves. OP insecticides are tested for their ability to cause OPIDN in adult hens as a result of their unique sensitivity to this effect. Also, pheasants and mallard ducklings are highly

susceptible to delayed neurotoxicity (Brown and Julian, 2003). Most OPIDN-inducing insecticides are no longer on the market (Hill, 2003).

As previously mentioned, OPs and carbamates inhibit cholinesterase enzymes. Inhibition of AChE is primarily responsible for the clinical signs associated with intoxication. Enzyme inhibition prevents the breakdown of ACh at the synapses and neuromuscular junctions. As a result ACh overstimulates muscarinic and nicotinic receptors in the central and peripheral nervous systems. Clinical signs include: convulsions, hyperexcitability, opisthotonus, lethargy, miosis or mydriasis, apparent blindness, ataxia, muscular weakness, tachypnea, dyspnea, emesis, defecation/diarrhea, piloerection, lacrimation, ptosis, and epistaxis (Friend and Franson, 1999). The onset of clinical signs and death can be rapid; birds are often found dead. Death is most often due to respiratory failure and hypoxia. Gross and histopathologic lesions in dead birds are usually minimal and non-specific. Gastrointestinal contents should be examined carefully, since the presence of granules or dye might suggest pesticide exposure. Often, given the rapidity of death, there is freshly ingested food in the upper gastrointestinal tract. OP inhibition of AChE activity can be irreversible, thus necessitating synthesis of new enzyme before recovery can occur. Carbamates do not irreversibly inhibit AChE and spontaneous regeneration of enzyme activity is rapid. Clinical signs can persist for days in OP intoxicated animals, whereas recovery from carbamate intoxication is generally completed within 2–3 days.

A diagnosis of intoxication is dependent on measuring reduced activity of cholinesterase along with identification of a specific insecticide in suitable antemortem or postmortem samples. In birds, plasma is suitable for cholinesterase activity determinations. Brain cholinesterase activity can be measured in dead birds. Depending on the size of the brain, either a whole brain or a right or left brain half should be submitted for analysis. It is important to point out that because carbamate inhibition of cholinesterase activity is readily reversible, even after death, care must be taken in interpreting laboratory results. In general, cholinesterase activity <50% of normal suggests significant exposure to an OP or carbamate (activity is often <20% of normal following lethal exposures) (Hill, 2003). Normal plasma/whole blood and brain cholinesterase activities are quite variable among bird species and interpretation of cholinesterase activity should be based on species specific, and when possible, laboratory specific reference ranges. Reference ranges for cholinesterase activity in plasma and brain from a variety of bird species have been published (Westlake *et al.*, 1983; Hill, 1988). Gastrointestinal contents and liver samples should be submitted for detection of a specific insecticide to confirm exposure. Urine should be submitted for detection of major metabolites of OPs (Jain, 2006).

Treatment of OP or carbamate intoxication can be effective. Atropine is used to block muscarinic receptor stimulation and its timely administration can provide rapid relief from life-threatening clinical signs. Atropine is given to effect and repeated administration is often required. Effective doses of atropine may need to be much higher in birds (25–50 mg/kg b.w. given intramuscularly (i.m.)) than generally recognized based upon experimental work in broiler chickens exposed to the OP, diazinon, or the carbamate, methomyl (Shlosberg *et al.*, 1997). Pralidoxime (2-PAM) is also given to promote AChE reactivation in OP poisoned animals. Again, effective doses in birds may need to be higher than generally recommended for mammals (up to 100 mg/kg b.w. given i.m. (Shlosberg *et al.*, 1997). Birds should be atropinized prior to administration of 2-PAM. 2-PAM is more effective when given as soon after OP exposure as possible due to the irreversible aging of AChE enzyme. 2-PAM is generally not indicated in carbamate intoxications due to the spontaneous regeneration of acetyl-cholinesterase.

Otherwise sublethal exposure to OPs or carbamates can result in mortality as a result of a variety of effects: increased vulnerability to trauma or predation, reduced ability to regulate body temperature and therefore increased susceptibility to hypothermia, and reduced activity leading to decreased feeding and weight loss (Friend and Franson, 1999; Hill, 2003). In addition, birds can be indirectly affected by reduced availability of food as a result of prey die-offs.

Fortunately, the overall adverse impact of agricultural pesticide use in general, and OPs and carbamate use more specifically, on avian wildlife has lessened over the last decade. This is due primarily to the replacement of older more toxic insecticides with newer, less toxic ones such as pyrethrins/pyrethroids and neonicotinoids (Mineau and Whiteside, 2006).

### *Pyrethrins/pyrethroids*

Naturally occurring pyrethrin (derived from chrysanthemum flowers) and synthetic pyrethroid insecticides are currently estimated to make up more than 25% of insecticide use worldwide (Volmer, 2004). Their popularity is due to their lack of environmental persistence and relatively low toxicity for birds and mammals (they are highly toxic for fish). They are used to control a variety of agricultural, home, and animal pests and are available in a large number of formulations including sprays, dusts, dips, shampoos, spot-ons, foggers, ear tags, wettable powders, granules, soluble powders, and emulsifiable concentrates. They are often combined with synergists such as piperonyl butoxide to enhance their insecticidal activity.

Pyrethrins and pyrethroids have low toxicity for birds. For example, the acute oral $LD_{50}$ of cypermethrin for mallard ducks is >4640 mg/kg and the dietary $LC_{50}$ for

mallard ducks and bobwhite quail is >20,000 ppm (Extoxnet). The 8-day $LC_{50}$ of deltamethrin for ducks and quail is >4650 mg/kg and >10,000 mg/kg, respectively. Permethrin is practically non-toxic to birds with acute oral $LD_{50}$s for one permethrin formulation (Pramex™) of >9900 mg/kg, >13,500 mg/kg, and >15,500 mg/kg for mallard ducks, pheasants, and Japanese quail, respectively (Extoxnet).

Pyrethrins and pyrethroids are neurotoxic as a result of their ability to alter sodium channels and cause repetitive nervous discharges or membrane depolarization. Clinical signs associated with acute intoxication are related to nervous system stimulation. There does not appear to be any adverse behavioral effects on avian wildlife following sublethal exposures to pyrethrins/pyrethroids, similar to those reported for OPs or carbamates, which would cause mortality due to increased predation or inability to obtain food. However, this possibility should be further investigated.

*Boric acid*

Boric acid is used in poultry litter to control darkling beetles (Brown and Julian, 2003). Recommended litter treatment concentrations are between 0.4 and 0.9 kg per 9.3 $m^2$ (Dufour *et al*., 1992). Boric acid has a relatively high acute oral $LD_{50}$ for 1-day-old chickens of 2.95 g/kg b.w. (Sander et al., 1991). Exposure of 1-day-old chicks to litter treated with up to 7.2 kg boric acid per 9.3 $m^2$ of litter for 15 days exhibited a dose-related feathering abnormality, but no effect on productivity and no lesions were noted. Exposure of poultry to litter treated with recommended amounts of boric acid is unlikely to be toxic.

*Nicotine*

Nicotine sulfate has been used to paint chicken roosts to control mites and has been given orally for internal parasites (Brown and Julian, 2003). Nicotine is a highly toxic alkaloid that stimulates nicotinic receptors at neuromuscular junctions. Initial receptor stimulation is followed by a depolarizing blockade. Death is due to hypoxia as a result of paralysis of the diaphragm and chest muscles. Currently, nicotine use as an insecticide is not common. Perhaps of more concern is the passive exposure of pet birds to cigarette, cigar, or pipe smoke. Chronic ocular, dermatologic, and respiratory diseases in pet birds have been associated with tobacco smoke exposure (Dumonceaux and Harrison, 1999).

Neonicotinoids, a new class of insecticide, are also nicotinic receptor agonists. They are considered to have low toxicity for vertebrates because of relatively low affinity for vertebrate nicotinic receptors compared to insect nicotinic receptors (Tomizawa and Casida, 2005). Neonicotinoids include acetamiprid, clothianidin, dinotefuran, imidicloprid, nitenpyram, thiacloprid, and thiamethoxam.

***Rodenticides***

*Anticoagulants*

Anticoagulant rodenticides are the most widely available and used rodenticides. They can be categorized as "first-generation" and "second-generation" anticoagulants. First-generation compounds include warfarin and indanediones such as chlorophacinone and diphacinone (Means, 2004). Because of rodent resistance to first-generation compounds, second-generation compounds such as brodifacoum, bromadiolone, difenacoum, and difethialone were developed and are now more commonly used than first-generation compounds. Products are formulated as pellets, wax blocks, and tracking powders. Warfarin products generally contain 0.025% active ingredient (AI) while second-generation compounds contain 0.05% AI (Means, 2004). Difethialone products usually contain 0.0025% AI. Second-generation anticoagulants are designed to kill rodents after one feeding and are more toxic and have much longer half-lives than warfarin.

Anticoagulant rodenticides have a common mechanism of toxic action. They block vitamin K-dependent clotting factor synthesis (factors II, VII, IX, and X) secondary to inhibition of vitamin-$K_1$ epoxide reductase. Thus, the extrinsic, intrinsic, and common coagulation pathways are affected in mammals, although in birds, affects on the extrinsic and common pathways may be more important (James *et al*., 1998). Clinical signs are often non-specific such as depression, anorexia, and weakness or are related to a coagulopathy such as subcutaneous (s.c.) hemorrhages, epistaxis, and oral petechiation (James *et al*., 1998). The onset of clinical signs is often delayed for several days after exposure to a toxic amount of anticoagulant since existing coagulation factors need to be depleted before hemorrhage occurs.

Available avian toxicity information is most often expressed as a median lethal concentration ($LC_{50}$) of anticoagulant included in feed over a defined period of time (Petterino and Paolo, 2001). A few $LD_{50}$ values are also available (see Table 56.3). There is little toxicity data available for species such as raptors which are likely to be exposed to the anticoagulants following ingestion of exposed and/or intoxicated prey.

There are almost no reported cases of anticoagulant rodenticide intoxication in poultry. There is one case report of coumafuryl toxicity in chickens <1 week of age (Munger *et al*., 1993). Exposure occurred as a result of contaminated wood-straw mats used to ship the birds. The low incidence of intoxication most likely reflects the relatively controlled environments associated with most commercial poultry production. Exposure is more likely in free-roaming poultry.

There are several documented cases of anticoagulant intoxication in captive wild birds including waterfowl, raptors, and bird-of-paradise (James *et al*., 1998; Poppenga, personal experience). Fortunately, most institutions

TABLE 56.3 Toxicity of anticoagulant rodenticides for birds

| Anticoagulant | Avian species | Acute, oral $LD_{50}$ | Dietary $LC_{50}$ |
|---|---|---|---|
| Warfarin | Japanese quail | 2000 mg/kg b.w. | |
| | Chickens | | >50 ppm b.w. – 96 h |
| Brodifacoum | Chicken | 4.5 mg/kg b.w. | |
| | Mallard duck | 0.31 mg/kg b.w. | 2.7 ppm – duration not specified |
| | Bobwhite quail | | 0.8 ppm – duration not specified |
| Bromodiolone | Mallard duck | | 110 ppm – 5 days |
| | Quail | 1600 mg/kg b.w. | |
| | Bobwhite quail | 138 mg/kg b.w. | |
| Difenacoum | Chicken | >50 mg/kg b.w. | |
| Difethialone | Mallard duck | | 1.95 ppm – 30 days |
| | Bobwhite quail | 0.264 mg/kg b.w. | 0.56 ppm – 30 days |
| Flocoumafen | Mallard duck | 24 mg/kg b.w. | 1.7 ppm – 5 days |
| | Japanese quail | >300 mg/kg b.w. | 37 ppm – 5 days |
| | Chicken | >100 mg/kg b.w. | 16.4 ppm – 5 days |
| Chlorophacinone | Mallard duck | | 204 ppm – 30 days |
| | Bobwhite quail | | 95 ppm – 30 days |
| Diphacinone | Mallard duck | 3158 mg/kg b.w. | |
| Pindone | Mallard duck | | 250 ppm – 8 days |
| | Bobwhite quail | | 1560 ppm – 8 days |

housing wild birds recognize risks associated with anticoagulant rodenticide use and either use alternative rodenticides or take precautions to avoid exposure.

Exposure of wild birds to anticoagulants either via bait ingestion or through ingestion of exposed and/or intoxicated prey is a major concern. The long half-lives of second-generation anticoagulants result in intoxication risks associated with repeated exposures to relatively low doses of the compounds. Avian species that feed primarily on small rodents are at greatest risk. Soon after the introduction of second-generation anticoagulants, the potential for secondary intoxication of raptor species such as owls was investigated (Mendenhall and Pank, 1980; Townsend *et al.*, 1981). Residues have been measured in a number of bird species (Merson and Byers, 1984; Hegdal and Colvin, 1988; Berny *et al.*, 1997; Stone *et al.*, 1999; Eason *et al.*, 2002; Stone *et al.*, 2003). Although anticoagulant intoxication has been documented in a number of individual birds, population impacts are less clear.

Diagnosis of intoxication is dependent on antemortem and/or postmortem evidence of a coagulopathy and the detection of an anticoagulant in blood, serum, or liver samples. Merely finding a residue of an anticoagulant in an animal is not sufficient for a diagnosis of intoxication since residues are often found in the absence of a coagulopathy.

Vitamin $K_1$ is antidotal treatment, although there is some delay before coagulopathy resolves. Dosing regimens have not been well defined in avian species, but vitamin $K_1$ at 0.2–2.2 mg/kg given i.m. or s.c. every 4–8 h until the bird is stabilized has been recommended (James *et al.*, 1998). Once stabilized, vitamin $K_1$ given at similar doses once daily for 2 weeks or longer is indicated.

***Avicides***

Avicides are marketed primarily to control pest bird species such as blackbirds, pigeons, and grackles, among others. They are designed to be placed on grain for baiting in such a way as to only affect a few individuals in a flock. The clinical signs exhibited by affected birds (distress cries and aerial distress displays) are such that unaffected birds are scared away. Two commonly used avicides are 4-aminopyridine and 3-chloro-*p*-toluidine hydrochloride (see Table 56.4).

A number of chemicals have been investigated for their ability to repel birds (Clark, 1998; Dolbeer *et al.*, 1998; Stevens and Clark, 1998). Repellants such as methyl anthranilate are generally not associated with lethal intoxication.

# ENVIRONMENTAL TOXICANTS

## Pentachlorophenol

Pentachlorophenol (PCP) is used primarily as a wood preservative. Poultry have been exposed to sawdust and shavings from PCP-treated wood (Brown *et al.*, 1997). Adverse effects associated with PCP include reductions in growth rates, kidney hypertrophy, and decreased humoral immune response (Stedman *et al.*, 1980; Prescott *et al.*, 1982). PCP exposure can also result in an off-taste to eggs and meat as a result of degradation of chlorophenols to chloroanisols (Frank *et al.*, 1983).

TABLE 56.4 Rodenticides, avicides, and molluscicides toxic for avian species

| Rodenticides/ avicides/ molluscicides | Uses | Formulations | Avian toxicity | Mechanism of toxic action | Clinical signs | Species affected | References |
|---|---|---|---|---|---|---|---|
| 4-aminopyridine (Avitrol®) | Control red winged blackbirds, blackbirds, grackles, pigeons, and sparrows | Powder concentrate; 0.5–3.0% grain baits; 3% cracked corn bait for agricultural use diluted to 0.03% with untreated grain; 1% whole corn diluted to 0.1% for crows; 0.5% bait for pigeons | Highly toxic for most species, including birds; $LD_{50}$s for birds range from 1.4 to 8.1 mg/kg | Blocks potassium ion channels and increases release of ACh | Distress cries, aerial distress displays, seizures, coma | Potentially all birds | Bischoff *et al*. (2001), Schell (2004a, b) |
| Bromethalin | Rodenticide | 0.01% tan or green grain-based pellets in 16–42.5 g place packs | Not determined | Uncouple oxidative phosphorylation | Not described in birds; clinical signs in mammals are dependent on ingested dose with CNS excitation noted with high doses and paralysis noted with lower doses | Potentially all birds | Dorman (2004) |
| 3-chloro-*p*-toluidine hydrochloride (Starlicide®) | Control ravens, starlings, crows, pigeons, cowbirds, grackles, magpies, and certain gull species | 98% powder used to prepare baits; 0.1% ready-to-use product | Highly toxic for most species including birds. $LD_{50}$s for birds of 1.8–3.8 mg/kg b.w. | Unknown | Decreased activity, tachypnea, dyspnea, renal failure | Potentially all birds | Schell (2004a, b) |
| Fluoroacetate (1080) | Rodenticide, coyote control | Use restricted to livestock protection collars (LPCs). LPCs for sheep and goats contain 30 ml at 1% | Oral $LD_{50}$s for magpies are 1.78–2.3 mg/kg b.w. | Blocks tricarboxylic acid cycle resulting in cell energy depletion | Vary depending on species; not well described in birds; CNS, gastrointestinal, and cardiovascular signs likely; skeletal muscle necrosis reported in mallard ducks | Potentially all birds; proper use of LPCs appears to present little to no intoxication risk to avian scavengers | Burns and Connolly (1995), Ataria *et al*. (2000), Parton (2004) |

(Continued)

TABLE 56.4 (Continued)

| Rodenticides/ avicides/ molluscicides | Uses | Formulations | Avian toxicity | Mechanism of toxic action | Clinical signs | Species affected | References |
|---|---|---|---|---|---|---|---|
| Metaldehyde | Control slugs and snails | Pelleted baits, granules, liquids, or wettable powders containing <5% metaldehyde | Minimum lethal doses for chickens and ducks are 500 and 300 mg/kg b.w., respectively | Decrease GABA in the brain alterations of other brain neurotransmitters | Restlessness, anxiety, tachypnea, seizures, hyperthermia | Potentially all birds; secondary poisoning not reported | Talcott (2004a, b) |
| Strychnine | Controlling ground squirrels, meadow, and deer mice, prairie dogs, porcupines chipmunks, rabbits and pigeons | Colored grain-based baits with 0.5–1.0% strychnine sulfate | Reported oral $LD_{50}$s for golden eagles, sage grouse, and pheasants are 5–10, 42.5 and 8.5–24.7 mg/kg, respectively | Blocks inhibitory actions of glycine of spinal cord anterior horn cells and inhibits neurotransmitter release from Renshaw cells | Nervousness, apprehension, anxiety, tachypnea, muscle spasms, stiffness, tonic extensor muscle contractions, death due to respiratory impairment Secondary toxicity reported | Potentially all birds | Redig *et al*. (1982) Wobeser and Blakley, (1987), Warnock and Schwarzbach (1995), Talcott (2004a, b) |
| Zinc phosphide | Used to control rats, mice, voles, ground squirrels, prairie dogs, nutrias, muskrats, rabbits, opossums, and gophers Aluminum phosphide is used as a fumigant | Grain-based bait, scrap bait, paste, or tracking powder; baits generally contain 0.5–2.0% zinc phosphide; paste up to 10% | Oral $LD_{50}$s for wild birds and ducks are reported to be 23.7–37.5 mg/kg; oral $LD_{50}$ in chickens reported to be 25 mg/kg | Phosphene released from zinc or aluminum phosphide blocks cytochrome oxidase | Often found dead Dullness, tachypnea, dyspnea, tremors paralysis, seizures, and death Secondary toxicity unlikely | Potentially all birds | Shivanandappa *et al*. (1979), Albretson, (2004), Poppenga *et al*., (2005), Tiwary *et al*., (2005) |

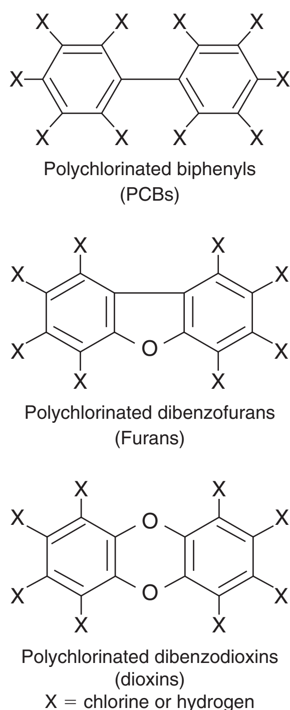


**FIGURE 56.1** Chemical structures of PCBs, PCDFs, and PCDDs.

## PCBs and dioxins

PCBs and dioxins are ubiquitous environmental contaminants. PCBs are chemicals that, historically, were used for a variety of industrial applications. They were probably best known for their use in electrical transformers.

Polychlorodibenzodioxins (PCDDs or dioxins) and related polychlorodibenzofurans (PCDFs), occur as trace contaminants in several chemical processes, primarily during the production of chlorinated phenols. They are also formed during bleaching of wood pulp and incineration of chlorine-containing waste materials (Andrews *et al*., 2001). The most well-known dioxin is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin or TCDD. The chemical structures of PCBs, dioxins, and furans are provided in Figure 56.1. Potentially, there are 209 different PCB congeners, 74 different PCDD congeners, and 134 different PCDF congeners. Not all congeners have equivalent toxicity. The most toxic PCBs are those whose phenyl rings are in a coplanar configuration and the most toxic PCDDs and PCDFs have four or more chlorine atoms. These compounds are lipophilic and environmentally stable. Like the OCs, they bioaccumulate and biomagnify within food webs. Restrictions on their production have resulted in slowly declining environmental concentrations, although they continue to cause adverse effects in wildlife. These groups of compounds are considered together given their common mechanisms of toxic action.

PCBs, PCDDs, and PCDFs are not considered to cause acute intoxication. However, chronic exposure can cause a number of adverse effects. Mechanistically, most toxic PCBs, PCDDs, and PCDFs are coplanar and bind to a cytosolic receptor called the aryl hydrocarbon or Ah receptor (AhR). The ligand–receptor complex is translocated to the nucleus where increased transcription of target genes ultimately results in a variety of responses. Adverse effects include weight loss, decreased immunocompetence, subcutaneous edema, reproductive and developmental effects, alterations in lipid metabolism and gluconeogenesis, thymic atrophy, hepatoxicity, endocrine disruption, hormone receptor modulation, and induction of liver enzymes (Bradbury, 1996; O'Hara and Rice, 1996). Non-coplanar PCBs that do not act via the AhR can alter cells in the nervous and immune systems resulting in cell death, stimulate insulin release, reduce synthesis of the neurotransmitter dopamine, and activate neutrophils to produce reactive oxygen species (Carpenter, 2006). Coplanar PCBs are anti-estrogenic, but most non-coplanar PCBs are estrogenic (Carpenter, 2006).

In avian species, perhaps the most significant impact of PCBs, PCDDs, and PCDFs is on reproduction. Avian piscivores are the most likely bird species to be affected due to bioaccumulation from dietary sources. The major effects are due to decreased egg production and embryo hatchability. The collective effects on avian embryos have been referred to as chick edema disease in chickens or Great Lakes Embryo Mortality, Edema, and Deformities Syndrome (GLEMEDS) in avian wildlife (O'Hara and Rice, 1996). Adverse effects include increased embryo and chick mortality, growth retardation, developmental abnormalities including bill deformities, club feed, missing eyes, and defective feathering, subcutaneous, pericardial, and peritoneal edema, liver enlargement and necrosis, and porphyria (Gilbertson, 1983; Gilbertson *et al*., 1991).

A diagnosis of PCB, PCDD, or PCDF intoxication can be difficult given the range of adverse effects that can be manifested. Also, clinical effects can be due to disease processes that are not a direct result of chemical exposure. For example, illness and death due to an infectious disease might occur as a result of impaired immune function. Concentrations of PCBs, PCDDs, and PCDFs in tissues such as brain and eggs have been correlated with specific adverse effects, so detection and quantification of these compounds in appropriate samples can be useful in establishing a diagnosis.

Poultry can be exposed to PCBs, PCDDs, and PCDFs via contaminated feedstuffs. Feed contaminated with PCBs and dioxin caused widespread contamination of poultry and poultry products in Belgium in 1999 (Bernard *et al*., 2002). The source was traced to recycled fat used in livestock feed production. Interestingly, diagnosis of chick edema disease in poultry receiving the contaminated feed resulted in the initial identification of the problem. Also, poultry and poultry products were found to be contaminated with TCDD as a result of inclusion of TCDD-contaminated ball clay used as a flowing agent in livestock

feed (Hayward *et al.*, 1999). No birds were clinically affected and the contamination was discovered as a result of a nationwide food survey conducted by the United States Environmental Protection Agency (USEPA).

## Ethylene glycol

Occasionally, birds are intoxicated by ethylene glycol (EG, antifreeze). There is one report of EG intoxication of ducks kept in an area used for automobile storage and repair (Stowe *et al.*, 1981). The authors has diagnosed EG intoxication in a turkey vulture; the source of exposure was not determined. A diagnosis relies on detection of EG in fluid or tissue (liver and kidney) samples and occurrence of calcium oxalate crystals in renal tubules. Another diagnostic clue in cases where EG is not suspected and kidneys are tested to assess potential exposure to metals, is a high kidney calcium concentration (often $>2000$ ppm wet weight with normal concentrations $<150$ ppm).

## Petroleum

Exposure of avian wildlife to crude oil is a significant cause of morbidity and mortality. Although oil spills involving large tanker accidents such as the grounding of the *Exxon Valdez* in Alaska receive most of the public's attention, other scenarios related to oil drilling and production, off-loading of oil from tankers, production, processing, and refining of oil, road transport, and improper disposal of waste oil and petroleum products result in considerable wildlife exposure (Jessup and Leighton, 1999). Because oil floats on the surface of water, birds that live on water or dive through water for food are more likely to be exposed in water spills. Marine birds and bird species that utilize environments near to shore are also likely to be exposed to oil if spills reach shorelines. Raptors such as peregrine falcons which feed on other birds can be exposed to oil from contaminated prey (Zuberogoitia *et al.*, 2006).

Crude oil and petroleum products are complex mixtures of chemicals, including a variety of aromatic and aliphatic compounds. The toxicity of crude oils and petroleum products varies depending on their chemical compositions. Another complicating factor in assessing the toxicity of crude oil is the fact that soon after environmental release, the process of "weathering" occurs which changes its chemical and physical properties.

Crude oil and other petroleum products affect birds in several ways. First, loss of insulating properties of the feathers results in rapid hypothermia. Oiled birds lose their ability to fly and they frequently die from starvation, exhaustion, or drowning (Friend and Franson, 1999). Second, crude oil and other petroleum products are irritating to skin, mucous membranes, and the respiratory tract. Aspiration of oil into the respiratory tract and lungs can cause aspiration pneumonia. Third, systemic absorption of chemicals found in crude oil or petroleum products causes adverse effects on several organ systems including reproductive, hematopoietic, nervous, immune, and hepatobiliary systems. Lastly, petroleum is extremely toxic to bird embryos.

Diagnosing petroleum intoxication as a cause of death can be challenging. There are no characteristic gross or histopathologic lesions specific to petroleum intoxication. A diagnosis is based upon a history or evidence of exposure and compatible clinical and postmortem findings. Exposed birds, especially those with white or light colored feathers, are easily identified because of the persistence of oil on feathers and skin. Birds are often emaciated, oil may be present in the respiratory or gastrointestinal tracts, there may be congestion along the intestines, and the salt glands may be swollen (Friend and Franson, 1999). Feathers, skin, or organs of birds which do not have external evidence of exposure can be analyzed for the presence of chemicals typically found in crude oil or petroleum products (Jessup and Leighton, 1996).

## Cyanide

Cyanide (hydrocyanic acid) is a rapidly acting cellular poison. Poisoning most often occurs following ingestion of a cyanide salt or inhalation of hydrogen cyanide gas. Avian wildlife intoxications have occurred following exposure to cyanide used in heap leach and carbon-in-pulp mill gold or silver mining processes (Friend and Franson, 1999). Birds are most often intoxicated by ingesting cyanide contaminated water. Between 1986 and 1995, 3000 cyanide-related mortalities involving 75 species of birds were reported to the National Wildlife Heath Center (Friend and Franson, 1999). Most mortality events occur in the spring and fall as migratory birds pass through mining areas. Large-scale cyanide spills have caused widespread contamination of rivers in Europe and South America (Koenig, 2000).

Cyanide binds to ferric (+3) iron of mitochondrial cytochrome oxidase. This enzyme mediates the transfer of electrons to molecular oxygen which is the last step in oxidative phosphorylation (Delaney, 2001). Cells are unable to use oxygen resulting in tissue anoxia, increased anaerobic metabolism, and rapid development of lactic acidosis.

The acute oral toxicity of sodium cyanide has been studied in several bird species. $LD_{50}$s ranged from 4.0 to 21 mg/kg b.w. (Wiemeyer *et al.*, 1986).

Cyanide is a rapidly acting toxicant; most intoxicated birds are found dead. Grossly, dead animals often have bright red blood and multi-organ congestion. A diagnosis is based upon the history of exposure and measurement of cyanide in blood, heart, liver, or brain. Samples should be stored and shipped frozen to avoid the loss of cyanide before

analysis. Denying birds access to cyanide-contaminated water is the best preventive approach.

## Disinfectants

### *Phenols*

Phenol, also known as carbolic acid, was commonly used as an antiseptic agent before the availability of other chemical antiseptics. Phenol acts as a general protoplasmic poison by causing cell wall disruption, protein denaturation, and coagulation necrosis (Sue and Delaney, 2001). Clinical signs occur as a result of local damage to the gastrointestinal tract or respiratory system. Systemic signs occur due to central nervous system (CNS), cardiac, hepatic, and renal damage.

### *Quarternary ammonium compounds*

Quarternary ammonium compounds are cationic surfactants that are used as disinfectants, detergents, and sanitizers. In general, diluted quarternary ammonium compounds have a low order of toxicity compared to other disinfectants such as phenol or formaldehyde. However, at sufficient concentrations, they are damaging to epithelial cells of the mouth, pharynx, and upper airway (Brown and Julian, 2003). Epithelial damage can progress to pseudomembrane and ulcer formation. Ulceration and epithelial thickening can occur in the upper gastrointestinal tract. There is one case report of intoxication of chickens exposed to drinking water with higher than recommended concentrations of alkyldimethylbenzyl ammonium chloride (Dhillon *et al*., 1982). The concentration in the water was not reported. Grossly, pale or white areas with focal areas of ulceration were noted on oral and pharyngeal mucosa. The same compound added experimentally to the water of turkey poults at 200 ppm did not cause an adverse effect; higher concentrations caused oral mucosal irritation and respiratory distress (Reuber *et al*., 1970).

### *Chlorine*

The toxicity of sodium hypochlorite added to drinking water for broiler chicks and laying hens has been investigated (Damron and Flunker, 1993). In chicks, 100 and 300 ppm chloride (derived from addition of sodium hypochlosite) reduced water intake and body weight, respectively. In hens, 40 ppm chloride reduced water consumption and 60 ppm reduced egg production when added to water in warm weather. Water consumption was reduced at 50 ppm, but 100 ppm had no effect on egg production or weight daily feed intake when added during cooler weather.

Seventeen of 35 psittacines housed in an aviary developed respiratory distress, depression, and anorexia after the aviary was cleaned with an undiluted solution of sodium hypochlorite (5% chlorine bleach) (Wilson *et al*., 2001). Seven birds subsequently died and were necropsied. Gross and histopathologic lesions included epithelial deciliation, ulceration, squamous metaplasia, and epithelial hyperplasia in the tracheas of the birds.

# TOXIC GASES

## Ammonia

High ambient air concentrations of ammonia (50–75 ppm) reduce feed consumption, growth rate, and egg production in poultry (Deaton *et al*., 1986). Ammonia readily dissolves in water to form ammonium hydroxide which is an alkaline irritant. Thus, contact with the moist mucous membranes of the eyes and respiratory tract results in corneal and epithelial cell damage. Toxic air concentrations cause kerato-conjunctivitis, corneal ulceration, blindness, photophobia, tracheitis, tachypnea, and dyspnea. Prolonged exposure can result in increased mucous secretion and hyperplasia of the bronchiolar and alveolar epithelium. Ambient ammonia air concentrations should be $<25$ ppm for poultry (Brown and Julian, 2003).

## Carbon monoxide

CO is an odorless, colorless, lighter-than-air gas that can reach toxic concentrations as a result of incomplete combustion of hydrocarbon-based fuels. Toxic concentrations of CO most often result from use of defective gas catalytic or open-flame brooders and furnaces or internal combustion engines in poorly or unventilated spaces. CO acts by competing with oxygen for a number of proteins, including hemoglobin. The affinity of hemoglobin for CO is approximately 250 times greater than for oxygen. Formation of carboxyhemoglobin reduces the ability of red blood cells to carry oxygen and the oxygen dissociation curve is shifted to the left resulting in tissue hypoxia. Affected birds exhibit drowsiness, labored breathing, weakness, and ataxia with seizures occurring before death (Brown and Julian, 2003). Birds may be found dead. Carboxyhemoglobin causes the blood to have a bright red appearance. Carboxyhemoglobin concentrations of 20% are associated with some motor impairment; death occurs when blood carboxyhemoglobin concentrations approach 60–70% (Osweiler *et al*., 1985). Ambient CO concentrations of 600 ppm for 30 min cause clinical signs in chickens; concentrations of 2000 ppm or greater are lethal within 1.5–2 h (Brown and Julian, 2003). Sublethal exposure to CO in poultry causes stunting.

Diagnosis of CO intoxication is based upon a history compatible with CO production, compatible clinical signs,

and measurement of ambient air CO and blood carboxyhemoglobin concentrations. Treatment is generally limited to provision of fresh air or oxygen; pet birds can be placed in oxygen cages.

Other gases such as hydrogen sulfide and carbon dioxide generally do not present significant intoxication risks for birds. Interestingly, chickens are less sensitive to hydrogen sulfide than humans or dogs (Brown *et al.*, 1997).

## Polytetrafluoroethylene: Teflon®

Polytetrafluoroethylene (PTFE) is a synthetic polymer that is widely used as a non-stick surface in cookware. It is also used in self-cleaning ovens and as a coating on heat lamp bulbs. Heated to high temperatures (⩾280°C or greater) PTFE releases toxic pyrolysis products that can cause rapid death of birds. There are no reported toxicities associated with the proper use of coated cookware since temperatures do not reach levels at which pyrolysis occurs. However, cookware without contents can reach critical temperatures within minutes.

While all birds are likely to be susceptible to intoxication, most spontaneous cases involve pet birds such as cockatiels, parrots, finches, and budgerigars (Blandford *et al.*, 1975; Wells, 1983; Stoltz *et al.*, 1992). There is one case report in which broiler chicks were intoxicated from heat lamp bulbs coated with PTFE (Boucher *et al.*, 2000) and another suspected intoxication of wild birds associated with industrial activity (Pennycott and Middleton, 1997). Quail have been exposed experimentally (Griffith *et al.*, 1973).

PTFE pyrolysis products contain particulates of respirable size (<1 μm diameter) that can penetrate to the alveoli (Wells *et al.*, 1982). The particulates are themselves toxic, but they also serve as a vehicle for other potentially damaging chemicals including hydrogen fluoride, carbonyl fluoride, and perfluoroisobutylene (Wells and Slocombe, 1982b). The fumes are acidic and cause direct damage to cell membranes of the lungs. However, it appears as though the particles are necessary for intoxication to occur since the gas phase of PTFE fumes alone does not cause damage (Seidel *et al.*, 1991). Lung damage is believed to be due to direct irritation and oxidative damage (Wells *et al.*, 1982, Johnston *et al.*, 1996).

Precise toxicity information is not available. However, experimentally, exposure of budgerigars to PTFE pyrolysis products for 9 min or longer resulted in severe clinical signs and lesions and the deaths of 31 of 32 budgerigars (Wells *et al.*, 1982). While there is little comparative toxicity information among avian species, budgerigars were more sensitive than quail in one experimental study (Griffith *et al.*, 1973).

After exposure, the onset of clinical signs is rapid. Eyelid blinking is an early sign and may be related to a direct irritant effect on the conjunctiva and cornea (Wells *et al.*, 1982). Eyelid movements could also be secondary to hypoxia-induced somnolence. Tachypnea, dyspnea, anxiety, cage wire biting, incoordination, and inability to stand may be due to hypoxia secondary to lung damage. In many cases, the bird may be found dead with no other clinical signs noted.

Grossly, the lungs are severely congested and hemorrhagic (Wells and Slocombe, 1982b). Histologically, there is extensive, severe, necrotizing, and hemorrhagic pneumonitis. Amorphous, elongate particles may also be noted (Wells and Slocombe, 1982b).

A diagnosis is generally based upon a history of exposure and characteristic postmortem lesions. Currently, there is no analytical test available to confirm exposure to the pyrolysis products. The rapidity of onset of severe signs and subsequent death most often precludes treatment. Awareness of the hazard and avoiding housing birds near PTFE coatings is the best prevention.

# REFERENCES


Albretson JC (2004) Zinc phosphide. In *Clinical Veterinary Toxicology*, 1st edn, Plumlee K (ed.). Mosby, St. Louis, MO, pp. 456–8.

Alfonso HA, Sanchez LM (1993) Intoxication due to *Crotalaria retusa* and *C spectabilis* in chickens and geese. *Vet Hum Toxicol* **35**: 539.

Alfonso HA, Sanchez LM, Merino N, *et al.* (1994) Intoxication due to *Nerium oleander* in geese. *Vet Hum Toxicol* **36**: 47.

Andrews JS, Needham LL, Patterson Jr DG (2001) Polychlorodibenzodioxins and polychlorodibenzofurans. In *Clinical Environmental Health and Toxic Exposures*, 2nd edn, Sullivan JB, Krieger GR (eds). Lippincott Williams and Wilkins, Philadelphia, PA, pp. 769–76.

Arai M, Stauber E, Shropshire CM (1992) Evaluation of selected plants for their toxic effect in canaries. *J Am Vet Med Assoc* **200**: 1329–31.

Arnouts S, Buyse J, Cokelaere MM, *et al.* (1993) Jojoba meal (*Simmondsia chinensis*) in the diet of broiler breeder pullets: physiological and endocrinological effects. *Poult Sci* **72**: 1714–21.

Ataria JM, Wickstrom M, Arthur D, *et al.* (2000) Biochemical and histopathological changes induced by sodium monofluoroacetate (1080) in mallard ducks. *NZ Plant Protect* **53**: 293–8.

Bailey TA, John A, Mensah-Brown EP, *et al.* (1998) Drug metabolizing enzymes in the houbara bustard (*Chlamydotis undulate*). *Comp Biochem Physiol Part C* **120**: 365–72.

Baker JR, Allen NK, Kleiss AJ (1967) Hypervitaminosis A in the chick. *Br J Exp Pathol* **48**: 507–12.

Barnes DM, Kirby YK, Oliver KG (2001) Effects of biogenic amines on growth and the incidence of proventricular lesions in broiler chicks. *Poult Sci* **80**: 906–11.

Barnes HJ (2003) Clostridial diseases. In *Diseases of Poultry*, 11th edn, Saif YM, Barnes HJ, Glisson JR, *et al.* (eds). Iowa State University Press, Ames, IA, 775–91.

Barr JR, Moura H, Boyer AE, *et al.* (2005) Botulinum neurotoxin detection and differentiation by mass spectrometry. *Emerg Infect Dis* **11**: 1578–83.

Berger LL (1993) *Salt and Trace Minerals for Livestock, Poultry and Other Animals*, Salt Institute, Alexandria, VA, pp. 1–52.

Bernard A, Broeckaert F, De Poorter G, *et al*. (2002) The Belgian PCB/dioxin incident: analysis of the food chain contamination and health risk evaluation. *Environ Res* **88**: 1–18.

Berny PJ, Buronfosse T, Buronfosse F, *et al*. (1997) Field evidence of secondary poisoning of foxes (*Vulpes vulpes*) and buzzards (*Buteo buteo*) by bromodiolone, a 4-year survey. *Chemosphere* **15**: 1817–29.

Bird JE, Walser MM, Duke GE (1983) Toxicity of gentamicin in red-tailed hawks. *Am J Vet Res* **44**: 1289–93.

Bischoff K, Morgan S, Chelsvig J, *et al*. (2001) 4-aminopyridine poisoning of crows in the Chicago area. *Vet Hum Toxicol* **43**: 350–2.

Blandford TB, Seamon PJ, Huges R, *et al*. (1975) A case of polytetrafluoroethylene poisoning in cockatiels accompanied by polymer fume fever in the owner. *Vet Rec* **96**: 175–6.

Blus LJ (2003) Organochlorine inesticides. In *Handbook of Ecotoxicology*, 2nd edn, Hoffman DJ, Rattner BA, Burton GA, *et al*. (eds). Lewis Publishers, Boca Raton, FL, pp. 313–39.

Blus LJ, Wiemeyer SN, Henny CJ (1996) Organochlorine pesticides. In *Noninfectious Diseases in Wildlife*, 2nd edn, Fairbrother A, Locke LN, Hoff GL (eds). Iowa State University Press, Ames, IA, pp. 61–70.

Bollinger TK, Mineau P, Wickstrom ML (2005) Toxicity of sodium chloride to house sparrows (*Passer domesticus*). *J Wildl Dis* **41**: 363–70.

Boothe DM (2001) Antimicrobial drugs. In *Small Animal Clinical Pharmacology and Therapeutics*, Boothe DM (ed.). W.B. Saunders Co., Philadelphia, PA, pp. 150–73.

Boucher M, Ehmler TJ, Bermudez AJ (2000) Polytetrafluoroethylene gas intoxication in broiler chickens. *Avian Dis* **44**: 449–53.

Bradbury SP (1996) 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. In *Noninfectious Diseases of Wildlife*, 2nd edn, Fairbrother A, Locke LN, Hoff GL. Iowa State University Press, Ames, IA, pp. 87–98.

Brown RE, Brain JD, Wang N (1997) The avian respiratory system: a unique model for studies of respiratory toxicosis and for monitoring air quality. *Environ Health Persp* **105**: 188–200.

Brown TP, Julian RJ (2003) Other toxins and poisons. In *Diseases of Poultry*, 11th edn, Saif YM, Barnes HJ, Glisson JR, *et al*. (eds). Iowa State University Press, Ames, IA, pp.1133–59.

Brown TP, Larsen CT, Boyd DL, *et al*. (1991) Ulcerative cholecystitis produced by 3-nitro-4-hydroxy-phenylarsonic acid toxicosis in turkey poults. *Avian Dis* **35**: 241–3.

Burger WP, Naude TW, Van Rensburg IB, *et al*. (1994) Cardiomyopathy in ostriches (*Struthio camelus*) due to avocado (*Persea americana var. quatemalensis*) intoxication. *J S Afr Vet Assoc* **65**: 113–18.

Burns RJ, Connolly GE (1995) Assessment of potential toxicity of compound 1080 from livestock protection collars to canines and scavenging birds. *Int Biodeterior Biodegrad* **36**: 161–7.

Burrows GE, Tyrl RJ (2001) *Toxic Plants of North America*. Iowa State University Press, Ames, IA.

Carmichael WW, Li R (2006) Cyanobacterial toxins in the Salton Sea. *Saline Syst* **2**: 5–17.

Carpenter DO (2006) Polybrominated biphenyls (PCBs): routes of exposure and effects on human health. *Rev Environ Health* **21**: 1–23.

Cattley RC, Barnett BD (1977) The effect of pokeberry ingestion on the immune response of turkeys. *Poult Sci* **56**: 246–8.

Cavaliere MJ, Calore EE, Haraguchi M, *et al*. (1997) Mitochondrial myopathy in *Senna occidentalis*-seed-fed chicken. *Ecotoxicol Environ Safe* **37**: 181–5.

Chowdhury SD (1988) Lathyrism in poultry – a review. *World's Poult Sci J* **44**: 7–16.

Chowdhury SR (2001) Effects of dietary 1,4-diaminobutane (putrescine) on eggshell quality and laying performance of hens laying thin-shelled eggs. *Poult Sci* **80**: 1702–9.

Clark L (1998) Review of bird repellants. *Proceedings of the 18th Vertebral Pesticides Conference*, Davis, CA, pp. 330–7.

Coulet M, Eeckhoutte C, Galtier P (1996) Ontogenic development of drug-metabolizing enzymes in male chicken liver. *Can J Physiol Pharmacol* **74**: 32–7.

Daft BM, Bickford AA, Hammarlund MA (1989) Experimental and field sulfaquinoxaline toxicosis in Leghorn chickens. *Avian Dis* **33**: 30–4.

Dalvi RR, Nunn VA, Juskevich J (1987) Studies on comparative drug metabolism by hepatic P-450-containing microsomal enzymes in quail, ducks, geese, chickens, turkeys and rats. *Comp Biochem Physiol* **87C**: 421–4.

Damron BL, Flunker LK (1993) Broiler chicks and laying hen tolerance to sodium hypochlorite in drinking water. *Poult Sci* **72**: 1650–5.

Davletov B, Bajohrs M, Binz T (2005) Beyond BOTOX: advantages and limitations of individual botulinum neurotoxins. *Trends Neurosci* **28**: 446–52.

Day EJ, Dilworth BC (1984) Toxicity of jimson weed seed and cocoa shell meal to broilers. *Poult Sci* **3**: 466–8.

Deaton JW, Reece FN, Thornberry FD (1986) Atmospheric ammonia and incidence of blood spots in eggs. *Poult Sci* **65**: 1427–8.

Delaney KA Cyanide (2001) In *Clinical Toxicology*, Ford MD, Delaney KA, Ling LJ, *et al*. (eds). W.B. Saunders Co. Philadelphia, PA, pp. 705–11.

Dhillon AA, Winterfield RW, Thacker HL (1982) Quarternary ammonium compound toxicity in chickens. *Avian Dis* **26**: 928–31.

Dolbeer RA, Seamans TW, Blackwell BF, *et al*. (1998) Anthraquinone formulation (Flight Control™) shows promise as avian feeding repellant. J *Wildl Manage* **62**: 1558–64.

Dorman D (2004) Bromethalin. In *Clinical Veterinary Toxicology*, 1st edn, Plumlee K (ed.). Mosby, St. Louis, MO, pp. 446–8.

Dufour L, Sander JE, Wyatt RD, *et al*. (1992) Experimental exposure of broiler chickens to boric acid to assess clinical signs and lesions of toxicosis. *Avian Dis* **36**: 1007–11.

Dumonceaux G, Harrison GJ (1999) Toxins. In *Avian Medicine: Principles and Practice*, 1st edn, Ritchie BW, Harrison GJ, Harrison LR (eds). HBD International, Delray Beach, FL, pp. 1030–49.

Eason CT, Murphy EC, Wright GRG, *et al*. (2002) Assessment of risks of brodifacoum to non-target birds and mammals in New Zealand. *Ecotoxicol* **11**: 35–48.

Ensley S (2004) Arsenic. In *Clinical Veterinary Toxicology*, 1st edn, Plumlee K (ed.). Mosby, St. Louis, MO, pp. 193–5.

Extoxnet Cypermethrin, http://extoxnet.orst.edu/

Extoxnet Permethrin, http://extoxnet.orst.edu/

Feed Additive Compendium(2005) Miller Publishing Corp, Memphis.

Fiedler HH, Perron RM (1994) Yew poisoning in Australian emus (*Dromaius novaehollandiae, Latham*). *Berl Munch Tierarztl Wochenschr* **107**: 50–2.

Fitzgerald SD, Sullivan JM, Everson RJ (1990) Suspected ethanol toxicosis in two wild cedar waxwings. *Avian Dis* **34**: 488–90.

Flory W, Hebert CD (1984) Determination of the oral toxicity of *Sesbania drummondii* seeds in chickens. *Am J Vet Res* **45**: 955–8.

Foreyt WJ, Abinanti FR (1980) Maggot-associated type C botulism in game farm pheasants. *J Am Vet Med Assoc* **177**: 827–8.

Frank AA, Reed WM (1987) *Conium maculatum* (poison hemlock) toxicosis in a flock of range turkeys. *Avian Dis* **31**: 386–8.

Frank R, Fish N, Sirons GJ, *et al*. (1983) Residues of polychlorinated phenols and anisoles in broilers raised on contaminated wood shavings. *Poult Sci* **62**: 1559–65.

Friend M, Franson JC (1999) *Field Manual of Wildlife Diseases*. Biological Resources Division, USGS, Information and Technology Report 1999–2001. US Government Printing Office, Washington, DC.

Garbarino JR, Bednar AJ, Rutherford DW, *et al*. (2003) Environmental fate of roxarsone in poultry litter. I. Degradation of roxarsone during composting. *Environ Sci Technol* **37**: 1509–14.

Gilbertson M (1983) Etiology of chick edema disease in herring gulls in the lower Great Lakes. *Chemosphere* **12**: 357–70.

Gilbertson M, Kubiak T, Ludwig J, *et al*. (1991) Great Lakes embryo mortality, edema and deformities syndrome (GLEMEDS) in colonial fish-eating birds: similarity to chick edema disease. *J Toxicol Environ Health* **33**: 455–520.

Gitter M, Lewis G, Crossman PJ, *et al*. (1979) Salt poisoning in turkey poults. *Br Vet J* **135**: 55–63.

Gopinath C, Ford EJ (1977) The effect of ragwort (*Senecio jacobea*) on the liver of the domestic fowl (*Gallus domesticus*): a histopathological and enzyme histochemical study. *Br Poult Sci* **18**: 137–41.

Gordus AG, Shivaprasad HL, Swift PK (2002) Salt toxicosis in ruddy ducks that winter on an agricultural evaporation basin in California. *J Wildl Dis* **38**: 124–31.

Griffith FD, Stephens SS, Tayfun FO (1973) Exposure of Japanese quail and parakeets to the pyrolysis products of fry pans coated with Teflon and common cooking oils. *Am Ind Hyg Assoc J* **34**: 176–8.

Gross WB, Smith LDS (1971) Experimental botulism in gallinaceous birds. *Avian Dis* **15**: 716–22.

Gupta RP, Abou-Donia MB (1998) Cytochrome P450 enzymes in chickens: characteristics and induction by xenobiotics. *Comp Biochem Physiol Part C* **121**: 73–83.

Haraguchi M, Dagli ML, Raspantini RC, *et al*. (2003) The effects of low doses of *Senna occidentalis* seeds on broiler chickens. *Vet Res Commun* **27**: 321–8.

Hargis AM, Stauber E, Casteel S, *et al*. (1989) Avocado (*Persea Americana*) intoxication in caged birds. *J Am Vet Med Assoc* **194**: 64–6.

Harper JA, Arscott GH (1962) Toxicity of common hairy vetch seed for poults and chicks. *Poult Sci* **41**: 1968–74.

Harry EG, Tucker JF (1976) The effect of orally administered histamine on the weight gain and development of gizzard lesions in chicks. *Vet Rec* **99**: 206–7.

Hayward DG, Nortrup D, Gardner A, *et al*. (1999) Elevated TCDD in chicken eggs and farm-raised catfish fed a diet with ball clay from a Southern United States mine. *Environ Res Sec A* **81**: 248–56.

Heckel JO, Sisson DC, Quist CF (1994) Apparent fatal snakebite in three hawks. *J Wildl Dis* **30**: 616–19.

Hegdal PL, Colvin BA (1988) Potential hazard to eastern screech-owls and other raptors of brodifacoum bait used for vole control in orchards. *Environ Toxicol Chem* **7**: 245–60.

Henriksen P, Carmichael WW, An J, *et al*. (1997) Detection of anatoxin-a(s)-like cholinesterase in natural blooms and cultures of cyanobacteria/blue green algae from Danish lakes and in the stomach contents of poisoned birds. *Toxicon* **35**: 901–13.

Heuser GF, Schumacher AE (1942) The feeding of corn cockle to chickens. *Poult Sci* **21**: 86–93.

Hill EF (1988) Brain cholinesterase activity of apparently normal wild birds *J Wildl Dis* **24**: 51–61.

Hill EF (2003) Wildlife toxicology of organophosphorus and carbamate pesticides. In *Handbook of Ecotoxicology*, 2nd edn, Hoffman DJ, Rattner BA, Burton GA, *et al*. (eds). Lewis Publishers, Boca Raton, FL, 281–312.

Hughes BL, Jones JE, Toler JE, *et al*. (1991) Effects of exposing broiler breeders to nicarbazin contaminated feed. *Poult Sci* **70**: 476–82.

Jain AV (2006) Analysis of organophosphate and carbamate pesticides and anticholinesterase therapeutic agents. In *Toxicology of Organophosphate and Carbamate Compounds*, Gupta RC (ed.). Academic Press/Elsevier, Amsterdam, pp. 681–701.

James SB, Raphael BL, Cook RA (1998) Brodifacoum toxicity and treatment in a white-winged wood duck (*Cairina scutulata). J Zoo Wildl Med* **29**: 324–7.

Jensen WI, Allen JP (1981) Naturally occurring and experimentally induced castor bean (*Ricinus communis*) poisoning in ducks. *Avian Dis* **25**: 184–94.

Jessup DA, Leighton FA (1996) Oil pollution and toxicity to wildlife. In *Noninfectious Diseases of Wildlife*, 2nd edn, Fairbrother A, Locke LN, Hoff GL (eds). Iowa State University Press, Ames, IA, pp. 141–56.

Johnston CJ, Finkelstein JN, Mercer P, *et al*. (1996) Pulmonary effects induced by ultrafine PTFE particles. *Toxicol Appl Pharm* **168**: 208–15.

Kamada Y, Oshiro N, Miyagi M, *et al*. (1998) Osteopathy in broiler chicks fed toxic mimosine in *Leucaena leucocephala*. *Biosci Biotechnol Biochem* **62**: 34–8.

Kim JS, Crichlow EC (1995) Clinical signs of ivermectin toxicity and the efficacy of antigabaergic convulsants as antidotes for ivermectin poisoning in epileptic chickens. *Vet Hum Toxicol* **37**: 122–6.

Kinde H (1988) A fatal case of oak poisoning in a double-wattled cassowary (*Casuarius casuarius*). *Avian Dis* **32**: 849–51.

Koenig R (2000) Wildlife deaths are a grim wake-up call in Eastern Europe. *Science* **287**: 1737–8.

Koutsos EA, Tell LA, Woods LW, *et al*. (2003) Adult cockatiels (*Nymphicus hollandicus*) at maintenance are more sensitive to diets containing excess vitamin A than to vitamin A-deficient diets. *J Nutr* **133**: 1898–1902.

Kovatsis A, Kotsaki-Kovatsi VP, Nikolaidis E, *et al*. (1994) The influence of *Datura ferox* alkaloids on egg-laying hens. *Vet Hum Toxicol* **36**: 89–92.

Krienitz L, Ballot A, Kotut K, Wiegand C, *et al*. (2003) Contribution of hot spring cyanobacteria to the mysterious deaths of Lesser Flamingoes at Lake Bogoria, Kenya. *FEMS Microbiol Ecol*. **43**: 141–8.

Kubota R, Kunito T, Tanabe S (2003) Occurrence of several arsenic compounds in the liver of birds, cetaceans, pinnipeds, and sea turtles. *Environ Toxicol Chem* **22**: 1200–7.

Lawal S, Abdu PA, Jonathan GB, *et al*. (1992) Snakebites in poultry. *Vet Hum Toxicol* **34**: 528–30.

Matsunaga H, Harada KI, Senma M, *et al*. (1999) Possible cause of unnatural mass death of wild birds in a pond in Nishinomiya, Japan: sudden appearance of toxic cyanobacteria. *Nat Toxins* **7**: 81–4.

McDermott CM, Nho CW, Howard W, *et al*. (1998) The cyanobacterial toxin, microcystin-LR can induce apoptosis in a variety of cell types. *Toxicon* **36**: 1981–96.

Mineau P (2005) Direct losses of birds to pesticides – beginnings of a quantification. In *Bird Conservation Implementation and Integration in the Americas: Third International Partners in Flight Conference 2002*, vol. 2, Ralph CJ, Rich TD (eds). US Department of Agriculture, Albany, CA, pp. 1065–70.

Mineau P, Whiteside M (2006) Lethal risk to birds from insecticide use in the United States – a spatial and temporal analysis. *Environ Toxicol Chem* **25**: 1214–22.

Means C (2004) Anticoagulant rodenticides. In *Clinical Veterinary Toxicology*, 1st edn, Plumlee K (ed.). Mosby, St. Louis, MO, pp. 444–6.

Medenhall VM, Pank LF (1980) Secondary poisoning of owls by anticoagulant rodenticides. *Wildl Soc Bull* **8**: 311–15.

Merson MH, Byers RE (1984) Residues of the rodenticide brodifacoum in voles and raptors after orchard treatment. *J Wildl Manage* **48**: 212–16.

Meteyer CU, Dubielzig RR, Dein FJ, *et al*. (1997) Sodium toxicity and pathology associated with exposure of waterfowl to hypersaline playa lakes of southeast New Mexico. *J Vet Diagn Invest* **9**: 269–80.

Munger LL, Su JJ, Barnes HJ (1993) Coumafuryl (Fumarin®) toxicity in chicks. *Avian Dis* **37**: 622–4.

Navarro JL (1992) Capsaicin effects on consumption of food by cedar wax-wings and house finches. *Wilson Bull* **104**: 549–51.

Newman MC, Unger MA (2003) Bioaccumulation from food and trophic transfer. In *Fundamentals of Ecotoxicology*, 2nd edn, Lewis Publishers, Boca Raton, FL, pp. 95–110.

O'Hara TM, Rice CD (1996) Polychlorinated biphenyls. In *Noninfectious Diseases of Wildlife*, 2nd edn, Fairbrother A, Locke LN, Hoff GL (eds). Iowa State University Press, Ames, IA, pp. 71–86.

Oaks JL, Gilbert M, Virani MZ, *et al*. (2004) Diclofenac residues as the cause of vulture population decline in Pakistan. *Nature* **427**: 630–3.

Ohishi I, Sakaguchi G, Riemann H, *et al*. (1979) Antibodies to *Clostridium botulinum* toxins in free-living birds and mammals. *J Wildl Dis* **15**: 3–9.

Onodera H, Oshima Y, Henriksen P, *et al*. (1997) Confirmation of anatoxin-a(s), in the cyanobacterium *Anabaena lemmermannii*, as the cause of bird kills in Danish lakes. *Toxicon* **35**: 1645–8.

O'Rourke K (2002) Euthanatized animals can poison wildlife: veterinarians receive fines. *J Am Vet Med Assoc* **220**: 145–146.

Osweiler GD, Carson TL, Buck WB, *et al*. (1985) *Clinical and Diagnostic Veterinary Toxicology*, 3rd edn, Kendall-Hunt Publishing, Dubuque, IO, pp. 369–77.

Pan HP (1978) Drug metabolism in birds. *Drug Metabol Rev* **7**: 1–253.

Parton KH (2004) Sodium fluoroacetate. In *Clinical Veterinary Toxicology*, 1st edn, Plumlee K (ed.). Mosby, St. Louis, MO, pp. 451–4.

Pennycott TW, Middleton JD (1997) Suspected PTFE toxicity in wild birds. *Vet Rec* **141**: 255.

Petterino C, Paolo B (2001) Toxicology of various anticoagulant rodenticides in animals. *Vet Hum Toxicol* **43**: 353–60.

Poppenga RH, Ziegler AF, Habecker PL, *et al*. (2005) Zinc phosphide intoxication of wild turkeys (*Meleagris gallopavo*). *J Wildl Dis* **41**: 218–23.

Prescott CA, Wilkie BN, Hunter B, *et al*. (1982) Influence of a purified grade of pentachlorophenol on the immune response of chickens. *Am J Vet Res* **43**: 481–7.

Puls R (1994) *Mineral Levels in Animal Health*, 2nd edn. Sherpa International Clearbrook, BC.

Ratanasethkul C, Riddell C, Salmon RE, *et al*. (1976) Pathological changes in chickens, ducks and turkeys fed high levels of rapeseed oil. *Can J Comp Med* **40**: 360–9.

Redig PT, Stowe CM, Arendt TD, *et al*. (1982) Relay toxicity of strychnine in raptors in relation to a pigeon eradication program. *Vet Hum Toxicol* **24**: 335–6.

Reece RL, Hooper PT (1984) Toxicity in utility pigeons caused by the coccidostat dinitolmide. *Aust Vet J* **61**: 259–61.

Reece RL, Barr DA, Forsyth WM, *et al*. (1985) Investigations of toxicity episodes involving chemotherapeutic agents in Victorian poultry and pigeons. *Avian Dis* **29**: 1239–51.

Reuber HW, Rude TA, Jorgenson TA (1970) Safety evaluation of a quarternary ammonium sanitizer for turkey drinking water. *Avian Dis* **14**: 203–430.

Riddell C (1984) Toxicity of dimetridazole in waterfowl. *Avian Dis* **28**: 974–7.

Rutherford DW, Bednar AJ, Garbarino JR, *et al*. (2003) Environmental fate of roxarsone in poultry litter. Part II. Mobility of arsenic in soils amended with poultry litter. *Environ Sci Technol* **37**: 1515–20.

Sakaguchi Y, Hayashi T, Kurokawa K, *et al*. (2005) The genome sequence of *Clostridium botulinum* type C neurotoxin-converting phage and the molecular mechanisms of unstable lysogeny. *Proc Nat Acad Sci* **102**: 17472–7.

Sander JE, Dufour L, Wyatt RD, *et al*. (1991) Acute toxicity of boric acid and boron tissue residues after chronic exposure in broiler chickens. *Avian Dis* **35**: 745–9.

Sander JE, Savage SI, Rowland GN (1998) Sodium sesquicarbonate toxicity in broiler chickens. *Avian Dis* **42**: 215–18.

Sarkar K, Narbaitz R, Pokrupa R, *et al*. (1981) The ultrastructure of nephrocalcinosis induced in chicks by *Cestrum diurnum* leaves. *Vet Pathol* **18**: 62–70.

Schell MM (2004a) 4-aminopyridine. In *Clinical Veterinary Toxicology*, 1st edn, Plumlee K (ed.). Mosby, St. Louis, MO, pp. 443–4.

Schell MM (2004b) 3-chloro-*p*-toluidine hydrochloride. In *Clinical Veterinary Toxicology*, 1st edn, Plumlee K (ed.). St. Louis, Mosby, p. 443.

Schlinger BA (1997) The activity and expression of aromatase in songbirds. *Brain Res Bull* **44**: 359–64.

Shultz S, Baral HS, Charman S, *et al*. (2004) Diclofenac poisoning is widespread in declining vulture populations across the Indian subcontinent. *Proc R Soc Lond B* **271**(Suppl.): S458–60.

Seidel WC, Scherer Jr KV, Cline Jr D, *et al*. (1991) Chemical, physical, and toxicological characterization of fumes produced by heating tetrafluoroethane homopolymer and its copolymers with hexafluoropropene and perfluoro (propyl vinyl ether). *Chem Res Toxicol* **4**: 22936.

Shivanandappa T, Ramesh HP, Krishnakumari MK (1979) Rodenticidal poisoning on non-target animals: acute oral toxicity of zinc phosphide to poultry. *B Environ Contam Toxicol* **23**: 452–5.

Shlosberg A, Egyed MN, Eilat A (1974) The comparative photosensitizing properties of *Ammi majus* and *Ammi visnaga* in goslings. *Avian Dis* **18**: 544–50.

Shlosberg A, Bellaiche M, Hanji V, *et al*. (1997) New treatment regimens in organophosphate (diazinon) and carbamate (methomyl) insecticide-induced toxicosis in fowl. *Vet Human Toxicol* **39**: 347–50.

Short CR, Flory W, Hsieh LC, *et al*. (1988) Comparison of hepatic drug metabolizing enzyme activities in several agricultural species. *Comp Biochem Physiol* **91C**: 419–24.

Shropshire CM, Stauber E, Arai M (1992) Evaluation of selected plants for acute toxicosis in budgerigars. *J Am Vet Med Assoc* **200**: 936–9.

Sousadias MG, Smith TK (1995) Toxicity and growth promoting potential of spermine when fed to chicks. *J Anim Sci* **73**: 2375–81.

Stedman TM, Booth NH, Bush PB (1980) Toxicity and bioaccumulation of pentachlorophenol in broiler chickens. *Poult Sci* **59**: 1018–26.

Stevens GR, Clark L (1998) Bird repellants: development of avian-specific tear gases for resolution of human–wildlife conflicts. *Int Biodeterior Biodegrad* **42**: 153–60.

Stolley DS, Meteyer CU (2004) Peracute sodium toxicity in free-ranging black-bellied whistling duck ducklings. *J Wildl Dis* **40**: 571–4.

Stoltz JH, Galey F, Johnson B (1992) Sudden death in ten psittacine birds associated with the operation of a self-cleaning oven. *Vet Hum Toxicol* **34**: 420–1.

Stone WB, Okoniewski JC, Stedelin JR (1999) Poisoning of wildlife with anticoagulant rodenticides in New York. *J Wildl Dis* **35**: 187–93.

Stone WB, Okoniewski JC, Stedelin JR (2003) Anticoagulant rodenticides and raptors: recent findings from New York, 1998–2001. *B Environ Contam Toxicol* **70**: 34–40.

Stowe CM, Barnes DM, Arendt TD (1981) Ethylene glycol intoxication in ducks, Philadelphia, PA. *Avian Dis* **25**: 538–41.

Sue Y, Delaney KA (2001) Antiseptics, disinfectants, and sterilizing agents. In *Clinical Toxicology*, Ford MD, Delaney KA, Ling LJ, *et al*. (eds). W.B. Saunders Co., Philadelphia, PA, pp. 749–56.

Takahashi S, Kaya K (1993) Quail spleen enlarged by microcystin RR as a blue-green algal hepatoxin. *Nat Toxin* **5**: 283–5.

Takeda M, Tsukamoto K, Kohda K, *et al*. (2005) Characterization of the neurotoxin produced by isolates associated with avian botulism. *Avian Dis* **49**: 376–81.

Talcott PA (2004a), Strychnine. In *Clinical Veterinary Toxicology*, 1st edn, Plumlee K (ed.). Mosby, St. Louis, MO, pp. 454–6.

Talcott PA (2004b) Metaldehyde. In *Clinical Veterinary Toxicology*, 1st edn, Plumlee K (ed.). Mosby, St. Louis, MO, pp. 182–3.

Tang KN, Rowland GN, Veltman Jr JR (1985) Vitamin A toxicity: comparative changes in bone of the broiler and leghorn chicken. *Avian Dis* **29**: 416–29.

Taylor DA (2004) Funky chicken. *Environ Health Persp* **112**: A50.

Temperton H (1944) Effect of green and sprouted potatoes on laying pullets. *Vet Med* **39**: 13–4.

Tiwary AK, Puschner B, Charlton BR, *et al.* (2005) Diagnosis of zinc phosphide poisoning in chickens using a new analytical approach. *Avian Dis* **49**: 288–91.

Tomizawa M, Casida JE (2005) Neonicotinoid insecticide toxicology: mechanisms of selective action. *Annu Rev Pharmacol* **45**: 247–68.

Townsend MG, Fletcher MR, Odam EM, *et al.* (1981) An assessment of the secondary poisoning hazard of warfarin to tawny owls. *J Wildl Manage* **45**: 242–7.

Trampel DW, Smith SR, Rocke TE (2005) Toxicoinfectious botulism in commercial caponized chickens. *Avian Dis* **49**: 301–3.

Volmer PA (2004) Pyrethrins and pyrethroids. In *Clinical Veterinary Toxicology*, 1st edn, Plumlee K (ed.). Mosby, St. Louis, MO, pp. 188–90.

Wages DP, Ficken MD, Cook ME, *et al*. (1995) Salt toxicosis in commercial turkeys. *Avian Dis* **39**: 158–61.

Warnock N, Schwarzbach SF (1995) Incidental kill of dunlin and killdeer by strychnine. *J Wildl Dis* **31**: 566–9.

Watkins JB, Klaassen CD (1986) Xenobiotic biotransformation in livestock: comparison to other species commonly used in toxicity testing. *J Anim Sci* **63**: 933–42.

Wells RE (1983) Fatal toxicosis in pet birds caused by an overheated cooking pan lined with polytetrafluoroethylene. *J Am Vet Med Assoc* **182**: 1248–50.

Wells RE, Slocombe RF (1982) Acute toxicosis of budgerigars (*Melopsittacus undulates*) caused by pyrolysis products from heated polytetrafluoroethylene: microscopic study. *Am J Vet Res* **43**: 1243–8.

Wells RE, Slocombe RF, Trapp AL (1982) Acute toxicosis of budgerigars (*Melopsittacus undulates*) caused by pyrolysis products from heated polytetrafluoroethylene: clinical study. *Am J Vet Res* **43**: 1238–42.

Westlake GE, Martin AD, Stanley PI, *et al*. (1983) Control enzyme levels in the plasma, brain and liver from wild birds and mammals in Britain. *Comp Biochem Physiol* **76C**: 15–24.

Wiemeyer SN, Hill EF, Carpenter JW, *et al*. (1986) Acute oral toxicity of sodium cyanide in birds. *J Wildl Dis* **22**: 538–46.

Wight PAL, Scougall RK, Shannon DWF (1987) Role of glucosinolates in the causation of liver hemorrhages in laying hens fed water-extracted or heat-treated rapeseed cakes. *Res Vet Sci* **43**: 313–19.

Wilde SB, Murphy TM, Hope CP, *et al*. (2005) Avian vacuolar myelinopathy linked to exotic aquatic plants and a novel cyanobacterial species. *Environ Toxicol* **20**: 348–53.

Wilson H, Brown CA, Greenacre CB, *et al*. (2001) Suspected sodium hypochlorite toxicosis in a group of psittacine birds. *J Avian Med Surg* **15**: 209–15.

Windingstad RM, Kartch FX, Stroud RK, *et al*. (1987) Salt toxicosis in waterfowl in North Dakota. *J Wildl Dis* **23**: 443–6.

Wobeser B, Blakley BR (1987) Strychnine poisoning of aquatic birds. *J Wildl Dis* **23**: 341–3.

Woolley SM, Wissman AM, Rubel EW (2001) Hair cell regeneration and recovery of auditory thresholds following aminoglycoside ototoxicity in Bengalese finches. *Hear Res* **153**: 181–5.

Yoder CA, Graham JK, Miller LA (2006) Molecular effects of nicarbazin on avian reproduction. *Poult Sci* **85**: 1285–93.

Zechmeister TC, Kirschner KT, Fuchsberger M, *et al*. (2004) Prevalence of botulinum neurotoxin C1 and its corresponding gene in environmental samples from low and high risk avian botulism areas. *Altex* **22**: 185–95.

Zuberogoitia I, Martinez JA, Iraeta A, *et al*. (2006) Short-term effects of the prestige oil spill on the peregrine falcon (*Falco peregrinus*). *Mar Pollut Bull* **52**: 1176–81.

Zurawell RW, Chen H, Burke JM, *et al*. (2005) Hepatotoxic cyanobacteria: a review of the biological importance of microcystins in freshwater environments. *J Toxicol Environ Health Part B* **8**: 1–37.

C H A P T E R

# 57

# Principles of ecotoxicology

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## INTRODUCTION

In this chapter we offer some background information on selected issues and toxicants of importance in ecotoxicology. Because of the breadth of this subject, readers are encouraged to capitalize on other sources as well. There are numerous scientific journals that publish papers on a broad array of topics in ecotoxicology, including *Ecotoxicology*, *Environmental Toxicology and Chemistry*, *Environmental Pollution*, *Journal of Environmental Quality*, *Environmental Health Perspectives*, *Archives of Environmental Contamination and Toxicology*, and others. In addition, there are numerous texts available on this subject, including *Fundamentals of Ecotoxicology* (Newman, 1998), *Handbook of Ecotoxicology* (Hoffman *et al.*, 2003), and *Principles of Ecotoxicology* (Walker *et al.*, 2001), to name just a few. There is also a growing number of books that are focused on specific issues or taxa, e.g. *Ecotoxicology of Amphibians and Reptiles* (Sparling *et al.*, 2000), *Radiotelemetry Applications for Wildlife Toxicology Field Studies* (Brewer and Fagerstone, 1998), *Principles and Practices for Evaluating Endocrine Disruption in Wildlife* (Kendall *et al.*, 1998), *Wildlife Toxicology and Population Modeling: Integrated Studies of Agroecosystems* (Kendall and Lacher, 1994), and many others.

### Defining ecotoxicology

Ecology is defined as the science of all living organisms, and all their interactions among one another and with the environment. We define toxicology as the science of all the adverse biochemically mediated effects of all chemicals on all life forms. Newman (1998:13) defined ecotoxicology as the "science of contaminants in the biosphere and their effects on constituents of the biosphere." More recently, Hoffman *et al.* (2003:1) defined ecotoxicology as "the science of predicting effects of potentially toxic agents on natural ecosystems and on nontarget species." For the purposes of this chapter, we define ecotoxicology as the science of all the adverse biochemically mediated effects of all chemicals on all living organisms including all their interactions within organisms and among species in the environment (Beasley, 1993). As such, ecotoxicology is the most encompassing specialty within the discipline of toxicology. The breadth of ecotoxicological studies is therefore immense. It ranges from: (a) tightly controlled laboratory studies of the pathophysiological effects of single chemicals on one strain of a native or surrogate microbe, plant, or animal species, to (b) studies of the transport and fate over time of one or more contaminants in the environment, (c) studies of the pathophysiological effects of single chemicals on a suite of organisms with various inanimate factors in microcosms or mesocosms, (d) studies of toxic interactions among groups of contaminants on one or more organisms in concert with inanimate factors in microcosms or mesocosms, and (e) small- and large-scale field research on impacts of one or more chemicals on suites of abiotic and biotic components in the environment.

### Successes and challenges

The necessity of an interdisciplinary approach and a combination of field and laboratory studies was illustrated in early ecotoxicological studies of spent lead shot pellets. Although lead poisoning resulting from lead shot ingestion was known to be a mortality factor in waterfowl in the late 1800s, the scope of the problem and the nature of the syndrome were not understood until Dr Frank Bellrose began comprehensively investigating this issue in the 1940s and 1950s. Bellrose (1959) and colleagues from the Illinois Natural History Survey addressed: the geographic distribution, frequency, and magnitude of die-offs; the accessibility

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of spent shot; ecological determinants of differential susceptibility; dose response; clinical signs in moribund individuals; lesions in exposed specimens; and the influence of diet on toxicity of lead shot. Others began examining lesions of lead poisoning in waterfowl and other species, and developing diagnostic criteria to support diagnoses of lead poisoning in birds (e.g. Longcore *et al.*, 1974a, b; Finley *et al.*, 1976a, b, c; Dieter and Finley, 1979; Friend, 1985). Their findings and others that followed eventually prompted restrictions and later a complete ban on lead shot for hunting waterfowl in the United States, Canada, and several other nations. Government regulations in these countries required the testing of potential non-toxic substitutes for lead shot. Some, such as iron (steel) (Sanderson and Irwin, 1976), bismuth (Sanderson *et al.*, 1997), and tungsten (Mitchell *et al.*, 2001), were deemed functionally non-toxic after ingestion by waterfowl and were approved for use in waterfowl hunting, whereas other candidates such as zinc were found to be toxic (Levengood *et al.*, 1999). Such restrictions soon led to decreased lead exposure (Samuel and Bowers, 2000) and reduced mortality (Anderson *et al.*, 2000) due to lead shot ingestion in some duck species.

The agricultural application of dichlorodiphenyltrichloroethane (DDT) began shortly after World War II (WWII). At that time, large amounts were sprayed on farm fields in the United States, Great Britain, and elsewhere. After noting increased incidence of raptor nests in the United Kingdom with broken eggs, Ratcliffe (1967) examined the thickness of eggshells, using the ratio of shell weight/size of eggs of three species of raptorial birds collected from 1900 through 1967. He found a precipitous decline in shell weight/size ratio from 1946 to 1950, after which shell thinning persisted. Shell thickness of eggs collected during 1900–1946 was significantly greater than of those collected during 1947–1967. Ratcliffe (1967) noted that the sharp decline in eggshell thickness coincided with beginning of widespread agricultural use of DDT (1945–1946), and Jefferies 1967 linked DDT and delayed ovulation in birds (suggesting a hormonal mechanism, i.e. endocrine disruption). This work was followed by many corroborative field and laboratory studies as discussed below, which, taken together provided an overwhelming weight of evidence as to the insidious and devastating impacts of DDT on avian reproduction. A combination of pest resistance (resulting in development of alternatives), decades of accumulated evidence on environmental effects, and publication of *Silent Spring* by Rachel Carson (1962) were necessary to drive a US ban on DDT use in 1972. Many other developed nations banned these compounds also, and pressured other, less-affluent nations to discontinue or curtail its use. Recently, the World Health Organization and Stockholm Convention on Persistent Organic Pollutants condoned the use of DDT for indoor mosquito control in tropical malaria-endemic (World Health Organization, 2005). Dichlorodiphenyldichloroethylene (DDE), the most persistent, bioactive environmental product of DDT, though declining in regions where its use was discontinued, is still widely disseminated in the environment.

The field of ecotoxicology has: helped terminate or reduce the manufacture and dissemination of some major environmental pollutants [e.g. lead, DDT, polychlorinated biphenyls (PCBs)]; prompted development of new, man-made chemicals of lower environmental risk (e.g. insect growth hormone mimics such as methoprene); helped applied ecology become problem driven; and is an important component of conservation medicine (Munson and Karesh, 2002; Tabor, 2002). In recent decades, ecotoxicology has begun to be applied in conjunction with other components of ecological restoration science to help reestablish ecological health in previously contaminated systems (Cairns, 2003; Linder *et al.*, 2003).

Despite benefits from ecotoxicology and other sciences that support rational actions to enhance ecosystem health, in many ways we have witnessed nearly 45 additional years of global mismanagement of ecological resources since the publication of *Silent Spring* in 1962. Although the general public in developed and developing nations has become more environmentally aware in recent decades, powerful economic interests e.g. mining, energy generation, chemical, construction, and agricultural and forestry industries have often hampered the application of sound ecological and ecotoxicological science to resource extraction and development.

Decisions sometimes fail to address long-term environmental needs because of misguided political pressure. Unlike progress seen recently in Europe, the United States has failed to maintain leadership in environmental stewardship with regard to contaminants. In fact, no new, major legislation in support of ecosystem health has been passed by the US Congress since 1990.

## Sources of pollution

Myriad toxic substances are released into our environment daily. Toxic agents are either deliberately manufactured (pesticides, drugs, construction chemicals, household chemicals) or accidentally produced (by-products in final formulations or in gaseous, particulate, liquid, and solid waste streams). Inappropriate siting and operation of chemical manufacturing and waste storage facilities can lead to "accidental" releases of chemicals into groundwater, surface water, soil, and air. Although it is increasingly recognized as ill-conceived, chemical and textile manufacturing plants, refineries, smelters, chemical storage facilities, and electricity generating plants were often located near waterways for ease of transportation of raw materials and finished products, because they use large

quantities of water in manufacturing, and/or to allow for dissemination of wastes. Such siting predisposes to deliberate (sometimes permitted), accidental, and even malicious releases of toxic chemicals into aquatic ecosystems.

Environmental contamination can emanate from a definable location (point-source pollution), such as atmospheric emissions from the stacks of a coal-burning power plant or liquid effluent from the discharge of a sewage treatment plant. Contamination can also be more widespread, emanating from a larger surface area (non-point source pollution), such as pesticides washed off the landscape by precipitation or airborne effluents from a host of automobiles.

## Chemical disasters

The stewardship of chemically mediated disasters ranges from responsible and efficient to neglect and indifference. Localized disasters involving a single, acutely toxic, short-lived chemical (e.g. a volatile solvent released from damaged rail tanker) can be relatively easy to address. Ecological recovery is also more likely when ecological resources (locally adapted microbes, plants, and animals) from the periphery are intact; local emergency agencies are well equipped, staffed, and responsive; funding is available from responsible parties or government to support containment, cleanup, and ecological rehabilitation; and the public is strongly engaged. Conversely, recovery is less likely when releases involve multiple long-lived chemicals (e.g. complex, halogenated, higher molecular-weight wastes) spread over a wide area when ecological resources from periphery are insufficient to support recovery, when responsible parties deny involvement, when neither the responsible parties nor the governments involved put forth needed funds, and the public is largely disengaged.

On March 24, 1989, the oil-tanker ship the *Exxon Valdez* hit a reef, spewing nearly 11,000,000 gallons of crude oil into the pristine Prince William Sound in Alaska. Exxon Corporation (now Exxon Mobil), the ship's owner, the Alyeska Pipeline Service Company, a consortium of oil companies with interests in Alaska, as well as state and federal disaster response planning, were criticized (Exxon and Alyeska were eventually sued) as being wholly unprepared to provide a quick and effective response for such a disaster, as was required by law. Containment and cleanup were to begin within 5 h, but crews and equipment did not begin arriving for 10–12 h after the incident, while oil continued to gush from the leaking tanker. Confusion, poor communications and coordination, lack of sufficient personnel, equipment and supplies for a spill of that magnitude, and the remoteness of the location hampered efforts to contain and begin effective cleanup during the first 2 days after the ship ran aground, after which rough seas created further difficulties.

Although the ecological and socioeconomic costs were tremendous, and could have been lessened, the response among the public, conservation organizations and agencies, academic institutions and media was unprecedented for an environmental disaster. Frustrated with a lack of action on the part of industry and government, local citizens began to clean up what oil they could with resources at hand. Within hours of the spill, scientists arrived to begin documenting the environmental impacts of the spill. Particularly sensitive environments were quickly identified and prioritized for protection and cleanup. Crews began to arrive from throughout the world to help clean oil-covered beaches, seabirds, and sea otters. An estimated 36,000 birds and 1000 sea otters eventually were treated for oil exposure; however, untold numbers of fish and wildlife died from direct impacts of oiling. According to the *Exxon Valdez* Oil Spill Trustee Council, the populations of only 4 of 11 bird species impacted were recovering or had recovered as of 2002. Impacts to sensitive intertidal communities may not be fully realized for many years to come.

The global news media followed many phases of the cleanup and recovery operations, focusing attention on the environmental and economic costs of this disaster, as well as on Exxon's initial response and failure to accept responsibility for the accident. The Oil Pollution Act of 1990, in response to the *Exxon Valdez* disaster, required increased spill preparedness and the phase-in of double-walled tanker ships, which greatly reduce the chances of a spill should a tanker run aground or hit an iceberg or reef. Unfortunately, the petroleum industry has found loopholes in the law and effectively delayed the deadline for new or retrofitted tankers until 2015. In the meantime, sensitive marine environments remain at high risk from oil spills. For more on the history, current activities, and future plans surrounding this disaster, see the *Exxon Valdez* Oil Spill Trustee Council's website (http://www.evostc.state.ak.us) and the US Environmental Protection Agency's *Exxon Valdez* Oil Spill Profile (http://www.epa.gov/oilspill/exxon.htm).

# ECOTOXICOLOGICAL STRESS IN CONCERT WITH OTHER MECHANISMS OF ECOSYSTEM DISEASE

Aquatic systems throughout the world are increasingly under stress from human activities. Wetlands, lakes, streams, rivers, and estuaries are being degraded due to loading with nutrients (Baird *et al*., 2004), metals, salts, synthetic organic chemicals, and combustion products, as well as a wide array of other anthropogenic stressors. For example, many of the world's coral reefs are being degraded through the use of destructive fishing and collecting methods (Hodgson, 1999; Jackson *et al*., 2001),

eutrophication (Diaz-Pulido and McCook, 2005), increasing ocean temperatures (Wolanski and De-ath, 2005), and coral diseases produced by bacteria, fungi, and cyanobacteria (Richardson, 1998). Although the role of environmental degradation in producing coral disease remains unclear, the vast majority of impacted reefs in the Caribbean were near areas of human activity (Green and Bruckner, 2000), and in the Netherlands Antilles, changes in the structure of coral reef bacterial, including cyanobacterial (blue-green algal), communities were observed in proximity to large inputs of contaminants (Klaus *et al*., 2004).

Surface runoff, coupled with stream channelization and subsurface tiling of fields, decreases groundwater recharge and increases the "flashiness" of streams, sometimes resulting in prolonged, catastrophic flooding. Rapid flow of water due to runoff from cropland, cleared forests, lawns, roofs, stockyards, roads, parking lots, demolition and construction sites, and poorly managed industrial and dump sites also carries pulses of complex pollutant mixtures to water bodies (Hyer *et al.*, 2001; Kolpin *et al.*, 2002; Gilliom *et al*., 2006). For example, precipitation events can mobilize pesticides from soils into runoff (Leu *et al.*, 2004a, b) and increase concentrations in surface waters (Stamer *et al*., 1994). Lack of water retention, due to a diminished wetland base, results in abnormally low flows during dry periods, resulting in decreased dilution and thus increased concentrations of contaminants (Kolpin *et al*., 2004). Reduced flows can also result in increased water temperatures in summer, thereby decreasing the oxygen-holding capacity of the water. Removal of tree canopies through logging, development, or other activities also increases stream temperatures to the detriment of aquatic species requiring cool, highly oxygenated water, such as trout and salmon (Kishi *et al*., 2004).

In addition to being sources of toxicants, road (Trombulak and Frissell, 2000) and bridge (Blettler and Marchese, 2005) construction, deforestation (Alin *et al.*, 2002; Klink and Machado, 2005; Silveira *et al*., 2005), agriculture (Uri, 2001), and mining (Hartman *et al*., 2005) erode soils and increase sedimentation of waterways. High suspended-sediment loads increase turbidity, shading plants so that primary productivity from photosynthesis, biomass, structure, and dissolved oxygen are reduced. Excess suspended sediments in waterways can also degrade spawning habitat (Burkhead and Jelks, 2001), produce lesions in gills (Herbert and Merkens, 1961), and modify animal behavior (Berg and Northcote, 1985), resulting in reduced fish productivity and altered fish community assemblages (Sutherland *et al*., 2002; Mol and Ouboter, 2004).

Prior to the advent of modern intensive agriculture, farms consisted of a mosaic of small fields of vegetables, grains, and forage crops, interspersed with fencerows, woodlots, pastures, and wetlands. Crop rotations offset the need for fertilization and pest control. By contrast, modern agricultural and forestry practices have produced vast monocultures of genetically similar plants that are susceptible to attack by plant pathogens such as fungi and herbivorous insects, triggering the use of toxic pesticides. Such monocultures deplete nutrients, prompting fertilization which increases the availability of free nutrients. High levels of nutrients such as nitrogen can be directly toxic (ionic forms, e.g. nitrite or nitrate) or have indirect impacts, e.g. cause harmful algal blooms that may produce toxins and usurp dissolved oxygen.

Modern agriculture and forestry, along with urbanization and suburban sprawl, have created a patchwork of habitat islands in a largely inhospitable landscape. Biota inhabiting such islands are prone to extirpation through environmental perturbations such as catastrophic weather events; competition with invasive, exotic species, habitat degradation that prompts starvation, desiccation, or predation; and mortality or reduced productivity via exposure to environmental contaminants. This loss of species from habitat patches, combined with reduced recolonization rates, can result in local or regional reductions in populations, leading to extirpation, and increasing the risks of extinction events.

Anthropogenic air pollution has been reported since 13th century London, when smog from coal fires blanketed the city; and the situation worsened with the Industrial Revolution of the 1700s. An estimated 12,000 people died in London during the Great Smog of the winter of 1952 (Wilkins, 1954). Pollutants from tall stacks of power plants may travel long distances, harming the environment far from the source. In addition to power generation, other major anthropogenic sources of atmospheric pollutants include waste incineration, oil refinement, industrial emissions, fires from slash and burn agriculture; large wildfires that follow misguided forest-fire suppression, and transportation sources. Widespread deforestation, especially in the tropics (the "lungs of the earth"), and coastal pollution that limits phytoplankton photosynthesis reduce the ability of the environment to remove $CO_2$ and clean the air.

Excessive reliance on fossil fuels (natural gas, petroleum, coal) and clearing of forests are increasing $CO_2$ and methane levels, which trigger global climate change. Small changes in average temperatures appear to be increasing the rate of glacial retreat and causing reductions in ice/snow packs, and increasing the frequency of extreme weather (e.g. drought, flooding, and hurricanes). Rising global temperatures are producing shifts in species tolerating warmer temperatures, with cold-dependent species moving nearer to the poles and into higher elevations. The United States is the leader in greenhouse emissions, according to the Oak Ridge National Laboratory (Marland *et al*., 2005). North American releases amounted to 1.73 billion tons in 2002 (approximately 92% of this generated in the United States), which was 26% of the world total. Half of the estimated 290 billion tons released due to human activities since 1751 have been since mid-1970s. Carbon dioxide emissions in the United States increased

an estimated 9-fold during 20th century. Of even greater concern at present is China, which has a much higher human population and has been rapidly industrializing, culminating in increased carbon dioxide emissions in the same time frame by 6000-fold (UKDTI, 2002).

Environmental and ecotoxicological problems related to fossil fuels go well beyond the aforementioned oil spills and global warming from carbon dioxide. Placing oilfields in sensitive arctic and marine environments, especially offshore in the Arctic, may have immense adverse impacts on marine mammal populations and indigenous human communities. Emissions during oil refining, and polycyclic aromatics produced through combustion from petroleum and coal have caused widespread pollution. Many of the polycyclic aromatic hydrocarbons are oxidized by P450 enzymes in lung, liver, and other tissues to form reactive epoxide derivatives that form adducts to DNA, resulting in mutations, with the potential to cause cancers as well as heritable defects. Among the best known polycyclic aromatic hydrocarbons is benzo(a)pyrene. Also of concern are volatile organic compounds (VOCs) such as benzene. Both benzo(a)pyrene and benzene known human carcinogens. Aluminum smelters using obsolete technologies have also polluted the environment with polycyclic aromatic hydrocarbons, and were incriminated in digestive tract cancers of beluga whales Martineau *et al*. (1991, 2002).

Through emissions of sulfur dioxide, coal-fired power plants in the United Kingdom have affected the environments of Sweden and Norway; and likewise those in the midwestern United States have impacted lakes and forests in the northeast part of that country as well as Canada. Oxidized nitrogen ($NO_x$) from burning gasoline, diesel fuel, heating oil, natural gas, and coal and oxidized sulfur from refining oil and burning coal form strong mineral acids when combined with precipitation. This can lead to changes in soil and water chemistry, including solubilization of aluminum, with adverse impacts on soil and aquatic flora and fauna. Aluminum is a ubiquitous constituent of soils, and excess concentrations in solution can precipitate out on gills of fishes and aquatic invertebrates to impair oxygen utilization (Glynn *et al*., 1992, Alexopoulos *et al*., 2003; Winter *et al*., 2005; Soucek, 2006). Highly acidic waters with excess aluminum can also reduce calcium uptake via gills, impacting growth and development in aquatic and invertebrate species (Malley and Chang, 1985).

Semi-volatile compounds such as PCBs are released into the atmosphere from soils at contaminated locations and are transported great distances before being deposited, often over cool surfaces such as large bodies of water. Thus, large lakes, seas, and oceans have become repositories for such chemicals. This process largely accounts for the comparatively high concentration of persistent organochlorines in polar ocean foodwebs. Other semi-volatile chemicals, such as halogenated dibenzodioxins and halogenated dibenzofurans, are toxic by-products of the incineration of solid wastes and manufacturing processes such as the Kraft paper bleaching process. Such compounds have multiple toxic effects including cognitive impairment, liver damage, and endocrine disruption.

Considering that milliseconds can greatly influence the likelihood of successful predation as well as avoiding becoming prey, that overall health is essential for competition to be among the breeding population in the wild, that high functioning is needed by many parents to feed and protect the next generation until it can survive on its own, it should be no surprise that biodiversity is being harmed not only by habitat loss, climate change, exotic species introduction, pathogen pollution, and noise pollution, but also by a host of chemical contaminants – both manmade and produced by anthropogenically altered populations of microbes, plankton, plants, and animals.

## ENVIRONMENTAL CONTAMINANTS

### Nutrients

Nutrients, primarily nitrogen and phosphorus, are released into the environment via chemical fertilizers, manure/sewage effluent, burning of fossil fuels, fires, and industrial processes such as pulp/paper milling and nitric acid production. Globally, the rate of transfer of atmospheric nitrogen (N) to the available nitrogen pool has doubled due to anthropogenic activities (Vitousek *et al*., 1997). Agriculture is the primary source of excess nutrients in the environment, both through soil management and livestock operations (Carpenter *et al*., 1998). World consumption of fertilizer in 2005/2006 totaled 154 million tons [International Fertilizer Industry Association, Statistics: Total fertilizer nutrient consumption = $N + P_2O_3 + K_2O$ (million tons nutrients)]. Fertilizer use in developed nations has plateaued, reaching a point where further inputs do not increase yield. The United States alone used 21 million tons of plant fertilizers in 2003, primarily as N, P, and K, which is about the same level as in 1976 (USDA Economic Research Service – US consumption of nitrogen, phosphate, and potash for 1960–2003). However, as populations and associated demand for cereal grains in developing nations grow, fertilizer use will likely increase.

Inorganic nitrogen, including ammonia, ammonium, nitrate, and nitrite, is toxic to aquatic life including fish, invertebrates, and amphibians. Ammonium nitrate fertilizer produced toxic effects in American toad (*Bufo americanus*), western chorus frog (*Pseudacris triseriata*), northern leopard frog (*Rana pipiens*), and green frog (*Rana clamitans*) tadpoles at concentrations found in the agricultural environment (Hecnar, 1995). High nitrogen concentrations contributed to lower amphibian reproductive success (Knutson *et al*., 2004) and species richness (Houlahan and Findlay, 2003; Knutson *et al*., 2004) in wetlands in agriculturalized areas. Rouse *et al*. (1999) reported that 20% of over 8000 water

samples from the Great Lakes Watershed exceeded concentrations considered toxic to amphibians. Nutrients may also interact with pesticides to produce toxic effects different than those of either alone. For example, a mixture of atrazine and nitrate had greater than expected impacts (synergistic effect) on snout-vent length of *Xenopus laevis* tadpoles (Sullivan and Spence, 2003). Conversely, the addition of higher nitrate concentrations to the same atrazine concentrations resulted in greater snout-vent lengths, an antagonistic effect. Much work remains to be done in examining the effects of complex mixtures of chemicals on amphibians and other biota.

In addition to direct toxic effects on amphibians, large inputs of nutrients into water bodies increase algal growth, which, at high levels, can result in decreased dissolved $O_2$ concentrations through algal respiration and decay, and shading, reducing light needed by submerged aquatic vegetation. Metabolism of dead vegetation further reduces dissolved oxygen concentrations, and a reduction in aquatic plants results in reduced nitrogen storage. Excess nutrient input promotes increased snail populations (Chase, 1998), due to increased algal and periphyton resources that comprise the main food source for snails. Johnson *et al*. (2002) found a positive relationship between the frequency of limb malformations and infection with *Ribeiroia* trematodes.

Trematode infections may produce limb deformities (Johnson *et al*., 1999, 2001; Schotthoefer *et al*., 2003a,b) or potentially debilitating kidney infections (Beaver, 1937; Fried *et al*., 1997; Beasley *et al*., 2005) in frogs. Infections by *Ribeiroia* and *Echinostoma* at the early stage in development can vastly reduce survival of amphibian tadpoles (Schotthoefer *et al*., 2003a,b; Beasley *et al*., 2005). Moreover, sublethal trematode infections may also make tadpoles less tolerant of other stressors. Higher snail densities coupled with increased waterbird (definitive hosts) use of a diminished wetland base and the reduced structural complexity of habits through use of herbicides and fertilizers appear to result in higher trematode infection rates and intensities. The presence and abundance of *Ribeiroia* infection were associated with the presence and abundance of snails that function as intermediate hosts for larval trematodes. These parasites undergo extremely high levels of asexual reproduction in snails. Furthermore, the presence and abundance of planorbid snails was associated with manmade wetlands (e.g. ponds) and higher orthophosphate concentrations.

Eutrophication can also promote growth of toxigenic algae such as certain blue-green algae (cyanobacteria), which produce neurotoxins, such as anatoxin-A, anatoxin-A(s), saxitoxin, and neosaxitoxin, and the yet-to-be-identified toxin believed to account for avian vacuolar myelopathy (AVM). A current theory is that acyanobacterium of the order stigonematales, a cyanobacteria that grows on the exotic invasive *Hydrilla*, which is consumed by avian species such as coots underlies AVM (Birrenkott *et al*., 2004). Eagles are exposed via the food web. In addition to producing neurotoxins, cyanobacteria may produce hepatotoxins, such as microcystins, nodularin, and cylindrospermopsin. Both neurotoxic and hepatotoxic cyanobacteria have been implicated in die-offs of lesser flamingos, an obligate algal feeder. However, additional research will be needed before the importance of cyanobacterial toxins in population trends of lesser flamingos can be deduced.

The causes of harmful marine algal blooms, which appear to be an emerging problem, increasing in frequency and distribution worldwide, are less well understood. For example, diatoms have shown to produce neurotoxic domoic acid. Other phytoplankton such as dinoflagellates have been shown to produce saxitoxins, ciguatoxins, and brevetoxins. Shellfish and finfish may accumulate these toxins and cause secondary poisoning in humans (Van Dolah, 2000), and have been implicated in mass mortalities of bottlenosed dolphins (*Tursiops truncates*) on the Atlantic coast of the United States (Flewelling *et al*., 2005). Brevetoxin exposures via aerosols and ingestion of sea grasses contaminated with toxins from dinoflagellates seem to account for a number of major death losses in manatees in Florida over the past decade.

## Organochlorine compounds

World pesticide use in 2001 totaled 2.3 billion kg. In that year, the United States alone used about 546 million kg, including herbicides (44%), insecticides (10%), fungicides (6%), and others (40%, including nematicides, fumigants, rodenticides, avicides, molluscicides, and piscicides) (US Environmental Protection Agency, 2000-2001 Pesticide Market Estimates). Agriculture accounts for the largest proportion of total pesticides applied (75-80% in United States, primarily glyphosate, atrazine, and metam sodium). The widespread use of organochlorine insecticides (OCIs), DDT, aldrin, chlordane, dieldrin, heptachlor, and others began after WWII. These compounds were initially a boon to agriculture and public health, as they were both effective and persistent. However, pests soon began to develop resistance, requiring higher application rates and the continual development of new compounds.

DDT increases sodium conductance across nerve cell membranes, increasing excitability and resulting in tremors and the potential for seizures (Hodgson *et al*., 1998). The cyclodiene pesticides such as aldrin, chlordane, dieldrin, endrin, and heptachlor, the structures of which differ markedly from DDT, exert their influence on $\gamma$-aminobutyric acid (GABA) receptors in the brain and are more acutely toxic than DDT. The slow biodegradation rate and high lipid solubility of many organochlorine compounds, which prompts accumulation in adipose tissue, causes vast increases in the net uptake as the chemical is passed from lower to higher trophic levels, resulting in biomagnification, or successively greater residue concentrations in consuming

organisms. Predatory animals thus often accumulate very high concentrations, and the nursing young of top predators feeding in contaminated aquatic food webs are often massively exposed. Among the species most at risk are marine mammals, the young of which may consume milk with extremely high fat content (e.g. up to 60% fat).

4,4′-(2,2,2-trichloroethane-1,1-diyl)bis(chlorobenzene), best known as DDT, was used extensively in agriculture and public health, at peak production in 1962, 85,000 tons were produced in the United States alone (ATSDR, 2002), and an estimated 675,000 tons were applied in that country during 1945–1972 [USEPA-DDT Regulatory History: A Brief Survey (to 1975)]. This pesticide was considered safe, as it has a low acute toxicity to most bird and mammal species. However, over time, it became apparent that DDT had insidious effects; its metabolic by-product *p,p′*-DDE inhibits prostaglandin synthetase, resulting in reduced calcium uptake by the shell gland mucosa (Lundholm, 1993, 1997) during eggshell formation. This results in thinning, so that adults crush eggs during incubation (e.g. herons and egrets – Faber *et al*., 1972; Cooke *et al.*, 1976; sparrowhawks – Burgers *et al*., 1986). Eggshell thinning resulting from DDE exposure, and subsequent impacts on productivity, has been observed in a variety of species of carnivorous birds, such as: herons and other wading birds (Henny *et al*., 1985; White *et al*., 1988); eagles (Wiemeyer *et al*., 1984, 1993; Bowerman *et al*., 1995); falcons (Newton *et al*., 1989); hawks (Burgers *et al*., 1986; Newton *et al*., 1986); gulls (Gilbertson, 1974); and others (e.g. see Grasman *et al*., 1998).

Some of the other OCIs, such as the cyclodienes aldrin and dieldrin, resulted in direct mortality in predatory birds, such as sparrowhawks and kestrels (Newton *et al*., 1992). Such mortality, when combined with sublethal effects such as starvation and accidents due to neurological deficits (Walker and Newton, 1998) and reduced productivity due to eggshell thinning caused by DDE exposure (Newton *et al*., 1986), soon led to population declines in such species (Sibly *et al*., 2000). DDE also may co-occur in egg contents with PCBs, which are embryotoxic and therefore contributed to decreased productivity in many waterbird populations (Gilbertson and Hale, 1974a, b; Bowerman *et al*., 2003). Exposure of Great Lakes fish-eating birds to organochlorine compounds including dioxins and dioxin-like compounds such as PCBs may result in embryo and chick mortality, growth retardation, and deformities associated with edema, hepatomegaly, gastroschisis, and other lesions, otherwise known as the Great Lakes Embryo Mortality, Edema, and Deformity Syndrome, or GLEMEDS (Gilbertson *et al*., 1991).

Populations of other vertebrates apart from birds may also have been negatively impacted by DDT. For example, Reeder *et al*. (2005) examined spatial and temporal patterns of intersexuality in cricket frogs collected in Illinois during 1852–2001. Compared with the preorganochlorine era (1852–1929), the percentage of intersex cricket frogs increased during the period of industrialization and initial uses of PCBs (1930–1945), was highest during the peak of manufacture and use of DDT and PCBs (1946–1959), began declining with increased public concern and environmental regulation that led restrictions and eventual ban on the use of DDT in the United States (1960–1979), and continued to decline through the period of gradual reductions in environmental residues of organochlorine pesticides and PCBs in the midwestern United States (1980–2001).

## Cholinesterase-inhibiting pesticides

Organophosphorous (OP) and carbamate insecticides such as diazinon, chlorpyrifos, malathion, and carbofuran are cholinesterase-inhibiting compounds. As such, they prevent breakdown of acetylcholine after transmission of a nerve impulse. The first OP cholinesterase inhibitors were developed as chemical weapons during WWII. After WWII, the use of OP compounds increased as pests developed resistance to OCIs and their use was restricted. The OP compounds are not persistent in the environment in comparison to OCIs, but they are highly acutely toxic and broad spectrum; human deaths from mishandling occur annually. Secondary poisoning can occur when predators consume invertebrates or vertebrate wildlife that have been poisoned by these chemicals. In one of the best-documented secondary-poisoning events, approximately 6000 wintering Swainson's hawks (*Buteo swainsoni*) were poisoned in Argentina during 1995–1996 after feeding on grasshoppers sprayed with the OP insecticide monocrotophos (Goldstein *et al*., 1999). In another incident, hundreds of laughing gulls (*Larus atricilla*) were poisoned after consuming insects killed or debilitated by application of parathion to cottonfields (White *et al*., 1979); this is especially noteworthy in that the mortality included nestlings which died after being fed contaminated prey by adult gulls. It is likely that large numbers of individual poisonings of birds and other species by OP insecticides go unnoticed, following both the intended use and misuse of licensed products. Organophosphorous insecticides have also been used illegally as avicides to protect grain crops from bird depredations (White *et al*., 1989), and in baits to kill livestock predators such as coyotes (Wobeser *et al*., 2004). Such misuse has resulted in additional mortalities from secondary poisoning of predatory birds and mammals eating carcasses of poisoned animals (Wobeser *et al*., 2004).

Two of the three previously most widely used OP insecticides, diazinon and chlorpyrifos (the third is malathion), are now severely restricted in the US. The removal of diazinon and chlorpyrifos from household products early in the 21st century led to almost immediate reduction in concentrations in umbilical cord blood and was associated with increased birth weights of babies of low-income families (Whyatt *et al*., 2004).

Granular carbofuran pellets have been consumed by birds that apparently mistook them for seeds; and one pellet of this carbamate insecticide is enough to kill a bird (Balcomb *et al*., 1984). Carbofuran was the insecticide most commonly associated with wildlife pesticide poisoning events (e.g. red-winged blackbirds (*Agelaius phoeniceus*), Augspurger *et al*., 1996; bald (*Haliaeetus leucocephalus*) and golden (*Aquila chrysaetos*) eagles, coyotes (*Canis latrans*), and red foxes (*Vulpes vulpes*), Wobeser *et al*., 2004; bald eagles and red-tailed hawks (*Buteo jamaicensis*), Elliot *et al*., 1996; buzzards (*Buteo buteo*), Dietrich *et al*., 1995; herons, Hunt *et al*., 1995), and was even implicated in declines of some species, e.g. burrowing owls (*Athene cunicularia*) (Blus, 1996). The phase-out of the granular formulation began in 1991 and registrations for most solid forms have been cancelled. In addition to direct toxic effects to avian wildlife, insecticides may reduce populations of non-target invertebrates used as food by birds (Martin *et al*., 2000; Moreby *et al*., 2001; Morris *et al*., 2005), which may require increased foraging effort (Martin *et al*., 2000; Morris *et al*., 2005) or prey switching (Martin *et al*., 2000) by adults with young to feed. Additional research in this topic area is needed.

## Other insecticides

Pyrethrins from chrysanthemums and structurally related synthetic insecticides (pyrethroids) bind to sodium channels preventing closure, or to GABA-mediated chloride channels inhibiting chloride influx at sodium channels resulting in tremors or seizures. These chemicals are generally of low toxicity to mammals and birds; however, they are highly acutely toxic to arthropods, including non-target taxa such as spiders, parasitoids, and bees. Also, they are often highly acutely toxic to tadpoles and fish. Although early formulations had a short half-life – measured in hours in direct sunlight – newer compounds are designed to be more photostable, extending their effectiveness for up to 10 days out-of-doors. Unfortunately this greater stability increases environmental risks associated with their use.

There are a variety of other classes of pesticides used for control of insects, arachnids (e.g. mites), and other "pests." Some are nerve poisons, others inhibit energy production, chitin synthesis, water balance, or growth. A number of lower-risk pesticides include microbes (e.g. the soil bacterium *Bacillus thuringiensis* (Bt) for mosquito control), microbial products (e.g. spinosyn to control caterpillars is a fermentation product of a soil actinomycete), a range of other "natural chemicals" (e.g. pheromones, florals, sulfur), and "plant-incorporated protectants" (e.g. transgenic crops that may produce toxins originally synthesized in bacteria, such as Bt). Many of these new methods of pest control are more environmentally benign alternatives to traditional insecticides. However, they are intended to disrupt normal physiological processes, and thus, each carries potential environmental risks, which are only partially understood.

## Herbicides

During 2000, herbicides represented 36% of the world pesticide market (Kiely *et al*., 2004); the agricultural sector accounted for 78% of the herbicide use in the United States in 2001. Atrazine is perhaps the world's most used agricultural herbicide, with an estimated 74–80 million pounds applied in the United States alone in 2001 (Kiely *et al*., 2004). Atrazine or its degradation products have been found in air (Kuang *et al*., 2003), rainwater (Goolsby *et al*., 1997; Kuang *et al*., 2003; Asman *et al*., 2005), surface water (Spalding and Snow, 1989; Battaglin and Goolsby, 1999; Scribner *et al*., 2000), and groundwater (Spalding *et al*., 2003; Mills *et al*., 2005).

Herbicides are often considered benign with regard to impacts on animals; however, these compounds can have toxic effects at concentrations found in the environment. Atrazine has come under increased scrutiny due to evidence that it may produce endocrine-disrupting effects in amphibians, and has been banned by several European Union countries. Reeder *et al*. (1998) associated detection of atrazine with the finding of intersex gonads in cricket frogs (*Acris crepitans*). Hayes *et al*. (2002a, b, 2003) found that concentrations of atrazine as low as 0.1 parts per billion (ppb) affected gonadal development, produced hermaphroditism, and reduced the laryngeal muscle (needed for calling to attract mates and ward off potential competing males) in larval male frogs. At a range of test concentrations that can be found in the environment, atrazine was associated with reduced survival of tadpoles (Storrs and Kiesecker, 2004), increased length of larval period (Coady *et al*., 2004), reduced size at metamorphosis (Diana *et al*., 2000), gonadal dysgenesis, and reduction of spermatogenesis (Tavera-Mendoza *et al*., 2001). However, there have been contrasting results from others studies, and authors have suggested that intersex may be found in unexposed frogs (Carr *et al*., 2003; Freeman and Rayburn, 2004; Hecker *et al*., 2005; Jooste *et al*., 2005). However, in most such reports to date, the animals in control groups or at reference sites were also exposed to low concentrations of atrazine. Additional, carefully designed studies are needed.

Glyphosate is a broad-spectrum herbicide that has become the most used herbicide in the United States. This chemical has a low toxicity to mammals; however, surfactants used in some formulations to cause glyphosate to adhere to plant surfaces greatly increases the chemical's toxicity (Goldstein *et al*., 2005). Thus, some formulations (those not intended for aquatic vegetation control) can result in direct mortality of larval and juvenile amphibians (Relyea, 2005a) and subsequent loss of biodiversity (Relyea, 2005b). Glyphosate readily adsorbs to soil or is rapidly degraded by bacteria, and therefore is not highly bioavailable to animals and has low potential for runoff (Malone *et al*., 2004). Nevertheless due to its high water solubility, glyphosate is sometimes detected in surface waters due to runoff (Battaglin *et al*., 2005). Moreover, some

formulations are intended for control of aquatic vegetation (Solomon and Thompson, 2003). Due to its effectiveness and non-specific herbicidal properties, use of glyphosate to control undesirable plant species put, non-target plants at risk (Matarczyk *et al*., 2002; Gettys and Sutton, 2004).

Studies conducted by our laboratory have revealed a complex set of interactions involving herbicides that affect the health of amphibians inhabiting agriculturalized regions. Beasley *et al.* (2005) studied impacts of herbicides on trematode infections on frogs. They found that, in herbicide-impacted farm ponds, recruitment of juvenile cricket frogs was reduced and trematode infections in the frogs were greatly increased. The reduced recruitment might have resulted from deaths due to the infection with trematode cercariae, because other species of frogs died when early stages were exposed to the parasite (Schotthoefer *et al*., 2003a,b). Sousa and Grosholz (1991) had previously suggested that a more complex habitat structure impedes parasite transmission, and Beasley *et al*. (2005) hypothesized that severe trematode infections noted in their studies were due to interacting factors related to fewer plants in the water. In such simplified ecosystems, predation would likely be facilitated so that the survivors would receive a greater infective load from trematodes in the water. Similarly, the motile cercariae would likely have less difficulty finding the tadpole intermediate host thereby facilitating infection.

Herbicide contamination of water bodies may also reduce dissolved oxygen and the algal food needed by tadpoles, which may slow the growth of tadpoles, possibly delaying metamorphosis in some species or reducing size at metamorphosis, which may reduce fitness (Diana *et al*., 2000). Both hypoxic water and reduced food may stress the tadpoles, so that they are less able to avoid cercarial infection and encystment. After a reduction in algal and macrophyte communities due to herbicide contamination, there is likely to be a subsequent rebound to produce higher than normal algal concentrations. This is because the competition for nutrients among surviving plant species is likely to be greatly reduced. Although algal food during a rebound might help later season tadpoles, it might also provide food for snails. Under conditions where snail numbers increase relative to tadpole numbers – and considering the immense amplification of infective load related to asexual reproduction of trematodes within snail intermediate hosts – it seems plausible that some wetlands impacted by herbicides may serve as sources of super-infection of tadpoles. This combination of events and nutrient loading as previously discussed may help explain a number of the outbreaks of supernumerary limbs in frogs at levels noted first in the 1990s (Helgen *et al*., 1998; Hoppe, 2000) that were previously seen only infrequently. In addition, pesticide exposure can lead to immunosuppression and increased susceptibility to trematode infections in tadpoles and frogs (Kiesecker, 2002; Linzey *et al*., 2003). Such stressors may result in prolongation of tadpole stage; longer time in the earlier tadpole stages may result in greater lethality due to trematodes, and higher body burdens of pesticides to pass to avian predators. In addition to herbicidal impacts on vegetation and periphyton, exposure to insecticides might influence trematode infections by impacting populations of benthic and plankton predators of cercariae. Members of our team (Labak and Schotthoefer, unpublished data) have demonstrated that invertebrates such as hydra, copepods, daphnids, and especially damselfly larvae and dragonfly larvae attack and consume cercariae in a controlled setting. Insecticide-mediated changes in invertebrate community structure might therefore release cercariae or other trematode life stages from this predation pressure. Whether these "micropredators" are important in determining infective loads of cercariae in natural and human-altered aquatic environments remains to be thoroughly investigated.

Herbicides typically occur in the environment along with other anthropogenic chemicals. Studies examining mixtures of herbicides and insecticides have found additive, synergistic, or even antagonistic effects on organisms, depending on the chemicals used, test organisms, and conditions (e.g. Sutton *et al*., 1971; Jin-Clark *et al*., 2001; Strachan *et al*., 2001; Anderson and Lydy, 2002; Anderson and Zhu, 2004; Green and Abdelghani, 2004). Other stress-ors may enhance the toxicity of pesticides. For example, Relyea (2005c) found that predatory stress increased the lethality of Roundup® (a popular glyphosate product) to larval amphibians. As with many chemicals, there is a need for further examination of the environmental distribution, fate and toxicity of herbicide and their transformation products, considering both direct and indirect adverse effects.

## Rodenticides

Anticoagulant rodenticides such as brodifacoum inhibit blood clotting through inhibition of vitamin K reductase, an enzyme essential to the reuse of the vitamin in producing clotting proteins. Many such compounds are highly and acutely toxic. These rodenticides may directly impact non-target small mammal populations (Brakes and Smith, 2005). Also, secondary poisonings (relay toxicoses) have had impacts on birds of prey (e.g. buzzards, Berny *et al*., 1997; barn owls (*Tyto alba*), Hegdal and Bloskiewicz, 1984; red kites (*Milvus milvus*), Ntampakis and Carter, 2005)), as well as carnivorous mammals such as mink (*Mustela vison*) (Fournier-Chambrillon *et al*., 2004), polecats (*Mustela putorius*) (Shore *et al*., 1996), and red foxes (Berny *et al*., 1997).

## Endocrine-disrupting compounds in sewage receiving waters

A variety of endocrine-disrupting compounds have been detected in sewage effluent, including steroidal hormones,

pesticides, breakdown products of surfactants and plasticizers, pharmaceuticals, and others (e.g. PCBs and dioxins). Kolpin *et al*. (2002) detected 82 compounds in 139 US streams, 34 of which are known or suspected to have estrogenic activity. In that study, 75% of the streams sampled had more than one of the organic compounds present in detectable concentrations. Non-hormonal compounds that influence estrogen receptors include those that mimic the effects of hormones (mimics) and those that interfere with normal hormonal activity (antagonists).

To our knowledge, the first study to establish a link between disruption of the endocrine system and exposure to both sewage effluent and synthetic estrogens was that of Purdom *et al*. (1994). Following reports from fishermen of hermaphroditic fish in sewage lagoons, caged rainbow trout (*Salmo gairdnari*) and carp (*Cyprinus carpio*) were placed in sewage effluent in the field, and others were exposed to an oral contraceptive formulation in the laboratory. Marked increases in plasma vitellogenin (VTG) concentrations were observed, particularly in the trout. VTG is an egg-yolk precursor protein produced in the liver under control of estrogen, and in adult fish, it is normally present in measurable amounts only in the blood or tissues of mature females. Thus, plasma VTG concentrations in males have been widely used as a marker of exposure to environmental estrogens (Hansen *et al*., 1998). Induction of VTG and increased incidence of intersexuality (presence of ova in testicular tissue) have been observed in male fish collected below sewage outfalls in numerous studies (e.g. Folmar *et al*., 1996; Harries *et al*., 1997; Jobling *et al*., 1998; Matthiessen, 1998; Larsson *et al*., 1999; Petrovic *et al*., 2002; Sole *et al*., 2002; Plesner and Christiansen, 2003). Young male roach (*Rutilus rutilus*) exposed to graded dilutions of sewage effluent experienced VTG induction and feminization of reproductive ducts, but no effects on germ cell development (Rodgers-Gray *et al*., 2001). Rodgers-Gray *et al*. (2000) found that VTG response was dose and time dependent; fish chronically exposed to low concentrations of effluent in river water had a lower threshold for VTG induction than did fish that were exposed for a shorter time period. Fish may show a sustained VTG response to estrogen exposure. High levels of plasma VTG in male fish may lead to reduced testicular growth and be accompanied by kidney and liver damage (Herman and Kincaid, 1988; Folmar *et al*., 2001). Plasma VTG concentrations in male fathead minnows remained elevated 21 days after exposure to estradiol (Panter *et al*., 2000). Also, reduced gonad mass relative to body mass (Harries *et al*., 1997; Jobling *et al*., 1998; Panter *et al*., 1998), increased relative liver mass (Harries *et al*., 1997), testicular damage (Plesner and Christiansen, 2003), reduced gonopodium length (the modified male anal fin, critical for sperm transfer in some species) (Batty and Lim, 1999), and reduced serum testosterone concentrations in males (Folmar *et al*., 1996) have been observed in fish collected downstream of wastewater treatment plants. In addition, gamete production and quality were reduced in intersex male roach collected from sites that received sewage effluent (Jobling *et al*., 2002).

Natural (e.g. 17β-estradiol, estrone) and synthetic (e.g. 17α-ethynylestradiol) steroid hormones are among the most commonly occurring and potent endocrine disrupting compounds in effluents from wastewater treatment plants handling domestic wastewater (Desbrow *et al*., 1998). However, sensitivity to estrogen exposure is species-specific (Purdom *et al*., 1994; Thompson *et al*., 2000), highlighting the need for proper selection of species for monitoring purposes.

Although mature female fish are not as responsive to estrogen exposure as are males, normal patterns of vitellogenesis in adult females may be altered by exposure to high concentrations of ethynylestradiol (e.g. Sole *et al*., 2002). Also, increased VTG production has also been documented in mussels exposed to sewage effluent, either directly (Gagne *et al*., 2001; Quinn *et al*., 2004) or in receiving streams (Gagne *et al*., 2001).

Research studies have documented a variety of endocrine-disrupting effects of steroidal hormones on fish, including reductions in gonadosomatic indices (organ weights as a proportion of body weight) and the number of males with milt and spawning tubercles (Bjerselius *et al*., 2001), reduced gonopodium (used as a phallus for sperm transfer) length (Doyle and Lim, 2002), and reduced fecundity (number of eggs) without a reduction in fertility (Oshima *et al*., 2003). Others have found changes in breeding behavior of male fish exposed to estradiol, including fewer courtship displays, chasing/following behaviors, and copulation attempts, and reduced aggressiveness toward other males (Bayley *et al*., 1999; Bell, 2001; Bjerselius *et al*., 2001; Doyle and Lim, 2002; Martinovic *et al*., 2003; Oshima *et al*., 2003). Male fathead minnows exposed to estradiol were able to acquire nests and spawn successfully in the absence of competing males (Martinovic *et al*., 2003). However, estradiol-treated males were less aggressive in encounters with control males, and subsequently acquired and held fewer nests and produced 5 times fewer offspring than controls. This decreased productivity of individual males could reduce their reproductive fitness (genes less likely to be passed to the next generation) and reduce the growth rates of exposed populations.

The long-term impacts of exposure to complex mixtures of endocrine disrupting compounds on populations of fish, frogs, and other aquatic life are not well understood. Grist *et al*. (2003) modeled the effects of ethynylestradiol exposure on populations of fathead minnows (*Pimephales promelas*) and determined that reductions in population growth rates due to reduced fertility could be anticipated. Questions such as whether males with subtle intersex characters breed successfully in the wild (impacting the reproductive fitness of affected individuals) and whether a population with fewer mature males can be sustainable long-term need to be addressed.

## Metals, metalloid and a non-metal

Metals are electropositive elements with metallic bonds that readily form cations. The heavy metals such as cadmium, lead, and mercury have high atomic weights, have no known physiological role, and can produce toxic effects at low concentrations. Others, such as the essential elements copper and zinc, are necessary for life, but they can be toxic at elevated concentrations. Metalloids are elements with properties intermediate between metals and non-metals. Some metalloids such as arsenic and non-metals such as selenium can be toxic at relatively low doses. Of course, selenium is an essential element needed in minute amounts for optimal health.

Metals are usually complexed with other elements in the environment, and the form, or species, of a metal, as well as its valence state, are important in determining its toxicity to organisms. Trivalent chromium occurs naturally, and small amounts are necessary for optimal health. In contrast, hexavalent chromium is produced and used in industry and is highly toxic.

Use of fertilizers, irrigation, and pesticides may increase the soil's toxic load of elements and salts. Early pesticides were metal salts such as lead arsenate; in some areas soils remain contaminated from early use of these compounds. Other anthropogenic activities such as processing and burning of fossil fuels, mining, smelting, and steel making have increased concentrations of metals in the biosphere.

Mercury is a highly toxic metal that is released naturally into the biosphere from volcanic eruptions, exposed bedrock, as well as through anthropogenic activities including chloralkali plants used to produce chlorine (now being phased out in much of the world), mining, reservoir development, burning fossil fuels, medical procedures and waste, metal processing, smelting, waste and incineration. Mercury is used in thermometers and barometers, electrical switches, fluorescent lights, dental amalgams and in the past was used in the making of felt hats. Mercury is also released during the burning of coal. Another important source has been use of liquid mercury metal to capture gold from stream bed deposits. Because human and wildlife populations are already experiencing the impacts of toxic exposures to methylmercury, the continued atmospheric transport and deposition of mercury are major concerns.

When mercuric salts and metallic mercury are metabolized by organisms in anaerobic sediments, they form the more toxic methylmercury. The rate of conversion of other forms of mercury to methylmercury is much faster in certain warm climates that have favorable geochemistry (e.g. parts of the Amazon and of Florida) than in areas more distant from the equator Veiga *et al*.,. Methylmercury bioaccumulates via the aquatic food chain, readily crosses the placenta and blood–brain barrier, and is highly toxic to the developing nervous system.

Cadmium is a toxic element used in Ni–Cd batteries and in certain pigments, plastics stabilizers, coatings, alloys, and electronics. It also is an impurity found in, or released during the processing of, other metals such as copper, iron, and zinc, manufacturing of steel, cement, fertilizers, and the combustion of fossil fuels. Cadmium is readily taken up by some plants (e.g. leafy vegetables, rice) and is then ingested by humans and other animals in their food. Among the principal target organs of cadmium are bone and kidneys.

Lead has been used in shotgun pellets, bullets, fishing sinkers, paint, batteries, gasoline, solder, water pipes, and other products. One of the largest sources of lead in the atmosphere is leaded gasoline; alkylated lead is added to reduce engine noises or "knocking." To help control harmful emissions, beginning in 1975 passenger cars and light trucks manufactured in the United States included catalytic converters that required lead-free gas. The US Environmental Protection Agency mandated a phase-down of lead concentrations from 2–3 to 1/10th g/gallon during 1977–1986; prohibitions on lead in paint, water pipes, and tin cans were also enacted during this period. Leaded gasoline was still available for use in passenger automobiles in some parts of the United States until 1996, and is still used in aviation, motorboat, farming, and racing vehicle gasoline. In the United States, reductions in lead use were reflected in a 37% decrease in average blood lead concentrations of human beings during 1976–1980 (Pirkle *et al.*, 1994), and 41% reduction between the periods 1988/ 1994 and 1999/2002 (Muntner *et al.*, 2005). However, lead exposures among children of some socioeconomic groups in the United States remain high due in part to contaminated dust, lead paint chips, lead dissolved from plumbing, and other sources (Muntner *et al.*, 2005). Worldwide, internal combustion engines are still a major source of environmental lead, although an increasing number of nations have banned or are beginning to restrict its usage in gasoline.

Mining of metals and minerals often carries large environmental impacts. Not only do these activities leave the land degraded and scarred, but processes to remove the product of interest often result in the release of toxic elements or other chemicals into the environment. For example, sulfides released from newly exposed rock may combine with water and oxygen to form deadly sulfuric acid, which often makes its way into soils, groundwater and streams. This acid also has the added effect of leaching or dissolution of toxic metals such as arsenic, cadmium, lead, iron, and mercury from mine tailings or "waste" rock. The legacy of the mining boom of the mid- to late-1800s in the American west are many miles of streams and rivers still contaminated with metals and sulfuric acid. In addition to devastating environmental impacts from effluents of mining in developing countries that are due to the lack, or poor enforcement, of environmental protection laws, the pursuit of the metals produced may also lead to armed conflict and human rights abuses (MMSD, 2002).

The use of chromated copper arsenic (CCA) in treating wood to prevent insect damage and rot has received much recent attention with regard to risks to children using treated playground equipment, as well as playing in areas where such equipment exists, due to leaching of the metals from wood into soil. The US Environmental Protection Agency has banned sales of CCA-treated wood for most residential uses. Nevertheless, much of the wood remains in use today, and CCA-treated wood produced before 2003 can still be sold. Of importance, animals attracted to the salty taste of the ash left from burning CCA-treated wood the pursuit of the metals produced may readily ingest a lethal dose of arsenic.

The process of removing metals from ore, or smelting, results in the deposition of aluminum, cadmium, fluoride, lead and zinc, and/or other elements that can be highly toxic to plants, soil-dwelling invertebrates, and vertebrates. Such toxicity can reduce the diversity of flora and soil fauna, leaving only more tolerate species, so that ecological function in contaminated landscapes is altered for centuries and likely millennia. An example is provided by two zinc smelters that operated near Palmerton in the mountains of eastern Pennsylvania for much of the 20th century. Approximately 485 ha of vegetation were severely impacted (Jordan, 1975), and damage extended 10 km downwind of the smelters. Drought and fire followed by erosion of as much as 30–60 cm of topsoil Oyler, 1988 exacerbated the damage to the mountainside. Areas that remained forested became dominated by species that re-sprouted from roots or stumps instead of seeds (Jordan, 1975). There was also a loss of diversity in moss (Nash, 1972) and lichen (Nash, 1975) communities. Retardation of decomposition due to greatly reduced populations of soil and litter macro- and microorganisms (Jordan and Lechevalier, 1975; Strojan, 1978a) resulted in a build-up of plant litter on top of mineral soil (Strojan, 1978b). These habitat changes, along with accumulations of toxic levels of cadmium, lead, and zinc (Beyer *et al.*, 1985, 1988), caused reductions in populations of forest birds, small mammals, and salamanders (forest floor salamanders were extirpated), especially insectivorous species. These impacts extended many kilometers downwind and beyond the area of obvious environmental impacts (Beyer and Storm, 1995). The sheer volume of contaminated soil, along with a cinder bank or "dross" pile containing an estimated 33 million tons of slag (waste ore), makes removal of contaminated material impractical. Efforts to re-vegetate the site began in the 1990s with the application of a mixture of fly ash, sewage sludge, and limestone to the barren mountainside, in an effort to control pH, prevent further erosion, and provide a medium for plant growth (Oyler, 1988). However, efforts to restore the steep, rocky, highly contaminated slopes have been largely unsuccessful to date.

Most natural soils contain low concentrations of toxic elements, although there are some areas where soils are naturally high in certain elements, e.g. seleniferous soils of the western United States. Arsenic and selenium can be present in levels of concern in ancient groundwater, and pumping from wells for drinking and irrigation makes them available at the surface. Due to the marine origins of the adjacent mountains, the soils of the San Joaquin Valley in California are high in salts and certain trace elements including selenium. Irrigation is necessary to leach salts from the soil to allow the production of vegetables and other crops away agricultural wastewater is then carried by a network of canals. In the past, the water was directed to a basin that formed 12 constructed wetlands on what became the Kesterson National Wildlife Refuge. These wetlands, located in an arid region with a diminished wetland base, were an attractive habitat to a variety of breeding waterbirds. High concentrations of selenium in wastewater became even more elevated over time in these wetlands through evaporation in the arid climate of central California, and by 1983 a high incidence of embryo mortality and deformities was noted in wetland birds such as grebes, coots, stilts, and ducks (Ohlendorf and Hothem, 1995). Because of the severely of these impacts, the ponds were closed and filled. Subsequently, 12 other water reclamation projects in the arid western United States were found to be contaminated with selenium in irrigation drainage water (Seiler *et al.*, 2003). Although this issue has received attention from planners and researchers, and some remediation has been undertaken, the underlying issues are socioeconomically, politically, and technically complex; thus, progress in finding solutions has been slow.

## Radiation/radionuclides

The nuclear age began with the discovery of X-rays in 1895. Fission was first demonstrated in 1938 by German scientists, which led to competition between Nazi Germany and the United States to develop an atomic weapon. The United States developed such a bomb in 1945, and then used two against Japan to end WWII. Nuclear weapons testing and the bombing of Hiroshima and Nagasaki, Japan, during the mid-20th century resulted in the global distribution of radioactive material.

The first reactor-produced radioisotopes for medical and industrial use were provided by the Oak Ridge National Laboratory in 1946. Since that time, nuclear medicine has been a boon for the diagnosis and treatment of many diseases.

By 1951, electricity was being produced via nuclear fission, and by July 1957 the first civilian reactor for power was on line. However, in October 1957, the first accidental release of radiation from a reactor occurred. Theoretically, nuclear energy can provide "cleaner" and cheaper electricity than burning of fossil fuels. However, in addition to small releases of radioactivity during normal operations and the transportation and storage of nuclear wastes there

have been accidental discharges of large amounts of radiation, such as the incidents at Chernobyl, Ukraine, and Three Mile Island, Pennsylvania, USA. In 1986, the explosion and fire at a poorly designed reactor at Chernobyl led to the hospitalization of 203 people and the death of 47 due to radiation poisoning. Also, as of 2004, 9 children from the area had died of thyroid cancer. Fallout containing short- and long-lived radioisotopes covered a wide area of northeastern Europe, and radiation from this incident ultimately was transported throughout the northern hemisphere. Residents of a 30 km radius were evacuated and few have returned. One source estimated that there would be an eventual 4000 additional human deaths, driven by a 2% increase in cancer rates. In spite of somatic and germ-cell mutations in birds, small mammals, and fish resulting in increased prevalence of aberrant phenotypic traits, native plants and wildlife have flourished, whereas invasive exotic species have declined, in the 30-mile exclusion zone around Chernobyl. This has been touted as "proof" that effects of radiation from this nuclear disaster were not as long-lasting as feared. However, these animals could represent the immigration of dispersing animals from surrounding areas into unoccupied habitat. Few detailed, longitudinal studies of wildlife populations and their age structures have been conducted.

Perhaps best studied in the Chernobyl area have been barn swallows, which are migratory insectivorous birds with a high degree of philopatry. Fourteen years after the incident, swallows nesting in the area around Chernobyl had high prevalence of abnormal sperm and reductions in antioxidant levels, compared to swallows from a reference area (Moller *et al*., 2004). The exposed birds also had a sustained high rate of partial albinism (Moller and Mousseau, 2001), and germline mutation rates that were 2- to 10-fold higher than distant reference sites (Ellegren *et al*., 1997). Immune suppression and reduction of carotenoid-based sexual coloration were also noted in this population (Camplani *et al*., 1999). Mutations produced phenotypic changes in secondary sexual traits (e.g. plumage coloration, feather lengths) that may influence reproductive success (Camplani *et al*., 1999; Moller and Mousseau, 2003).

Small mammal populations in some areas near Chernobyl were reduced by as much as 90% following the reactor incident. Increases in populations in the following spring were attributed to immigration from less-contaminated adjacent areas (Sokolov *et al*., 1990) and the cessation of tillage and crop harvest (Sokolov *et al*., 1993). Voles collected from the Chernobyl area had the highest radiocesium burdens and dose rates ever recorded for mammals (Baker *et al*., 2001). Researchers have reported high genetic diversity in voles from the highly contaminated zone (Matson *et al*., 2000; Baker *et al*., 2001; Wickliffe *et al*., 2006), which could be indicative of high mutation rates and/or immigration from less-contaminated areas. Cytogenetic damage was documented in voles in Belarus (Goncharova and Ryabokon, 1995) and Sweden (Cristaldi *et al*., 1991), both of which were in the direction of prevailing winds in the first few days following the disaster.

## ENVIRONMENTAL FATE OF CHEMICALS IN THE ENVIRONMENT

Our environmental contamination problems are complex but controllable, by limiting environmental contamination so that concentrations are not high enough to harm prokaryotic and eukaryotic life forms (e.g. beyond the intended target site, such as an agricultural plot selected for treatment because an exotic insect pest has been recognized). The assimilative capacity of most natural environments is tremendous; however, it is essential to ensure that pollutants are not produced and used at rates that undermine the capacity of the purposefully exposed site to detoxify itself.

Environmental contaminants are degraded, or transformed into simpler, often (though not always) less toxic forms, in several ways. Photodegradation involves transformation of chemicals often involving oxidation to reduce toxicity. Many manmade chemicals are rapidly photodegraded by sunlight. Some chemicals may become more toxic to organisms through the process of photoactivation. For example, dechlorination of hexachlordibenzo-*p*-dioxin may produce lower chlorinated but more toxic dioxin analogs.

Biotransformation involves the modification of chemicals by the physiological processes (e.g. enzymes in the liver that catalyze hydrolysis, oxidation, reduction, or conjugation) in living organisms – usually in an effort to detoxify and eliminate them. But biotransformation also occurs in soil microbes such as bacteria and fungi under aerobic or anerobic conditions. Plants and microbes within digestive tracts are also important in the biotransformation of environmental contaminants. Of course, metabolic changes can also bioactivate chemicals to produce (by definition) metabolites that are more toxic than their parent compound. For example, the organochlorine pesticide DDT is not itself highly toxic to birds. However, its metabolite *p,p'*-DDE can cause thinning of eggshells due to disruption of calcium metabolism. Also, during microbial metabolism, inorganic mercury is methylated, producing a potent neurotoxic substance responsible for cognitive, fine motor, and visual-spatial disabilities, especially in developing organisms. Efforts by organisms to detoxify exogenous compounds can sometimes produce temporary bioactivation manifested in reactive species such as singlet oxygen or hydroxy radicals. These free-radicals or oxidants can cause oxidative stress, damaging cells and may promote tumor formation. More research on fate and toxicity of metabolites and environmental degradation products is needed.

## AVAILABILITY OF CHEMICALS IN THE ENVIRONMENT

Because a contaminant is present in the environment does not mean that an organism will have contact with it. For example, surface-dwelling animals may not have direct access to a contaminant buried under of many centimeters of topsoil. However, they may gain exposure by consuming earthworms or plants that bring contaminants to the surface. Thus, contaminants and receptors (i.e. organisms of interest) must overlap in both time and space for there to be a potential for exposure. Should pollutants and receptors co-occur, there are a number of potential pathways of exposure: dermal, oral (dietary and grooming), inhalation, via gills and rarely, injection.

The bioavailability of pollutants in the environment refers to the proportion of a substance that is absorbed across the gut, skin, or other portals to enter the bloodstream and other tissues where it can cause a physiological reaction in an organism. Bioavailability can be determined by the amount of organic matter, pH, and cation-exchange capacity of soil or sediment, the presence of antagonistic elements of chemicals, and the nutritional status of an organism.

## THE FUTURE OF ECOTOXICOLOGY

The science of ecotoxicology should exist within a balanced ecological and biomedical context. In the view of the authors, ecotoxicology needs to become more of a preventive science and less of a remediative enterprise. Although diagnostic laboratories must become far more involved in forensic ecotoxicology, simply waiting on animals to come to diagnostic laboratories is insufficient to protect wildlife and ecosystems from chemically induced damage. Thus, ecotoxicology must also be pro-active, involving trips to the field for monitoring of exposures and impacts, and trips to the laboratory for exploratory and confirmatory research.

In the authors' view, there needs to be better testing of products before marketing to insure not only the health of humans, but also of pets, livestock, wildlife, plants, and the environment as a whole. For most toxicants, detailed and comprehensive ecotoxicological information exists for very few plant and animal species. Risk assessment therefore often relies upon the use of a few surrogate species, with extrapolation to a huge array of organisms that will likely be exposed when a product is used or an effluent is released.

Of concern is that much information from industry on testing of products is buried in reports to the US Environmental Protection Agency and difficult to obtain. We believe that, upon granting of patents, industry scientists should be required to publish results of toxicity testing in the scientific literature and via the Internet so that the information is fully available to scientists and the interested public. Stakeholders should share in decision-making with regard to risk management and longer-term product registration.

Finally, we are of the view that considerable effort in regulatory ecotoxicology should be devoted to removal of problematic compounds and formulations, with their replacement, when warranted, with products unlikely to present undue ecological harm.

Ecotoxicology initially was focused largely on determining chemical residues in the environment and not enough on mechanisms and effects, though this has begun to change. Ecotoxicological research typically extends beyond the health effects on individuals, to examine how these changes manifest as population-, community-, and ecosystem-level impacts. However, if environmental mismanagement places greater numbers of species at risk for extinction consideration of toxic effects on individuals will be increasingly necessary.

Far greater attention needs to be devoted to complex mixtures of chemicals, indirect effects of contaminants, fate and toxicity of chemical degradation products, development of more endpoints of exposure and their role in disease, and the validation of risk assessments.

There is a continuing need for more integrative ecotoxicology research, i.e. comprehensive, large-scale field studies examining interactions among biotic and abiotic factors, complemented by microcosm and mesocosm studies. More research incorporating reproductive toxicology including multi-generational studies is needed. More studies should examine how contaminant exposure affects animal behavior, since subtle changes in behavior can have immense negative impacts on an animal's survival and reproductive fitness. There is a need for more studies designed to identify cause and effect, including assessments of clinical signs, histological lesions, and residues over time at relevant exposures, to enhance diagnostic and forensic capabilities. Animals subjected to toxicity testing to determine $LC_{50}$ or $LD_{50}$ or minimum toxic dose or maximum tolerated dose should consistently be evaluated with clinical pathology assays, gross and histological pathology studies, and analyses to determine residues. All of these endpoints should be evaluated at doses with no impact, threshold toxic reactions, marked toxic reactions, and overt lethal toxicity.

"Veterinary ecotoxicologists" can participate in meeting critical data gaps by specializing in a variety of disciplines with regard to environmental contaminants, including: epidemiology, pathology, immunology, environmental risk assessment, environmental chemistry/fate, environmental law, ecological rehabilitation, and others. Veterinary ecotoxicologists can also specialize in a particular toxicant group (e.g. metals, hazardous wastes), animal species group (marine mammals, birds, invertebrates), given habitat type

or biome (aquatic, terrestrial, desert, forest, estuaries, coral reefs), or given region (midwestern United States, Illinois, north Africa, tropics, polar).

The future of a healthy environment for humans, domestic animals, and wild biota depends to a large extent on the degree to which we learn to use and control naturally occurring and synthetic chemicals. Although there have been successes, and some environments are cleaner now than in the recent past, the continually expanding number of chemicals released into the environment increasing free nutrients that prompt biotoxin production and the adverse impacts noted in the environment demonstrate the striking need for vigilance and accountability through research, education, environmental law and enforcement, and development of prudent science-driven environmental policy.

## REFERENCES

Agency for Toxic Substances and Disease Registry (ATSDR) (2002) *Toxicological Profile for DDT, DDE, and DDD*. Division Toxicology/Toxicology Information Branch, Atlanta, GA.

Alexopoulos E, McCrohan CR, Powell JJ, Jugdaohsingh R, White KN (2003) Bioavailability and toxicity of freshly neutralized aluminium to the freshwater crayfish *Pacifastacus leniusculus. Arch Environ Contam Toxicol* **45**, 509–13.

Alin SR, O'Reilly CM, Cohen AS, Dettman DL, Palacios-Fest MR, McKee BA (2002) Effects of land-use change on aquatic biodiversity: a view from the paleorecord at Lake Tanganyika. East Africa. *Geology* **30**: 1143–6.

Anderson WL, Havera SP, Zercher BW (2000) Ingestion of lead and nontoxic shotgun pellets by ducks in the Mississippi Flyway. *J Wildl Manage* **64**: 848–57.

Anderson TD, Lydy MJ (2002) Increased toxicity to invertebrates associated with a mixture of atrazine and organophosphate insecticides. *Environ Toxicol Chem* **21**: 1507–14.

Anderson TD, Zhu KY (2004) Synergistic and antagonistic effects of atrazine on the toxicity of organophosphorodithioate and organophosphorothioate insecticides to *Chironomus tentans* (Diptera: Chironomidae). *Pestic Biochem Physiol* **80**: 54–64.

Asman WAH, Jorgensen A, Bossi R, Vejrup KV, Mogensen BB, Glasius M (2005) Wet deposition of pesticides and nitrophenols at two sites in Denmark: measurements and contributions from regional sources. *Chemosphere* **59**: 1023–31.

Augspurger T, Smith MR, Meteyer CU, Converse KA (1996) Mortality of passerines adjacent to a North Carolina cornfield treated with granular carbofuran. *J Wildl Dis* **32**: 113–16.

Baird D, Christian RR, Peterson CH,. Johnson GA (2004) Consequences of hypoxia on esturarine ecosystem function: energy diversion from consumers to microbes. *Ecol Appl* **14**: 805–22.

Baker RJ, Bickham AM, Bondarkov M, Gaschak S, Matson CW, Rodgers BE, Wickliffe JK, Chesser RK (2001) Consequences of polluted environments on population structure: the bank vole (*Clethrionomys glareolus*) at Chornobyl. *Ecotoxicology* **10**: 211–16.

Balcomb R, Stevens R, Boiwen CA (1984) Toxicity of 16 granular insecticides to wild–caught songbirds. *Bull Environ Contam Toxicol* **33**: 302–7.

Battaglin WA, Goolsby DA (1999) Are shifts in herbicide use reflected in concentration change sin midwestern rivers? *Environ Sci Tech* **33**: 2917–25.

Battaglin WA, Kolpin DW, Scribner EA, Kuivila KM, Sandstrom MW (2005) Glyphosate, other herbicides, and transformation products in midwestern streams, 2002. *J Am Water Resour Assoc* **41**: 323–32.

Batty J, Lim R (1999) Morphological and reproductive characteristics of male mosquitofish (*Gambusia affinis holbrooki*) inhabiting sewage–contaminated waters in New South Wales, Australia. *Arch Environ Contam Toxicol* **36**: 301–7.

Bayley M, Nielsen JR, Baatrup E (1999) Guppy sexual behavior as an effect biomarker of estrogen mimics. *Ecotoxicol Environ Safety* **43**: 68–73.

Beasley VR (1993) Ecotoxicology and ecosystem health: roles for veterinarians; goals for the Envirovet program. *J Am Vet Med Assoc* **203**: 617–28.

Beasley VR, Faeh SA, Wikoff B, Staehle C, Eisold J, Nichols D, Cole R, Schotthoefer AM, Greenwell M,Brown LE (2005) Risk factors and declines in northern cricket frogs (Acris crepitans). In *Amphibian Declines: The Conservation Status of United States Species*, Lannoo M (ed.). University of California Press, Berkeley, CA, pp. 75–86.

Beaver PC (1937) Experimental studies with Echinostoma revolutum (Foelich), a fluke from birds and mammals. *IL Biol Mono* **34**:1–96.

Bell A (2001) Effects of an endocrine disruptor on courtship and aggressive behaviour of male three–spined stickleback, *Gasterosteus aculeatus*. *Anim Behav* **62**: 775–80.

Bellrose FC (1959) Lead poisoning as + a mortality factor in waterfowl populations. *IL Nat Hist Survey Bull* **27**: 235–88.

Berg L, Northcote TG (1985) Changes in territorial, gill–flaring, and feeding behavior in juvenile coho salmon (Onchorynchus kisutch) following short–term pulses of suspended sediment. *Can J Fish Aquat Sci* **42**:1410–17.

Berny PJ, Buronfosse T, Buronfosse F, Lamarque F, Lorgue G (1997) Field evidence of secondary poisoning of foxes (*Vulpes vulpes*) and buzzards (*Buteo buteo*) by bromadiolone, a 4–year survey. *Chemosphere* **35**: 1817–29.

Beyer WN, Pattee OH, Sileo L, Hoffman DJ, Mulhern BM (1985) Metal contamination in wildlife living near two zinc smelters. *Environ Pollut Ser A Ecol Biol* **38**: 63–86.

Beyer WN, Spann JW, Sileo L, Franson JC (1988) Lead poisoning in six captive avian species. *Arch Environ Contam Toxicol* **17**: 121.

Beyer WN, Storm G (1995) Ecotoxicological damage from zinc smelting at Palmerton, Pennsylvania. In *Handbook of Ecotoxicology* Hoffman DJ, Rattner BA Burton GA Jr, Cairns J Jr. (eds.), Lewis Publishers, *Boca Raton*, pp. 596–608.

Birrenkott AH, Wilde SB, Hains JJ, Fischer JR, Muphy TM, Hope CP, Parnell PG, Bowerman WW (2004) Establishing a food-chain link between aquatic plant material and avian vacuolar myelinopathy in mallards (*Anas platyrhynchos*) *J Wildl Dis* **40**: 485–92.

Bjerselius R, Lundstedt-Enkel K, Olsén H, Mayer I, Dimberg K (2001) Male goldfish reproductive behaviour and physiology are severely affected by exogenous exposure to 17-estradiol. *Aquat Toxicol* **53**: 139–52.

Blettler MCM, Marchese MR (2005) Effects of bridge construction on the benthic invertebrates structure in the Parana River delta. *Interciencia* **30**: 60–6

Blus LO (1996) Effects of pesticides on owls in North America. *J Rap Res* **30**: 198–206.

Bowerman WW, Giesy JP, Best DA, Kramer VJ (1995) A review of factors affecting productivity of bald eagles in the Great Lakes region: implications for recovery. *Environ Health Perspec* **103**(Suppl. 4): 51–9.

Bowerman WW, Best DA, Giesy JP, Shieldcastle MC, Meyer MW, Postupalsky S, Sikarskie JG (2003) Associations between regional differences in polychlorinated biphenyls and dichlorodiphenyldichloroethylene in blood of nestling bald eagles and reproductive productivity. *Environ Toxicol Chem* **22**: 371–6.

Brakes CR, Smith RH (2005) Exposure of non-target small mammals to rodenticides: short-term effects, recovery, and implications for secondary poisoning. *J Appl Ecol* **42**: 118–28.

Brewer L, Fagerstone K (1998) *Radiotelemetry Applications for Wildlife Toxicology Field Studies*. SETAC Press, Pensacola, FL.

Burgers J, Opdam P, Mueskens G, de Ruiter E (1986) Residue levels of DDE in eggs of Dutch sparrowhawks *Accipiter nisus* following the ban on DDT. *Environ Pollut B Chem Phys* **12**: 29–40.

Burkhead NM, Jelks HL (2001) Effects of suspended sediment on the reproductive success of the tricolor shiner, a crevice-spawning minnow. *Trans Am Fish Soc* **130**: 959–68.

Cairns Jr J (2003) Restoration ecology and ecotoxicology. In *Handbook of Ecotoxicology*, 2nd edn, Hoffman DG, Rattner BA, Burton Jr GA, Cairns Jr J (eds). Lewis Publishers, CRC Press, Boca Raton, FL pp. 1015–29.

Camplani A, Saino N, Moller AP (1999) Carotenoids, sexual signals and immune function in barn swallows from Chernobyl. *Proc Biol Sci* **266**: 1111–6.

Carpenter SR, Caraco NF, Correll DL, Howarth RW, Sharply AN, Smith VH (1998) Nonpoint pollution of surface waters with phosphorus and nitrogen. *Ecol Appl* **8**: 559–68.

Carr JA, Gentles A, Smith EE, Coleman WL, Urquidi LJ, Thuett K, Kendall RJ, Giesy JP, Gross TS, Solomon KR, Van der Kraak G (2003) Response of larval *Xenopus laevis* to atrazine: Assessment of growth, metamorphosis, and gonadal and laryngeal morphology. *Environ Toxicol Chem* **22**: 396–405.

Carson R (1962) *Silent Spring*. Houghton Mifflin Co., Boston, MA.

Chase JM (1998) *Size-Structured Interactions and Multiple Domains of Attraction in Pond Food Webs.* Ph.D. Dissertation, University of Chicago, Chicago,IL.

Coady KK,. Murphy MB, Jones PD, Carr JA, Solomon KR, Smith EE, Van der Kraak G, Kendall RJ, Giesy JP (2004) Effects of atrazine on metamorphosis, growth, and gonadal development in the green frog (*Rana clamitans*) *J Toxicol Environ Health A Curr Iss* **67**: 941–57.

Cooke AS, Bell AA, Prestt I (1976) Egg shell characteristics and incidence of shell breakage for gray herons *Ardea cinerea* exposed to environmental pollutants. *Environ Pollut* **11**: 59–84.

Cristaldi M, Ierdi LA, Mascanzoni D, Mattei V (1991) Environmental impact of `the Chernobyl accident: Mutagenesis in bank voles from Sweden. *Int J Rad Biol* **59**: 31–40.

Desbrow C, Routledge EJ, Brighty GC, Sumpter JP, Waldock M (1998) Identification of estrogenic chemicals in STW effluent. I. Chemical fractionation and *in vitro* biological screening. *Environ Sci Technol* **32**: 1549–58.

Diana SG, Resetarits Jr WJ, Schaeffer DJ, Beckmen KB, Beasley VR (2000) Effects of atrazine on amphibian growth and survival in artificial aquatic communities. *Environ Toxicol Chem* **19**: 2961–7.

Diaz-Pulido G, McCook LJ (2005) Effects of nutrient enhancement on the fecundity of a coral reef macroalga. *J Exp Marine Bio Ecol* **317**:13–24.

Dieter MP,Finley MP (1979) Delta-aminoleuvulinic acid dehydratase enzyme activity in blood, brain, and liver of lead-dosed ducks. *Environ Res* **19**: 127–35.

Dietrich DR, Schmid P, Zweifel U, Schlatter Ch, Jenni-Eiermann S, Bachmann H, Bühler U, Zbinden N (1995) Mortality of birds of prey following field application of granular carbofuran: a case study. *Arch Environ Contam Toxicol* **29**: 140–5.

Doyle CJ, Lim RP (2002) The effect of 17-estradiol on the gonopodial development and sexual activity of *Gambusia holbrooki*. *Environ Toxicol Chem* **21**: 2719–24.

Elliot JE, Langelier KM, Mineau P, Wilson LK (1996) Poisoning of bald eagles and red-tailed hawks by carbofuran and fensulfothion in the Fraser Delta of British Columbia, Canada. *J Wildl Dis* **32**: 486–91.

Ellegren H, Lindgren G, Primer CR, Moller AP (1997) Fitness loss and germline mutations in barn swallows breeding in Chernobyll. *Nature* **389**: 593–6.

Faber RA, Risebrough RW, Pratt HM (1972) Organochlorines and mercury in common egrets and great blue herons. *Environ Pollut* **3**: 111–22.

Finley MT, Dieter MP, Locke LN (1976a) Lead in tissues of mallard ducks dosed with two types of lead shot. *Bull Environ Contam Toxicol* **16**: 261–9.

Finley MT, Dieter MP, Locke LN (1976b) Delta-aminolevulinic acid dehydratase inhibition in ducks dosed with lead shot. *Environ Res* **12**: 243–9.

Finley MT, Dieter MP, Locke LN (1976c) Sublethal effects of chronic lead ingestion in mallard ducks. *J Toxicol Environ Health* **1**: 929–37.

Flewelling L, Naar JP, Abbott JP, Baden DG, Barros NB, Bosssart GD, Marie JD, Hammond DG, Haubold EM, Heil CA, Henry MS, Jacoks HM, Leighfield TA, Pierce RH, Pitchford TD, Rommel SA, Scott PS, Steidinger KA, Truby EW, Van Dolah FM, Landsberg JH (2005) Brevetoxicosis: red tides and marine mammal mortalities. *Nature* **435**: 755–6.

Folmar LC, Denslow ND, Rao V, Chow M, Crain DA, Enblom J, Marcino J, Guillette Jr LJ (1996) Vitellogenin induction and reduced serum testosterone concentrations in feral male carp (*Cyprinus carpio*) captured near a major metropolitan sewage treatment plant. *Environ Health Perspec* **104**: 1096–1101.

Folmar LC, Gardner GR, Schreibman MP, Magliulo-Cepriano L, Mills LJ, Zaroogian G, Gutjahr-Gobell R, Haebler R, Horowitz DB, Denslow ND (2001) Vitellogenin-induced pathology in male summer flounder (*Paralichthys dentatus*). *Aquat Toxicol* **51**: 431–41.

Fournier-Chambrillion C, Berny PJ, Coiffier O, Barbedienne P, Dasse B, Delas G, Galineau H, Mazet A, Pouzenc P, Rosoux R, Fournier P (2004) Evidence of secondary poisoning of free-ranging mustelids by anticoaguleant rodenticides in France: implications for conservation of European mink (*Mustela lutreola*) *J Wildl Dis* **40**: 688–95.

Freeman JL, Rayburn AL (2004) *In vivo* genotoxicity of atrazine to anuran larvae. *Mutat Res Genet Toxicol Environ Mutag* **560**: 69–78.

Fried P, Pane PL, Reddy A (1997) Experimental infection of *Rana pipiens* tadpoles with Echinostoma trivolvis cercariae. *Parasit Res* **83**: 666–9.

Friend M (1985) Interpretation of criteria commonly used to determine lead poisoning problem areas. *Fish Wildl Leaflet* **24**: USFWS, Washington, DC.

Gagne F, Blaise C, Salazar M, Salazar S,Hansen PD (2001) Evaluation of estrogenic effects of municipal effluents to the freshwater mussel Elliptio complanata. *Comp Biochem Phys* **128**: 213–25.

Gettys LA, Sutton DL (2004) Comparison of torpedograss and pickerelweed susceptibility to glyphosate. *J Aquat Plant Manage* **42**: 1–4.

Gilbertson M (1974) Pollutants in breeding herring gulls in the lower Great Lakes. *Can Field Nat* **88**: 273–80.

Gilbertson M, Hale R (1974a) Characteristics of the breeding failure of a colony of herring gulls on Lake Ontario. *Can Field Nat* **88**:, 356–8.

Gilbertson M, Hale R (1974b) Early embryonic mortality in a herring gull colony in Lake Ontario. *Can Field Nat* **88**: 354–6.

Gilbertson M, Kubiak T, Ludwig J, Fox G (1991) Great Lakes embryo mortality, edema, and deformities syndrome (GLEMEDS) in colonial fish-eating birds: Similarity to chick-edema disease. *J Toxicol Environ Health* **33**: 455–520.

Gilliom RJ, Barbash JE, Crawford CG, Hamilton PA, Martin JD, Nakagaki N, Nowell LH, Scott JC, Stackelberg PE, Thelin GP, Wolcock DM (2006) *The Quality of our Nation's Waters – Pesticides in the Nation's Streams and Ground Water, 1992–2001*. US Geological Survey Circular 1291.

Glynn AW, Norrgren L, Malmborg O (1992) The influence of calcium and humic substances on aluminum toxicity and accumulation in

the minnow, *Phoxinus phoxinus*, at low pH. *Comp Biochem Physiol* **102C**: 427–32.

Goldstein MI, Lacher TE Jr, Woodbridge B, Bechard MJ, Canavelli SB, Zaccagnini ME, Cobb GP, Scollon EJ, Tribolet R, Hooper MJ (1999) Monocrotophos-induced mass mortality of Swainson's hawks in Argentina, 1995-96 *Ecotoxicology* **8**: 201–14.

Goldstein DA, Farmer DL, Levine SL, Garnett RP (2005) Mechanism of toxicity of commercial glyphosate formulations: How important is the surfactant? *J Toxicol Clin Toxicol* **43**: 423–4.

Goncharova RI, Ryabokon NI (1995) Dynamics of cytogenetic injuries in natural populations of bank vole in the Republic of Belarus. *Rad Protect Dosim* **62**: 37–40.

Goolsby DA, Thurman EM, Pomes ML, Meyer MT, Battaglin WA (1997) Herbicides and their metabolites in rainfall: origin, transport, and deposition patterns across the Midwestern and northeastern United States, 1990–1991. *Environ Sci Tech* **31:** 1325–33.

Grasman KA, Scanlon PF, Fox GA (1998) Reproductive and physiologic effects of environment al contaminants in fish-eating birds of the Great Lakes: a review of historical trends. *Environ Monit Assess* **53**: 117–45.

Green EP, Bruckner AW (2000) The significance of coral disease epizootiology for coral reef conservation. *Biol Conserv* **96**: 347–61.

Green RM, Abdelghani AA (2004) Toxicity of a mixture of 2,4-dichlorophenoxyacetic acid and monosodium methanearsonate to the red swamp crawfish, *Procambus clarkii*. *Int J Env Res Publ Health* **1**: 35–8.

Grist EPM, Wells NC, Whitehouse P, Brighty G, Crane M (2003) Estimating the effects of 17-ethinylestradiol on populations of the fathead minnow *Pimephales promelas*: are conventional toxicological endpoints adequate? *Environ Sci Techn* **37**: 1609–16.

Hansen P-D, Dizer H, Hock B, Marx A, Sherry J, McMaster M, Blaise Ch (1998) Vitellogenin – a biomarker for endocrine disruptors. *Trends Analyt. Chem.* **17**: 448–51.

Harries JE, Sheahan DA, Jobling S, Matthiessen P, Neall P, Sumpter JP, Tylor T, Zaman N (1997) Estrogenic activity in five United Kingdom rivers detected by measurement of vitellogenesis in caged male trout. *Environ Toxicol Chem* **16**: 534–42.

Hartman KJ, Kaller MD, Howell JW, Sweka JA (2005) How much do valley fills influence headwater streams? *Hydrobiologica* **532**: 91–102.

Hayes T, Haston K, Tsui M, Hoang A, Haeffele C, Vonk A (2002a) Feminization of male frogs in the wild. *Nature* **419**: 895–6.

Hayes TB, Collins A, Lee M, Mendoza M, Noriega N, Stuart AA, and. Vonk AA (2002b) Hermaphroditic, demanusculinized frogs after exposure to the herbicide atrazine at low ecologically relevant doses. *Proc Nat Acad Sci* **99**: 5476–80.

Hayes T, Haston K, Tsui M, Hoang A, Haeffele C, Vonk A (2003) Atrazine-induced hermaphroditism at 0.1 ppb in American leopard frogs (*Rana pipiens*): laboratory and field evidence. *Environ Health Perspect* **111**: 568–75.

Hecker M, Kim WJ, Park JW, Murphy MB, Villeneuve D, Coady KK, Jones PD, Solomon KR, Van der Kraak G, Carr JA, Smith EE, Preez LD, Kendall RJ, Giesy JP (2005) Plasma concentrations of estradiol and testosterone, gonadal aromatase activity and ultrastructure of the testis in *Xenopus laevis* exposed to estradiol or atrazine. *Aquat Toxicol* **72**: 383–96.

Hecnar SJ,. (1995) Acute and chronic toxicity of ammonium nitrate fertilizer to amphibians from southern Ontario. *Environ Toxicol Chem* **14**: 2131–7.

Hegdal PL, Blaskiewicz RW (1984) Evaluation of the potential hazard to barn owls of Talon (brodifacoum bait) used to control rats and house mice. *Environ Toxicol Chem* **3**: 167–79.

Helgen J, McKinnell JR, Gernes MC (1998) Investigations of malformed northern leopard frogs in Minnesota. In *Status and Conservation of Midwestern Amphibians*, Lannoo MJ (ed.). University of Iowas Press, Iowa City, pp. 288–97.

Henny CJ, Blus LJ, Hulse CS (1985) Trends and effects of organochlorine residues on Oregon and Nevada wading birds, 1979–83. *Colon Waterbird* **8**: 117–28.

Herbert DWM, Merkens JC (1961) The effect of suspended mineral solids on the survival of trout. *Int J Air Water Pollut* **5**: 46–55.

Herman RL,Kincaid HL (1988) Pathological effects of orally administered estradiol to rainbow trout. *Aquaculture* **72**: 165–72.

Hoppe DM (2000) History of Minnesota frog abnormalities: do recent findings represent a new phenomenon? In *Investigating Amphibian Declines: Proceedings of the 1998 Midwest Declining Amphibians Conference*, vol. 107 , Kaiser H, Casper GS, Bernstein N (eds). Iowa Academy of Science, Cedar Falls, pp. 86–9.

Hodgson E, Mailman RB, Chambers J, eds. (1998) *Dictionary of toxicology*. MacMillan Reference LTD: London.

Hodgson G (1999) A global assessment of human effects on coral reefs. *Marine Pollut Bull* **38**: 345–55.

Hoffman DG, Rattner BA, Burton Jr GA, Cairns Jr J (2003) *Handbook of Ecotoxicology*,2nd edn. Lewis Publishers, CRC Press, Boca Raton, FL.

Houlahan JE, Findlay CS (2003) The effects of adjacent land use on amphibian species richness and community composition. *Can J Fish Aquat Sci* **60**:1078–94.

Hunt KA, Hooper MJ, Littrell EE (1995) Carbofuran poisoning in herons: Diagnosis using cholinesterase reactivation techniques *J Wild Dis* **31**: 186–92.

Hyer KE, Hornberger GM, Herman JS (2001) Processes controlling the episodic streamwater transport of atrazine and other agrichemicals in a agrichemical watershed. *J Hydrol* **254**:47–66.

Jackson JBC, Kirby MX,. Berger WH, Bjorndal KA, Botsford WL, Bourque BJ, Bradbury RH, Cooke R, Erlandson J, Estes JA, Hughes TP, Kidwell S, Lange CB, Lenihan HS, Pandolfi JM, Peterson CH, Steneck RS, Tegner MJ, Warner RR (2001) Historical overfishing and the recent collapse of coastal ecosystems. *Science* **293**: 629–38.

Jefferies DJ (1967) The delay in ovulation produced by p, pp'DDT and its possible significance in the field. *Ibis* **109**: 266–72.

Jin-Clark Y, Lydy MJ, Zhu KY (2001) Effects of atrazine and cyanazine on chlorpyrifos toxicity in *Chironomus tentans* (Diptera: Chironomidae). *Environ Toxicol Chem* **21**: 598–603.

Jobling S, Nolan M, Tyler CR, Brighty G, Sumpter JP (1998) Widespread sexual disruption in wild fish. *Environ Sci Tech* **32**: 2498–506.

Jobling S, Coey S, Whitmore JG, Kime DE, Van Look KJW, McAllister BG, Beresford N, Henshaw AC, Brighty G, Tyler CR, Sumpter JP (2002) Wild intersex roach (*Rutilus rutilus*) have reduced fertility. *Bio Repro* **67**: 515–24.

Johnson PTJ, Lunde KB, Ritchie EG,Launer AE (1999) The effect of trematode infection on amphibian limb development and survivorship. *Science* **284**: 802–4.

Johnson PTJ, Lunde KB, Haight RW, Bowerman J,Blaustein AR (2001) *Ribeiroia ondatrae* (Trematoda: Digena) infection induces severe limb malformations in western toads (*Bufo boreas*). *Can J Zool* **79**: 370–9.

Johnson PTJ, Lunde KB, Thurman EM, Ritchie EG, Wray SN, Sutherland DR, Kapper JM, Frest TJ, Bowerman J, Blaustein AR (2002) Parasite (*Ribeiroia ondatrae*) infection linked to amphibian malformations in the western United States. *Ecol Monogr* **72**: 151–68.

Jooste AM, DuPreez LH, Carr JA, Giesy JP, Gross TS, Kendall RJ, Smith EE, Van der Kraak GL, Solomon KR (2005) Gonadal development of larval male *Xenopus laevis* exposed to atrazine in outdoor microcosms. *Environ Sci Tech* **39**: 5255–61.

Jordan MJ (1975) Effects of zinc smelter emissions and fire on a chestnut-oak woodland. *Ecology* **56**: 78–91.

Jordan MJ, Lechevalier MP (1975) Effects of zinc-smelter emissions on forest soil microflora. *Can J Microbiol* **21**: 1855–965.

Kendall R, Dickerson R, Giesy J, Suk W (1998) *Principles and Processes for Evaluating Endocrine Disruption in Wildlife*. SETAC Press, Pensacola, FL.

Kendall RJ, Lacher Jr TE (1994) *Wildlife Toxicology and Population Modeling: Integrated Studies of Agroecosystems*. Lewis Publishers, SETAC Press, Boca Raton, FL.

Kiely T, Donaldson D, Grube A (2004) *Pesticides Industry Sales and Usage: 2000 and 2001 Market Estimates*. US Environmental Protection Agency, Washington, DC.

Kiesecker JM (2002) Synergism between trematode infection and pesticide exposure: a link to amphibian limb deformities in nature? *Proceed Nat Acad Sci* **99**: 9900–4.

Kishi D, Murakami M, Nakano S, Tangiguchi Y (2004) Effects of forestry on the thermal habitat of Dolly Varden (*Salvelinus malma*). *Ecol Res* **19**:283–90.

Klaus JS, Frias-Lopez J, Bonheyo GT, Heikoop JM, Fouke BW (2004) Bacterial communities inhabiting the healthy tissues of two Caribbean reef corals: interspecific and spatial variation. *Coral Reefs* **24**: 129–37.

Klink CA, Machado RB (2005) Conservation of the Brazilian Cerrado. *Cons Biol* **19**: 707–13.

Knutson MG, Richardson WB, Rieneke DM, Gray BR, Parmelee JR, Weick SW (2004) Agricultural ponds support amphibian populations. *Ecol Appl* **14**: 669–84.

Kolpin DW, Furlong ET, Meyer MT, Thurman EM, Zaugg SD, Barber LB, Buxton HT (2002) Pharmaceuticals, hormones, and other organic wastewater contaminants in US streams, 1999-2000: a national reconnaissance. *Environ Sci Technol* **365**: 1202–11.

Kolpin DW, Skopec M, Myer MT, Furlong ET, Zaugg SD (2004) Urban contribution of pharmaceuticals and other organic wastewater contaminants to streams during differing flow conditions. *Sci Total Environ* **328**:119–30.

Kuang K, McConnell LL, Torrents L, Meritt D, Tobash S (2003) Atmospheric deposition of pesticides to an agricultural watershed of the Chesapeake Bay. *J Environ Qual* **32**: 1611–22.

Larsson DGJ, Adolfsson-Erici M, Parkkonen J, Pettersson M, Berg AH, Olsson P-E, Förlin L (1999) Ethinyloestradiol – an undesired fish contraceptive? *Aquat Toxicol* **45**: 91–7.

Leu C, Singer H, Stamm C, Mueller SR, Schwarzenbach RP (2004a) Simultaneous assessment of sources, processes, and factors influencing herbicide losses to surface waters in a small agricultural catchment. *Environ Sci Tech* **38**: 3827–34.

Leu C, Singer H, Stamm C, Mueller SR, Schwarzenbach RP (2004b) Variability of herbicide losses from 13 fields to surface waters within a small catchment after a controlled herbicide application. *Environ Sci Tech* **38**: 3835–41.

Levengood JM, Sanderson GC, Anderson WL, Foley GL, Skowron LM, Brown PW, Seets JW (1999) Acute toxicity of ingested zinc shot to game-farm mallards. *IL Nat Hist Surv Bull* **36**:1–36.

Linder G, Henderson G, Ingham E (2003) Wildlife and remediation of contaminated soils: extending the analysis of ecological risks to habitat restoration. In *Handbook of Ecotoxicology*, 2nd edn, Hoffman DG, Rattner BA, Burton Jr GA,Cairns Jr J (eds). Lewis Publishers, CRC Press, Boca Raton, FL, pp. 167–90.

Linzey DW, Burroughs J, Hudson L, Marini M, Robertson J, Bacon JP, Nagarkatti M, Nagarkatti PS (2003) Role of environmental pollutants on immune function, parasitic infections and limb malformations in marine toads and whistling frogs from Bermuda. *Int J Environ Health Res* **13**:125–48.

Longcore JR, Andrews R, Locke LN, Bagley GE, Young LT (1974a) Toxicity of lead and proposed substitute shot to mallards. US Fish and Wildlife Science, Special Scientific Report No. 183.

Longcore JR, Locke LN, Bagley GE, Andrews R (1974b) Significance of lead residues in mallard tissues. *US Fish and Wildlife Science, Special Scientific Report* No 182.

Lundholm CE (1993) Inhibition of prostaglandin synthesis in eggshell gland mucosa as a mechanism for *p,p'*-DDE-induced eggshell thinning in birds-a comparison of ducks and domestic fowls. *Comp Biochem Physiol C. Pharmacol Toxicol* **106**: 389–94.

Lundholm CE (1997) DDE-induced eggshell thinning in birds: effects of *p,p'*-DDE on the calcium and prostaglandin metabolism of the eggshell gland. *Comp Biochem Physiol Part C Pharmacol, Toxicol Endocrinol* **118**:113–28.

Malley DF, Change PSS (1985) Effects of aluminum and acid on calcium uptake by the crayfish *Orconectes virilis*. *Arch Environ Contam Toxicol* **14**: 739–47.

Malone RW, Shipitalo MJ, Wauchope RD, Sumner H (2004) Residual and contact herbicide transport through field lysimiters via preferential flow. *J Environ Qual* **33**: 2141–8.

Marland G, Boden TA, Andres RJ (2005) Global, regional, and national $CO_2$ emissions. In *Trends: A Compendium of Data on Global Change*. Carbon Dioxide Information Analysis Center, Oak Ridge National Laboratory, US Department of Energy, Oak Ridge, Tenn.

Martin PA, Johnson DL, Forsyth DJ, Hill BD (2000) Effects of two grasshopper control insecticides on food resources and reproductive success of two species of grassland songbirds. *Environ Tox Chem* **19**: 2987–96.

Martineau D, Lemberger K, Dallaire A, Labelle P, Liscombe TP, Michel P, Mikaelian I (2002) Cancer in wildlife, a case study: Beluga from the St. Lawrence Estuary, Quebec, Canada. *Environ Health Perspec* **110**: 282–92.

Martineau D, Lair S, De Guise S, Lipscomb TP, Beland P (1999) Cancer in Beluga whales from the St. Lawrence Estuary, Quebec, Canada: A potential biomarker of environmental contamination. *J Cetac Res Mgt* **suppl p.I**: 249–65.

Martinovic D, Schoenfuss HL, Sorensen PW (2003) Exposure to low levels of water-borne estrogen suppresses the competitive spawning ability of male fathead minnows. *Proceeding of the 3rd International Conference on Pharmaceuticals and Endocrine Disrupting Chemicals in Water.* Minneapolis, MN.

Matarczyk JA, Willis AJ, Vranjic JA, Ash JE (2002) Herbicides, weeds, and endangered species: management of bitou bush (*Chrysanthemoides monilifera* ssp. *Rotundata*) with glyphosate and impacts on the endangered shrub, Pimelea spicata. *Biol Conserv* **108**: 133–41.

Matthiessen P (1998) Effects on fish of estrogenic substances in English rivers. In *Principles and Processes for Evaluating Endocrine Disruption in Wildlife*. Kendall R, Dickerson R, Giesy J, Suk W (eds). SETAC Press, Pensacola, FL, pp. 239–47.

Matson CW, Rodgers BE, Chesser RK, Baker RJ (2000) Genetic diversity of Clethrionomys glareolus populations from highly contaminated sites in the Chernobyl region, Ukraine. *Environ Toxicol Chem* **19**: 2130–5.

Mining, Minerals, and Sustainable Development (MMSD) (2002) Report of the experts meeting on human rights issues in the mining and minerals sector. Offices of Transparency International, Berlin, September 6, 2001. No. 222.

Mitchell RR, Fitzgerald SD, Aulerich RJ, Balander RJ, Powell DC, Tempelman RJ, Stickle RL, Stevens W,Bursian SJ (2001) Health effects following chronic dosing with tungsten–iron and tungsten-polymer shot in adult game-farm mallards. *J Wildl Dis* **37**: 451–8.

Mills PC, Kolpin WD, Scribner EA,Thurman EM (2005) Herbicides and degredates in shallow aquifers of Illinois: spatial and temporal trends. *J Am Water Res Assoc* **41**: 537–47.

Mol JH, Ouboter PE (2004) Downstream effects of erosion from small-scale gold mining on the instream habitat and fish community of a small neotropical rainforest stream. *Cons Bio* **18**: 201–14.

Møller AP, Mousseau TA (2001) Albinism and phenotype of barn swallows (*Hirundo rustica*) from Chernobyl. *Evolution* **55**: 2097–104.

Møller A P, Mousseau TA (2003) Mutation and sexual selection: a test using barn swallows from Chernobyl. *Evolution* **57**: 2139–46.

Møller AP, Surai P, Mosseau TA (2004) Antioxidants, radiation, and mutation as revealed by sperm abnormality in barn swallows from Chernobyl. *Proc Royal Soc* **272**: 247–52.

Moreby SJ, Southway S, Barker A, Holland JM (2001) A comparison of the effect of new and established insecticides on nontarget invertebrates of winter wheat fields. *Environ Toxicol Chem* **20**: 2243–54.

Morris AJ, Wilson JD, Whittington MJ, Bradbury RB (2005) Indirect effects of pesticides on breeding yellowhammer (*Emberiza citrinella*). *Agric Ecosyst Environ* **106**: 1–16.

Munson L, Karesh WB (2002) Disease monitoring for the conservation of terrestrial animals. In *Conservation Medicine: Ecological Health in Practice*, Aguirre AA, Ostfeld RS, Tabor GM, House C, Pearl MC (eds). Oxford University Press, New York, pp. 95–103.

Muntner P, Menke A, DeSalvo KB, Rabito FA, Bautman V (2005) Continued decline on blood lead levels among adults in the United States. *Arch Internal Med* **165**: 2155–61.

Nash TH III (1972) Effects of effluents from a zinc smelter on mosses. Ph.D. thesis. Rutgers, The State University, Brunswick, NJ.

Nash TH III (1975) Influence of effluents from a zinc factory on lichens. *Ecol Monogr* **45**: 183–98.

Newman MC (1998) *Fundamentals of Ecotoxicology*. Sleeping Bear/Ann Arbor Press, Chelsea, MI.

Newton I, Bogan JA, Rothery P (1986) Trends and effects of organochlorine compounds in sparrowhawk eggs. *J Appl Ecology* **23**: 461–78.

Newton I, Bogan JA, Hass MB (1989) Organochlorines and mercury in the eggs of British peregrines Falco peregrinus. *Ibis* **131**: 355–76.

Newton I, Wyllie I, Asher A (1992) Mortality from the pesticides alldrin and dieldrin in British Sparrowhawks and Kestrels. *Ecotoxicology* **23**: 461–78.

Ntampakis L, Carter I.(2005) Red kites and rodenticides: a feeding experiment. *British Birds* **98**: 411–16.

Ohlendorf HM, Hothem RL (1995) Agricultural drainwater effects on wildlife in central California. In *Handbook of Ecotoxicology* Hoffman DJ, Rattner BA Burton GA Jr, Cairns J Jr. (eds.), Lewis Publishers, *Boca Raton*, pp. 577–95.

Oshima Y, Kang IJ, Kobayashi M, Nakayama K, Imada N, Honjo T (2003) Suppression of sexual behavior in male Japanese medaka (*Oryzias latipes*) exposed to 17–estradiol. *Chemosphere* **50**: 429–36.

Oyler JA (1988) Remediation of metals-contaminated site near a zinc smelter using sludge/fly ash amendments: herbaceous species. *Trace Elements in Environmental Health*, vol 22. University Missouri–Columbia.

Panter GH, Thompson RS, Sumpter JP (1998) Adverse reproductive effects in male fathead minnows (*Pimephales promelas*) exposed to environmentally relevant concentrations of the natural oestrogens, oestradiol and oestrone. *Aquatic Tox* **42**: 243–53.

Panter GH, Thompson RS, Sumpter JP (2000) Intermittent exposure of fish to estradiol. *Environ Sci Tech* **34**: 2756–60.

Petrovic M, Solé M, López de Alda MJ, Barceló D (2002) Endocrine disruptors in sewage treatment plants, receiving river waters, and sediments: integration of chemical analysis and biological effects on feral carp. *Environ Toxicol Chem* **21**: 2146–56.

Pirkle JL, Brody DJ, Gunter EW, Kramer RA, Paschal DC, Flegal KM, Matte TD (1994) The decline in blood lead levels in the United States. The National Health and Nutrition Examinations Surveys (NHANES). *J Amer Med Assoc* **272**: 284–91.

Plesner T, Christiansen LB (2003) Testicular damages in brook trout (*Salmo trutta* L.) and intersex in roach (*Rutilus rutilus* L.) in waste water impacted Danish streams. *Proceeding of the 3rd International Conference on Pharmaceuticals and Endocrine Disrupting Chemicals in Water.* Minneapolis, MN.

Purdom CE, Hardiman PA, Bye VJ, Eno NC, Tyler CR, Sumpter JP (1994) Estrogenic effects of effluents from sewage treatment works. *Chem Ecol* **8**: 275–85.

Quinn B, Gagne F, Costello M, McKenzie C, Wilson J, Mothersill C (2004) The endocrine-disrupting effect of municipal effluent on the zebra mussel (*Dreissena polymorpha*). *Aquat Toxicol* **66**: 279-92.

Ratcliffe D (1967) Decreases in eggshell weight in certain birds of prey. *Nature* **215**: 208–10.

Reeder AL, Foley GL, Nichols DK, Hansen LG, Wikoff B, Faeh S, Eisold J, Wheeler MB, Warner R, Murphy JE, Bealey VR (1998) Forms and prevalence of intersexuality and effects of environmental contaminants on sexuality in cricket frogs (*Acris crepitans*). *Environ Health Perspect* **106**: 261–6.

Reeder AL, Ruiz MO, Pessier A, Brown LE, Levengood JM, Phillips CA, Wheeler MB, Warner RE, Beasley VR (2005) Intersexuality and the cricket frog decline: historic and geographic trends. *Environ Health Persp* **113**: 261–5.

Relyea RA (2005a) The lethal impact of roundup on aquatic and terrestrial amphibians. *Ecol Appl* **15**: 1118–24.

Relyea RA (2005b) The impact of insecticides and herbicides on the biodiversity and productivity of aquatic communities. *Ecol Appl* **15**: 618–27.

Relyea RA (2005c) The lethal impacts of roundup and predatory stress on six species of North American tadpoles. *Environ Contam Toxicol* **48**: 351–7.

Rodgers-Gray TP, Jobling S, Morris S, Kelly C, Kirby S, Janbakhsh A, Harries JE, Waldock MJ, Sumpter JP, Tyler CR (2000) Long–term temporal changes in the estrogenic composition of treated sewage effluent and its biological effects on fish. *Environ Sci Techn* **34**: 1521–8.

Rodgers-Gray TP, Jobling S, Kelly C, Morris S, Brighty G, Waldock MJ, Sumpter JP, Tyler CR (2001) Exposure of juvenile roach (*Rutilus rutilus*) to treated sewage effluent induces dose-dependent and persistent disruption in gonadal duct development. *Environ Sci Techn* **35**: 462–70.

Rouse JD, Bishop CA, Struger J (1999) Nitrogen pollution: an assessment of its threat to amphibian survival. *Environ Health Perspect* **107**: 799–803.

Samuel MD, Bowers EF (2000) Lead exposure in American black ducks after implementation of non–toxic shot. *J Wildl Manage* **64**: 947–53.

Sanderson GC, Irwin JC (1976) Effects of various combinations and numbers of lead:iron pellets dosed in wild-type captive mallards. Final Report Contract No. 14-16-0008-914, US Fish Wildlife Service.

Sanderson GC, Anderson WL, Foley GL, Skowron LM, Brawn JD, Seets JW (1997) Acute toxicity of ingested bismuth alloy shot in game-farm mallards. *IL Nat Hist Surv Bull* **35**: 185–216.

Schotthoefer AM, Koehler AV, Meteyer CU, Cole RA (2003a) Influence of *Ribeiroia ondatrae* (Trematoda: Digenea) infection on limb development and survival of northern leopard frogs (*Rana pipiens*): effects of host stage and parasite–exposure level. *Can J Zool* **81**:1144–53.

Schotthoefer AM, Cole RA, Beasley VR (2003b) Relationship of tadpole stage to location of echinostome cercariae encystment and the consequences for tadpole survival. *J Parasit* **89**: 475–82.

Scribner EA, Battaglin WA, Goolsby DA, Thurman EM (2000) Changes in herbicide concentrations in Midwestern streams in relation to use, 1989-1998. *Sci Total Environ* **248**: 255–63.

Seiler RL, Skorupa JP, Naftz DL, Nolan BT (2003) Irrigation-induced contamination of water, sediment, and biota in the western United States- Synthesis of data from the National Irrigation Water Quality Program. US Geological Survey Professional Paper 1655.

Shore RF, Birks JDS, Freestone P, Kitchner AC (1996) Second-generation rodenticides and polecats (*Mustela putorius*) in Britain. *Environ Pollut* **91**: 279–82.

Sibly RM, Newton I, Walker CH (2000) Effects of dieldrin on population growth rates of sparrowhawks 1963-1986. *J Appl Ecology* **37**: 540–6.

Silveira MP, Baptista DF, Buss DF, Nessimian JL, Egler M (2005) Application of biological measures for stream integrity assessment in south-east Brazil. *Environ Monit Assess* **101**: 117–28.

Sokolov VE, Krivolutsky DA, Ryabov IN, Taskaev AE, Shevchenko VA (1990) Bioindication of biological after-effects of the Chernobyl atomic power station accident in 1986–1987. *Biol Int* **18**: 6–11.

Sokolov VE, Ryabov IN, Ryabtsev IA, Tikhomirov FA, Shevchenko VA, Taskaev AE (1993) Ecological and genetic consequences of the Chernobyl atomic power plant accident. *Vegetation* **109**: 91–9.

Solé M, Barceló D, Porte C (2002) Seasonal variation of plasmatic and hepatic vitellogenin and EROD activity in carp, *Cyprinus carpio*, in relation to sewage treatment plants. *Aquat Toxicol* **60**: 233–48.

Solomon K, Thompson D (2003) Ecological risk assessment for aquatic organisms from over-water uses of glyphosate. *J Toxicol Environ Health, Pt B Crit Rev* **6**: 289–324.

Soucek DJ (2006) Effects of freshly neutralized aluminum on oxygen consumption by freshwater invertebrates. *Arch Environ Contam Toxicol* **50**: 353–60.

Sousa WP, Grosholz ED (1991) The influence of habitat structure on the transmission of parasites. In *Habitat Structure: The Physical Arrangement of Objects in Space*, Bell SS, McCoy ED, Mushinsky HR (eds). Chapman and Hall, London, pp. 300–24.

Spalding RF, Snow DD (1989) Stream levels of agrichemicals during a spring discharge event. *Chemosphere* **19**: 1129–40.

Spalding RF, Exner ME, Snow DD, Cassada DA, Burbach ME, Monson SJ (2003) Herbicides in ground water beneath Nebraska's Management Systems Evaluation Area. *J Environ Quality* **32**: 92–9.

Sparling DW, Linder G, Bishop CA (2000) *Ecotoxicology of Amphibians and Reptiles*. SEATC Press, Pensacola, FL.

Stamer JK, Swanson RB, Jordan PR (1994) Atrazine in spring runoff as related to environmental setting in Nebraska, 1992. *Water Resour Bull* **30**: 823–31.

Storrs SI, Kiesecker JM (2004) Survivorship patterns of larval amphibians exposed to low concentrations of atrazine. *Environ Health Perspect* **112**: 1054–7.

Strachan G, Preston S, Maciel H, Porter AJR, Paton GI (2001) Use of bacterial biosensors interpret the toxicity and mixture toxicity of herbicides in freshwater. *Water Res* **35**: 3490–5.

Strojan CL (1978a) The impacts of zinc smelter emissions on forest litter arthropods. *Oikos* **31**: 41–6.

Strojan CL (1978b) Forest leaf litter decomposition in the vicinity of a zinc smelter. *Oecologica* **32**: 203–12.

Sullivan KB, Spence KB (2003) Effects of sublethal concentrations of atrazine and nitrate on metamorphosis of the African clawed frog. *Environ Toxicol Chem* **22**: 627–35.

Sutherland AB, Myer JL, Gardiner EP (2002) Effects of land cover on sediment regime and fish assemblage structure in four southern Appalachian streams. *Fresh Bio* **47**: 1791–805.

Sutton DL, Blackburn RD, Barlowe WC (1971) Response of aquatic plants to combinations of endothall and copper. *Weed Sci* **19**: 643–6.

Tabor GM (2002) Defining conservation medicine. In *Conservation Medicine: Ecological Health in Practice*, Aguirre AA, Ostfeld RS, Tabor GM, House C, Pearl MC (eds). Oxford University Press, New York, pp. 8–16.

Tavera-Mendoza L, Ruby S, Brousseau P, Fournier M, Cyr D, Marcogliese D (2001) Response of the amphibian tadpole (*Xenopus laevis*) to atrazine during sexual differentiation of the testis. *Environ Toxicol Chem* **21**: 527–31.

Richardson LL (1998) Coral diseases: what is really known? *Trends Ecol Evol* **13**: 438–43.

Thompson S, Tilton F, Schlenk D, Benson WH (2000) Comparative vitellogenic responses in three teleost species: extrapolation to *in situ* field studies. *Marine Environ Res* **51**: 185–9.

Trombulak SC, Frissell CA (2000) Review of ecological effects of roads on terrestrial and aquatic communities. *Cons Bio* **14**:18–30.

United Kingdom Department of Trade and Industry (UKDTI) (2002) Energy: its impact on the environment and society. URN No. 05/1274.

Uri ND (2001) A note on soil erosion and its environmental consequences in the United States. *Water Air Soil Pollut* **129**:181–97.

Van Dolah FM (2000) Marine algal toxins: origins, health effects, and their increased occurrence. *Environ Health Perspect Supple S1* **108**: 133–41.

Veiga MM, Hinton J, Lilly C (1999) Mercury in the Amazon: A comprehensive review with special emphasis on bioaccumulation and bioindicators. *Proc NIMD Forum*, Minamata, Japan, pp. 19–39.

Vitousek PM, Aber J, Howarth RW, Likens RE, Matson PA, Schindler DW, Schlesinger WH, Tilman GD (1997) Human alteration of the global nitrogen cycle: causes and consequences. *Iss in Ecol* **1**: 2–16.

Walker CH, Newton I (1998) Effects of cyclodiene insecticides on the sparrowhawk (*Accipiter nisus*) in Britain – a reappraisal of evidence. *Ecotoxicology* **7**: 185–9.

Walker CH Hopkin SP, Sibly RM, Peakall DB (2001) *Principles of Ecotoxicology*. Taylor and Francis, London and New York.

White DH, King KA, Mitchell CA, Hill EF, Lamont TG (1979) Parathion causes secondary poisoning in a laughing gull colony. *Bull Environ Contam Toxicol* **23**: 281–4.

White DH, Hayes LE, Bush PB (1989) Case histories of wild birds killed intentionally with famphur in Georgia and West Virginia. *J Wildl Dis* **25**: 184–8.

White DH, Fleming WJ, Ensor KL (1988) Pesticide contamination and hatching success of waterbirds in Mississippi. *J Wildl Manage* **52**: 724–9.

Whyatt RM, Rauh V, Barr DB, Camann DE, Andrews HF, Garfinkel R, Hoepner LA, Diaz D, Dietrich J, Reyes A, Tang D, Kinney PL, Perera FP **(**2004) Prenatal insecticide exposures and birth weight and length among an urban minority cohort**.** *Environ Health Perspec* **112**: 1125–32**.**

Wickliffe JK, Dunina-Barkovskaya YV, Gaschak SP, Rodgers BE, Chesser RK, Bondarkov M (2006) Variation in mitochondrial DNA control region haplotypes in populations of the bank vole, *Clethrionomys glareolus*, living in the Chernobyl environment, Ukraine. *Environ Toxicol Chem* **25**: 503–8.

Wiemeyer SN, Lamont TG, Bunck C.M, Sindelar CR, Gramlich FJ, Fraser JD, Byrd MA (1984) Organochlorine pesticide polychlorobiphenyl and mercury residues in bald eagle *Haliaeetus leucocephalus* eggs 1969–1979 and their relationships to shell thinning and reproduction. *Arch Environ Contam Toxicol* **13**: 529–50.

Wiemeyer SN, Bunck CM, Stafford CJ (1993) Environmental contaminants on bald eagle eggs: 1980–1984 and further interpretations of relationships to productivity and shell thickness. *Arch Environ Contam Toxicol* **24**: 213–27.

Wilkins ET (1954) Air pollution and the London fog of December 1952. *J Royal Sanitary Inst* **74**: 1–21.

Winter AR, Nichols JW, Playle RC (2005) Influence of acidic to basic water pH and natural organic matter on aluminum accumulation by gills of rainbow trout (*Onchorhynchus mykiss*). *Can J Fish Aqua Sci* **62**: 2303–11.

Wobeser G, Bollinger T, Leighton FA, Blakley B, Mineau P (2004) Secondary poisoning of eagles following intentional poisoning of coyotes with anticholinesterase pesticides in western Canada. *J Wildl Dis* **40**: 163–72.

Wolanski E, De'ath G (2005) Predicting the impact of present and future human land-use on the Great Barrier Reef. *Estuar Coast Shelf Sci* **64**: 504–8.

World Health Organization (2005) Frequently asked questions on DDT use for disease vector control. Geneva, Switzerland. http://www.who.int/malaria/docs/FAQonDDT.pdf.

CHAPTER

# 58

# Aquatic toxicology

*Robert W. Coppock and P.N. Nation*

## INTRODUCTION

Aquaculture is the production of aquatic animals for human food. In aquaculture aquatic animals are grown under water in an aquatic environment. The water may be a sheltered oceanic bay containing pens of aquatic organisms, or the aquatic organisms may be raised in inland ponds or in a tank system. Chemical causes of disease in aquatic organisms are generally linked to water and food because the primary pathways of toxic substances to animals in aquaculture systems are generally feedstuffs and water. Residues of metals and persistent organic compounds can occur.

## BACKGROUND

Aquaculture is an expanding and continually developing industry. Aquatic animals are grown in a confined space, i.e. a pen, pond or tank. The aquatic animals generally are dependent on humans for all or part of their feed. Commercial feed is generally in a granular or pelleted form and contains a binding agent. Inland aquaculture generally requires at least some mechanical management of the aquatic environment. Large capital investment and high operational costs are incentives to increase stocking densities in aquaculture systems. Increased stocking densities increase the demand for water management.

## WATER

### Nitrogen cycle

The nitrogen cycle in aquatic systems is important (Boyd and Tucker, 1998; Hargreaves, 1998). An excellent review on the geobiochemistry of the nitrogen cycle has been written (Hargreaves, 1998). The nitrogen cycle transforms organic nitrogen, ammonia and other forms of nitrogen to nitrogen gas. Important sources of nitrogen in the aquatic system are feedstuff residues, feces and ammonia excreted from aquatic animals. The nitrogen cycle in an aquatic system is primarily regulated by biota. The organisms regulating the different steps in the aquatic nitrogen cycle are remarkably independent, and the products from the different steps can accumulate in the system. Species of *Nitrosomonas* oxidize ammonia to nitrite ($NO_2^{1-}$). The next step is the oxidation of $NO_2^{1-}$ to nitrate ($NO_3^{1-}$) and species of *Nitrobacter* are the primary organisms. Species of *Nitrosomonas* and *Nitrobacter* are dissimilarly affected by oxygen tension, pH, temperature, etc. and species of *Nitrobacter* are the most sensitive allowing the level of $NO_2^{1-}$ to increase. The end step in the nitrogen cycle is reduction with production of nitrogen gas ($N_2$) released into the atmosphere. The most toxic compounds in the aquatic nitrogen cycle are ammonia and $NO_2^{1-}$. Changes in the nitrogen cycle can also be seasonal, related to weather conditions. For example, nitrate poisoning (brown blood disease) in the southern USA is more common in the fall and spring (Durborow *et al.*, 1997). When the concentration of nitrite is $<1\,\mu$mol, the water is generally considered unpolluted (Jensen, 2003).

#### *Ammonia*

The economics of tank-rearing of fish requires high densities. Recirculation systems have densities as high as 0.3 kg of fish/kg of water. Estimated ammonia production by fish is 0.02 kg of ammonia nitrogen produced for each kilogram of feed fed (Masser *et al.*, 1999). Total ammonia nitrogen should be monitored in the aquatic system because ammonia is the primary nitrogen waste excreted by freshwater fish and is the most toxic form. At a given pH of the water, ammonia ($NH_3$) is in equilibrium with

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ammonium ($NH_4^{1+}$). The unionized ammonia can radially diffuse across cell membranes. The toxicity of ammonia is dependent on the pH of water, water temperature and the species of aquatic animals.

The mechanisms of ammonia toxicity in fish have not been elucidated. Amino acid metabolism is disrupted (Arillo *et al.*, 1984; Smutna *et al.*, 2002). There appear to be metabolic dysfunctions in the liver and links to disruption of neurotransmitters and electrochemistry in skeletal muscle (Randall and Tsui, 2002). Increasing water ammonia decreases swimming ability (Wicks *et al.*, 2002). The decrease in swimming ability was linked to depolarization of white muscles, and increasing water calcium ions ameliorated ammonia toxicity. Feeding fish appears to provide some protection from ammonia toxicity (Wicks and Randall, 2002).

### Nitrite

Aquatic animals, especially freshwater fish and crustacea, are more at risk for nitrite ($NO_2^{1-}$) poisoning that are terrestrial organisms (Boyd, 1982; Boyd and Tucker, 1998). Nitrite intoxication of aquatic animals can occur when conditions exist for dyssenergy in the nitrogen cycle. Freshwater fish are more susceptible to $NO_2^{1-}$ than saltwater fish. In freshwater fish $NO_2^{1-}$ is rapidly absorbed across the gills. The generally accepted mechanism for $NO_2^{1-}$ absorption is by competing for chloride ($Cl^{1-}$) uptake across the gills. The chloride ion is exchanged for bicarbonate ($HCO_3^{1-}$) and this exchange occurs in the apical part of the gill epithelial cell. Nitrite has affinity for the chloride/$HCO_3^{1-}$ exchange protein and replaces $Cl^{1-}$ in the exchange process. Increasing the water concentration of $Cl^{1-}$ reduces the toxicity of $NO_2^{1-}$. Fish with high $Cl^{1-}$ uptake (rainbow trout, perch and pike) are more susceptible to $NO_2^{1-}$. Millimolar $NO_2^{1-}$ levels in the fish, especially if $Cl^{1-}$ in the water are low, can result from micromolar levels of $NO_2^{1-}$ exposure from water. Nitrite causes a net loss of $Cl^{1-}$ because there is a reduction in the influx of $Cl^{1-}$. Nitrite also stimulates a net loss of potassium ions from muscle and this causes an extracellular hyperkalemia and increased excretion of potassium. Nitrite also enters the red blood cells and oxidizes $Fe^{2+}$ to $Fe^{3+}$ and methemoglobin (metHb) is formed. The metHb cannot carry oxygen and oxygen tensions in blood approach the values for dissolved oxygen. The oxidation of iron changes oxygen affinity of hemoglobin. Changes in oxygen affinity and blood pH increase movement of oxygen to the swim bladder. Buoyancy problems can occur. The stress response in teleost (bony) fish is $NO_2^{1-}$-mediated disruption of the $Na^{1+}/H^{1+}$ exchange mechanism in the red blood cells. This can further decrease the affinity of hemoglobin for oxygen. Decreased oxygen tensions increase anarobic metabolism and acidosis occurs. Exposure of rainbow trout to 1 mmol $NO_2^{1-}$ causes vasodilation and an increased cardiac workload (Aggergaard and Jensen, 2001; Jensen and Agnisola, 2005). The fish died when the metHg was >70% and plasma $NO_2^{1-}$ level was 2.9 mmol at the time of death. Liver cell damage linked to mitochondrial pathology occurs and hepatic stores of glycogen are depleted. Necrosis of the retina can occur. Hepatocytes from rainbow trout have been shown to detoxify $NO_2^{1-}$ by converting $NO_2^{1-}$ to considerable less toxic nitrate ($NO_3^{1-}$) (Doblander and Lackner, 1996). Recovery from nitrite poisoning appears to take several weeks and compensatory gain may or may not occur.

Species susceptibility to $NO_2^{1-}$ is variable and is linked to $Cl^{1-}$ uptake by the gills (Durborow *et al.*, 1997; Jensen, 2003). Largemouth and smallmouth bass, bluegill and green sunfish are resistant to high concentrations of $NO_2^{1-}$ and catfish, goldfish and fathead minnows and tilapia are sensitive and coldwater fish like rainbow trout are highly sensitive to $NO_2^{1-}$.

Nitrite poisoning (brown blood disease) can be prevented by adding $Cl^{1-}$ to the water (Durborow *et al.*, 1997). The most common option is to add $Cl^{1-}$ to the water to achieve a ratio of $Cl^{1-}$ to $NO_2^{1-}$ of 10:1. Decreased feeding rates and increased throughput of non-recycled water are alternative methods of controlling $NO_2^{1-}$. Bacterial and parasitic disease increase the sensitivity of fish to $NO_2^{1-}$ poisoning. The presence of concurrent disease requires increasing the concentration of $Cl^{1-}$. Catfish producers commonly maintain 100 ppm $Cl^{1-}$ in the pond or tank water as insurance against a bump in $NO_2^{1-}$ levels or to counteract the effects of concurrent infectious disease.

## Metals

### Copper

There are many sources of copper ions including surface water that potentially can enter aquaculture systems. Copper is commonly used in aquaculture as an algicide and as a treatment for parasites. There is generally a small margin of safety for many aquatic species. Cupric ions ($Cu^{+2}$) disrupt the energy-dependent sodium/potassium pump located in the gill chloride cells. This allows increased efflux of sodium ions. Cupric ions also replace calcium ions at the tight junctions resulting in an efflux of sodium ions. The net loss of sodium ions results in cardiovascular collapse. The uptake of copper ions across the gill of fish is dependent on many parameters including water hardness (levels of calcium and magnesium ions). Copper ions are neurotoxic in fish. These are disruptive of the olfactory and mechanosensory systems (Baldwin *et al.*, 2003; Linbo *et al.*, 2006). Loss of neuromasts occurred at concentrations >20 μg/l, and peripheral olfactory function is inhibited at levels as low as 4 μg/l. Exposure to copper also decreases immune function. Copper levels can be tricky to interpret because pH, carbonate ions and dissolved organic carbon are interactive in forming unavailable forms of copper and $Mg^{+2}$ and $Ca^{+2}$

compete for copper uptake by the fish (Sciera *et al.*, 2004). Changes in these parameters can cause 60-fold difference in lethal toxicity. Decreasing the pH increases the toxicity and a 100 times increase in toxicity can occur with each unit decrease in pH. Warmwater fish are more tolerant of copper than coldwater fish. In a study in fathead minnows, increasing sodium chloride and calcium chloride levels in the water decreased copper toxicity and increasing potassium chloride increased sensitivity to copper (Erickson *et al.*, 1996).

## Non-metals

### *Chlorine*

Water is not considered safe for fish if a measurable level of chlorine ($Cl_2$) is present. Chlorine and chlorine compounds are used as disinfection agents in municipal water and in aquaculture to disinfect ponds and tanks (Boyd and Tucker, 1998). Chlorine gas added to water forms a number of compounds (hypochlorous acid, hydrochloric acid and hypochlorite) and the concentration of dissociated ion depends on the pH of the water. Chloramine is formed by the reaction of chlorine with ammonia in the water. The toxicity of chlorine residues is variable due to effects of water temperature. Residual chlorine in the water is generally oxidative and causes irritation and damage to the gills. The gill lesion can be hypertrophy and hyperplasia with necrosis occurring at higher concentrations. Gill lesions cause hypoxia.

# FEED

## Mycotoxins

### *Fumonisins and moniliformin*

Fumonisin $B_1$ ($FB_1$) or moniliformin (MON) have been shown to reduce the productivity of fish. Nile tilapia fingerlings were fed $FB_1$ or MON at 0, 10, 40, 70 and 150 ppm for 8 weeks (Tuan *et al.*, 2003). The $FB_1$ and MON were extracted from cultures of *Fusarium moniliforme* and *F. proliferatum*, respectively. Mortalities in all treatment groups were low and were not dose related. Feeding diets containing 150 ppm $FB_1$ or MON decreased hematocrit. Both mycotoxins disrupted metabolism. Feeding MON increased serum pyruvate. There was evidence that sphingolipid metabolism was disrupted in fish fed $FB_1$. For fish fed MON, decreased weight gains were observed at the 70 and 150 ppm levels, and for fish fed $FB_1$ decreased weight gains were observed at the 40, 70 and 150 ppm levels. Fish fed 10 ppm $FB_1$ had decreased weight gains for the first 2 weeks, but body weights at 4 weeks were not significantly different from controls. Histopathology linked to treatments was not observed in the fish. Studies on the combination of $FB_1$ and MON showed interactions between the two toxins (Yildirim *et al.*, 2000). Channel catfish are more sensitive to $FB_1$ and MON than tilapia and $FB_1$ is more toxic in channel catfish than MON.

### *Aflatoxins*

Aflatoxins (AF) were identified in the early 1960s as causing hepatic cancer in fish (Sinnhuber and Wales, 1978). Rainbow trout are sensitive to AF and dietary levels as low as 0.4 ppb fed for less than a year will cause hepatic cancer. Salmon are more resistant that rainbow trout to the carcinogenic effects of AF. Aflatoxin $B_1$ ($AFB_1$) is metabolized by the P450 enzyme system in the liver of fish to form the $AFB_1$-8,9-epoxide. The $AFB_1$-8,9-epoxide forms adducts with DNA, RNA and proteins. The formation of adducts is considered to be the primary mechanism of action for the carcinogenicity of $AFB_1$. *In vitro* cultures of rainbow trout peripheral blood leukocytes incubated with $AFB_1$ showed the trout leukocytes to be 1000 times more sensitive to $AFB_1$ than murine (mouse) leukocytes (Ottinger and Kaattari, 1998). Exposure of trout embryos to $AFB_1$ causes long-term immunodysfunction (Ottinger and Kaattari, 2000). $AFB_1$ has been shown to be immunosuppressive in India major carp (*Labeo rohita*) (Sahoo and Mukherjee, 2003). Feeding a diet containing 500 ppm $AFB_1$ altered immune parameters and decreased the native resistance to disease in India major carp. Dietary vitamin C was shown to provide protection against $AFB_1$. $AFB_1$ has been studied in tilapia (Nguyen Anh *et al.*, 2002). One-month-old tilapia fingerlings were fed diets containing 0, 0.25, 2.5, 10 or 100 ppm $AFB_1$ for 8 weeks. Fish fed with 10 and 100 ppm $AFB_1$ consumed less feed and expelled the feed after ingestion suggesting feed refusal occurred. At 6 weeks of feeding, the survival rate for the fish fed the 100 ppm level of $AFB_1$ was 55%. Weight gains for the fish fed the diets containing $\geqslant$2.5 ppm $AFB_1$ were decreased. Hematocrit was reduced in the fish-fed diets $\geqslant$0.25 ppm $AFB_1$. Fish fed diets containing 10 and 100 ppm $AFB_1$ had liver lesions. Pleiomorphic liver nuclei were present in 86% of the fish fed the diet containing 10 ppm $AFB_1$ and 100% of the fish fed the diet containing 100 ppm $AFB_1$. Catfish have high resistance to the carcinogenic effects of $AFB_1$ and this is linked to a low rate of formation of the $AFB_1$-8,9-epoxide (Gallagher and Eaton, 1995).

### *Ochratoxins*

Early studies of parenteral exposure of rainbow trout to ochratoxins showed that ochratoxin A (OA) was ~10 times more toxic that Ochratoxin B (OB). OA extracted from cultures of *Aspergillus ochraceus* was added to channel catfish diets at 0, 0.5, 1.0, 2.0, 4.0 or 8.0 ppm (Manning *et al.*, 2003).

The average initial weight was 6 g/fish. Decreased body weight gain was observed at 2 weeks and at 8 weeks in the catfish fed the diet containing 1.0 ppm OA. At 8 weeks, a decrease in body weight gains was observed for the catfish fed the 2, 4 and 8 ppm diets. Hematocrit was decreased in the fish fed the 8 ppm diet and white blood cell numbers were not changed. At 8 weeks, there was 80% survival in the fish fed the diet containing 8 ppm OA. Histopathology lesions were observed in fish fed the OA diets at the 1, 2, 4 and 8 ppm levels. The lesions were described as enlarged melanomacrophage centers replacing the hepatopancreatic cells and the melanomacrophage centers were present in the posterior kidney. These findings suggest the hepatopancreatic cells are the target for OA in catfish.

## Contaminants in aquaculture fish

### *Chlorinated dioxins*

Aquaculture animals are a source of polychlorinated dibenzodioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and polychlorinated biphenyl (PCBs) compounds. Each of these groups is a mixture of congeners. These groups of compounds are collectively referred to as persistent organic carbons (POCs). Health concerns exist because these compounds are bioconcentrated in body fat of animals. These compounds are also transferred to the fetus during pregnancy and excreted in breast milk during lactation. Dietary animal protein–fat and clays are the predominant source of the POCs for aquaculture animals. Catfish have been identified as an important source of PCDDs for persons consuming them, and in these individuals, this can exceed the dietary exposure from diary products (Jensen and Bolger, 2001). In a study on the transfer of PCDDs and PCDFs from diet to muscle fat, it was found that in the first 13.5 months of feeding ~30% of the dietary PCDDs and PCDFs in a diet fed to rainbow trout were transferred to fat located in muscle tissue (Karl *et al.*, 2003). At 19 months, the transfer rate increased slightly to ~34% for male trout and decreased to ~27% for egg-laying female trout. Congener-specific pattern of PCDDs and PCDFs accumulation in the fish was not identified. The concentrations of PCDDs and PCDFs in diet were directly related to the concentrations of PCDDs and PCDFs in muscle fat. The levels of PCDDs and PCDFs in muscle fat increased over the 19-month feeding period.

### *Methylmercury*

Methylmercury (meHg) is a concern for human foods from aquatic sources. The primary source of meHg in aquaculture is the use of fish by-products in feedstuffs. meHg is formed by biota in the benthic region of the aquatic system, and is biomagnified in the food web. In fish, meHg has affinity for skeletal muscle and is bioaccumulated in skeletal muscle (Berntssen *et al.*, 2004). Approximately 23% of the dietary meHg and ~6% of dietary inorganic mercury (Hg) is absorbed by fish. Fish fed diets containing 5 and 10 ppm meHg for 4 months had 1.1 and 3.1 ppm of meHg in muscle (freeze-dried), respectively. The threshold toxic level for Atlantic salmon is estimated at 0.5 ppm meHg in diet. Studies in fathead minnows showed that the threshold level of dietary meHg on spawning parameters is $<$0.88 ppp meHg (Hammerschmidt *et al.*, 2002).

# TOXICOLOGY INVESTIGATIONS

The triggers for a toxicological investigation generally are decreased performance in reproduction, feed conversion and growth, and increased mortality. It is important to document when the toxicological incident occurred. Review of records can be helpful to determine when the incident started. A complete history is important to direct the diagnostic procedures and analytical toxicology. Important questions to be asked are:

- Was the incident an acute event or did it occur over a period of time?
- What are the size, age and location of the animals that are affected?
- Were clinical signs observed?
- Can the onset be linked to changes in feed sources, a particular feed storage area?
- Are there links to changes in water handling, filtering or a rainfall event?
- Are there links to ambient and water temperature?
- Pesticide use and location of use?
- Did deaths also occur in terrestrial animals?
- Is the death of aquatic animals linked to a die-off of aquatic plants and phytoplankton?
- Previous land use in the area where the ponds have been constructed?
- Is pathology data available?

The time of day that mortalities occur is important. These can be linked to changes in water temperature and a decrease in dissolved oxygen. Sporadic mortality is more likely to be related to parasites or changes within the tank and pond system. As a general rule small fish are more resistant to low dissolved oxygen and are less resistant to chemical substances.

# CONCLUDING REMARKS AND FUTURE DIRECTIONS

Aquaculture is a growing industry. Toxicological causes of diseases in aquatic organisms are in two categories.

These are toxic substances in water and toxic substances in feedstuffs. Toxic substances in feedstuffs are generally easier to control. The manufactures and suppliers of feedstuffs to the aquaculture industry have the responsibility for quality assurance and quality control that ensure toxic substances are not present in feedstuffs. Toxic substances can bioconcentrate in aquatic organisms and present human food safety issues for the aquaculture industries. Herein animal feed safety and human food safety are linked to quality assurance, and quality control programs and due diligence in the animal feed industries.

Water safety issues are linked to chemical cause of disease in aquatic organisms. In pond- and tank-reared animals, the operator controls the aquatic environment. This requires monitoring of water for levels of toxic substances and ensuring that natural drainage of surface water does not introduce toxic substances to the aquaculture system. Multiple uses of limited water resources will continue to present challenges to water resources available to the aquaculture industry. Aquaculture also has increasing capital investment and operating costs. Water availability and economics are incentives to increase animal numbers per volume of water. Increased animal densities increase the demands on water safety for aquatic animals, and increase the need for research on water recirculation systems.

## REFERENCES


Aggergaard S, Jensen FB (2001) Cardiovascular changes and physiological response during nitrite exposure in rainbow trout. *J Fish Biol* **59**: 13–27.

Arillo A, Gaino E, Margiocco C, Mensi P, Schenone G (1984) Biochemical and ultrastructural effects of nitrite in rainbow trout: liver hypoxia as the root of the acute toxicity mechanism. *Environ Res* **34**: 135–54.

Baldwin DH, Sandahl JF, Labenia JS, Scholz NL (2003) Sublethal effects of copper on coho salmon: impacts on nonoverlapping receptor pathways in the peripheral olfactory nervous system. *Environ Toxicol Chem* **22**: 2266–74.

Berntssen MHG, Hylland K, Julshamn K, Lundebye AK, Waagbo R (2004) Maximum limits of organic and inorganic mercury in fish feed. *Aquacult Nut* **10**: 83–97.

Boyd CE (1982) *Water Quality Management for Pond Fish Culture.* Elsevier Scientific Publishing Company, New York, p. 318.

Boyd CE, Tucker CS (1998) *Pond Aquaculture Water Quality Management.* Kluwer Academic Publishers, Boston, MA, p. 700.

Doblander C, Lackner R (1996) Metabolism and detoxification of nitrite by trout hepatocytes. *Biochim Biophys Acta* **1289**: 270–4.

Durborow RM, Crosby DM, Brunson MW (1997) *Nitrate in Fish Ponds.* Southern Regional Aquaculture Center, Stoneville, MS, p. 4.

Erickson RJB, Benott DA, Mattson VRJ, Nelson HP, Leonard EN (1996) The effects of water chemistry on the toxicity of copper to fathead minnows. *Environ Toxicol Chem* **15**: 181–93.

Gallagher EP, Eaton DL (1995) *In vitro* biotransformation of aflatoxin $B_1$ ($AFB_1$) in channel catfish liver. *Toxicol Appl Pharmacol* **132**: 82–90.

Hammerschmidt CR, Sandheinrich MB, Wiener JG, Rada RG (2002) Effects of dietary methylmercury on reproduction of fathead minnows. *Environ Sci Technol* **36**: 877–83.

Hargreaves JA (1998) Nitrogen biogeochemistry of aquaculture ponds. *Aquaculture* **166**: 181–212.

Jensen E, Bolger PM (2001) Exposure assessment of dioxins/furans consumed in dairy foods and fish. *Food Addit Contam* **18**: 395–403.

Jensen FB (2003) Nitrite disrupts multiple physiological functions in aquatic animals. *Comp Biochem Physiol, Part A Mol Integr Physiol* **135**: 9–24.

Jensen FB, Agnisola C (2005) Perfusion of the isolated trout heart coronary circulation with red blood cells: effects of oxygen supply and nitrite on coronary flow and myocardial oxygen consumption. *J Exp Biol* **208**: 3665–74.

Karl H, Kuhlmann H, Ruoff U (2003) Transfer of PCDDs and PCDFs into the edible parts of farmed rainbow trout, *Oncorhynchus mykiss* (Walbaum), via feed. *Aquacult Res* **34**: 1009–14.

Linbo TL, Stehr CM, Incardona JP, Scholz NL (2006) Dissolved copper triggers cell death in the peripheral mechanosensory system of larval fish. *Environ Toxicol Chem* **25**: 597–603.

Manning BB, Ulloa RM, Li MH, Robinson EH, Rottinghaus GE (2003) Ochratoxin A fed to channel catfish (*Ictalurus punctatus*) causes reduced growth and lesions of hepatopancreatic tissue. *Aquaculture* **219**: 739–50.

Masser MP, Rakocy J, Losordo TM (1999) *Recirculating Aquaculture Tank Production Systems. Management of Recirculating Systems.* Southern Regional Aquaculture Center, Stoneville, MS, p. 12.

Nguyen Anh T, Grizzle JM, Lovell RT, Manning BB, Rottinghaus GE (2002) Growth and hepatic lesions of Nile tilapia (*Oreochromis niloticus*) fed diets containing aflatoxin $B_1$. *Aquaculture* **212**: 311–19.

Ottinger CA, Kaattari SL (1998) Sensitivity of rainbow trout leucocytes to aflatoxin $B_1$. *Fish Shellfish Immunol* **8**: 515–30.

Ottinger CA, Kaattari SL (2000) Long-term immune dysfunction in rainbow trout (*Oncorhynchus mykiss*) exposed as embryos to aflatoxin $B_1$. *Fish Shellfish Immunol* **10**: 101–6.

Randall DJ, Tsui TKN (2002) Ammonia toxicity in fish. *Marine Pollut Bull* **45**: 17–23.

Sahoo PK, Mukherjee SC (2003) Immunomodulation by dietary vitamin C in healthy and aflatoxin B1-induced immunocompromised rohu (*Labeo rohita*). *Comp Immunol Microbiol Infect Dis* **26**: 65–76.

Sciera KL, Isely JJ, Tomasso Jr JR, Klaine SJ (2004) Influence of multiple water-quality characteristics on copper toxicity to fathead minnows (*Pimephales promelas*). *Environ Toxicol Chem* **23**: 2900–5.

Sinnhuber RO, Wales JH (1978) The effects of mycotoxins in aquatic animals. *In Mycotoxic Fungi. Mycotoxins, Mycotoxicoses. An Encyclopedic Handbook*, vol. 2, Wyllie TD, Morehouse LG (ed.). Marcel Dekker, New York, pp. 489–509.

Smutna M, Vorlova I, Svobodova Z (2002) Pathobiochemistry of ammonia in the internal environment of fish (review). *Acta Vet Brno* **71**: 169–81.

Tuan NA, Manning BB, Lovell RT, Rottinghaus GE (2003) Responses of Nile tilapia (*Oreochromis niloticus*) fed diets containing different concentrations of moniliformin or fumonisin $B_1$. *Aquaculture* **217**: 515–28.

Wicks BJ, Randall DJ (2002) The effect of feeding and fasting on ammonia toxicity in juvenile rainbow trout, *Oncorhynchus mykiss*. *Aquat Toxicol* **59**: 71–82.

Wicks BJ, Joensen R, Tang Q, Randall DJ (2002) Swimming and ammonia toxicity in salmonids: the effect of sub lethal ammonia exposure on the swimming performance of coho salmon and the acute toxicity of ammonia in swimming and resting rainbow trout. *Aquat Toxicol* **59**: 55–69.

Yildirim M, Manning BB, Lovell RT, Grizzle JM, Rottinghaus GE (2000) Toxicity of moniliformin and fumonisin $B_1$ fed singly and in combination in diets for young channel catfish *Ictalurus punctatus. J World Aquacult Society* **31**: 599–607.

C H A P T E R

# 59

# Cyanobacterial (blue-green algae) toxins

*Birgit Puschner and Jean-François Humbert*

## INTRODUCTION

Cyanobacterial proliferations in freshwater ecosystems, also known as blooms, can have a significant impact on the health of animals and humans living in or using these systems for drinking water and/or recreational purposes. Among the 2000 species identified on morphological criteria, 40 of them are known to be toxigenic. The first scientific report of cyanotoxin poisoning in animals was made in Australia by Francis in 1878, but a much earlier indication of mammalian cyanotoxin poisoning is estimated to date back to the Pleistocene age, i.e. about 150,000 years BC (Braun and Pfeiffer, 2002). Since this first publication in 1878, numerous case reports describing animal morbidity and mortality exposed to cyanotoxins have been published (Fitzgerald and Poppenga, 1993; Naegeli *et al.*, 1997; Puschner *et al.*, 1998; Gugger *et al.*, 2005). The frequency of blue-green algae poisoning in animals is likely to be underestimated because of the lack of methods to confirm exposure. There are most likely many unreported cases. Proper diagnostic workup of suspect blue-green algae poisoning cases of humans and animals can demand extensive efforts from toxicologists and clinicians. New algal toxins are being discovered continuously and for many, toxicity data, especially after oral exposure, is unavailable. It is possible that blue-green algae poisonings are more common in animals than humans as a result of their behavior and tendency for accidental exposure.

In 1998, after several cases of major human intoxications in Australia, Europe and Brazil (Falconer and Humpage, 2005), the World Health Organization (WHO) proposed a guideline value for the concentration in water of one of the most common cyanotoxin, microcystin-LR. This guideline for drinking water has now been adopted by sanitary authorities of many countries in the European community, and North (Canada) and South America (Brazil). However, the health risks for wild and domestic terrestrial vertebrates resulting from the exposure to cyanotoxins in water are still largely ignored, despite an increasing number of reports of poisoning in the veterinary literature.

## BACKGROUND

The degradation of numerous aquatic ecosystems throughout the world, due to nutrient pollution (phosphorus and nitrogen especially), has led to significant eutrophication ($>$40% in Europe, Asia and America) (Bartram *et al.*, 1999). As a major consequence, photosynthetic microorganisms have ample nutrients available to reach very high biomasses. In addition, the diversity of the phytoplanktonic communities decreases drastically, resulting in the dominance of a very few, generally one or two, numbers of species. In such conditions, cyanobacteria are often the dominant species. Indeed, these microorganisms have developed many adaptive processes to outcompete other phytoplanktonic species and to avoid predation by zooplanktonic organisms (Mur *et al.*, 1999; Oliver and Gant, 1999).

Cyanobacteria are oxygenic photosynthetic prokaryotes that are probably among the oldest form of microorganisms found on earth, some 3 billion years ago. They can be observed as isolated cells (e.g. *Synechococcus*), organized in filaments (e.g. *Planktothrix*) or in colonies (e.g. *Microcystis*). More than 2000 species belonging to four orders are described in the botanical code based on

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morphological and morphometrical criteria (Anagnostidis and Komarek, 1985). But cyanobacteria are also classified under the bacterial code into five sections defined on the combined use of genetic data, morphological criteria and cellular fission (Rippka *et al.*, 1979).

In aquatic ecosystems, different species can be found in the pelagic (water column) or in the benthic (e.g. on the sediments) domains. Typically, blooms of pelagic species occur in mesotrophic and eutrophic ecosystems (concentrations in phosphorus $>30\,\mu g/l$) during the summer, when the

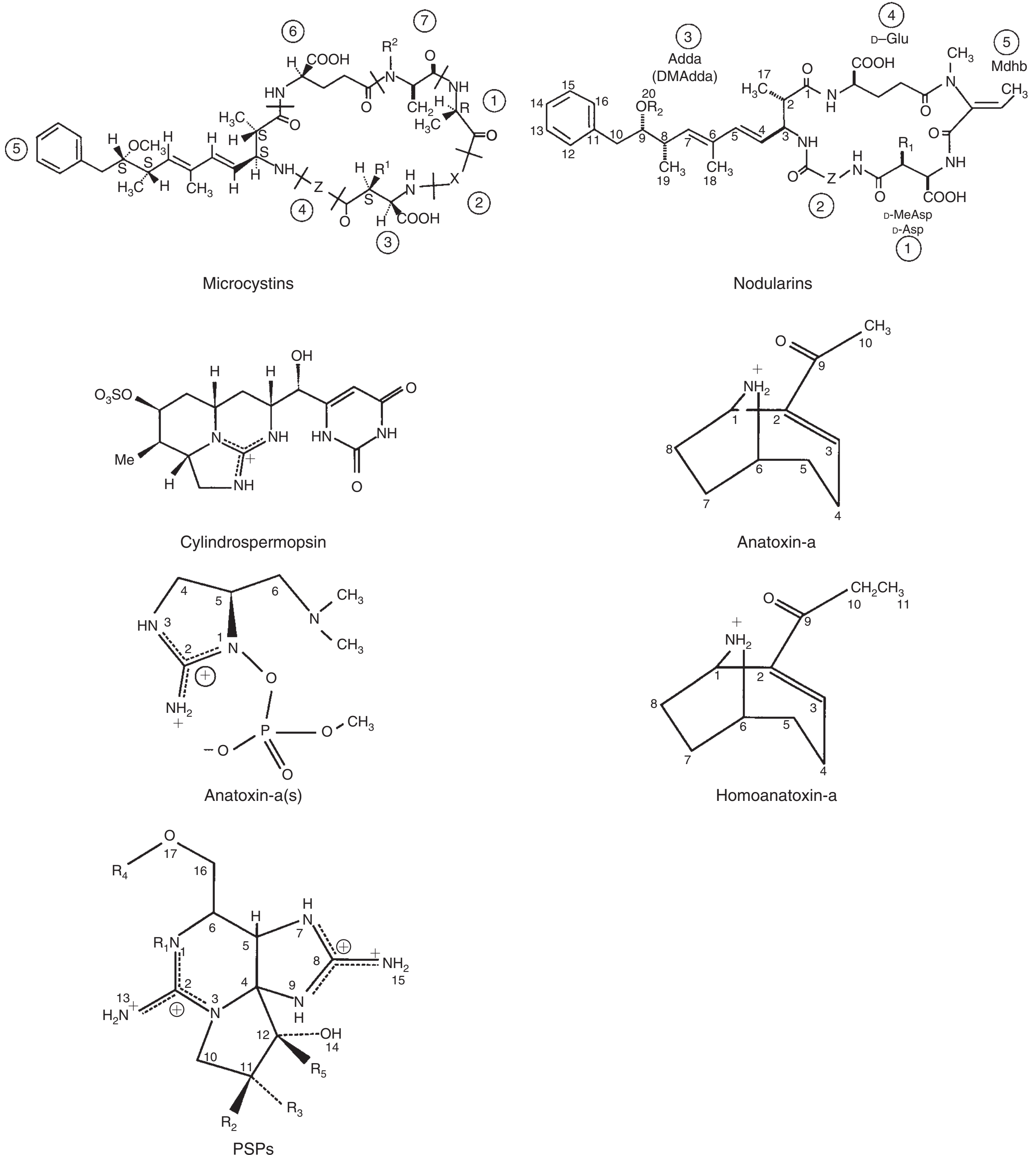


**FIGURE 59.1** Structural formulas of cyanobacterial toxins.

water temperature is warm (>20°C) and in low turbulence conditions (Reynolds and Walsby, 1975). These proliferations are usually easy to detect at the water surface of lakes or ponds, because of evident coloration of the water or the accumulation of scum. Proliferations of benthic species generally occur on the surfaces of sediment, stones or macrophytes in small oligotrophic rivers or in oligotrophic lakes (Mez *et al*., 1997) during the summer.

In the past, most cases were diagnosed by positive identification of the algae in the suspect water source along with the occurrence of consistent clinical signs and pathological findings. However, new analytical methods can now be applied to detect toxins in biological specimensof animals or humans with suspect exposure to toxic algal blooms (Figure 59.1). These capabilities will allow for in-depth diagnostic investigations and a better estimate of the true frequency of blue-green algae poisonings in livestock, pets and wildlife. Table 59.1 provides an overview of cyanobacterial species that are known to produce a large number of toxins. Some species can produce a variety of cyanotoxins and thus, it is difficult to predict the nature and the level of the toxin production during a bloom event. While not inclusive of all cyanotoxins known, this chapter is organized by the various types of toxins, providing detailed information on their toxic mechanisms, toxicokinetics, diagnostic and therapeutic approaches with a focus on veterinary medicine.

**TABLE 59.1 Potential toxins produced by the different cyanobacterial species**

| Cyanobacterial species | Toxins |
|---|---|
| *Anabaena circinalis* | Anatoxin-a, saxitoxins, microcystins |
| *Anabaena flos-aquae* | Anatoxins, microcystins |
| *Anabaena lemmerman* | Microcystins, anatoxin-a(s) |
| *Anabaena planctonica* | Anatoxin-a |
| *Anabaena spiroides* | Anatoxin-a, microcystins |
| *Anabaena* sp. | Anatoxin-a |
| *Anabaenopsis milleri* | Microcystins |
| *Aphanizomenon flos-aquae* | Anatoxin-a, saxitoxins |
| *Aphanizomenon ovalisporum* | Cylindrospermopsin |
| *Aphanizomenon* sp. | Anatoxin-a |
| *Cylindrospermopsis raciborskii* | Cylindrospermopsin, saxitoxins |
| *Cylindrospermum* sp. | Anatoxin-a |
| *Hapalosiphon hibernicus* | Microcystins |
| *Lyngbya gracilis* | Debromoaplysiatoxin |
| *Lyngbya majuscula* | Lyngbyatoxin-a |
| *Lyngbya wollei* | Saxitoxins |
| *Microcystis aeruginosa* | Microcystins |
| *Microcystis botrys* | Microcystins |
| *Microcystis farlowian* | Ichtyotoxin |
| *Microcystis flos-aquae* | Microcystins |
| *Microcystis panniformis* | Microcystins |
| *Microcystis viridis* | Microcystins, microviridin |
| *Microcystis wesenbergii* | Microcystins |
| *Microcystis* sp. | Anatoxin-a |
| *Nodularia spumigena* | Nodularins |
| *Nostoc* sp. | Microcystins |
| *Oscillatoria formosa* | Homoanatoxin-a |
| *Oscillatoria limosa* | Microcystins |
| *Oscillatoria tenuis* | Microcystins |
| *Oscillatoria nigroviridis* | Oscillatoxin-a |
| *Oscillatoria* sp. | Anatoxin-a |
| *Phormidium favosum* | Anatoxin-a |
| *Planktothrix agardhii* | Microcystins |
| *Planktothrix mougeotii* | Microcystins |
| *Planktothrix rubescens* | Microcystins |
| *Planktothrix* sp. | Anatoxin-a |
| *Pseudanabaena* sp. | Neurotoxin |
| *Raphidiopsis* sp. | Cylindrospermopsin |
| *Schizothrix calcicola* | Aplysiatoxins |
| *Scytonema hofmanni* | Scytophycins a and b |
| *Scytonema pseudohofmanni* | Scytophycins a and b |
| *Symploca muscorum* | Aplysiatoxin |
| *Trichodesmium erythraeum* | Neurotoxin |
| *Umezakia natans* | Cylindrospermopsin |
| *Woronichinia naegeliana* | Anatoxin-a |

## MICROCYSTINS

Microcystins are produced by several cyanobacteria, including species within the genera *Microcystis*, *Anabaena*, *Planktothrix*, *Nostoc*, *Oscillatoria* and *Anabaenopsis* and have been found worldwide (e.g. Fromme *et al*., 2000; Hitzfeld *et al*., 2000; Ballot *et al*., 2004; Briand *et al*., 2005; Karlsson *et al*., 2005a; Ndetei and Muhandiki, 2005; Agrawal *et al*., 2006). Not all strains produce microcystins and only those that possess genes for microcystin production are considered toxicologically significant. In addition to genetic factors, the toxin production is influenced by a variety of environmental factors, such as pH, nutrient concentrations and water temperature. The production of microcystins increases with increased water temperature and elevated concentrations of phosphorus and nitrogen, and globally with the growth rate (Orr and Jones, 1998; Briand *et al*., 2005; Downing *et al*., 2005). Therefore, microcystin prevalence may be greatest in July and August when the growth of cyanobacteria is high, but the toxins may be detected any time of year. While most microcystin-producing algal blooms are most commonly found in freshwater, they have also occurred in saline ecosystems (Atkins *et al*., 2001; Carmichael and Li, 2006).

Microcystins are cyclic heptapeptides that cause hepatotoxicosis (Falconer *et al*., 1988; Falconer and Yeung, 1992). Under field conditions, the toxins are normally confined within the algal cell and are released only when the cell is damaged leading to cell lysis and death. Interestingly, the amount of microcystins may increase after a large decrease in the number of algal mats since the toxin is released after destruction of the algal cell wall (Park *et al*., 1998). After oral exposure to microcystin-containing algae, the acidic environment of the stomach can result in the release of microcystins. Recently, there has been increased concern

with the potential exposure to microcystins via blue-green algae food supplements (Schaeffer *et al.*, 1999; Dietrich and Hoeger, 2005).

Over the past years, over 80 different microcystins were isolated from various genera of cyanobacteria (Luukkainen *et al.*, 1994; Lawton *et al.*, 1995; Welker and von Döhren, 2006). The general basic structure for all microcystins is the same and involves an amino acid called ADDA (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid) and six other amino acids, of which two are variable (Figure 59.1). Microcystin-LR is the most extensively studied, has been found worldwide and has caused acute, lethal hepatotoxicoses in farm animals (Carbis *et al.*, 1994; Mez *et al.*, 1997; Puschner *et al.*, 1998) and hepatic injury in humans (Carmichael *et al.*, 2001; Rao *et al.*, 2002). This congener is also known to characterize more than 70% of the microcystins present in a sample (Lawton *et al.*, 1995).

## Pharmacokinetics/toxicokinetics

There is limited data available. Most studies have been conducted in mice after intravenous (i.v.) and intraperitoneal (i.p.) administration of cyanobacteria, their filtrates and in some instances, purified microcystins. After i.v. and i.p. administration in mice and rats, microcystins are rapidly distributed to the liver (Falconer *et al.*, 1986; Runnegar *et al.*, 1986; Robinson *et al.*, 1991). Plasma half-lives of microcystin-LR in mice after i.v. administration were 0.8 and 6.9 min for the alpha- and beta-phases of elimination (Robinson *et al.*, 1991). Interestingly, the hepatic concentration of 3H-microcystin-LR remained constant throughout the 6-day study indicating the accumulation in this target organ. This study also demonstrated that approximately 9% and 14% of the dose were excreted in urine and feces after 12 h, with 60% of it being excreted unchanged. To our knowledge, there are very few studies about the metabolism of microcystins and results are conflicting (Robinson *et al.*, 1991; Rinehart *et al.*, 1994). Glutathione and cysteine conjugation with microcystins has been confirmed and may represent major detoxification pathways (Kondo *et al.*, 1996; Pflugmacher *et al.*, 1998). In addition, other metabolites have been identified, but further work is needed.

Definite data on bioavailability for microcystins is lacking, although it has been shown that absorption takes place in the small intestine (Ito *et al.*, 2000). Therefore, the integrity of the small intestinal mucosa can significantly affect the degree of absorption. In mice, aging has shown to result in altered epithelial cell permeability of the small intestine, making aged mice much more susceptible to orally administered microcystins than young animals (Ito *et al.*, 1997). Once absorbed, microcystins are rapidly distributed to the liver (Runnegar *et al.*, 1981; Fischer *et al.*, 2000), but can also reach lung, heart and capillaries (Ito *et al.*, 2000). Recently, it was shown that there is considerable absorption of microcystin via the respiratory route (Ito *et al.*, 2001) leading to lethality in mice.

## Mechanism of action

Microcystins are specifically toxic to liver, causing liver enlargement, progressive centrilobular hepatocyte rounding, dissociation and necrosis. Breakdown of the sinusoidal endothelium and intrahepatic hemorrhage ultimately result in death (Hooser *et al.*, 1991a; Falconer and Yeung, 1992). Microcystins are unable to cross cell membranes and enter hepatocytes via the bile acid transporter mechanism (Hooser *et al.*, 1991b). Once inside the hepatocytes, microcystins are potent inhibitors of protein phosphatases 1 and 2A (Falconer and Yeung, 1992; Runnegar *et al.*, 1993). The disruption of the cytoskeletal components and the associated rearrangement of filamentous actin within hepatocytes account for the morphological changes, although other mechanisms play a role in the development of liver lesions. Recently, it was shown that microcystins can induce apoptosis of hepatocytes (McDermott *et al.*, 1998) via induction of free radical formation and mitochondrial alterations (Ding Nam Ong, 2003). In addition, microcystins are classified as tumor-promoting compounds (Humpage and Falconer, 1999). Clinical signs of microcystin poisoning have been described in a number of reports in livestock, humans and wildlife in the United States (Galey *et al.*, 1987; Kerr *et al.*, 1987; DeVries *et al.*, 1993; Fitzgerald and Poppenga, 1993; Puschner *et al.*, 1998) and other countries (Odriozola *et al.*, 1984; Done and Bain, 1993; Van Halderen *et al.*, 1995; Mez *et al.*, 1997; Naegeli *et al.*, 1997; Azevedo *et al.*, 2002; Ballot *et al.*, 2004; Ndetei and Muhandiki, 2005). While genetic and environmental conditions are important for the production of high microcystin concentrations, laboratory animals preferred water contaminated with a microcystin-producing algal bloom (Lopez Rodas and Costas, 1999) over uncontaminated water. This preference increases the risk for microcystin toxicosis in animals. Microcystin intoxication should be suspected in cases of acute hepatotoxicosis with clinical signs of diarrhea, vomiting, weakness, pale mucous membranes and shock. While most animals die within a few hours of exposure, some animals may live for several hours and develop hyperkalemia, hypoglycemia, nervousness, recumbency and convulsions. Animals that survive the acute intoxication may develop hepatogenous photosensitization. Nephrotoxic effects have been described in laboratory animals after chronic microcystin exposure (Milutinovic *et al.*, 2003). In humans, primary liver cancer as well as colorectal cancer has been associated with microcystin-contaminated drinking water (Ueno *et al.*, 1996; Zhou *et al.*, 2002). In mice, subchronic exposure by i.p. injection of microcystin-LR (20 μg/kg) causes the appearance of hepatic nodules, which is not observed after oral administration (Ito *et al.*, 1997).

## Toxicity

The lethal doses 50 ("$LD_{50}$"s) for microcystins vary between 50 and 11,000 μg/kg, depending on the microcystin analog, the species affected and the route of administration. In mice, the oral $LD_{50}$ value for microcystin-LR is 10.9 mg/kg, while the i.p. $LD_{50}$ is 50 μg/kg. Considering that most blooms contain a number of structural variants of microcystins, it is difficult to estimate the toxicity potential of a bloom. The NOAEL (no observed adverse effect level) for orally administered microcystin-LR to mice is 40 μg/kg/day (Fawell *et al.*, 1994). In pigs, the LOAEL (lowest observed adverse effect level) for microcystin-LR is 100 μg/kg/day (Falconer *et al.*, 1994) and in rat 50 μg/kg/day (Heinze, 1998). The WHO set the tolerable daily intake (TDI) for human ingestion of microcystin-LR at 0.04 μg/kg/day (Kuiper-Goodman *et al.*, 1999). The potential risk to humans by ingesting food products derived from animals exposed to microcystins was evaluated in beef (Orr *et al.*, 2003) and dairy cattle (Orr *et al.*, 2001). From these studies, it is unlikely that consumption of milk, meat or liver poses a significant health risk to humans. It might be prudent to establish specific guidelines for non-lethal, chronic microcystin exposure in livestock.

## Treatment

There is no specific antidote for microcystins. Despite the evaluation of numerous treatment options, no specific therapy has been proven to be effective. In many cases, the rapid onset of acute hepatotoxicosis does not allow for timely, therapeutic intervention and mortality rates are very high. In mice, the i.p. administration of rifampin was considered an effective treatment after exposure (i.p.) to microcystin-LR (Hermansky *et al.*, 1990b). In contrast, many of the other tested compounds, such as glutathione, silymarin and cyclosporine A, were only beneficial if administered prior to microcystin administration (Hermansky *et al.*, 1990a; Hermansky *et al.*, 1991; Rao *et al.*, 2004). This is important to consider when trying to protect against microcystin intoxication in chronic exposures. Because microcystins can enhance oxidative stress, antioxidants such as vitamin E and selenium appear to be beneficial, but best protection is likely to be achieved if given prior to microcystin exposure (Gehringer *et al.*, 2003a, b). While the adsorption of microcystins by activated charcoal is used successfully to decontaminate drinking water (Warhurst *et al.*, 1997), this decontamination procedure was not protective in mice dosed with microcystins (Mereish and Solow, 1989). Diagnosis of microcystin toxicosis is aided by identification of microcystin-containing water in the environment of the animal. Identification of algae material in water and gastric contents is an important component of the diagnostic workup, but does not confirm intoxication. As described, the toxicity of the cyanobacteria is strain specific, and morphological observations alone cannot predict the hazard level. Detection of microcystins in gastric contents is confirmatory, but these tests are not routinely available at diagnostic laboratories. In the past, the mouse bioassay was used to determine the toxicity of crude algal biomass in suspicious blue-green algae poisonings. While many assays are available to analyze water samples for microcystins (Zweigenbaum *et al.*, 2000; Maizels Budde, 2004; McElhiney and Lawton, 2005; Frias *et al.*, 2006), there are only limited methods available to reliably and accurately detect microcystins in biological specimens collected from animals suspected to have died from microcystin intoxication (Bogialli *et al.*, 2005; Karlsson *et al.*, 2005b). Recently, an ESI-LC-MS (electrospray ionization with liquid chromatography-mass spectroscopy) method was developed to determine the bound microcystin concentrations in animal tissues, which provides an estimate of the total microcystin burden in exposed animals (Ott and Carmichael, 2006).

Differential diagnoses in animals with a clinical presentation of liver failure include other toxic ingestions such as amanitins, cocklebur, cycad palm, aflatoxin, certain heavy metals and acetaminophen overdose. Careful evaluation of the history, feed and environment of the animal can help eliminate most of the toxicant differentials on the list.

# ANATOXINS

Anatoxins are mainly produced by cyanobacteria in the *Anabaena* genus (Beltran and Neilan, 2000), but also by other genera, such as *Planktothrix*, *Oscillatoria*, *Microcystis*, *Aphanizomenon*, *Cylindrospermum* and *Phormidium*. Reports of anatoxin poisoning are less frequent than microcystin toxicosis; however, poisoning has occurred worldwide (Edwards *et al.*, 1992; Gunn *et al.*, 1992; Beltran and Neilan 2000; Fromme *et al.*, 2000; Gugger *et al.*, 2005; Yang and Boyer, 2005). Anatoxins are neurotoxins and can generally be divided into anatoxin-a, homoanatoxin-a and anatoxin-a(s). Anatoxin-a is a secondary amine (Figure 59.1) and has been detected in blooms worldwide. Homoanatoxin-a is a methyl derivative of anatoxin-a (Figure 59.1) and has been identified in blooms in Japan (Namikoshi *et al.*, 2004) and Ireland (Furey *et al.*, 2003). Anatoxin-a(s) is a unique *N*-hydroxyguanidine methyl phosphate ester (Figure 59.1) that has been detected in the Americas (Matsunaga *et al.*, 1989; Monserrat *et al.*, 2001) and Europe (Henriksen *et al.*, 1997).

## Pharmacokinetics/toxicokinetics

Definite data on the toxicokinetics of anatoxin-a, homoanatoxin-a and anatoxin-a(s) have not been

established. Based on the rapid onset of clinical signs after oral exposure, rapid absorption of the toxins is suspected.

## Mechanism of action

Anatoxin-a is potent cholinergic agonist at nicotinic acetylcholine receptors in neurons and at the neuromuscular junctions (Amar *et al.*, 1993; Thomas *et al.*, 1993). Anatoxin-a has two enantiomers and the protonated (+) anatoxin-a form has higher binding affinity than the (−) form (Spivak *et al.*, 1980; Zhang and Nordberg, 1993). Compared to nicotine, anatoxin-a is approximately 20 times more potent than acetylcholine. After continuous electrical stimulation at the neuromuscular junctions, a nerve block may follow and result in death due to respiratory paralysis. Further, anatoxin-a has modulatory action at presynaptic neuronal nicotinic acetylcholine receptors, which can lead to dopamine as well as noradrenaline release (Wonnacott *et al.*, 2000; Barik and Wonnacott, 2006). Clinical signs of anatoxin-a poisoning include a rapid onset of rigidity and muscle tremors followed by paralysis, cyanosis and death. Death usually occurs within minutes to a few hours. Anatoxin-a poisonings have been reported in dogs in Europe (Edwards *et al.*, 1992; Gunn *et al.*, 1992; James *et al.*, 1997; Gugger *et al.*, 2005) and the United Staes (Puschner, personal communication). Anatoxin-a is also considered a contributing factor in the deaths of Lesser Flamingos in Kenya (Krienitz *et al.*, 2003). Homoanatoxin-a is a methyl derivate of anatoxin-a with similar pharmacological and toxicological properties (Wonnacott *et al.*, 1992). In addition to being a nicotinic agonist, homoanatoxin-a can increase the release of acetylcholine from peripheral cholinergic nerves through opening of endogenous voltage-dependent neuronal L-type $Ca^{2+}$ channels (Aas *et al.*, 1996). Poisonings caused by natural algal blooms containing homoanatoxin-a have not been reported in the veterinary literature.

Anatoxin-a(s) is different from anatoxin-a and homoanatoxin-a. This neurotoxin has a unique chemical structure and is a natural occurring irreversible acetylcholinesterase inhibitor. The increased concentrations of acetylcholine in the synapse lead to persistent stimulation, followed by a neuromuscular block (Matsunaga *et al.*, 1989; Cook *et al.*, 1990; Hyde and Carmichael, 1991). The mechanism of toxic action is similar to that of carbamate or organophosphorus insecticides/chemical warfare agents. However, one of the main differences is that anatoxin-a(s) only acts in the periphery, while the insecticides inhibit acetylcholinesterase in the brain and retina (Cook *et al.*, 1989b). Animals poisoned with anatoxin-a(s) show a rapid onset of excessive salivation ("s" stands for salivation), lacrimation, diarrhea and urination. Clinical signs of nicotinic overstimulation include tremors, incoordination, and convulsions. Recumbency and respiratory arrest are most commonly observed in cases with a lethal outcome. The survival time of animals with anatoxin-a(s) poisoning is very short and animals may die within 30 min of exposure. Animals that die from either anatoxin-a or anatoxin-a(s) toxicosis do not show specific gross or microscopic lesions. Anatoxin-a(s) poisoning has been reported in pigs, birds, dogs and calves in the United States and Europe (Mahmood *et al.*, 1988; Cook *et al.*, 1989a; Onodera *et al.*, 1997). Because of the lack of specific detection methods for anatoxin-a(s), the natural occurrence of this neurotoxin has not been fully evaluated.

## Toxicity

In mice, the i.p. $LD_{50}$ of anatoxin-a is 200 μg/kg (Stevens and Krieger, 1991), while the i.v. $LD_{50}$ is estimated at <100 μg/kg. The oral toxicity of anatoxin-a is much higher, with an oral $LD_{50}$ in mice reported as >5000 μg/kg. Several studies have shown that there are significant species differences with regards to anatoxin-a toxicity. While an anatoxin-a containing *A. flos-aquae* bloom was toxic to sheep after i.p. administration, oral administration failed to induce toxicity (Runnegar *et al.*, 1988). In contrast, calves developed toxicity after oral administration of an anatoxin-a containing *A. flos-aquae* (Carmichael *et al.*, 1977). The i.p. $LD_{50}$ of homoanatoxin-a in mice is 250 μg/kg (Skulberg *et al.*, 1992). Anatoxin-a(s) is much more toxic than anatoxin-a or homoanatoxin-a with an i.p. $LD_{50}$ in mice of 20 μg/kg (Mahmood and Carmichael, 1986; Matsunaga *et al.*, 1989; Briand *et al.*, 2003).

## Treatment

There is no specific antidote for anatoxin-a. Because of the rapid onset of clinical signs, emesis is not likely to be useful. Although there are no studies that evaluate the efficacy of specific decontamination procedures, administration of activated charcoal has been recommended. In addition, artificial respiration may be of benefit along with general supportive care. Specific measures to control seizures include benzodiazepines, phenobarbital or pentobarbital. If given, they may cause central nervous system (CNS) and respiratory depression and careful monitoring of the animal is necessary. In any seizuring animal, control of body temperature is an important part of the symptomatic care.

Treatment of animals poisoned with anatoxin-a(s) is primarily symptomatic and supportive. Decontamination procedures can be considered but have not been evaluated. It has been shown that pralidoxime (2-PAM) is not able to reactivate the inhibited acetylcholinesterase and is therefore not recommended (Hyde and Carmichael, 1991). Atropine should be given at a test dose to determine its efficacy in animals with life-threatening clinical signs. After the test dose, atropine can be given repeatedly until

cessation of salivation. It is important to carefully monitor the animal for anticholinergic effects, and to reduce or discontinue atropine should adverse effects develop.

As with other cyanobacteria toxins, toxicity is strain specific and identification of the cyanobacteria alone cannot predict the toxicity level. Therefore, detection of anatoxin-a in biological specimens is confirmatory, but these tests are not routinely available (James *et al*., 1998; Furey *et al*., 2003). Recently anatoxin-a was confirmed in the stomach contents and livers of dogs in France (Gugger *et al*., 2005) and in the stomach contents of dogs in the United States (Puschner, personal communication). Initial difficulties with the analysis for anatoxin-a have been overcome by using tandem mass spectrometry with multiple mass spectrometry experiments (Furey *et al*., 2005). In suspect cases, environmental samples should also be saved for further analysis.

Diagnosis of anatoxin-a(s) toxicosis is aided by the determination of blood acetylcholinesterase activity. However, organophosphorus and carbamate insecticides can also inhibit acetylcholinesterase and additional diagnostic workup is needed to establish a firm diagnosis. These include the determination of brain acetylcholinesterase activity assay postmortem (unchanged in cases of anatoxin-a(s) poisoning), screening of gastrointestinal contents for insecticides, morphological observation of stomach contents (possible identification of cyanobacteria) and a careful evaluation of the environment (access to freshwater, access to insecticides). Detection methods for anatoxin-a(s) are rare. Recently, a biosensor method was developed that allows the quantitation of anatoxin-a(s) in environmental samples (Devic *et al*., 2002). New analytical methods for anatoxin-a(s) are necessary to better document the distribution of this neurotoxin in freshwater worldwide.

## MISCELLANEOUS FRESHWATER CYANOBACTERIAL TOXINS

Although microcystin and anatoxin poisonings make up the majority of cases reported in animals, other cyanotoxins are of concern. Saxitoxins and derived forms (PSPs; see Figure 59.1) belong to the group of paralytic shellfish toxins and have been produced by a number of freshwater cyanobacteria, including *Aphanizomenon flos-aquae*, *Cylindrospermopsis raciborskii*, *Anabanea circinalis*, *Lyngbya wollei* and *Planktothrix* sp. (Humpage *et al*., 1994; Carmichael *et al*., 1997; Sivonen and Jones, 1999; Kaas and Henriksen, 2000; Molica *et al*., 2005). In humans, saxitoxins are associated with the development of a neurological disease after the ingestion of shellfish contaminated with saxitoxins. All saxitoxin analogs have a high toxicity in mammals and act by binding to the voltage-gated sodium channel, followed by inhibition of the influx of sodium ions. This results in a blockade of neuronal transmission leading to respiratory arrest, neuromuscular weakness and cardiovascular shock. While saxitoxin poisonings have not been documented in animals, the risk for exposure certainly exists. Another cyanotoxin of concern to animals is cylindrospermopsin. This alkaloid (Figure 59.1) has resulted in deaths in cattle (Saker *et al*., 1999) and has caused severe gastrointestinal disease in humans. Cylindrospermopsin is a potent inhibitor of protein synthesis and can lead to various degrees of injury to the liver, kidneys, adrenal gland, intestine, lung, thymus and heart (Griffiths and Saker, 2003). Furthermore, this cyanotoxin is of particular concern because of its mutagenic and possibly carcinogenic activities. As to date, cylindrospermopsin has been found in Europe, Australia, New Zealand and Asia (Hawkins *et al*., 1997; Saker and Griffiths, 2001; Fastner *et al*., 2003), but it should be considered a potential worldwide problem. Cylindrospermopsin, deoxycylindrospermopsin and 7-epicylindrospermopsin have been produced by *Cylindrospermopsis raciborskii* for the first two listed (Ohtani *et al*., 1992; Norris *et al*., 1999), and by *Aphanizomenon ovalisporum* for the third (Banker *et al*., 1997). After oral exposure, the $LD_{50}$ of cylindrospermopsin obtained with culture extracts of *C. raciborskii*, ranges from 4.4 to 6.9 mg/kg in equivalent cylindrospermopsin (Seawright *et al*., 1999; Shaw *et al*., 2000). Nodularins are cyclic pentapeptides (Figure 59.1) that lead to severe hepatotoxicosis in the same way as microcystins (Harding *et al*., 1995). In addition, nodularin is a more potent tumor promoter than microcystin (Sueoka *et al*., 1997). The only cyanobacterium species known to produce nodularin is *Nodularia spumigena*. This cyanobacterium can form extensive blooms in the Baltic Sea and in brackish waters in the summer (Francis, 1878; Sivonen *et al*., 1989; Sivonen and Jones, 1999). The risk of nodularin intoxication is 2-fold, because toxin exposure cannot only occur through recreational or drinking water, but also via contamination of seafood (Van Buynder *et al*., 2001). In cases of acute hepatotoxicity, exposure to microcystins as well as nodularins must be considered.

## CONCLUDING REMARKS AND FUTURE DIRECTIONS

Worldwide, the frequency and intensity of harmful cyanobacterial bloom appears to increase in relation with the degradation of the water quality (eutrophication) in numerous freshwater ecosystems. As stated in Table 59.1, numerous cyanobacterial species are potentially able to produce a large range of toxins, but for each of these species, it is very difficult to predict, during a bloom event, the nature and the level of the toxin production. While new detection methods have allowed for better monitoring of potentially harmful blooms over the past few years, there is still a need to develop new methods

and to reliably confirm intoxications. Some of the recently developed methods have been useful in analyzing biological specimens in order to confirm a diagnosis of poisoning, but this is not routinely available or pursued in suspect cases. The lack of methods to confirm exposure is most likely responsible for the low number of reported cases in the veterinary literature over the past 20–30 years. The new analytical methods will provide insight into the true frequency of cyanotoxin poisoning in animals. For example in France, several dog intoxications due to anatoxin-a have been recently identified in different regions, in relation with the development of an analytical method using high-performance liquid chromatography (HPLC) mass spectrometry analysis (Gugger *et al.*, 2005; Mejean, personal communication). Furthermore, more information is needed on the efficacy of therapeutic measures. Until then, in the same way as for human populations, it would be prudent to take safety measures for avoiding animal intoxication, when cyanobacterial blooms occur in ponds used for their water supply.

## REFERENCES

Aas P, Eriksen S, Kolderup J, *et al.* (1996) Enhancement of acetylcholine release by homoanatoxin-a from *Oscillatoria formosa*. *Environ Toxicol Pharmacol* **2**: 223–32.

Agrawal MK, Ghosh SK, Bagchi D, *et al.* (2006) Occurrence of microcystin-containing toxic water blooms in central India. *J Microbiol Biotechnol* **16**: 212–18.

Amar M, Thomas P, Johnson C, *et al.* (1993) Agonist pharmacology of the neuronal alpha 7 nicotinic receptor expressed in *Xenopus* oocytes. *FEBS Lett* **327**: 284–8.

Anagnostidis K, Komarek J (1985) Modern approach to the classification system of cyanophytes. *Arch Hydrobiol Suppl 7*, *Algol Stud* **38/39**: 291–302.

Atkins R, Rose T, Brown RS, *et al.* (2001) The *Microcystis* cyanobacteria bloom in the Swan River – February 2000. *Water Sci Technol* **43**: 107–14.

Azevedo SM, Carmichael WW, Jochimsen EM, *et al.* (2002) Human intoxication by microcystins during renal dialysis treatment in Caruaru, Brazil. *Toxicology* **181–182**: 441–6.

Ballot A, Krienitz L, Kotut K, *et al.* (2004) Cyanobacteria and cyanobacterial toxins in three alkaline rift valley lakes of Kenya – Lakes Bogoria, Nakuru and Elmenteita. *J Plankton Res* **26**: 925–35.

Banker R, Carmeli S, Hadas O, *et al.* (1997) Identification of cylindrospermopsin in *Aphanizomenon ovalisporum* (*Cyanophyceae*) isolated from lake Kinneret, Israel. *J Phycol* **33**: 613–16.

Barik J and Wonnacott S (2006) Indirect modulation by alpha 7 nicotinic acetylcholine receptors of noradrenaline release in rat hippocampal slices: interaction with glutamate and GABA systems and effect of nicotine withdrawal. *Mol Pharmacol* **69**: 618–28.

Bartram J, Carmichael WW, Chorus I, *et al.* (1999) Introduction. In *Toxic Cyanobacteria in Water – a Guide to Their Public Health Consequences, Monitoring and Management*, Chorus I, Bartram J (eds). E & FN Spon, London/New York, pp. 1–14.

Beltran EC, Neilan BA (2000) Geographical segregation of the neurotoxin-producing cyanobacterium *Anabaena circinalis*. *Appl Environ Microbiol* **66**: 4468–74.

Bogialli S, Bruno M, Curini R, *et al.* (2005) Simple assay for analyzing five microcystins and nodularin in fish muscle tissue: hot water extraction followed by liquid chromatography-tandem mass spectrometry. *J Agric Food Chem* **53**: 6586–92.

Braun A, Pfeiffer T (2002) Cyanobacterial blooms as the cause of a Pleistocene large mammal assemblage. *Paleobiology* **28**: 139–4.

Briand JF, Jacquet S, Bernard C, *et al.* (2003) Health hazards for terrestrial vertebrates from toxic cyanobacteria in surface water ecosystems. *Vet Res* **34**: 361–77.

Briand JF, Jacquet S, Flinois C, *et al.* (2005) Variations in the microcystin production of *Planktothrix rubescens* (Cyanobacteria) assessed from a four-year survey of Lac du Bourget (France) and from laboratory experiments. *Microb Ecol* **50**: 418–28.

Carbis CR, Simons JA, Mitchell GF, *et al.* (1994) A biochemical profile for predicting the chronic exposure of sheep to *Microcystis aeruginosa*, an hepatotoxic species of blue-green alga. *Res Vet Sci* **57:** 310–16.

Carmichael WW, Gorham PR, and Biggs DF (1977) Two laboratory case studies on oral toxicity to calves of freshwater cyanophyte (blue-green-alga) *Anabaena flos-aquae* Nrc-44-1. *Can Vet J* **18**: 71–5.

Carmichael WW, Evans WR, Yin QQ, *et al.* (1997) Evidence for paralytic shellfish poisons in the freshwater cyanobacterium *Lyngbya wollei* (Farlow ex Gomont) comb. nov. *Appl Environ Microbiol* **63**: 3104–10.

Carmichael WW, Azevedo SM, An JS, *et al.* (2001) Human fatalities from cyanobacteria: chemical and biological evidence for cyanotoxins. *Environ Health Perspect* **109**: 663–8.

Carmichael WW, Li R (2006) Cyanobacteria toxins in the Salton Sea. *Saline Ecosystems* **2**: 5.

Cook WO, Beasley VR, Lovell RA, *et al.* (1989a) Consistent inhibition of peripheral cholinesterases by neurotoxins from the fresh-water cyanobacterium A*nabaena flos-aquae* – studies of ducks, swine, mice and a steer. *Environ Toxicol Chem* **8**: 915–22.

Cook WO, Dellinger JA, Singh SS, *et al.* (1989b) Regional brain cholinesterase activity in rats injected intraperitoneally with anatoxin-a(s) or paraoxon. *Toxicol Lett* **49**: 29–34.

Cook WO, Iwamoto GA, Schaeffer DJ, *et al.* (1990) Pathophysiologic effects of anatoxin-a(s) in anaesthetized rats: the influence of atropine and artificial respiration. *Pharmacol Toxicol* **67**: 151–5.

Devic E, Li DH, Dauta A, *et al.* (2002) Detection of anatoxin-a(s) in environmental samples of cyanobacteria by using a biosensor with engineered acetylcholinesterases. *Appl Environ Microbiol* **68**: 4102–6.

DeVries SE, Galey FD, Namikoshi M, *et al.* (1993) Clinical and pathologic findings of blue-green algae (*Microcystis aeruginosa*) intoxication in a dog. *J Vet Diagn Invest* **5**: 403–8.

Dietrich D, Hoeger S (2005) Guidance values for microcystins in water and cyanobacterial supplement products (blue-green algal supplements): a reasonable or misguided approach? *Toxicol Appl Pharmacol* **203**: 273–9.

Ding WX, Nam Ong C (2003) Role of oxidative stress and mitochondrial changes in cyanobacteria-induced apoptosis and hepatotoxicity. *FEMS Microbiol Lett* **220**: 1–7.

Done SH, Bain M (1993) Hepatic necrosis in sheep associated with ingestion of blue-green algae. *Vet Rec* **133**: 600.

Downing TG, Sember CS, Gehringer MM, *et al.* (2005) Medium N:P ratios and specific growth rate comodulate microcystin and protein content in *Microcystis aeruginosa* PCC7806 and *M. aeruginosa* UV027. *Microb Ecol* **49**: 468–73.

Edwards C, Beattie KA, Scrimgeour CM, *et al.* (1992) Identification of anatoxin-a in benthic cyanobacteria (blue-green algae) and in associated dog poisonings at Loch Insh, Scotland. *Toxicon* **30**: 1165–75.

Falconer IR, Buckley T, Runnegar MTC (1986) Biological half-life, organ distribution and excretion of I-125 labeled toxic

peptide from the blue-green-alga *Microcystis aeruginosa*. *Aust J Biol Sci* **39**: 17–21.

Falconer IR, Smith JV, Jackson AR, *et al*. (1988) Oral toxicity of a bloom of the cyanobacterium *Microcystis aeruginosa* administered to mice over periods up to 1 year. *J Toxicol Environ Health* **24**: 291–305.

Falconer IR, Yeung DS (1992) Cytoskeletal changes in hepatocytes induced by microcystis toxins and their relation to hyperphosphorylation of cell proteins. *Chem Biol Interact* **81**: 181–96.

Falconer IR, Burch MD, Steffensen DA, *et al*. (1994) Toxicity of the blue-green algae (Cyanobacterium) *Microcystis aeruginosa* in drinking water to growing pigs, as animal model for human injury and risk assessment. *Environ Toxic Water* **9**: 131–9.

Falconer IR, Humpage AR (2005) Health risk assessment of cyanobacterial (blue-green algal) toxins in drinking water. *Int J Environ Res Public Health* **2**: 43–50.

Fastner J, Heinze R, Humpage AR, *et al*. (2003) Cylindrospermopsin occurrence in two German lakes and preliminary assessment of toxicity and toxin production of *Cylindrospermopsis raciborskii* (Cyanobacteria) isolates. *Toxicon* **42**: 313–21.

Fawell JK, James CP, James HA (1994) *Toxins from Blue-Green Algae: Toxicological Assessment of Microcystin-LR and a Method for Its Determination in Water*. Water Research Centre, Medmenham, UK, pp. 1–46.

Fischer WJ, Hitzfeld BC, Tencalla F, *et al*. (2000) Microcystin-LR toxicodynamics, induced pathology, and immunohistochemical localization in livers of blue-green algae exposed rainbow trout (*Oncorhynchus mykiss*). *Toxicol Sci* **54**: 365–73.

Fitzgerald SD, Poppenga RH (1993) Toxicosis due to microcystin hepatotoxins in three Holstein heifers. *J Vet Diagn Invest* **5**: 651–3.

Francis G (1878) Poisonous Australian Lake. *Nature* **18**: 11–12.

Frias HV, Mendes MA, Cardozo KH, *et al*. (2006) Use of electrospray tandem mass spectrometry for identification of microcystins during a cyanobacterial bloom event. *Biochem Biophys Res Commun* **344**: 741–6.

Fromme H, Kohler A, Krause R, *et al*. (2000) Occurrence of cyanobacterial toxins – microcystins and anatoxin-a – in Berlin water bodies with implications to human health and regulations. *Environ Toxicol* **15**: 120–30.

Furey A, Crowley J, Hamilton B, *et al*. (2005) Strategies to avoid the mis-identification of anatoxin-a using mass spectrometry in the forensic investigation of acute neurotoxic poisoning. *J Chromatogr A* **1082**: 91–7.

Furey A, Crowley J, Shuilleabhain AN, *et al*. (2003) The first identification of the rare cyanobacterial toxin, homoanatoxin-a, in Ireland. *Toxicon* **41**: 297–303.

Galey FD, Beasley VR, Carmichael WW, *et al*. (1987) Blue-green algae (*Microcystis aeruginosa*) hepatotoxicosis in dairy cows. *Am J Vet Res* **48**: 1415–20.

Gehringer MM, Downs KS, Downing TG, *et al*. (2003a) An investigation into the effect of selenium supplementation on microcystin hepatotoxicity. *Toxicon* **41**: 451–8.

Gehringer MM, Govender S, Shah M, *et al*. (2003b) An investigation of the role of vitamin E in the protection of mice against microcystin toxicity. *Environ Toxicol* **18**: 142–8.

Griffiths DJ, Saker ML (2003) The palm island mystery disease 20 years on: a review of research on the cyanotoxin cylindrospermopsin. *Environ Toxicol* **18**: 78–93.

Gugger M, Lenoir S, Berger C, *et al*. (2005) First report in a river in France of the benthic cyanobacterium *Phormidium favosum* producing anatoxin-a associated with dog neurotoxicosis. *Toxicon* **45**: 919–28.

Gunn GJ, Rafferty AG, Rafferty GC, *et al*. (1992) Fatal canine neurotoxicosis attributed to blue-green algae (cyanobacteria). *Vet Rec* **130**: 301–2.

Harding WR, Rowe N, Wessels JC, *et al*. (1995) Death of a dog attributed to the cyanobacterial (blue-green algal) hepatotoxin nodularin in South Africa. *J South Afr Vet Assoc – Tydskrif Van Die Suid-Afrikaanse Veterinere Vereniging* **66**: 256–9.

Hawkins PR, Chandrasena NR, Jones GJ, *et al*. (1997) Isolation and toxicity of *Cylindrospermopsis raciborskii* from an ornamental lake. *Toxicon* **35**: 341–6.

Heinze R (1998) Toxicity of the cyanobacterial toxin microcystin-LR to rats after 28 days intake with the drinking water. *Environ Toxicol* **14**: 57–60.

Henriksen P, Carmichael WW, An JS, *et al*. (1997) Detection of an anatoxin-a(s)-like anticholinesterase in natural blooms and cultures of cyanobacteria/blue-green algae from Danish lakes and in the stomach contents of poisoned birds. *Toxicon* **35**: 901–13.

Hermansky SJ, Casey PJ, Stohs SJ (1990a) Cyclosporin A – a chemoprotectant against microcystin-LR toxicity. *Toxicol Lett* **54**: 279–86.

Hermansky SJ, Wolff SN, Stohs SJ (1990b) Use of rifampin as an effective chemoprotectant and antidote against microcystin-LR toxicity. *Pharmacology (Basel)* **41**: 231–6.

Hermansky SJ, Stohs SJ, Eldeen ZM, *et al*. (1991) Evaluation of potential chemoprotectants against microcystin-LR hepatotoxicity in mice. *J Appl Toxicol* **11**: 65–74.

Hitzfeld BC, Lampert CS, Spaeth N, *et al*. (2000) Toxin production in cyanobacterial mats from ponds on the McMurdo ice shelf, Antarctica. *Toxicon* **38**: 1731–48.

Hooser SB, Beasley VR, Waite LL, *et al*. (1991a) Actin filament alterations in rat hepatocytes induced *in vivo* and *in vitro* by microcystin-LR, a hepatotoxin from the blue–green alga, *Microcystis aeruginosa*. *Vet Pathol* **28**: 259–66.

Hooser S B, Kuhlenschmidt MS, Dahlem AM, *et al*. (1991b) Uptake and subcellular localization of titrated dihydro-microcystin-LR in rat liver. *Toxicon* **29**: 589–601.

Humpage AR, Rositano J, Bretag AH, *et al*. (1994) Paralytic shellfish poisons from Australian cyanobacterial blooms. *Aust J Mar Fresh Res* **45**: 761–71.

Humpage AR., Falconer IR (1999) Microcystin-LR and liver tumor promotion: Effects on cytokinesis, ploidy, and apoptosis in cultured hepatocytes. *Environ Toxicol* **14**: 61–75.

Hyde EG, Carmichael WW (1991) Anatoxin-a(s), a naturally occurring organophosphate, is an irreversible active site-directed inhibitor of acetylcholinesterase (EC 3.1.1.7). *J Biochem Toxicol* **6**: 195–201.

Ito E, Kondo F, Harada K (1997) Hepatic necrosis in aged mice by oral administration of microcystin-LR. *Toxicon* **35**: 231–9.

Ito E, Kondo F, Harada K (2000) First report on the distribution of orally administered microcystin-LR in mouse tissue using an immunostaining method. *Toxicon* **38**: 37–48.

Ito E, Kondo F, Harada K (2001) Intratracheal administration of microcystin-LR, and its distribution. *Toxicon* **39**: 265–71.

James KJ, Sherlock IR, Stack MA (1997) Anatoxin-a in Irish freshwater and cyanobacteria, determined using a new fluorimetric liquid chromatographic method. *Toxicon* **35**: 963–71.

James KJ, Furey A, Sherlock IR, *et al*. (1998) Sensitive determination of anatoxin-a, homoanatoxin-a and their degradation products by liquid chromatography with fluorimetric detection. *J Chromatogr A* **798**: 147–57.

Kaas H, Henriksen P (2000) Saxitoxins (PSP toxins) in Danish lakes. *Water Res* **34**: 2089–97.

Karlsson KM, Kankaanpaa H, Huttunen M, *et al*. (2005a) First observation of microcystin-LR in pelagic cyanobacterial blooms in the northern Baltic Sea. *Harmful Algae* **4**: 163–6.

Karlsson KM, Spoof LE, Meriluoto JA (2005b) Quantitative LC-ESI-MS analyses of microcystins and nodularin-R in animal tissue – matrix effects and method validation. *Environ Toxicol* **20**: 381–9.

Kerr LA, McCoy CP, Eaves D (1987) Blue-green algae toxicosis in five dairy cows. *J Am Vet Med Assoc* **191**: 829–30.

Kondo F, Matsumoto H, Yamada S, *et al*. (1996) Detection and identification of metabolites of microcystins formed *in vivo* in mouse and rat livers. *Chem Res Toxicol* **9**: 1355–9.

Krienitz L, Ballot A, Kotut K, *et al*. (2003) Contribution of hot spring cyanobacteria to the mysterious deaths of Lesser Flamingos at Lake Bogoria, Kenya. *FEMS Microbiol Ecol* **43**: 141–8.

Kuiper-Goodman T, Falconer I, Fitzgerald J (1999) Human health aspects. In *Toxic Cyanobacteria in Water – a Guide to Their Public Health Consequences, Monitoring and Management*, Chorus I, Bartram J (eds). World Health Organization, London, UK, pp. 113–53.

Lawton LA, Edwards C, Beattie KA, *et al*. (1995) Isolation and characterization of microcystins from laboratory cultures and environmental samples of *Microcystis aeruginosa* and from an associated animal toxicosis. *Nat Toxins* **3**: 50–7.

Lopez Rodas V, Costas E (1999) Preference of mice to consume *Microcystis aeruginosa* (toxin-producing cyanobacteria): a possible explanation for numerous fatalities of livestock and wildlife. *Res Vet Sci* **67**: 107–10.

Luukkainen R, Namikoshi M, Sivonen K, *et al*. (1994) Isolation and identification of 12 microcystins from four strains and two bloom samples of *Microcystis* spp.: structure of a new hepatotoxin. *Toxicon* **32**: 133–9.

Mahmood NA, Carmichael WW (1986) The pharmacology of anatoxin-a(s), a neurotoxin produced by the freshwater cyanobacterium *Anabaena flos-aquae* NRC 525-17. *Toxicon* **24**: 425–34.

Mahmood NA, Carmichael WW, Pfahler D (1988) Anticholinesterase poisonings in dogs from a cyanobacterial (blue-green algae) bloom dominated by *Anabaena flos-aquae*. *Am J Vet Res* **49**: 500–3.

Maizels M, Budde WL (2004) A LC/MS method for the determination of cyanobacteria toxins in water. *Anal Chem* **76**: 1342–51.

Matsunaga S, Moore RE, Niemczura WP, *et al*. (1989) Anatoxin-a(s), a potent anticholinesterase from *Anabaena flos-aquae*. *J Am Chem Soc* **111**: 8021–3.

McDermott CM, Nho CW, Howard W, *et al*. (1998) The cyanobacterial toxin, microcystin-LR, can induce apoptosis in a variety of cell types. *Toxicon* **36**: 1981–96.

McElhiney J, Lawton LA (2005) Detection of the cyanobacterial hepatotoxins microcystins. *Toxicol Appl Pharmacol* **203**: 219–30.

Mereish KA, Solow R (1989) Interaction of microcystin-LR with SuperChar: water decontamination and therapy. *J Toxicol Clin Toxicol* **27**: 271–80.

Mez K, Beattie KA, Codd GA, *et al*. (1997) Identification of microcystin in benthic cyanobacteria linked to cattle deaths on alpine pastures in Switzerland. *Eur J Phycol* **32**: 111–17.

Milutinovic A, Zivin M, Zorc-Pleskovic R, *et al*. (2003) Nephrotoxic effects of chronic administration of microcystins – LR and YR. *Toxicon* **42**: 281–8.

Molica RJR, Oliveira EJA, Carvalho PVC, *et al*. (2005) Occurrence of saxitoxins and an anatoxin-a(s)-like anticholinesterase in a Brazilian drinking water supply. *Harmful Algae* **4**: 743–53.

Monserrat JM, Yunes JS, Bianchini A (2001) Effects of *Anabaena spiroides* (cyanobacteria) aqueous extracts on the acetylcholinesterase activity of aquatic species. *Environ Toxicol Chem* **20**: 1228–35.

Mur LR, Skulberg OM, Utkilen H (1999) Cyanobacteria in the environment. In *Toxic Cyanobacteria in Water – a Guide to Their Public Health Consequences, Monitoring and Management*, Chorus I, Bartram J (eds). E & FN Spon, London/New York, pp. 15–40.

Naegeli H, Sahin A, Braun U, *et al*. (1997) Sudden deaths of cattle on Alpine pastures in South-Eastern Switzerland. *Schweiz Archiv Tierheilk* **139**: 201–9.

Namikoshi M, Murakami T, Fujiwara T, *et al*. (2004) Biosynthesis and transformation of homoanatoxin-a in the cyanobacterium *Raphidiopsis mediterranea* Skuja and structures of three new homologues. *Chem Res Toxicol* **17**: 1692–6.

Ndetei R, Muhandiki VS (2005) Mortalities of Lesser Flamingos in Kenyan Rift Valley saline lakes and the implications for sustainable management of the lakes. *Lakes Reservoirs Res Manag* **10**: 51–8.

Norris RL, Eaglesham GK, Pierens G, *et al*. (1999) Deoxycylindrospermopsin, an analog of cylindrospermopsin from *Cylindrospermopsis raciborskii*. *Environ Toxicol* **14**: 163–5.

Odriozola E, Ballabene N, Salamanco A (1984) Poisoning in cattle caused by blue-green algae. *Rev Argent Microbiol* **16**: 219–24.

Ohtani I, Moore RE, Runnegar MTC (1992) Cylindrospermopsin: a potent hepatotoxin from the blue-green alga *Cylindrospermopsis raciborskii*. *J Am Chem Soc* **114**: 7941–2.

Oliver RL, Gant GG (1999) Freshwater blooms. In *The ecology of cyanobacteria*, Whitton BA, Potts M (eds). Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 149–94.

Onodera H, Oshima Y, Henriksen P, *et al*. (1997) Confirmation of anatoxin-a(s), in the cyanobacterium *Anabaena lemmermannii*, as the cause of bird kills in Danish lakes. *Toxicon* **35**: 1645–8.

Orr PT, Jones GJ (1998) Relationship between microcystin production and cell division rates in nitrogen-limited *Microcystis aeruginosa* cultures. *Limnol Oceanogr* **43**: 1604–14.

Orr PT, Jones GJ, Hunter RA, *et al*. (2001) Ingestion of toxic *Microcystis aeruginosa* by dairy cattle and the implications for microcystin contamination of milk. *Toxicon* **39**: 1847–54.

Orr PT, Jones GJ, Hunter RA, *et al*. (2003) Exposure of beef cattle to sub-clinical doses of *Microcystis aeruginosa*: toxin bioaccumulation, physiological effects and human health risk assessment. *Toxicon* **41**: 613–20.

Ott JL, Carmichael WW (2006) LC/ESI/MS method development for the analysis of hepatotoxic cyclic peptide microcystins in animal tissues. *Toxicon* **47**: 734–41.

Park HD, Iwami C, Watanabe MF, *et al*. (1998) Temporal variabilities of the concentrations of intra- and extracellular microcystin and toxic microcystis species in a hypertrophic lake, Lake Suwa, Japan (1991–1994). *Environ Toxic Water* **13**: 61–72.

Pflugmacher S, Wiegand C, Oberemm A, *et al*. (1998) Identification of an enzymatically formed glutathione conjugate of the cyanobacterial hepatotoxin microcystin-LR: the first step of detoxication. *BBA-Gen* **1425**: 527–33.

Puschner B, Galey FD, Johnson B, *et al*. (1998) Blue-green algae toxicosis in cattle. *J Am Vet Med Assoc* **213**: 1571, 1605–7.

Rao PV, Gupta N, Bhaskar AS, *et al*. (2002) Toxins and bioactive compounds from cyanobacteria and their implications on human health. *J Environ Biol* **23**: 215–24.

Rao PV, Jayaraj R, Bhaskar AS (2004) Protective efficacy and the recovery profile of certain chemoprotectants against lethal poisoning by microcystin-LR in mice. *Toxicon* **44**: 723–30.

Reynolds CS, Walsby AE (1975) Water-blooms. *Biol Rev* **50**: 437–81.

Rinehart KL, Namikoshi M, Choi BW (1994) Structure and biosynthesis of toxins from blue-green-algae (cyanobacteria). *J Appl Phycol* **6**: 159–76.

Rippka R, Deruelles J, Waterbury JB, *et al*. (1979) Generic assignments, strain histories and properties of pure cultures of cyanobacteria. *J Gen Microbiol* **111**: 1–61.

Robinson NA, Pace JG, Matson CF, *et al*. (1991) Tissue distribution, excretion and hepatic biotransformation of microcystin-LR in mice. *J Pharmacol Exp Ther* **256**: 176–82.

Runnegar MTC, Falconer IR, Silver J (1981) Deformation of isolated rat hepatocytes by a peptide hepatotoxin from the blue-green alga *Microcystis aeruginosa*. *Naunyn Schmiedebergs Arch Pharmacol* **317**: 268–72.

Runnegar MTC, Falconer IR, Buckley T, *et al*. (1986) Lethal potency and tissue distribution of I-125 labeled toxic peptides from the blue-green-alga *Microcystis aeruginosa*. *Toxicon* **24**: 506–9.

Runnegar MTC, Jackson ARB, Falconer IR (1988) Toxicity to mice and sheep of a bloom of the cyanobacterium (blue green-alga) *Anabaena circinalis*. *Toxicon* **26**: 599–602.

Runnegar MT, Kong S, Berndt N (1993) Protein phosphatase inhibition and *in vivo* hepatotoxicity of microcystins. *Am J Physiol* **265**: G224–30.

Saker ML, Thomas AD, Norton JH (1999) Cattle mortality attributed to the toxic cyanobacterium *Cylindrospermopsis raciborskii* in an outback region of north Queensland. *Environ Toxicol* **14**: 179–82.

Saker ML, Griffiths DJ (2001) Occurrence of blooms of the cyanobacterium *Cylindrospermopsis raciborskii* (Woloszynska) Seenayya and Subba Raju in a north Queensland domestic water supply. *Mar Freshwater Res* **52**: 907–15.

Schaeffer DJ, Malpas PB, Barton LL (1999) Risk assessment of microcystin in dietary *Aphanizomenon flos-aquae*. *Ecotoxicol Environ Safe* **44**: 73–80.

Seawright AA, Nolan CC, Shaw GR, *et al*. (1999) The oral toxicity for mice of the tropical cyanobacterium *Cylindrospermopsis raciborskii* (Woloszynska). *Environ Toxicol* **14**: 135–42.

Shaw GR, Seawright AA, Moore MR, *et al*. (2000) Cylindrospermopsin, a cyanobacterial alkaloid: evaluation of its toxicologic activity. *Ther Drug Monit* **22**: 89–92.

Sivonen K, Himberg K, Luukkainen R, *et al*. (1989) Preliminary characterization of neurotoxic cyanobacteria blooms and strains from Finland. *Toxic Assess* **4**: 339–52.

Sivonen K, Jones G (1999) Cyanobacterial toxins. In *Toxic Cyanobacteria in Water – a Guide to Their Public Health Consequences, Monitoring and Management*, Chorus I, Bartram J (eds). E & FN Spon, London/New York, pp. 41–111.

Skulberg OM, Carmichael WW, Andersen RA, *et al*. (1992) Investigations of a neurotoxic oscillatorialean strain (*Cyanophyceae*) and its toxin – isolation and characterization of homoanatoxin-A. *Environ Toxicol Chem* **11**: 321–9.

Spivak CE, Witkop B, Albuquerque EX (1980) Anatoxin-a: a novel, potent agonist at the nicotinic receptor. *Mol Pharmacol* **18**: 384–94.

Stevens DK, Krieger RI (1991) Effect of route of exposure and repeated doses on the acute toxicity in mice of the cyanobacterial nicotinic alkaloid anatoxin-a. *Toxicon* **29**: 134–38.

Sueoka E, Sueoka N, Okabe S, *et al*. (1997) Expression of the tumor necrosis factor alpha gene and early response genes by nodularin, a liver tumor promoter, in primary cultured rat hepatocytes. *J Cancer Res Clin Oncol* **123**: 413–19.

Thomas P, Stephens M, Wilkie G, *et al*. (1993) (+)-Anatoxin-a is a potent agonist at neuronal nicotinic acetylcholine receptors. *J Neurochem* **60**: 2308–11.

Ueno Y, Nagata S, Tsutsumi T, *et al*. (1996) Detection of microcystins, a blue-green algal hepatotoxin, in drinking water sampled in Haimen and Fusui, endemic areas of primary liver cancer in China, by highly sensitive immunoassay. *Carcinogenesis* **17**: 1317–21.

Van Buynder PG, Oughtred T, Kirkby B, *et al*. (2001) Nodularin uptake by seafood during a cyanobacterial bloom. *Environ Toxicol* **16**: 468–71.

Van Halderen A, Harding WR, Wessels JC, *et al*. (1995) Cyanobacterial (blue-green algae) poisoning of livestock in the Western Cape Province of South Africa. *J S Afr Vet Assoc* **66**: 260–4.

Warhurst AM, Raggett SL, McConnachie G L, *et al*. (1997) Adsorption of the cyanobacterial hepatotoxin microcystin-LR by a low-cost activated carbon from the seed husks of the pan-tropical tree, *Moringa oleifera*. *Sci Total Environ* **207**: 207–11.

Welker M, von Döhren H (2006) Cyanobacterial peptides – nature's own combinatorial biosynthesis. *FEMS Microbiol Rev* **38**: 530–63.

Wonnacott S, Swanson KL, Albuquerque EX, *et al*. (1992) Homoanatoxin: a potent analogue of anatoxin-A. *Biochem Pharmacol* **43**: 419–23.

Wonnacott S, Kaiser S, Mogg A, *et al*. (2000) Presynaptic nicotinic receptors modulating dopamine release in the rat striatum. *Eur J Pharmacol* **393**: 51–8.

Yang X, Boyer GL (2005) Couf in Ann Arbor, MI Occurrence of the cyanobacterial neurotoxin, anatoxin-a, in lower Great Lakes. Ann Arbor, MI. *IAGLR Conf Program Abstract* **48**: 203–4.

Zhang X, Nordberg A (1993) The competition of (-)-[3H]nicotine binding by the enantiomers of nicotine, nornicotine and anatoxin-a in membranes and solubilized preparations of different brain regions of rat. *Naunyn Schmiedebergs Arch Pharmacol* **348**: 28–34.

Zhou L, Yu H, Chen K (2002) Relationship between microcystin in drinking water and colorectal cancer. *Biomed Environ Sci* **15**: 166–71.

Zweigenbaum JA, Henion JD, Beattie KA, *et al*. (2000) Direct analysis of microcystins by microbore liquid chromatography electrospray ionization ion-trap tandem mass spectrometry. *J Pharm Biomed Anal* **23**: 723–33.

CHAPTER

# 60

# Toxicology of marine toxins

*Aurelia Tubaro and James Hungerford*

## INTRODUCTION

Most marine toxins, tetrodotoxins being the exception, are produced by microalgae. These are the same primary producers that make up the basis of the marine food chain. Just as with many plants, bacteria and other eukaryotic organisms, many microalgae produce compounds with potent biological activity. These compounds are generally considered secondary metabolites, not essential for the basic metabolism and growth of the producing species. The role of secondary metabolites in these organisms is not yet completely clear and many functions have been hypothesized, for instance as chemical defences. This has been suggested for algal toxins, a class of secondary metabolites produced primary from three classes of unicellular algae: dinoflagellates, diatoms and cyanobacteria. Dinoflagellates are responsible for the production of the majority of these toxins, although only a few dozen species out of the several thousands of dinoflagellates identified so far are known to produce toxic metabolites. Among the diatoms, only the genus *Pseudonitzschia* produces toxic compounds. Cyanobacteria toxins are discussed elsewhere in this book.

Of the many marine algal toxins identified so far, some are responsible for human poisonings, marine mammals and birth morbidity and mortality, as well as for extensive fish kills, causing sanitary, ecological and economic problems (Van Dolah, 2000). An important distinction between envenoming and poisoning is necessary to marine poisoning. Venomous animals have a specialized gland that produces venom, generally applied or injected parenterally. Other marine animals like those described here accumulate toxic compounds from the environment, without possessing any specialized toxin producing gland. These animals can provoke human poisonings after consumption (Isbister and Kiernan, 2005).

Shellfish contamination represents a sanitary and economical problem all over the world. Although viral and bacterial infections resulting from shellfish ingestion are more common, shellfish contaminated by algal toxins can cause severe and life-threatening poisonings. These poisonings account for 7.4% of marine intoxications in the United States (Isbister and Kiernan, 2005). Human poisonings associated with marine algal toxins follow ingestion of (primarily molluscan) shellfish. These vectors accumulate the toxic compounds during their filter feeding activity.

Marine toxins are not exclusively shellfish toxins however. Fish can also be contaminated after eating toxic phytoplankton, as in the case of domoic acid (DA)-laden anchovies, or ciguatoxic herbivores inhabiting tropical reefs. The marine toxins also move through food webs, and in the process can be transformed to other more potent toxins as hypothesized for highly ciguatoxic predators that dwell near impacted reefs, eating those same herbivores. In spite of these biologically mediated changes in the natural world, the toxins are otherwise resilient. Most marine toxins are heat and gastric-acid-stable compounds.

The marine toxins represent a structurally and pharmacologically diverse group. Most, but not all of the marine toxins exert their toxic effects by disrupting the nervous system. Other toxins impact protein phosphorylation or other critical processes. Their relative toxicities also vary considerably. Among the neurotoxins, there is again considerable diversity, but those causing the greatest numbers of illnesses and some deaths, the ciguatoxins (CTXs) and the saxitoxins (STXs), have one trait in common, which is their binding and modulation of the voltage gated sodium channel (VGSC). One group enhances sodium permeability while the other blocks it. All however are of public health concern.

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No matter what is the specific mechanism of toxicity, many of the marine toxins present the same limitations and challenges in both toxicological studies and in their management. Specifically, toxicological studies and other efforts addressing these and other algal toxins are limited by the reduced availability of large quantities of purified toxins and especially of certified reference materials. Often these are not commercially available. Therefore, in many of the toxicological studies a reduced number of animals are used and sometimes, to maximize the toxicological effects of the test material, parenteral routes of administration are employed. On the contrary, very few studies have examined the effects of oral administration of these toxins, the usual exposure route. In this chapter we will focus on the toxicology of the most dangerous marine toxins for humans. Specifically, the major clinical poisonings due to the consumption of fish and shellfish will be covered.

## SAXITOXINS

Saxitoxins (STXs), a family of hydrophilic toxins based on the parent STX were discovered in the last century and can contaminate seafood and drinking water. The name of this toxin group is derived from *Saxidomus giganteus*, the clam from which it was purified for the first time. The ingestion of STXs-contaminated seafood provokes a paralytic syndrome known as paralytic shellfish poisoning (PSP), one of the most common and most dangerous forms of shellfish poisoning. Although PSP cannot be classified as a major public health problem due to its relatively low incidence rate, it is of considerable concern because a fatal dose for humans can be obtained eating a single highly contaminated shellfish. There is no available antidote, although supportive treatment, if done promptly, is generally sufficient for this potent but reversibly binding group of toxins (see Treatment below). Furthermore, STX is the only marine natural product that is a declared chemical weapon (Llewellyn, 2006).

### Background

Persons suffering PSP can develop symptoms within 5–30 min after contaminated shellfish consumption. The first effect is typically paraesthesia, with burning or tingling of the tongue and lips, which spreads to the face, neck, fingers and toes. In more severe cases, this is followed by a feeling of numbness in fingertips and toes, which progresses to the arms, legs and neck within 4–6 h. Death is usually caused by respiratory paralysis and, without mechanical respiratory support, the fatality rate is 5–10%. There is a good prognosis for individuals surviving beyond 12 h, although muscular weakness can persist following recovery (Llewellyn, 2006).

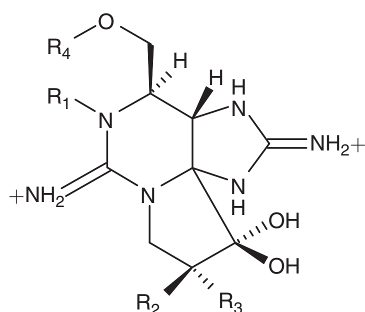
Other minor symptoms reported are: a feeling of dizziness or "floating" (owing to distortion of sensation and proprioception), generalized paraesthesia, arm and leg weakness, and ataxia. Headache, nausea and vomiting, also can occur in the initial phase of the poisoning (Isbister and Kiernan, 2005; Llewellyn, 2006). Reflexes may be normal or absent, and patients may remain conscious and alert throughout the poisoning. In patients with mild to moderate poisoning, the symptoms resolve in 2–3 days, but in more severe cases weakness may persist for a week (Isbister and Kiernan, 2005).

PSP diagnosis is based on characteristic neurological clinical signs and on a history of recent ingestion of seafood (most often bivalve shellfish, hence the term PSP). The symptoms of PSP are difficult to differentiate from those of tetrodotoxin poisoning, due to the (pharmacological) similarities of the toxins.

The toxins responsible for PSP, STX and its derivatives, including gonyautoxins (GTXs) are water soluble, heat stable, tetrahydropurine compounds that are among the most potent known neurotoxins (Isbister and Kiernan, 2005). The chemical structure of STX is represented by two guanidinium moieties joined in a stable azaketal linkage (Figure 60.1) (Rogers and Rapoport, 1980; Llewellyn, 2006; Sapse *et al.*, 2006). More than 30 natural STX analogues, that vary by different combination of hydroxyl and sulphate substitutions at four sites on the molecule, have been isolated so far. STXs can be divided into four groups based on substitution at $R_4$, the carbamate toxins, the sulphocarbamoyl toxins, the decarbamoyl toxins and the deoxydecarbamoyl toxins. The guanidinium group at positions C-7, C-8 and C-9 and the hydroxyl group at C-12 are essential for binding of STXs to $Na^+$ channels and the substitution at $R_4$ leads to substantial changes in toxicity, the carbamate toxins representing the most potent group (Ciminiello and Fattorusso, 2004).

The toxins are produced by toxic marine dinoflagellates, including *Alexandrium* ssp., *Pyrodinium bahamense* var. *compressum* and *Gymnodinium catenatum.* Shellfish accumulate the toxins by filter feeding the toxic microlagae. Paralytic toxins have been identified also in different species of freshwater blue-green algae (cyanobacteria), but no human poisoning has been reported so far (Isbister and Kiernan, 2005). Also red algae species of the genus *Jania* were reported to produce STX. Controversial is the production of STXs by a symbiotic bacteria isolated from *A. tamarense* (Llewellyn, 2006).

STX and its analogues are concentrated by bivalve shellfish such as clams, oysters, mussels and scallops, but also by crabs, puffer fish, gastropods and cephalopods. Shellfish contamination can persist for a long time after exposure to the toxic microalgae, although in most cases bivalve




| Toxin | $R_1$ | $R_2$ | $R_3$ | $R_4$ |
|---|---|---|---|---|
| STX | H | H | H | |
| neoSTX | OH | H | H | |
| GTX–1 | OH | H | $OSO_3^-$ | $H_2N$–CO |
| GTX–2 | H | H | $OSO_3^-$ | |
| GTX–3 | H | $OSO_3^-$ | H | |
| GTX–4 | OH | $OSO_3^-$ | H | |
| dcSTX | H | H | H | |
| dcNEO | OH | H | H | |
| dcGTX–1 | OH | H | $OSO_3^-$ | H |
| dcGTX–2 | H | H | $OSO_3^-$ | |
| dcGTX–3 | H | $OSO_3^-$ | H | |
| dcGTX–4 | OH | $OSO_3^-$ | H | |
| B1 (GTX–5) | H | H | H | |
| B2 (GTX–6) | OH | H | H | |
| C–3 | OH | H | $OSO_3^-$ | $^-O_3S$–NH–COO |
| C–1 | H | H | $OSO_3^-$ | |
| C–2 | H | $OSO_3^-$ | H | |
| C–4 | OH | $OSO_3^-$ | H | |

**FIGURE 60.1** Chemical structures of saxitoxin and some of its analogues (from Luckas *et al.*, 2003).

molluscs clear the toxin quickly enough to allow later reopening of harvest areas.

While Paralytic shellfish intoxication is widely distributed, crustacean-induced PSP seems to be concentrated in the tropics, where predator crabs of the Xanthidae family often are contaminated by paralytic toxins and have caused human deaths. Recently in Florida, puffer fish thought to be *Sphoeroides nephelus*, provoked the poisoning of over 12 people due to the presence of large amounts of paralytic toxins (Llewellyn, 2006). Occurrence of STX as the major toxin in another puffer fish (*Arothron firmamentum*) has also been recently reported (Nakashima *et al.*, 2004).

Shellfish contamination by STXs is a global concern, being endemic in north-western America. PSP cases also occurred in Chile, Taiwan, Japan, South Africa, Australia, India, England, Guatemala, Costa Rica, Singapore, Canada, Spain and Mexico (Gessner, 2000; Llewellyn, 2006).

## Pharmacokinetics/toxicokinetics

Although the mechanism of action of STXs is well established at the molecular level, little is known about their absorption, distribution, biotransformation and elimination. The charged nature of STXs makes these molecules poor candidates for oral availability, but their uptake from the gut occurs within minutes from ingestion, and no toxin is eliminated in the faeces of test animals (Llewellyn, 2006). Studies in animals and evidences from PSP poisonings in humans revealed that STXs are eliminated mainly through the urine (Stafford and Hines, 1995; Gessner *et al.*, 1997; Andrinolo *et al.*, 1999, 2002). The half-life of urinary elimination of a sublethal dose of STX in rats (2 μg/kg, intravenously (i.v.)) is about 90 min (Stafford and Hines, 1995). Urinary elimination of STX was demonstrated also in cats: after i.v. injection of 2.7 or 10 μg/kg STX excretion involved glomerular filtration which dropped to low levels at the highest dose of STX due to its hypotensive effect. The study also showed the presence of the toxin in liver, spleen and brain (Andrinolo *et al.*, 1999). After oral administration in cats, GTX 2/3 epimers (70 μg/kg) were completely absorbed at intestinal level and, similarly to STX, the excretion involved the glomerular filtration (Andrinolo *et al.*, 2002).

Metabolic transformation of paralytic shellfish toxins by mammals has been little studied. Serum and urine of poisoned humans revealed the occurrence of GTX2 sulphation, which is in contrast with the findings in rats, where no metabolism of STX occurs during passage in urine (Llewellyn, 2006). In another case report, STX was found mainly in the gut contents whereas the urine contained only 50% STX, with neoSTX and dcSTX becoming quite prominent (Llewellyn, 2006). The marked difference between the toxin compositions in the ingested mussels and in human sera, as well as the similar toxin profile between sera and urine samples suggest that PSP toxins are metabolized at gastrointestinal and/or hepatic levels (Gessner *et al.*, 1997). Anyway, it has to be considered that the transformations of the PSP toxins in mammals can be the consequence of both biochemical metabolism and the conditions to which the toxins are exposed, such as the gastric juice, which can convert small amounts of the less toxic sulphated PSP toxins to more potent compounds (Llewellyn, 2006). Cleareance of PSP toxins from human serum was evident within 24 h (Gessner *et al.*, 1997).

## Mechanism of action

STX is a potent neurotoxin that selectively blocks the sodium channels, preventing the entry of $Na^+$ ions into cells, preventing nerve conduction and resulting in motor and sensory nerve abnormalities. All the STX analogues bind to the same (site 1) receptor, although with different affinities (Isbister and Kiernan, 2005).

## Toxicity

### *Human toxicity*

STXs provokes potentially lethal PSP. The most common symptom of PSP, occurring in almost all affected people, is perioral paraesthesias, described as either numbness or tingling. Among people displaying more severe illness, in some cases gastrointestinal symptoms (nausea, vomiting, abdominal pain and diarrhoea) were reported. More severe intoxication leads to a variety of neurological symptoms, including weakness, dysarthria, diplopia, ataxia and vertigo or dizziness, and, in some cases, in respiratory arrest or death. It is unclear whether PSP provokes significant direct effects on the myocardium (Lagos and Andrinolo, 2000). Both diastolic and systolic hypertension have been reported in almost all the 11 PSP intoxicated patients studied by Gessner *et al.* (1997). Another previous report referred hypertension in three PSP patients from England. Experimental studies in cats and dogs emphasized a hypotensive effect after i.v. exposure to high doses of STX and an hypotensive effect at low doses (Lagos and Andrinolo, 2000). Deaths result from respiratory arrest and collapse, the terminal symptoms in patients who do not have access to medical care. No long-term clinical effects of PSP have been reported so far (Gessner, 2000).

Several PSP incidents have been recorded all over the world. The exposure to STX was estimated, examining about 20 incidents of poisoning occurred in Canada, between 1970 and 1990, and involving about 60 persons (age 3–72 years). The symptoms were classified as mild, moderately severe or extremely severe. Mild cases were generally associated with an STX exposure of 2–30 μg/kg, whereas in the more severe cases an STX exposure >10–300 mg/kg was involved. Based on these data a lowest observable adverse effect level (LOAEL) of 2 μg/kg was established (FAO/IOC/WHO, 2005).

### *Experimental toxicity*

#### *Single administration*

The mouse $LD_{50}$ (median lethal dose) values of STX by intravenous (i.v.), intraperitoneal (i.p.) and oral routes are 3.4, 10 and 263 μg/kg, respectively. Significant species differences in oral toxicity have not been observed (Andrinolo *et al.*, 1999; Llewellyn, 2006). The potency of different STXs varies widely. After i.p. administration in mice, the carbamates and the decarbamoyl derivatives are the most toxic PSP toxins, while the sulphocarbamoyls derivatives exert the lowest acute toxicity (Llewellyn, 2006).

## Treatment

The treatment for PSP is only supportive. Admission to an intensive care unit is necessary in moderate to severe cases to prevent respiratory failure: artificial respiration must be prompt and is the most vital treatment in many cases. Patients have to be carefully observed in the early stages of poisoning to immediately recognize and treat the progressing paralysis and respiratory failure.

## Concluding remarks

PSP due to the intake of toxins from the STXs group has provoked many fatalities. Since a specific antidote is not available for PSP, prevention is important and monitoring programmes are carried out in many countries. A regulatory level of 0.8 mg STX equivalents/kg shellfish meat has been set up both in North America, in European Community as well as in many other countries worldwide. The AOAC Int. validated mouse bioassay (MBA) has been widely used in monitoring programmes and has provided health protection (FAO/IOC/WHO, 2005). Three mice are intraperitoneally (i.p.) injected with the contaminated shellfish extracts, and their survival time compared to that of mice injected with different concentrations of STX. The detection limit is only 0.4 mg STX equivalents/g shellfish meat (Luckas, 1992). Various other methods have been developed to detect the paralytic toxins (such as receptor binding assay, immunoassay and instrumental methods). The recent AOAC validated Lawrence pre-column liquid chromatography (LC) fluorescence method (Lawrence *et al.*, 2005) can provide nearly full coverage of STXs and complete coverage of the most toxic forms. In Europe it was approved in 2006, with some minor restrictions, as an alternative method to the MBA in monitoring programmes.

# TETRODOTOXIN

## Background

Puffer fish are an esteemed delicacy in Japan, despite the presence of a lethal toxin, tetrodotoxin, in the gonad, liver,

viscera and skin. Tetrodotoxin poisoning is the commonest lethal marine poisoning. Its clinical effects develop rapidly: early symptoms are sensory, including perioral and distal numbness and paraesthesia. In *mild* poisoning cases, only sensory symptoms associated with minor gastrointestinal effects (mainly nausea and vomiting) occur. In *moderately severe* poisonings, patients develop distal muscle weakness and facial weakness, and later, ataxia and incoordination with normal reflexes. Dizziness can also be accompanied by sensation of floating. *Severe* poisoning provokes generalized flaccid paralysis, respiratory failure and aphonia. In *severe and life-threatening* poisoning cardiovascular effects (bradycardia, hypotension and dysrhythmias) occur together with respiratory failure and coma. Patients with severe poisoning rapidly progress to flaccid paralysis. Most moderate and severe cases generally resolve after 5 days or longer in severe poisoning. In minor cases the recovery can be complete in few hours (Isbister and Kiernan, 2005).

The symptoms are very similar to those of PSP, due to the similar mechanism of action of the involved toxins. The anamnesis (if possible) and the analysis of uneaten fish or patient's urine or serum confirms diagnosis for tetrodotoxin. Tetradotoxin (TTX) and its analogues are water soluble and heat stable heterocyclic compounds with a guanidinium group which is positively charged in the biological pH range (Figure 60.2) (Yotsu-Yamashita *et al.*, 2003) that are not produced by microalgae, but by other microorganisms. A bacterium initially classified as *Pseudomonas* sp. and later amended as *Shewanella alga* was confirmed to produce TTX (Yasumoto, 2000). A list of bacteria known to produce TTX is reported by Yu *et al.* (2004). Recently, Yu *et al.* (2004) and Wu *et al.* (2005) referred other species of TTX-producing bacteria, *Microbacterium arabinogalactanolyticum, Serratia marcescens* and the actinomycete, *Nocardiopsis dassonvillei*.

Tetrodotoxin concentrates in fish mainly of the Tetraodontidae family, which include puffer fish. TTX has also been found in xanthid crabs, horse-shoe crabs, in some frogs newts and as well as in the blue-ringed octopus (Isbister and Kiernan, 2005).

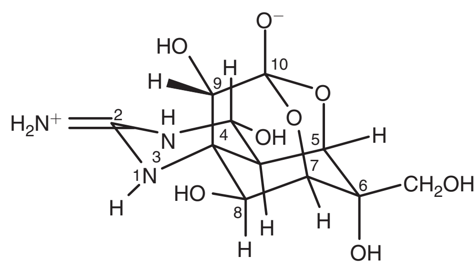



**FIGURE 60.2** Chemical structure of some tetrodotoxin (from Yasumoto, 2000).

Puffer fish poisoning mainly occurs in Asia where *fugu* is a delicacy. In the first half of the 20th century, in Japan about 100 deaths per year from ingestion of *fugu* have been reported. The mortality rate substantially decreased with improved legislation of *fugu* preparation and marketing, although TTX poisoning remains the major cause of fatal food poisoning in Japan (Isbister and Kiernan, 2005).

## Pharmacokinetics/toxicokinetics

Although the pharmacokinetics in humans is not fully understood, serum concentrations of TTX fall rapidly and may be undetectable after 12–24 h. In contrast, TTX can be detected in urine for up to 5 days after ingestion (Isbister and Kiernan, 2005).

After single subcutaneous injection in rats, TTX was detected in kidneys, heart, liver, lungs, intestine, brain and blood, reaching a peak in 20 min. The concentrations were highest in kidneys and heart, and lowest in brain and blood. A rapid appearance of high concentration of TTX in the kidneys and a slow disappearance from these organs were observed, suggesting that an appreciable amount of the toxin is excreted in the urine in unchanged form (Kao, 1966).

## Mechanism of action

Tetrodotoxin affects the nervous system by preventing the propagation of the nerve impulse. TTX blocks $Na^+$ conductance by extracellular binding at receptor-site 1 of $Na^+$ channels to occlude the outer pore and thereby prevent access of monovalent cations to the pore, blocking the membrane depolarization (Isbister and Kiernan, 2005).

## Toxicity

### *Human toxicity*

Onset of TTX poisoning generally occurs within minutes, seldom more than 6 h after consumption of contaminated fish. TTX intoxication has been divided into four stage of progression. Stage 1 includes oral paraesthesias with or without gastrointestinal symptoms. Stage 2 includes paraesthesias in other areas and motor paralysis. Stage 3 includes muscular incoordination, aphonia, dysphagia, respiratory distress, cyanosis and hypotension. Stage 4 includes respiratory paralysis and severe hypotension.

The mortality rate in TTX poisoning depends, among other things, on access to intensive care facilities. Patients, who have not died within 24 h, generally recover

completely. As with PSP, symptoms of TTX poisoning generally are completely resolved within 1–2 days (Gessner, 2000).

### *Experimental toxicity*

*Single administration*

Although the mechanism of action of TTX is well known, only outdated and limited data on its toxicity in animals are available. The effects of TTX have been tested on a variety of animal species. After i.p. injection in mice, the minimal lethal dose of TTX was 8 μg/kg, while the $LD_{50}$ was estimated to be 10 μg/kg and doses ranging from 12 to 14 μg/kg killed all the mice. After oral and subcutaneous administration in mice, the $LD_{50}$ is 332 and 16 μg/kg, respectively (Kao, 1966). Similar results were observed in a recent study, when $LD_{50}$ values after intraperitoneal, subcutaneous and oral administration in mice were determined as 10.7, 12.5 and 532 μg/kg, respectively. In rabbits, the minimal lethal doses and the lethal doses for all the animals were 5.3 and 3.1 μg/kg after intramuscular injection, or 5.8 and 3.8 μg/kg, after i.v. injection (Xu *et al.*, 2003). In almost all animal species, the observed symptoms were similar and comparable. The toxic effects involve mainly the peripheral neuromuscular system, which paralysed to different extent. Furthermore, TTX is a highly potent haemetic agent, so that vomiting was frequently observed in both cats and dogs. Moreover, a pronounced and long-lasting hypotension was observed as well as hypothermia (Kao, 1966).

## Treatment

There is no antidote available for clinical use, so far. To appropriately treat respiratory failure or cardiac effects, a careful observation of the poisoned patient is recommended. Admission to an intensive care unit is necessary in moderate to severe cases to prevent complications. In case of severe poisoning, atropine can be used to treat bradycardia. Respiratory support may be necessary for 24–72 h. Since the patient may be fully conscious during the poisoning, sedation is important when the patient is paralysed.

## Concluding remarks

Due to the severity of TTX intoxication, prevention is very important: public education is essential to reduce puffer fish poisoning (How *et al.*, 2003). In Japan, low mortality rates have been achieved after the introduction of legislation related to the preparation and marketing of *fugu* (Isbister and Kiernan, 2005). The official method to detect TTX in puffer fish in Japan is MBA, as detailed in Hungerford (2006). In United States, no regulatory limits for TTX have been established, but personal importation of puffer fish is prohibited (Gessner, 2000).

# CIGUATOXINS AND MAITOTOXINS

In tropical regions many species of fish may become toxic, provoking neurological, gastrointestinal and, sometimes, cardiovascular symptoms, when ingested. Eating these contaminated fish, humans can develop ciguatera, a marine fish poisoning that causes diverse and often long-lasting health problems. Although this poisoning is rarely fatal (0.1%), it has been estimated that more than 25,000 persons are affected every year.

# BACKGROUND

Ciguatera fish poisoning (CFP) is characterized by moderate to severe gastrointestinal symptoms (vomiting, diarrhoea and abdominal cramps), neurological signs (myalgia, paraesthesia, cold allodynia and ataxia) pruritus and, less common, cardiovascular effects (Isbister and Kiernan, 2005). Both gastrointestinal and neurological symptoms are typical of CFP, but the proportion of each type depends on the region (Isbister and Kiernan, 2005). Gastrointestinal symptoms characterize the first stage of this poisoning and are predominant in Caribbean cases of ciguatera. Generally, they appear within 2–12 h of contaminated fish ingestion and are represented mainly by nausea, vomiting, diarrhoea, but also by abdominal pain (Farstad and Chow, 2001).

Neurological symptoms predominate in the Pacific area. Neurological effects develop over 24 h, although the onset of symptoms can be very different, even in patients eating the same fish. Cold allodynia, a dysestesia that induces a burning sensation on contact with cold objects, is one of the most typical symptoms of ciguatera and is commonly incorrectly referred as “temperature reversal” (Isbister and Kiernan, 2005).

Persistent effects of ciguatera poisoning have been reported, although no prospective studies with definitive cases of ingestion have been set up. Reported chronic clinical effects include fatigue, arthralgia, myalgia, headache and pruritus. Also depression and anxiety have been associated with chronic ciguatera poisoning (Isbister and Kiernan, 2005). Cardiovascular symptoms, not very frequent, include bradycardia and hypotension (Farstad and Chow, 2001).

Initially this poisoning can present similar symptoms to those observed in Diarrhoeic Shellfish Poisoning (DSP) or in microbiological food poisoning, so the anamnesis of the patient is important. Mild ciguatera cases are often misdiagnosed as a common illness such as the flu. Whereas symptoms of cold allodynia are indicative of ciguatera poisoning, differential diagnosis of neuropathy can be necessary (Glaziou and Legrand, 1994; Isbister and Kiernan, 2005).

The toxin isolated from the ciguatera implicated fish are produced by the epiphytic benthic dinoflagellate *Gambierdiscus toxicus*. This dinoflagellate is common in coral reef waters and lives at temperature ranging from 20°C to 34°C, in conditions of low salinity and depths (3–15 m). Its growth is increased in places where there is a reef degradation by human or natural factors (Lewis and Holmes, 1993). Different strains of *G. toxicus* produce chemically distinct lipo- and water-soluble toxins, named CTXs and MTXs (maitotoxins), respectively (Lewis, 2001). Since the actual role of MTX in CFP is still unclear, the two groups of toxins will be treated separately.

The accumulation of these toxins through the food chain involves the ingestion of *G. toxicus* by herbivorous fishes which are eaten by carnivorous species (Lewis and Holmes, 1993; Lehane and Lewis, 2000). CTXs are concentrated both into viscera (such as liver, intestines and gonads) and into fish muscle (Lehane and Lewis, 2000), whereas MTXs seem to be confined only to the viscera (Yasumoto, 2001). Pacific and Caribbean reef-fish species associated with ciguatera include *Lutjanids* (red bass and snappers), *Serranids* (coral trout from the Great Barrier Reef, sea bass and groupers), *Epinephelids* (cod, including flowery cod and spotted cod), *Lethrinids* (emperors and scavengers), *Muraenids* (moray eels), *Scombrids* (mackerel, including Spanish mackerel, and tunas), *Carangids* (jacks and scads) and *Sphyraenids* (barracuda) (Lehane and Lewis, 2000; Isbister and Kiernan, 2005).

Ciguatera is endemic in subtropical and tropical regions of the western Indian and Pacific Ocean regions and in the Caribbean Sea (Lehane and Lewis, 2000). However, due to the increase in trade of these fish, CFP is not confined to the tropics. Epidemiological characterization of ciguatera has been limited by the lack of laboratory test to confirm the presence of the toxins, so far (Lewis *et al.*, 2000).

## CIGUATOXINS

Toxins responsible for CFP are mainly CTXs (Figure 60.3), lipid-soluble and heat-stable polyether compounds with a backbone composed of 10–14 rings transfused by ether linkages into a ladder-like structure that reminds the brevetoxins (PbTxs) (Dechraoui *et al.*, 1999; Lehane and Lewis, 2000).

CTXs seem to be oxidation products of their less toxic precursors, gambiertoxins, through cytochrome enzymes in the liver of fish (Lehane and Lewis, 2000; Yasumoto, 2001; Cembella, 2003). Structural differences have been found in CTXs isolated in different regions, so that Pacific (P-CTXs) and Caribbean (C-CTXs) CTXs are usually referred to separately (Danaras *et al.*, 2001; Yasumoto, 2001).

### Pharmacokinetics/toxicokinetics

Only limited data are available on absorption, distribution, metabolism and excretion of CTXs. Most of the toxicokinetic information is not obtained from actual pharmacokinetic studies, but often derives either from direct clinical observations or are indirectly hypothesized on the basis of the chemical properties of CTXs. The oral absorption of these toxins should be complete or almost complete due to their lipophilicity; it has been hypothesized that CTXs can also penetrate the skin and mucous membranes, due to the observed human effects after local exposure (Lehane and Lewis, 2000). CTXs are able to cross the placenta (Pearn *et al.*, 1982) as well as they are excreted in breast milk, although hyperaestesia of the nipples of a lactating

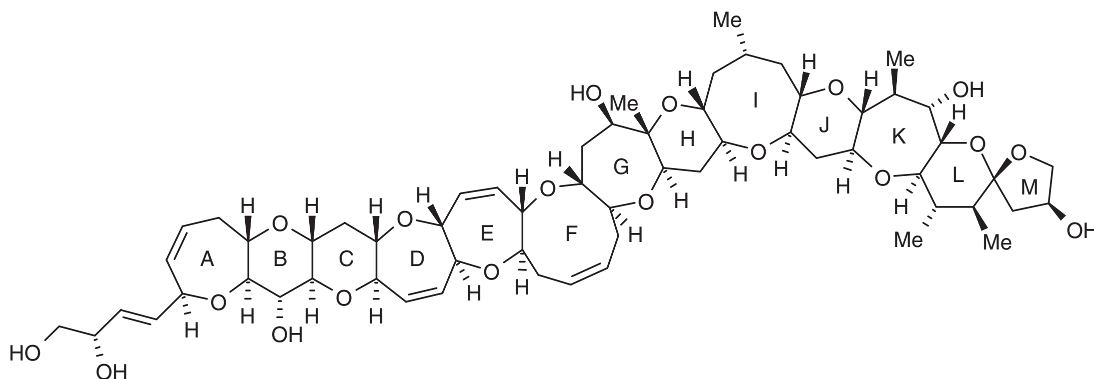



**FIGURE 60.3** Chemical structure of ciguatoxin-1 (from Lewis *et al.*, 2000).

mother may interfere with breast feeding (Bagnis and Legrand, 1987). In a recent study, the blood collected from mice exposed to a sublethal dose of C-CTX-1 was found to contain toxin concentrations ranging from 0.25 to 0.12 ng/ml, 30 min and 12 h after exposure, respectively (Bottein Dechraoui *et al.*, 2005).

## Mechanism of action

CTXs increase cell $Na^+$ permeability by binding to site 5 neuronal voltage-sensitive sodium channels which open at normal resting membrane potential. Therefore, CTXs affect various $Na^+$-dependent mechanisms to enhance membrane excitability, induce mobilization of intracellular $Ca^{2+}$, and provoke cell swelling. The effects of CTXs are most prominent in nerves (Lewis *et al.*, 2000).

It has been hypothethized that the cardiovascular effects can result from a positive inotropic effect of the toxin on the myocardium, responding to the increased intracellular $Ca^{2+}$ (Lehane and Lewis, 2000).

## Toxicity

### *Human toxicity*

Few fatal cases of CFP are reported. Postmortem analysis evidenced acute visceral congestion with eosinophilic necrotic lesions in the liver at light microscope and ultrastructural changes in the sural nerve with swelling Schwann cells, axonal compression and vesicular degeneration of myelin (Lehane and Lewis, 2000; Terao, 2000). Both P-CTX-1 and C-CTX-1 pose a health risk at concentrations above 0.1 ppb (Pearn, 2001).

In its typical form, ciguatera is characterized initially by the onset of intense vomiting, diarrhoea and abdominal pain within hours after ingestion of toxic fish, although the neurological symptoms tend to be the most distinctive and permanent ones. They include sensory disturbances such as generalized pruritus, circumoral numbness, long-lasting weakness and fatigue and the unusual and perhaps pathognomic sensory discomfort triggered by cold stimuli ("cold allodynia") (Lehane and Lewis, 2000). Patients with bradycardia and/or hypotension may require urgent care, because the infrequent cardiovascular symptoms may indicate a poor prognosis (Lewis *et al.*, 2000).

### *Experimental toxicity*

#### *Single administration*

After single administration in mice, the toxicity is similar either after oral or i.p. administration, suggesting that oral absorption is almost complete in this species. P-CTXs and C-CTXs show a different potency by i.p. injection. The i.p. $LD_{50}$ values in mice are 0.25, 2.3 and 0.9 μg/kg for P-CTX-1, P-CTX-2 and P-CTX-3, respectively, whereas $LD_{50}$ values of 3.6 and 1.0 μg/kg were recorded for C-CTX-1 and C-CTX-2 (Lehane and Lewis, 2000).

The acute i.p. injection or oral administration of P-CTX-1 or P-CTX-4C (0.7 μg/kg) in mice provokes similar toxicity, initially characterized by severe diarrhoea. Target organs were heart, medulla of adrenal glands, autonomic nerves and penis. Light microscopy examination shows marked swelling and focal necrosis of cardiac muscle cells. Degeneration of cells in the medulla of the adrenal glands was also observed. A marked lung oedema, with congestion at alveolar spaces and bronchioles, was reported in mice with severe dyspnoea. Continuous erection of the penis was observed in about 15% of the mice suffering from ciguatoxicosis (Lehane and Lewis, 2000; Terao, 2000).

#### *Repeated administration*

After repeated i.p. and oral administrations (100 ng/kg of P-CTX-1 or P-CTX-4C) for 15 days, marked swelling of cardiac cells and endothelial lining cells of blood capillaries in the heart were observed. Although single doses of the same toxins did not provoke any discernible change at macroscopic, light microscopic and at even ultrastructural level in the hearts of mice, the repeated administration of CTXs resulted in severe morphological, but reversible, cardiac changes. At the ultrastructural level, the changes induced by repeated CTXs administration were similar to those of mice receiving a CTXs single dose (700 ng/kg or more) (Terao, 2000).

## Treatment

For ciguatera poisoning there is only a supportive and symptomatic treatment such as the control of fluid and electrolyte balance, since no effective antidote for ciguatera is available, so far. The infrequent cardiovascular complications, such as symptomatic bradycardia and severe hypothension, may require treatment.

The most common treatment during the acute phase of CFP is i.v. infusion of mannitol (Lehane and Lewis, 2000; Terao, 2000), although controversial data are reported about its actual efficacy. In particular, a double-blind randomized, controlled trial found no difference between mannitol and saline treatment. Local anaesthetics and antidepressants may also be useful in some cases (Lehane and Lewis, 2000; Terao, 2000; Lewis, 2001; Isbister and Kiernan, 2005).

## Concluding remarks

Since CFP still remains a significant problem, prevention is important in endemic regions. The most widely used method for monitoring purposes is the MBA, based on

clinical signs (marked hypothermia) and death observed for up to 48 h after i.p. injection of a 20 mg ether extract from fish muscle. Due to the poor sensitivity, this assay cannot detect the presence of CTXs in low-contaminated ciguateric fish (Lehane and Lewis, 2000). Although various immunoassays, such as radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), as well as binding and cytotoxicity assays have been developed, there is still a need for easy diagnostic and screening methods for ciguatera. In the United States ciguatera outbreaks are investigated using laboratory-based cytotoxicity screening and confirmatory liquid chromatography/mass spectrometry (LC-MS) methods (Hungerford, 2006).

## MAITOTOXINS

MTX, a water-soluble compound isolated together with CTXs, was considered as a biotoxin involved in CFP (Yasumoto, 2001). Anyway, its content being insignificant in the fish flesh and having low oral toxicity, MTX seems to play a limited role in CFP (Estacion, 2000; Yasumoto, 2000).

MTX, named also MTX-1 (Figure 60.4), is a polycyclic polyether and, except biopolymers, the largest natural compound isolated and structurally elucidated so far ($C_{164}H_{256}O_{68}S_2Na_2$; molecular weight (m.w.): 3422) (Yasumoto, 2001). It is produced by the epiphytic dinoflagellate *Gambierdiscus toxicus* and accumulates in the liver of fishes such as surgeonfish and parrotfish. MTX was first detected in viscera of *Ctenochaetus striatus* (*maito*), from which MTX derived its name. MTX analogues (MTX-2; m.w.: 3298 and MTX-3; m.w.: 1060) have been purified from *G. toxicus* strains (Holmes and Lewis, 1994; Bouaïcha *et al.*, 1997; Estacion, 2000; Terao, 2000).

### Pharmacokinetics/toxicokinetics

No data on MTXs absorption, distribution, biotransformation and elimination are available, so far.

### Mechanism of action

MTX is a powerful activator of $Ca^{2+}$ entry via non-selective cation channels in wide variety of cells. Thus, it causes an increase of cytosolic $Ca^{2+}$ levels which stimulate a broad spectrum of calcium-dependent processes, including cell death (Escobar *et al.*, 1998; Estacion, 2000; Morales-Tlalpan and Vaca, 2002).

### Toxicity

#### *Human toxicity*

The toxicity of MTXs in humans is unclear as its role in CFP is not completely understood (Estacion, 2000).

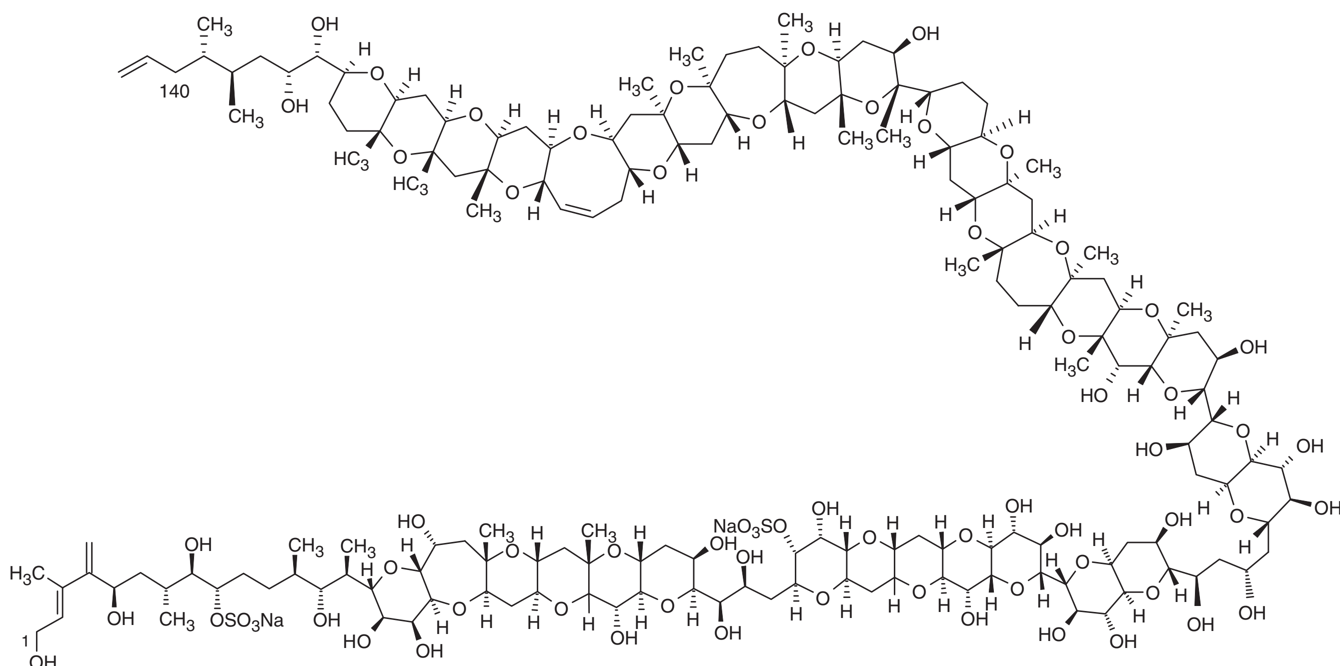



**FIGURE 60.4** Chemical structure of maitotoxin (from Yasumoto, 2001).

***Experimental toxicity***

*Single administration*

After i.p. injection in mice, the $LD_{50}$ of MTX was determined as 500 ng/kg (Yasumoto, 2000). An i.p. injection of MTX in mice (200–400 ng/kg) and rats (400 ng/kg) induced severe alterations at the gastric mucosa, cardiac muscle and lymphoid tissues, as well as an increase of plasma cortisol, which was probably involved in the lymphoid tissues involution (Terao, 2000). A hypothermic effect after i.p. injection in rats (338 ng/kg) was reported too (Gordon and Ramsdell, 2005).

*Repeated administration*

Repeated daily i.p. injection of MTX to mice (45 ng/kg, for 13 days) resulted in marked atrophy of lymphoid tissues, reduced circulating lymphocytes and serum immunoglobulin M levels, as well as increased calcium content of adrenal glands and plasma cortisol (Terao, 2000).

## Concluding remarks

Although MTX is accumulated mainly in viscera of herbivorous fishes and seems to play a marginal role in CFP, the possible contribution of MTX should not be disregarded, considering the practice of eating small fish without eliminating their viscera in some areas of the world (Yasumoto, 2000). Therefore, the possibility of MTXs detection in fish and phytoplankton is of particular importance so that biological assays and chemical methods based on high-performance liquid chromatography (HPLC) and zone electrophoresis with ultraviolet and/or mass spectrometry detection have been set up (Fessard *et al.*, 1994; Lewis *et al.*, 1994; Van Dolah *et al.*, 1994; Bouaïcha *et al.*, 1997).

# DOMOIC ACID AND ANALOGUES

## Background

Amnesic shellfish poisoning (ASP) is a toxic syndrome provoked by DA-contaminated shellfish consumption. ASP differs from most other neurotoxic marine poisonings because the main effect is on the central nervous system (CNS) (Isbister and Kiernan, 2005). It was reported for the first time in Canada in 1987 when, after consumption of contaminated mussels, people developed gastrointestinal symptoms within 24 h (vomiting, abdominal cramps and diarrhoea) and/or neurological symptoms within 48 h (headache, loss of short-term memory, disorientation, lethargy, seizures and, sometimes, convulsions and coma). Mortality was also recorded. Since the most relevant clinical effects were memory loss, the condition was termed ASP (Nijjar and Nijjar, 2000). The loss of memory in patients intoxicated with DA-contaminated mussels appeared to be similar to patients with Alzheimer's disease. However, whereas symptoms of Alzheimer's disease are generally present in older people and intensify with advancing age, loss of memory in mussel-intoxicated patients was not affected by the age of patients. Further, the findings that higher cortical functions, such as intellect, were not affected by DA poisoning distinguishes ASP from Alzheimer's disease (Nijjar and Nijjar, 2000).

The toxin responsible for ASP is DA (Figure 60.5), an amino acid belonging to the kainoid class of compounds (Wright *et al.*, 1990) and 10 DA isomers (isodomoic acids A to H and DA 5′-diastereoisomer), that are less toxic than the parent compound. DA and its isomers are water-soluble and heat-resistant tricarboxylic amino acid.

Previously DA was only known from the red macroalgae *Chondria armata*. After 1987, marine diatoms of the genus *Pseudonitzschia*, such as *P. multiseries*, *P. pseudodelicatissima* and *P. australis* were shown to produce DA. Recently, *P. navis-varingica* was found to produce isodomoic acids A and B as major toxins (Kotaki *et al.*, 2005).

DA has been shown to accumulate in blue mussels (*Mytilus edulis, Perna canaliculus* and *Mytilus galloprovincialis*) as well as in other shellfish, such as cockles (*Cerastoderma edule*), razor clams (*Siliqua patula*), scallops (*Pecten maximus*), crabs (*Cancer magister*) in furrow shell (*Scrobicularia plana*) (Jeffery *et al.*, 2004) and in anchovies.

DA contaminated shellfish and potentially toxic *Pseudonitzschia* ssp. have been detected worldwide. Although DA has caused bird (via toxic anchovies) and other animal mortalities, the only toxic episode with confirmed human illnesses is that of Canada.

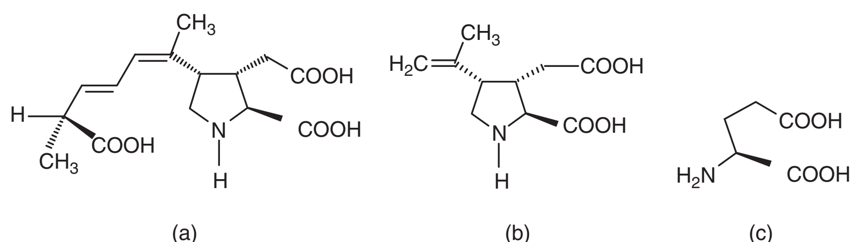



**FIGURE 60.5** Chemical structure of (a) domoic acid, (b) kainic acid and (c) glutamic acid (from Nijjar and Nijjar, 2000).

## Pharmacokinetics/toxicokinetics

Very limited data are available on the absorption, distribution, metabolism and excretion of DA, showing not appreciable differences in the effects observed after i.p. and oral administration. It has been suggested that DA is poorly absorbed after the usual exposure route (Jeffery *et al.*, 2004). Furthermore, in the two studies comparing the intraperitoneal and oral administration administration in rodents, involving a small group size ($n=1$ or 2/dose) (Iverson *et al.*, 1989), DA was almost completely excreted in the faeces supporting the hypothesis that DA is not well absorbed. Truelove *et al.* (1996) reported that only 2% of the orally administered dose of DA (5 mg/kg/day) in rats ($n=10$) was excreted in the urine. Similar results (mean DA urinary excretion of the daily dose ranged from 4% to 7%) were obtained in cynomolgus monkeys ($n=3$), treated with a subtoxic dose of DA for 32 days (Truelove *et al.*, 1997). Once absorbed, DA appears to be poorly metabolized. In fact, approximately 75% of a dose of $^{3}$H-labelled DA i.v. administered in rats ($n=8$) was excreted unchanged in the urine within 160 min, suggesting that it is not significantly metabolized before the rapid elimination. In the same study, evidences for some metabolism to compounds of greater hydrophilicity were hypothesized, due to remaining radiolabel eluted earlier during LC than DA (Suzuki and Hierlihy, 1993). In another study on the distribution and excretion of i.v. administered DA in rats (500 and 1000 μg/kg) and monkeys, half-lives of about 20 and 110 min, apparent volume of distribution of approximately 300 and 180 ml/kg and apparent clearances of approximately 10 and 1 ml/min/kg were reported. Although the limited number of animals used in the study, these data suggest that DA, after i.v. administration, is well distributed into the body fluids in both species and is rapidly eliminated (Truelove and Iverson, 1994).

After DA i.v. administration in rats, DA poorly permeates across the blood–brain barrier and appears not to cross via a specific transport carrier (Preston and Hynie, 1991). However, despite this limited transfer across the blood–brain barrier, the brain is the primary organ of DA-induced toxicity.

## Mechanism of action

The toxicity of DA in the nervous system is known to occur on excitatory amino acid receptors and on synaptic transmission. Two amino acids, L-glutamate and L-aspartate are considered to be neurotransmitters and act upon several receptor types. Three receptor subtypes have been described for excitatory amino acids, the kainic acid (KA) and the *N*-methyl-D-aspartate (NMDA) receptors being best characterized.

DA produces its action through pre- and post-synaptic receptors in a manner similar to KA opening the channel to $Ca^{++}$ and inducing cellular lethality (Wright *et al.*, 1990; Todd, 1993). Due to the structural similarity of DA to glutamic acid and, in particular, to KA (Figure 60.5), DA has a strong affinity for subclasses of kainate receptors. Kainate receptors are widely distributed in the mammalian brain and are particularly concentrated in the CA1–3 regions of the hippocampus in rodents, in the CA2 and CA3 regions in non-human primates as well as in the CA3 region in humans (Jeffery *et al.*, 2004). The high affinity of DA to kainate receptors and the apparent co-localization of these receptors at sites where DA induced damage in the brains of rodents and primates suggest that DA-kainate receptor interactions play a major role in the toxic response. This binding of DA to glutamate receptor subtypes appears to stimulate neuronal firing, eliciting an excitatory response both *in vitro* and *in vivo* (Jeffery *et al.*, 2004).

Although the exact mechanism of neuronal stimulation leading to tissue brain damage is not completely understood, and influx of $Ca^{2+}$ into cells was observed in brain tissue slices exposed to DA, as well as an increased cytosolic $Ca^{2+}$ levels in hippocampal pyramidal neurons exposed to DA were reported (Jeffery *et al.*, 2004). It seems that DA binding to glutamate receptors in specific brain regions leads to excitation of neurons, giving rise to an influx of $Ca^{2+}$ resulting in a failure to maintain intracellular ion homeostasis and in neuronal cell death (Jeffery *et al.*, 2004).

## Toxicity

### *Human toxicity*

The acute ASP outbreak occurred in 1987 in Canada after the ingestion of blue mussels (*Mytilus edulis*) was characterized by gastrointestinal and unusual neurological symptoms. Although 150 reports of this illness were recorded, only 107 individuals met the clinical definition of the poisoning (Jeffery *et al.*, 2004). The most common gastrointestinal symptoms were vomiting (76%), abdominal cramps (50%) and diarrhoea (42%); the most common neurological symptoms were headache (43%) and loss of short-term memory (25%) (Nijjar and Nijjar, 2000).

The poisoning was particularly severe in 19 people who were hospitalized: 12 individuals with particularly severe symptoms (e.g. seizures, coma, profuse respiratory secretions or unstable blood pressure) required treatment in intensive care unit. Although in most patients decreased arousal and somnolence were seen, in some cases leading to coma, in less severely affected patients, agitation was observed. Cardiovascular symptoms (tachycardia, hypotension and cardiac arrhythmias) may have been a consequence of dysfunction of the central autonomic centres, since there was no evidence of primary cardiovascular impairment (Doble, 2000).

In most patients, symptoms resolved in a few weeks, but in some of them a residual memory impairment persisted (Doble, 2000).

Three hospitalized patients died 11–24 days after consumption of mussels. A fourth patient died of myocardial infarction within 3 months of mussel consumption. Postmortem histological examination showed neuronal necrosis and astrocytosis which was most prominent in the hippocampus and amygdala nucleus (Nijjar and Nijjar, 2000).

In nine cases of poisoning, estimates of DA exposure were carried out from analysis of leftover mussels collected from households or restaurants. The amount of DA ingested ranged from 60 to 290 mg in poisoned patient. From this evaluation, it seems that 60 mg of DA/person (≈1 mg DA/kg) is sufficient to provoke gastrointestinal symptoms, whereas ingestion of 270 mg/DA/kg bw (≈4.5 mg DA/kg) provokes neurological effects (Jeffery *et al.*, 2004).

### *Experimental toxicity*

*Single administration*

After i.p. administration in mice $DL_{50}$ values of 2.4 and 3.6 mg DA/kg were reported in two different studies using DA-contaminated mussel extracts. Clinical symptoms such as scratching, tremors and seizures at both lethal and sublethal doses of the extracts were observed. $LD_{50}$ values have been recorded for newborn mice after i.p. administration at different postnatal days: the results suggest that they may be much more sensitive to DA than the adult mice (Jeffery *et al.*, 2004).

Neuropathological studies were carried out in both mice and rats: dose-related lesions in the brains (oedema in the hypothalamus and hypothalamic arcuate nucleus and neuronal degeneration in different areas of the hippocampus) of mice and rats were found after i.p. and after *per os* administration of mussel extracts containing DA (Iverson *et al.*, 1989).

Similar effects have been reported in studies of non-human primates, after i.p. and i.v. administration. Severe vomiting and scratching were reported after single i.v. DA administration (0.24, 0.5, 1.0, 1.25, 1.5, 2 and 4 mg/kg) in cynomolgus monkey, with a dose-related latency in the appearance of the symptoms. DA doses ⩾1.0 mg/kg provoked death by respiratory failure in 4/7 animals. Postmortem examination revealed lesions in the hippocampus (Scallet, 1995) and in another similar study, utilizing specialized histochemistry, also the thalamus appeared affected (Schmued *et al.*, 1995).

*Repeated administration*

After repeated (64 days) *per os* exposure to DA (0.01 or 5 mg/kg/day) of male and female rats, neither clinical abnormalities nor differences in haematology, clinical chemistry or histopathology were observed in treated animals (Jeffery *et al.*, 2004).

Neither clinical symptoms nor significant changes in body weight, haematology, clinical chemistry or brain histology were observed in cynomolgus monkey orally treated with low DA doses (0.5 mg/kg/day for 15 days and 0.75 mg/kg/day for a further 15 days) (Truelove *et al.*, 1997).

*Teratogenicity*

The effects of DA (0–2 mg/kg) i.p. daily administered to pregnant rats from gestational days 7 to 16 were studied both on mothers and their offspring at gestational day 22. Mother death was recorded only at the higher DA doses (6/12 and 6/9 at 1.75 and 2.0 mg/kg, respectively). At doses ⩾ 0.5 mg/kg/day, a reduction in the alive foetuses number at term was recorded, but the number of deaths was not increased in a dose-dependent way. A non-significant increase in the number of foetuses with visceral and skeletal anomalies was reported (Khera *et al.*, 1994).

*Genotoxicity*

Since the structure of DA contains a butadiene moiety, there is the possibility for the formation of DNA-reactive epoxides *in vivo*. Although only limited data are available about its possible genotoxicity, DA (87 or 174 μM) did not increase mutation frequency in V79 Chinese hamster lung cells *in vitro* measured by thioguanine or ouabain resistance, sister chromatid exchange or micronucleus assays, nor does it give rise to DNA-reactive metabolites (Jeffery *et al.*, 2004). *In vivo* data about carcinogenicity of DA are not available, so far.

## Treatment

The diagnosis is based on the history of ingestion of bivalve molluscs followed by characteristic symptoms. Environmental surveillance programmes of phytoplanktons in risk regions may be suggestive of a possibility of poisoning.

Due to the risk of convulsions, emesis should not be induced. Severe cases should be admitted to the intensive care unit and monitored for convulsions, CNS depression, cardiovascular collapse or gastric haemorrhages. Treatment is symptomatic and no antidote is available (Gulland *et al.*, 2002).

## Concluding remarks

After the human outbreak of ASP in 1897 a regulatory limit for shellfish flesh was introduced in Canada and

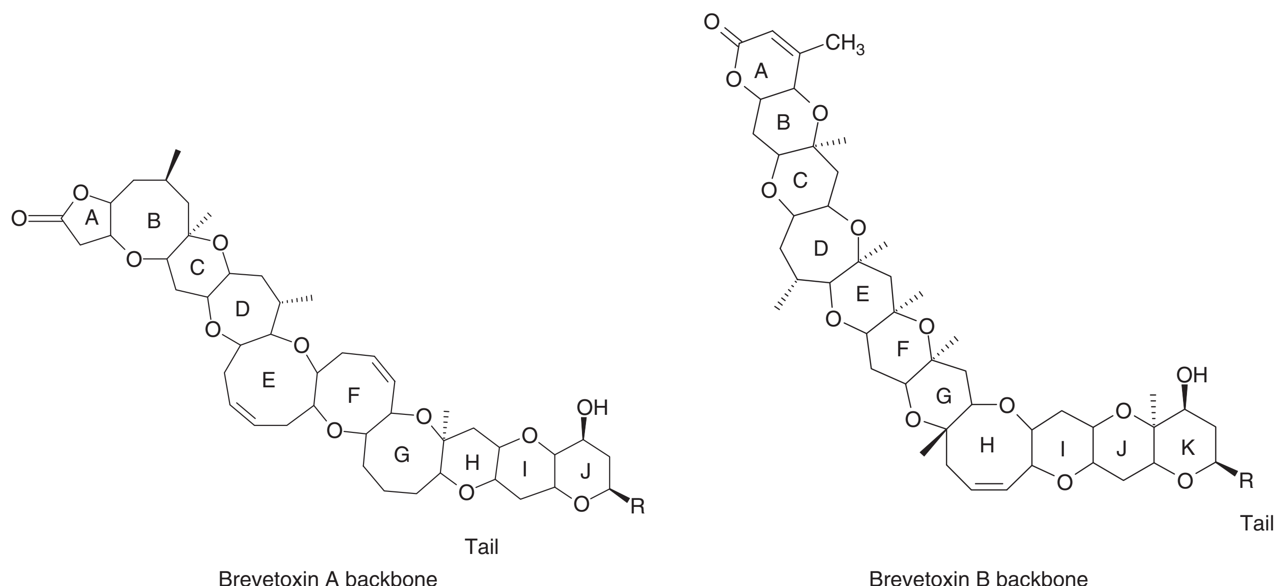



**FIGURE 60.6** Chemical structure of brevetoxins – type A and type B (from Baden and Adams, 2000).

consequently in other parts of the world. The limit, 20 mg/kg is based on a retrospective estimate of the quantity of DA in mussels that resulted in human illness in the 1987 Canadian ASP and incorporates a 10-fold safety factor. Due to the worldwide distribution of the diatoms producing DA, the action limit employed by Canada has been adopted elsewhere and is the limit enforced in the European Union, United States, New Zealand and Australia (Jeffery *et al.*, 2004). An HPLC-UV method has gained favor in various countries (FAO/IOC/WHO, 2005).

# BREVETOXINS

## Background

Brevetoxins (PbTxs) are neurotoxic polyethers produced by the dinoflagellates of *Karenia* genus (mainly *K. brevis*; formerly known as *Gymnodinium breve* or *Ptychodiscus breve*), which forms "red tide" blooms along the Florida coast and the Gulf of Mexico. These harmful blooms cause mass mortality of fish and other marine organisms, episodes of respiratory distress in humans after inhalation of the sea spray, and eyes and skin irritation also after swimming in the sea. As shellfish are resistant to PbTxs and accumulate these compounds, their ingestion may provoke an illness called neurotoxic shellfish poisoning (NSP) (Landsberg, 2002). NSP is characterized by acute gastrointestinal and neurological symptoms, including nausea, vomiting, diarrhoea, chills, sweats, headache, muscle weakness and joint pain, paraesthesia, arrhythmias, difficult breathing, mydriasis, double vision, and troubles in talking and swallowing. Recovery occurs in 2–3 days and no fatal cases from NSP have been reported (Baden and Adams, 2000; Hallegraeff, 2003; Isbister and Kiernan, 2005).

PbTxs are lipid-soluble and heat-stable polycyclic ether compounds grouped in two types (A and B), according to their backbone structures consisting of 10 (type A) or 11 (type B) transfused rings (Figure 60.6) (Baden and Adams, 2000). At least nine PbTxs are produced by *K. brevis*, while several metabolites of these compounds have been identified in shellfish (Ishida *et al.*, 2004; Plakas *et al.*, 2004; Baden *et al.*, 2005). Recently other *Karenia* species were involved in NSP, whereas some raphidophytes (*Chattonella marina, C. antiqua, Fibrocapsa japonica* and *Heterostigma akashiwo*) were reported to produce brevetoxin-like compounds, but no documented cases of NSP were caused by these species (Landsberg, 2002; Hallegraeff, 2003; Ciminiello and Fattorusso, 2004).

Blooms of *Karenia* occurred mainly in the Gulf of Mexico, where NSP has historically been limited, but occasional blooms associated with NSP were reported also along the mid- and south Atlantic coast of the United States and in New Zealand. Shellfish involved in NSP were mainly oysters, clams, cockles and mussels (Landsberg, 2002).

## Pharmacokinetics/toxicokinetics

After oral administration of $^{3}$H-labelled PbTx-3 to rats, the toxin was shown to distribute to all organs, and concentrated mainly in the liver. It was eliminated in equivalent quantities through urine and faeces: about 80% of the

dose was excreted within 7 days (during the first 48 h PbTx-3 was cleared mainly through the faeces, and afterwards mostly through urine) (Cattet and Geraci, 1993).

After i.p. injection of PbTx-3 in mice, blood concentration of the toxin was maximal between 0.5 and 4 h. After 24 h it was reduced by one-third, being still detectable after 7 days (Fairey *et al.*, 2001; Woofter *et al.*, 2003). A significant part of PbTx-3 in mouse plasma binds to high-density lipoproteins (Woofter *et al.*, 2005). In rats, after i.p. injection of PtPx-2 and PtBx-3, the toxins were detected in blood within 1 h and, for PbTx-2, a rapid metabolism to polar cysteine conjugates, eliminated in urine over 24 h, was demonstrated (Radwan *et al.*, 2005).

An i.v. administration of $^{3}$H-labelled PbTx-3 to rats showed that the toxin cleared rapidly from the blood (<10% remained after 1 min) and distributed mainly to the liver, skeletal muscle and gastrointestinal tract (18%, 70% and 8% of the dose after 30 min, respectively). Within 24 h, PbTx-3 concentration in skeletal muscle decreased to 20% of the dose, while that in liver remained constant and increased in gastrointestinal tract and faeces, probably because of biliary excretion. By day 6, about 14% of the toxin had been excreted through urine and 75% in faeces, also in the form of more polar metabolites (Poli *et al.*, 1990).

Intratracheal instillation of $^{3}$H-labelled PbTx-3 to rats revealed that over 80% of the dose was cleared within 0.5 h from the lung and distributed throughout the body, chiefly to the carcass (49%), intestine (32%) and liver (8%). Blood, brain and fat contained the lowest levels of the toxin. About 20% of the initial level in tissues was retained for 7 days. The majority of PbTx-3 was excreted within 48 h in faeces (60%) and urine (30%) (Benson *et al.*, 1999). Also in mice, after intratracheal instillation, PbTx-3 distributed rapidly to tissues, mainly in liver and gastrointestinal tract, while about 90% of excretion occurred within 4 days in urine (11%) and faeces (64%) (Tibbetts *et al.*, 2006). After repeated inhalation of PbTx-3 by rats for 5 or 22 days, small amounts of the toxins were detected in splenic and peribronchiolar lymphoid tissue as well as in liver, where no accumulation of brevetoxin occurred (Benson *et al.*, 2004, 2005).

Cutaneous application of $^{3}$H-labelled PbTx-3 on pig skin revealed a rapid penetration of the toxin to the dermis with maximal dermal accumulation at 4 h (Kemppainen *et al.*, 1991).

## Mechanism of action

PbTxs bind to site 5 on the α-subunit of voltage-sensitive sodium channels in the cell membranes. Normally, these channels open in response to membrane depolarization and subsequentially inactivate returning to closed configuration during the membrane repolarization. Binding of PbTxs opens the voltage-sensitive sodium channels, determining a sustained influx of $Na^{+}$ and a membrane depolarization, induces a shift in activation potential (towards more negative values) and alters the normal changes of the channels configuration during the depolarization/repolarization processes. These actions affect the membrane properties of excitable cells, and are the basis of the neurotoxic effects of PbTxs. A structural feature of these compounds (Figure 60.6) required for this activity is the lactone in the A ring ("head" of the molecule) as well as the conserved structure on the "tail" rings (Ciminiello and Fattorusso, 2004; Baden *et al.*, 2005).

Respiratory problems associated with the inhalation of aerosolized PbTxs are believed to be due in part to opening of sodium channels. Other actions involved in the bronchoconstriction and/or in the immunological effects at the respiratory tract seem to be related to a stimulation of neurotransmitters release and mast cells degranulation, as well as to an inhibition of the phagocytic cells lysosomal proteinases known as cathepsins (Abraham *et al.*, 2005; Baden *et al.*, 2005).

## Toxicity

### Human toxicity

Ingestion of PbTxs contaminated shellfish can cause NSP, a syndrome that include some symptoms similar to CFP, but less severe. The symptoms occur within 30 min to 3 h, last a few days and include nausea, vomiting, diarrhoea, chills, sweats, headache, muscle weakness and joint pain, paraesthesia, arrhythmias, difficulty breathing, mydriasis, double vision, and troubles in talking and swallowing. Sometimes coma, but no mortality or chronic symptoms, were reported (Baden and Adams, 2000; Hallegraeff, 2003; Isbister and Kiernan, 2005).

Due to the fragility of *Karenia* cells, PbTxs can be released in seawater and aerosolized by wind and surf with the possibility of inhalation exposure to these compounds, causing respiratory distress as well irritation of the eyes and respiratory tract mucosa. Normally, these symptoms are rapidly reversible by leaving the beach area. The main brevetoxin responsible for respiratory discomfort seems to be PbTx-3 (Benson *et al.*, 1999; Landsberg, 2002). During swimming also a direct contact with toxic blooms with consequent skin, nasal and eye irritations can occur (Landsberg, 2002).

### Experimental toxicity

#### Single administration

After i.p. injection in mice, the $LD_{50}$ of PbTx-1, -2 and -3 were calculated to be 100, 200 and 170 μg/kg, respectively. The symptoms include immediate irritability, followed by hind-quarter paralysis, dyspnoea, salivation, lachrymation, urination, defecation and death from respiratory paralysis (Landsberg, 2002; FAO/IOC/WHO, 2005).

The oral $LD_{50}$ in mice ranges from 520 μg/kg for PbTx-3 to 6600 μg/kg for PbTx-2 (FAO/IOC/WHO, 2005). PbTx-3 caused tremors followed by marked muscular contractions or fasciculations, tail elevation, laboured breathing and death (van Apeldorn *et al.*, 2001).

*Repeated administration*

Studies on repeated exposure to PbTxs were carried out in rats after inhalation of PbTx-3. Exposure to 500 μg PbTx-3/$m^3$ by nose-only inhalation for 0.5 or 2 h/day, for 5 days (corresponding to 8.3 and 33 μg/kg/day, respectively), provoked a reduction of body weight at the highest dose, but no tissue lesions or signs of cytotoxicity and inflammation in bronchoalveolar lavage fluid were observed. In contrast, the humoral-mediated immunity was suppressed (Benson *et al.*, 2004). A more prolonged inhalation exposure of rats (22 days) to PbTx-3 (corresponding to 0.9 and 5.8 μg/kg/day, respectively) showed similar results: reduced body weight in both PbTx-3 dosed groups of rats, suppression of humoral-mediated immunity, as well as minimal alveolar macrophage hyperplasia and increase of blood reticulocytes (Benson *et al.*, 2005).

***Toxicity for fish and other marine animals***

PbTxs are potent ichthyotoxins, being responsible for the deaths of billions of fish over the years. These toxins are thought to be absorbed through the gills but also by ingestion and mortality can occur in presence of about $2.5 \times 10^2$ *K. brevis* cells/ml. Signs of intoxication in fish include violent twisting and corkscrew swimming, defaecation and regurgitation, pectoral fin paralysis, caudal fin curvature, loss of equilibrium, quiescence, vasodilatation, convulsions and death due to respiratory failure. Chronically intoxicated fish show little pathology aside from slight precipitate haemolysis. Chronic haemolysis was detected via anaemia, cyanosis, viscous blood, splenomegaly, hepatic haemosiderosis and dehydration. Frequently, also birds were found moribund or dead, particularly double-crested cormorants, red-breasted mergansers and lesser scaup, and also dolphins and manatees (Landsberg, 2002).

## Treatment

The treatment of NSP is symptomatic and supportive and normally patients recovery within 2–3 days (van Apeldorn *et al.*, 2001).

## Concluding remarks

In the United States and other countries, the regulatory level of 80 μg of PbTx-3 equivalents/100 g shellfish was established. The risk assessment of NSP toxins is performed by the *in vivo* MBA, based on the observation of the survival time of mice after i.p. injection of an ether extract of shellfish. Because of the higher polarity, some of the known brevetoxin metabolites are not extracted from shellfish by the regulatory protocol for shellfish monitoring (Hungerford, 2006).

Alternatively, *in vitro* functional assays (assay on neuroblastoma cells, receptor binding assays), immunochemical methods (ELISA, RIA) and instrumental methods (spectroscopy-coupled HPLC, LC-MS) can be used for PbTxs detection. Validation studies are underway to meet all the criteria for a reference method (Hungerford, 2006).

# DIARRHOEIC TOXINS

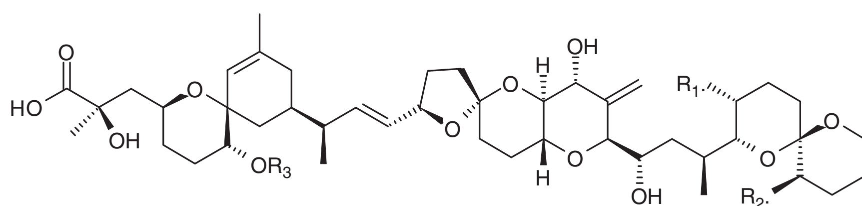
Diarrhoeic toxins induce gastrointestinal symptoms and serious diarrhoea after consumption of contaminated shellfish. They are grouped in two families of compounds: okadaic acid (OA) and its derivatives, responsible for DSP, and azaspiracids, responsible for azaspiracids shellfish poisoning (AZP).

# OA AND DERIVATIVES

## Background

Toxins from the OA group have been known to provoke human poisonings since the late 1970s. The illness was called Diarrheic Shellfish Poisoning (DSP) due to its main symptom. Symptoms of DSP are mainly gastrointestinal distress, diarrhoea, nausea, vomiting and abdominal cramps, occurring between 30 min and several hours after contaminated shellfish consumption. It is not a fatal illness and complete recovery occurs within 3 days, generally without any pharmacological treatment. Nevertheless, DSP is an important cause of morbidity worldwide, having a serious impact on the shellfish industries (Yasumoto, 1990).

The toxins responsible for DSP are lipid-soluble and heat-stable polyether compounds including OA and its derivatives dinophysistoxins (DTXs), the most frequent of which are DTX-1, DTX-2 and DTX-3 (Figure 60.7). OA, the main DSP toxin, originally isolated from the sponge *Halichondria okadai*, is produced by dinoflagellates of the genus *Dinophysis*, similar to DTX-1 and DTX-2. These compounds are produced also by cultured dinoflagellates of *Prorocentrum* genus, but these algal species were sporadically detected in marine phytoplankton during DSP




| | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|
| Okadaic acid | $CH_3$ | H | H |
| DTX-1 | $CH_3$ | $CH_3$ | H |
| DTX-2 | H | $CH_3$ | H |
| DTX-3 | H or $CH_3$ | H or $CH_3$ | Acyl |

**FIGURE 60.7** Chemical structure of okadaic acid and dinophysystoxin-1, -2 and -3 (DTX-1, -2 and -3) (from Quilliam, 2003).

outbreaks. On the other hand, DTX-3, a mixture of OA, DTX-1 and/or DTX-2 esters containing a fatty acid generally at the 7-hydroxy group, is believed to be a metabolic product of shellfish rather than direct algal metabolites (Quilliam, 2003).

The first documented dinoflagellate species involved in DSP intoxications was *Dinophysis fortii*, in Japan (Yasumoto *et al.*, 1980), and later it was the causative organism of diarrhoeic poisoning in many other areas. Other species, such as *D. acuminata*, *D. acuta*, *D. mitra*, *D. norvegica*, *D. rotundata* and *D. tripos* were reported also as the sources of DSP toxins too (Lee *et al.*, 1989). The toxins production may vary considerably among these species and among regional and seasonal morphotypes in one species (Hallegraeff, 2003). Several bivalves, such as mussels, scallops, oysters and clams, filtering the seawater containing the toxic phytoplankton, can accumulate the toxins and cause the poisoning to humans after their ingestion.

DSP cases have been reported in many areas of the world and the global distribution of the poisoning involved mainly Japan, Europe, Chile, Thailand, Canada, Australia and New Zealand (Hallegraeff, 2003). The DSP incidences, or at least the presence of DSP, appear to be increasing, probably also because of increasing knowledge of the disease and better surveillance programmes.

## Pharmacokinetics/toxicokinetics

Few toxicokinetic studies of OA have been performed in mice. Twenty-four hours after single oral $[^3H]$OA administration (50 or 90 μg/kg), the toxin was detected in all organs and fluids, including skin, being more concentrated in urine, intestinal contents and intestine. The slow elimination of OA suggests an enterohepatic circulation of the toxin (Matias *et al.*, 1999).

After oral OA administration in mice (75–250 μg/kg), the toxin was absorbed very quickly from the small intestine, mainly from its middle part (jejunum), and reached the liver within 5 min. The distribution of the toxin, evaluated by an immunostaining method, involves the whole body, being detected in lungs, liver, heart, kidneys and intestine, where was still detected after 2 weeks (Ito *et al.*, 2002b).

After intramuscular injection of $[^3H]$OA in mice (25 μg/kg), the toxin was detected in bile and intestinal contents 1 h later and its elimination pattern showed biliary excretion and enterohepatic circulation (Matias and Creppy, 1996a). Transplacental passage of OA in pregnant mice was observed too (Matias and Creppy, 1996b).

## Mechanism of action

OA and its derivative DTX-1 are inhibitors of protein phosphatases 1 and 2A, two enzymes dephosphorylating serine/threonine residues of proteins in eukaryotic cells, through their specific binding to a receptorial site (Bialojan and Takai, 1988; Cohen, 1989). As a consequence, a rapid increase of phosphorylated proteins occurs in cells. The diarrhoea and the degenerative changes in absorptive epithelium of small intestine induced by these toxins had been attributed to accumulation of the phosphorylated proteins controlling ions secretion in intestinal cells and to inhibition of dephosphorylation of cytoskeletal or junctional elements that regulate the permeability to solutes, both phenomena resulting in a passive loss of fluids (Aune and Yndestad, 1993; Tripuraneni *et al.*, 1997). Inhibition of protein phosphatases is involved also in the tumour promoting properties of these toxins (Fujiki and Suganuma, 1993).

A free carboxyl group in the DSP molecule is essential for inhibition of protein phosphatase activity, since their

methyl and diol esters did not show inhibition (Mountfort *et al.*, 2001).

## Treatment

The treatment of the diarrhoeic poisoning is symptomatic and supportive with regards to short-term diarrhoea and accompanying fluid and electrolyte losses. In general, hospitalization is not necessary. Other diarrhoeic illnesses associated with shellfish consumption, such as bacterial or viral contamination, should be ruled out on the basis of anamnesis of the patients (Aune and Yndestad, 1993).

## Toxicity

### *Human toxicity*

Symptoms of DSP are mainly diarrhoea, nausea, vomiting and abdominal pain. Although the intoxication can be highly debilitating for some days, no human mortalities from DSP were reported, so far. Recovery is usually complete in 3 days, despite the pharmacological treatment. An LOAEL of 1.2–1.6 μg/kg was determined after human poisoning in Japan (eight persons from three families, age 10–68 years). In Norway, 38 of 70 adults were affected at levels ranging from 1.0 to 1.5 μg/kg (FAO/IOC/WHO, 2005).

### *Experimental toxicity*

*Single administration*

After single i.p. injection in mice, $LD_{50}$ values of OA were estimated in the range from 192 to 225 μg/kg (Tachibana *et al.*, 1981; Dickey *et al.*, 1990; Tubaro *et al.*, 2003).

OA or DTX-1 injection in rodents (mice and rats) induced damages at intestinal mucosa, particularly at duodenum and upper portion of jejunum, within 15 min. The injuries can be divided into three consecutive stages: increase of capillary permeability and extravasion of serum into the lamina propria of villi; degeneration of absorptive epithelium of villi; and desquamation of the degenerated epithelium from the lamina propria. At sublethal doses, these alterations are reversible and the recovery process, observed already 2 h after the toxin administration, was complete within 24 h (Terao *et al.*, 1986; Terao *et al.*, 1993; Ito and Terao, 1994). Morphological alterations induced by DTX-3 were less pronounced and consisted only in dilatation of the cysternae of Golgi apparatus and presence of vesicles in the cytoplasm of the absorptive epithelium (Terao *et al.*, 1993). An i.p. administration of OA, DTX-1 or DTX-3 to rodents induced also liver damages, with vacuolization and/or necrosis of hepatocytes (Terao *et al.*, 1993; Ito and Terao, 1994; Aune *et al.*, 1998; Tubaro *et al.*, 2003).

OA was observed to induce liver damage also after i.v. administration to rats, with congestion of blood in the liver, and dissolution of hepatic bile canalicular actin sheaths (Berven *et al.*, 2001).

OA, DTX-1 and DTX-3 are less toxic after oral administration than after i.p. injection. In particular, by oral route, the $LD_{50}$ of OA in mice ranged between 1 and 2 mg/kg (Tubaro *et al.*, 2003). Besides diarrhoea, the signs of toxicity are similar to those observed after i.p. administration. In addition, DTX-1 and DTX-3 caused also the degeneration of surface cells of the gastric mucosa (Terao *et al.*, 1993; Ito and Terao, 1994; Ito *et al.*, 2000; Berven *et al.*, 2001).

*Repeated administration*

Daily repeated oral administration of OA to mice (1 mg/kg/day, for 7 days) induced diarrhoea, body weight loss, reduced food consumption and the death of 2/5 mice. The toxic effects were observed at forestomach and liver and, at the ultrastructural level, OA induced alterations of mitochondria and fibres of myocardiocytes (Tubaro *et al.*, 2004).

*Mutagenic and genotoxic activity*

Although OA did not induce mutations in *Salmonella typhimurium* in the absence or in presence of metabolic activation, it was mutagenic in various eucaryotic cell lines *in vitro* (Aune and Yndestad, 1993; Fessard *et al.*, 1996). No genotoxicity data are available for DTX-2 and DTX-3. Genotoxic effects of OA, such as micronuclei formation, mitotic arrest and polyploidy, were observed in human Caco-2 cells (Le Hégarat *et al.*, 2006).

*Tumour promoting activity*

OA and DTX-1 are tumour promoters, as evidenced in two-stage experiments on mouse skin (Fujiki and Suganuma, 1993) and in the rat glandular stomach (Suganuma *et al.*, 1992).

## Concluding remarks

OA and DTXs are involved in DSP and possess tumour promotion activity through their inhibition of protein phosphatases. DSP risk is managed by monitoring seafood for toxicity: frequency of sampling should ensure that contamination does not rise to dangerous levels in temporal or spatial gaps between sampling times or locations. Monitoring programmes need also the assessment of the toxin-producing phytoplankton organisms in the water column in order to provide an early warning of the potential for DSP toxin contamination in shellfish.

Various methods have been developed for DSP toxins analysis. They include *in vivo* biological assays and *in vitro*

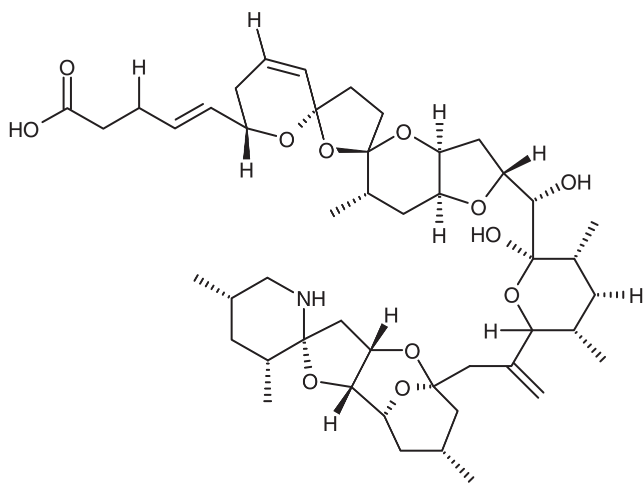



**FIGURE 60.8** Chemical structure of azaspiracid-1 (from James *et al.*, 2004).

biochemical or biological assays as well as chemical methods of analysis. The MBA is at the time of writing the accepted method for DSP toxins determination in shellfish as it can detect the presence of all DSP toxins, although other lipophilic compounds can interfere. It evaluates the survival time of mice injected i.p. with a suspension of a shellfish extract usually over a 24 h observation period (Fernández *et al.*, 2003). *In vitro* assays include enzyme or radioactivity-based immunoassays, mainly for OA and DTX-1 determination. Protein phosphatase 2A inhibition assay is a particularly sensitive *in vitro* functional assay, being based on the mechanism of action of OA (Cembella *et al.*, 2003). Chemical methods of analysis include high-performance liquid chromatography (HPLC), capillary electrophoresis (CE) and liquid chromatography-mass spectrometry (LC-MS) techniques (Quilliam, 2003). For functional, ELISA and instrumental methods, the results should include the toxins release by hydrolysis of their ester forms.

Regarding the control of DSP toxins, the regulation in most countries is based on the negative results of shellfish samples at the MBA. In the European Union, besides the MBA, alternative detection methods can be used for the assessment of DSP risk and the maximum tolerated level of diarrhoeic toxins in shellfish was set at 160 μg of OA equivalents/kg of edible part (EC, 2002).

## AZASPIRACIDS

### Background

AZP is the most recently discovered toxic syndrome from algal contaminated shellfish consumption. The illness was reported for the first time in 1995 in the Netherlands, when eight people, after ingestion of blue mussels from Ireland, developed symptoms very similar to those of DSP, even though the concentration of OA and derivatives was low. Subsequently, the azaspiracid toxin group was discovered. The structural elucidation of the first toxin of this group, Azaspiracid1 (AZA1), showed that it had a polyether backbone with a unique spiral ring assembly, a cyclic amine and a carboxylic acid (Figure 60.8). Two analogues of AZA1 (AZA2 and AZA3) were discovered in mussels by liquid chromatography-tandem electrospray mass spectrometry (Ofuji *et al.*, 1999). Others analogues are AZA4 and AZA5, these structures were elucidated by electrospray ionization-mass spectrometry (Ofuji *et al.*, 2001). Recently, five hydroxylated analogues were discovered in mussels (*Mytilus edulis*) using multiple tandem mass spectrometry (James *et al.*, 2003b).

AZAs, first detected in blue mussels (*Mytilus edulis*), have been found in other bivalves, including oysters (*Crassostrea gigas*, *Ostrea edulis*), scallops (*Pecten maximus*), clams (*Tapes phillipinarium*), cockles (*Cardium edule*) and razor clams (*Ensis siliqua*) (Furey *et al.*, 2003). Reports of AZA poisonings and/or contaminated shellfish have been documented from several European Countries, including Ireland, United Kingdom, Norway, the Netherlands, France and Spain. The source of AZAs is considered to be the heterotropic dinoflagellate *Protoperidinium crassipes*. Although AZAs have not been identified outside of Western Europe so far, *Protoperidinium* species occur throughout the world's oceans. Therefore, the AZP syndrome is probably widespread, but generally unreported because its gastrointestinal symptoms are similar to those of DSP or of bacterial enterotoxin poisoning (James *et al.*, 2003a).

### Pharmacokinetics/toxicokinetics

No data are available, so far, about absorption, distribution, metabolism and excretion of AZAs.

### Mechanism of action

Studies on neuroblastoma cells demonstrated that AZA1 disrupts the cytoskeletal structure decreasing F-actin pools (Román *et al.*, 2002). AZA1 was also found to affect the arrangement of F-actin in Jurkat cells, with the concurrent loss of pseudopodia (Twiner *et al.*, 2005). It has been suggested that the actin-disrupting effect of OA in cultured cells could be associated with its diarrhogenic activity, reflecting the loosening of tight junctions *in vivo* (Fiorentini *et al.*, 1996). Although the AZAs effect on F-actin is lower than that of OA, this link between F-actin changes and diarrhogenic activity could be relevant to

explain the AZAs gastrointestinal toxicity, very similar to that of DSP. AZA1 causes also a significant increase in intracellular calcium ions levels ($[Ca^{2+}]_i$) in lymphocytes (Román *et al.*, 2002), whereas AZA4 appears to inhibit $Ca^{2+}$ entry in human T lymphocytes (Alfonso *et al.*, 2005).

## Toxicity

### *Human toxicity*

The human symptoms included nausea, vomiting, severe diarrhoea and stomach cramps (Magdalena *et al.*, 2003).

### *Experimental toxicity*

Due to the lack of commercially available toxins, only few *in vivo* toxicological studies are available for AZAs, so far.

#### *Single administration*

After i.p. administration, the lethal dose of AZA1 in mice has been shown to be 200 μg/kg, whereas AZA2 and AZA3 are more toxic (140 μg/kg). AZA4 and AZA5 are less toxic (James *et al.*, 2004). Although diarrhoea is the main symptom in humans, AZAs did not cause diarrhoea after i.p. injection: mice showed progressive paralysis of the limbs, dyspnoea, and convulsions before death.

After *per os* administration of sublethal doses of AZA1 in mice, necrosis in the lamina propria of the small intestine and in lymphoid tissues (thymus, spleen and Peyer's patches) was observed. Both T and B lymphocytes were injured. Fatty changes in the liver were also observed (Ito *et al.*, 2000). The authors hypothesized that the oral toxicity is 2.5 times higher than that after i.p., but no data on $LD_{50}$ are available. No data about oral toxicity are available for AZA analogues.

#### *Repeated administration*

After repeated oral intake of sublethal doses of AZA1 (250–450 μg/kg) in ICR mice, death in some mice was observed as well as serious gastrointestinal, pulmonary and hepatic effects that persisted for a prolonged period in surviving mice. Repeated oral administration of AZA1 once or twice a week (20–50 μg/kg, for 10–20 weeks) caused interstitial pneumonia, shortening of intestinal villi as well as death of some mice. Lung tumours were developed by 4 out of 20 mice treated with 20 or 50 μg AZA1/kg but further studies are needed to confirm the carcinogenicity of this toxin (Ito *et al.*, 2002a). No genotoxicity data are available and no definitive conclusions regarding relevance to humans can be drawn, so far (FAO/IOC/WHO, 2005).

## Treatment

As for DSP, a specific antidote is not available for AZP: therefore, the treatment is only symptomatic and supportive.

## Concluding remarks

The maximum overall level of azaspiracid was set by the European Union at 160 μg of AZAs equivalents/kg of edible mollusc part (EC, 2002). Mouse or rat bioassay can detect AZAs with a limit of detection of about 160 μg/kg, but other lipophilic toxins can potentially interfere in this assay. Analytical methods like LC-MS (MS) are preferred for accuracy in measuring the concentration of AZAs in contaminated shellfish.

# OTHER TOXINS

# YESSOTOXINS

## Background

Yessotoxin (YTX) (Figure 60.9) is a ladder-shaped polycyclic ether toxin, named for the scallop *Patinopecten yessoensis*, from which it was isolated (Murata *et al.*, 1987). YTX and its analogues were initially included in the DSP toxins because they were found together with OA and other lipophilic toxins during the shellfish extraction procedure for DSP MBA. Recently, YTXs have been classified and regulated separately from the DSP toxins, since they do not induce diarrhoea (Ogino *et al.*, 1997). More than 30 YTX analogues have been isolated both from scallops and microlagae, so far. Nevertheless, no human toxicity has been reported for YTXs, although many of these compounds are highly toxic to mice, after i.p. administration (Bowden, 2006).

YTXs were initially found to be produced by dinoflagellates, such as *Protoceratium reticulatum* (=*Gonyaulax grindley*) (Satake *et al.*, 1997) and *Lingulodinium polyedrum* (=*Gonyaulax polyedra*) (Paz *et al.*, 2004). Because both algal species are common in coastal waters of many regions. The YTXs have widespread occurrence (Yasumoto and Satake, 1998). Recently, in New Zealand also *Gonyaulax spinifera* was identified as a YTX producer (Rhodes *et al.*, 2006).

During their filter-feeding activity, YTXs are concentrated in bivalve shellfish.YTXs-contaminated shellfish are found mainly in Japan, Chile, Italy, Norway and New Zealand (Ciminiello and Fattorusso, 2004; Aasen *et al.*, 2005a; Koike *et al.*,2006).

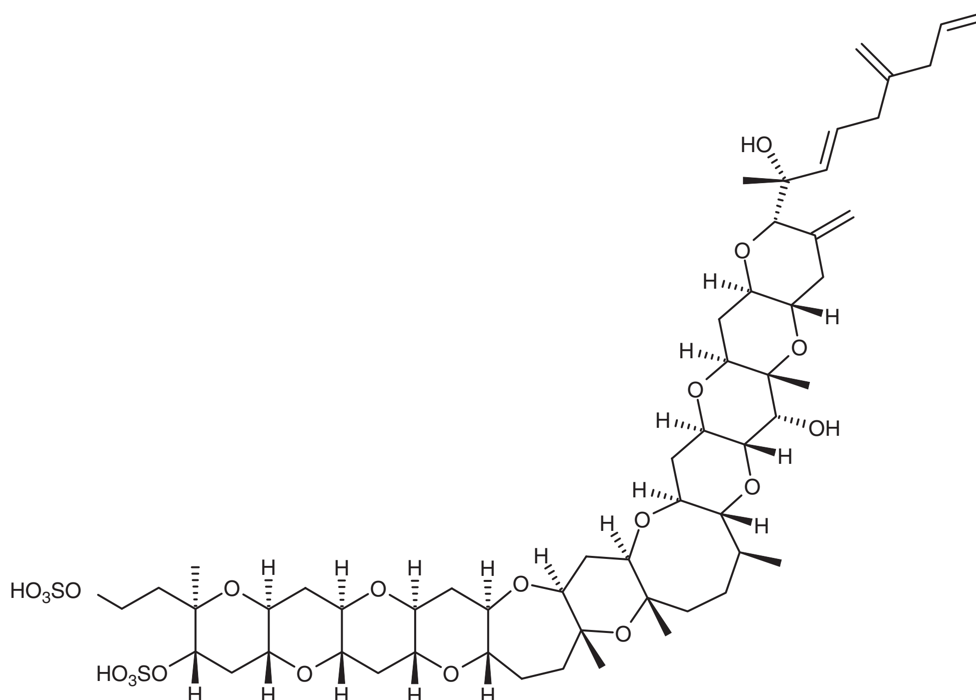



**FIGURE 60.9** Chemical structure of yessotoxin (from Quilliam, 2003).

## Pharmacokinetics/toxicokinetics

No data are available for about absorption, distribution, metabolism and excretion of YTXs, although toxicity data in mice suggest a poor absorption after oral intake.

## Mechanism of action

Based on available data, very low concentrations of YTX appear to affect many systems *in vitro*, but some of these effects are not confirmed *in vivo* after *per os* exposure (Bowden, 2006). Bianchi *et al.* (2004) demonstrated that YTX opens the permeability transition pore of the inner mitochondrial membrane at nanomolar concentrations. YTX is cytotoxic against several cell lines (Glioma C6, HeLa $S_3$, BE(2)-M17, L6 rat myoblast and BC3H1 mouse myoblast) and induces apoptotic effects (Ogino *et al.*, 1997; Leira *et al.*, 2002a; Malaguti *et al.*, 2002; Suarez-Korsnes *et al.*, 2006). Furthermore, YTX disrupt cell adhesion and causes E-cadherin fragmentation at concentrations ranging from $10^{-8}$ to $10^{-10}$ M (Ronzitti *et al.*, 2004). Furthermore, YTX seems to modulate intracellular calcium movements and cyclic AMP (cAMP) levels at higher concentration (ranging from $10^{-7}$ to $10^{-5}$ M) (de la Rosa *et al.*, 2001).

## Toxicity

### *Human toxicity*

There are no reports of human poisoning induced by YTXs, so far (FAO/IOC/WHO, 2005).

### *Experimental toxicity*

Although more than 30 YTXs were isolated, only YTX, desulpho-YTX, homo-YTX and 45-OH-homoYTX were submitted to actual toxicological studies.

#### *Single administration*

After i.p. injection in mice, $LD_{50}$ values ranging from 100 to 750 μg/kg are reported for YTX and homoYTX, while 45-OH-homoYTX did not induced lethality at 750 μg/kg (Terao *et al.*, 1990; Ogino *et al.*, 1997; Aune *et al.*, 2002, Tubaro *et al.*, 2003). Light microscopy revealed only slight intracellular oedema in the heart of animals treated with 750 and 1000 μg/kg (Aune *et al.*, 2002). Electron microscopy analysis revealed swelling of cardiomyocytes and rounded mitochondria in some areas of these cells closed to capillaries (Terao *et al.*, 1990; Aune *et al.*, 2002; Tubaro *et al.*, 2003). Damages to cerebellar Purkinje cells after i.p. injection of YTX (420 μg/kg) were also observed, but not confirmed after *per os* administration (Hungerford, 2006).

After oral administration (the usual human exposure route), YTXs are much less toxic. No lethality or changes in mice behaviour was observed after acute administration of YTX (0.5–10 mg/kg). Only ultrastructural changes were observed at myocardium level, similar to that described after i.p. administration (Terao *et al.*, 1990; Aune *et al.*, 2002; Tubaro *et al.*, 2003). Although YTX induces apoptosis *in vitro* in various cell lines, no apoptotic changes were observed in the myocardium of treated animals using *in situ* TUNEL staining (Tubaro *et al.*, 2003).

*Repeated administration*

After repeated *oral administration* no deaths, notable changes in behaviour or growth, macroscopic abnormalities were observed after daily treatment for 7 days with YTX (1 and 2 mg/kg), homoYTX and 45-OH-homoYTX (1 mg/kg). Only ultrastructural changes at cardiac level (cardiomyocytes swelling, protrusion of cardiac cells into pericapillary space with rounding mitochondria) were observed (Tubaro *et al.*, 2004).

## Concluding remarks

Due to the lack of evidence of adverse effects in humans and the significant reduction in potency after oral administration compared to i.p. injection in mice, so far a regulatory level of 1 mg YTX equivalents/kg shellfish has been fixed in some countries. For regulatory purposes, MBA for lipophilic toxins is used in European countries (EC, 2002), but LC-MS methods, suitable for screening and confirmation, have been developed for YTXs (FAO/ IOC/WHO, 2005).

Since, *in vitro* very low concentration of YTX provokes disruption of the tumour suppressor E-cadherin, a possible risk that YTX may favour tumour spreading and metastasis formation *in vivo* has been hypothesized (Ronzitti *et al.*, 2004). However, further *in vivo* experiments are necessary to verify this hypothesis.

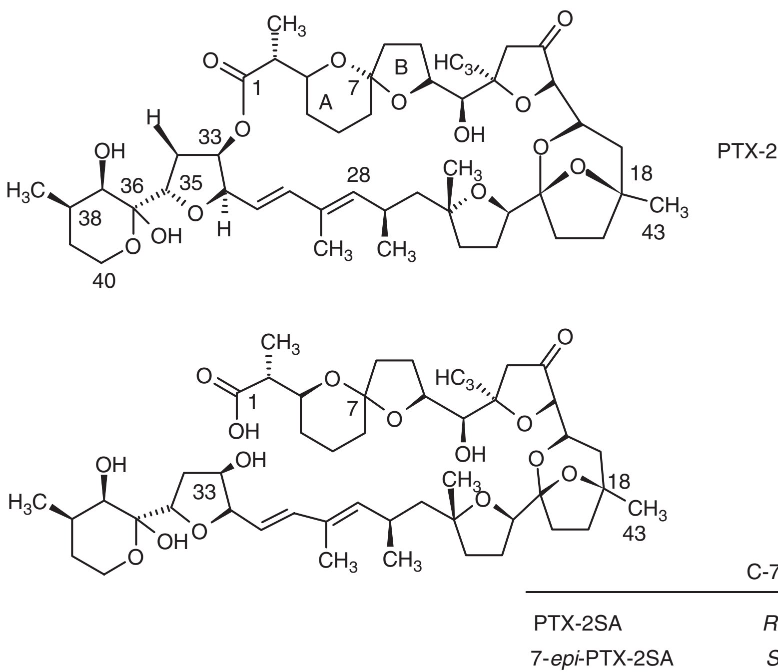
# PECTENOTOXINS

## Background

Pectenotoxins (PTXs) are hepatotoxic compounds, initially classified as DSP toxins, being often detected in shellfish and phytoplankton together with these compounds. Nevertheless, although a PTX derivative was associated with severe diarrhoeic illnesses in Australia, the actual diarrhogenic potential of PTXs is still unclear as is their actual health threat to consumers of contaminated mussels (Burgess and Shaw, 2001; FAO/IOC/ WHO, 2005).

PTXs are polyether macrolides containing a spiroacetal, three substituted tetrahydrofurans and 19 (20 in the case of PTX-11) stereocentres within a 40-carbon chain. To date, more than 10 PTXs and two PTX-2 derivatives (PTX-2 seco-acid and 7-epi-PTX-2 seco acid) (Figure 60.10) have been isolated from phytoplankton or shellfish. Recently, fatty acid esters of PTX-2 seco acids were detected in shellfish too (Miles *et al.*, 2006; Wilkins *et al.*, 2006).

PTX-2, produced by the dinoflagellate species *Dinophysis acuta* and *D. fortii*, is thought to be the precursor of the other PTXs, formed through biotransformation reactions in shellfish (Draisci *et al.*, 2000; Leira *et al.*, 2002b). Only PTX-11, -12 and -13 were identified as biosynthetic compounds in algae (*D. acuta*), besides PTX-2 (Miles *et al.*, 2004, 2006; Suzuki *et al.*, 2006). PTXs are accumulated in the digestive glands of filter-feeding bivalves, such as




| | C-7 |
|---|---|
| PTX-2SA | *R* |
| 7-*epi*-PTX-2SA | *S* |

**FIGURE 60.10** Chemical structure of pectenotoxin-2 (PTX-2) and of its seco acids (from Draisci *et al.*, 2000).

scallops (*Patin-opecten yessoensis*), green-shell mussels (*Perna canaliculus*), blue mussels (*Mytilus galloprovincialis* and *Mytilus edulis*), cockles (*Cerastoderma edule*) and pipis (*Donax delatoides*), after filtration of *Dinophysis* cells (Yasumoto *et al.*, 1985; Draisci *et al.*, 2000; Suzuki *et al.*, 2001; Vale and de Sampayo, 2002). These organisms can transfer the toxins to humans after their consumption.

PTXs were detected in shellfish and/or phytoplankton from Japan, New Zealand and Europe (Sasaki *et al.*, 1999; Draisci *et al.*, 2000; Burgess and Shaw, 2001; Pavela-Vrančič *et al.*, 2001; Leira *et al.*, 2002b; Miles *et al.*, 2004; Suzuki *et al.*, 2006).

## Pharmacokinetics/toxicokinetics

No data on absorption, distribution, metabolism and excretion of PTXs are available.

## Mechanism of action

The mechanism of hepatotoxic action of PTXs requires clarification. PTXs are cytotoxic against several cancer cell lines and PTX-1 and PTX-2 were shown to induce apoptotic effects (Draisci *et al.*, 2000; Chae *et al.*, 2005). *In vitro* studies revealed a depolymerizing action of F-actin by PTX-6 (Leira *et al.*, 2002b). Furthermore, PTX-6 modified cAMP levels in human lymphocytes (Leira *et al.*, 2002b).

## Toxicity

### *Human toxicity*

PTXs had been associated with severe diarrhoeic illness in Australia, resulting in hospitalization. However, although the shellfish responsible for the poisoning contained PTX-2 seco acid, the gastrointestinal effects were later attributed to OA esters. Consequently, no evidence of gastrointestinal or other adverse effects in humans for PTXs is available (FAO/IOC/WHO, 2005).

### *Experimental toxicity*

*Single administration*

After i.p. injection in mice, $LD_{50}$ values of PTXs were determined to be 250 μg/kg (PTX-1), 219–260 μg/kg (PTX-2), 350 μg/kg (PTX-3), 770 μg/kg (PTX-4) and 500 μg/kg (PTX-6), whereas $LD_{50}$ of PTX-7, PTX-8, PTX-9, PTX-2 seco acid and 7-epi-PTX-2 seco acid was higher than 5 mg/kg. Thus, it seems that the oxidation of substituent at C18, occurring in the digestive glands of shellfish, reduces the toxicity of PTXs (Draisci *et al.*, 2000; Miles *et al.*, 2004, 2006).

A hepatotoxic effect was observed in suckling mice for PTX-1 as well as for PTX-1 and PTX-2 in adult mice and rats. None of the two toxins caused diarrhoea and no damage at the intestinal level was observed in suckling mice after i.p. injection of up to 1 mg PTX-1/kg. On the other hand, PTX-1 and -2 induced an increased permeability of capillaries in the digestive tract, ascites and fluid accumulation in the thorax and pericardium of adult mice and rats at the dose level of 375 μg/kg (Terao *et al.*, 1986, 1993).

Although some literature data on PTXs toxicity after oral administration are controversial, Terao *et al.* (1993) observed that PTX-1 and PTX-2 (750 μg/kg) did not induce changes at intestinal level of mice and rats. Recently, no lethality and no diarrhogenic effects were evidenced for PTX-2 and PTX-2 seco acid in mice, at the dose of 5 mg/kg (Miles *et al.*, 2004).

## Concluding remarks

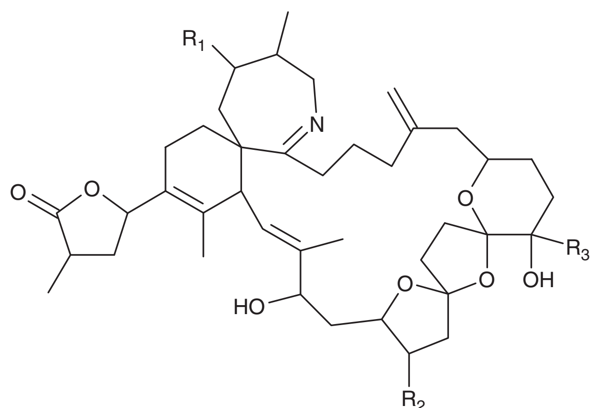
The European Union did not set a maximum overall level for PTXs alone in shellfish since the established level of 160 μg/ kg of edible part of shellfish is referred to the sum of PTXs and diarrhoeic toxins of the OA group (EC, 2002). MBA can detect PTXs, but not the seco acids, the presence of other lipophilic toxins, such as azaspiracids, OA and its derivatives can be detected by the MBA. Since PTXs are synthesized by the same microalgae producing OA and its derivatives, it is important to distinguish the two toxin groups. Immunochemical and LC-MS methods are preferred for their sensitivity and accuracy in detecting PTXs in shellfish (FAO/IOC/WHO, 2005).

# CYCLIC IMINE TOXINS

## Background

In recent years, a variety of seafood toxins with a cyclic imine function and macrocyclic structure had been discovered through their fast lethality in mice using the MBA for lipophilic shellfish toxins detection. These toxins include spirolides, gymnodimines, prorocentrolides and pinnatoxins, which harmful potential for humans is unclear (Quilliam, 2003; FAO/IOC/WHO, 2005).

Several spirolides, characterized by a spiro-linked tricyclic ether structure and an imine or amine function, had been identified in shellfish and phytoplankton: spirolides A–G and their derivates, among which spirolides E and F are non-toxic metabolites formed in shellfish (Hu *et al.*, 1996; Falk *et al.*, 2001; Aasen *et al.*, 2005b) (Figure 60.11). Spirolides, produced by the dinoflagellate *Alexandrium ostenfeldii*, are accumulated in filter-feeding bivalves, such as mussels and oysters (Cembella *et al.*, 2001). They occur




| Spirolide | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|
| A | H | $CH_3$ | $CH_3$ |
| B | H | $CH_3$ | $CH_3$ |
| C | $CH_3$ | $CH_3$ | $CH_3$ |
| D | $CH_3$ | $CH_3$ | $CH_3$ |

**FIGURE 60.11** Chemical structure of spirolides A–D (from Ciminiello and Fattorusso, 2004).

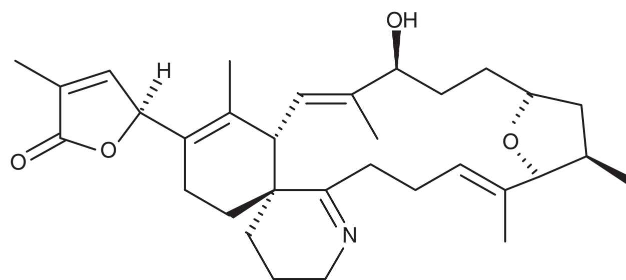



**FIGURE 60.12** Chemical structure of gymnodimine (from Quilliam, 2003).

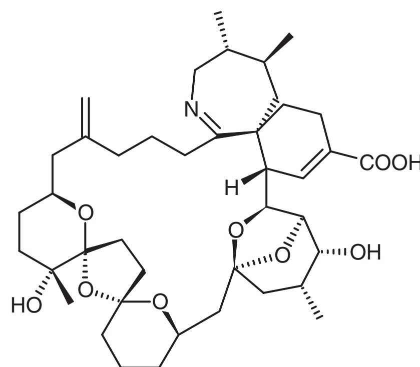



**FIGURE 60.13** Chemical structure of pinnatoxin A (from Quilliam, 2003).

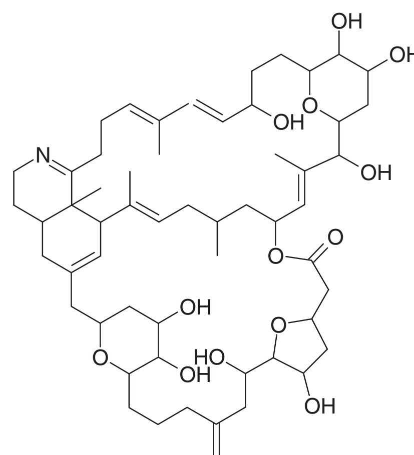



**FIGURE 60.14** Chemical structure of prorocentrolide (from Torigoe *et al.*, 1988).

in different areas of the world, being detected in the United States, Canada, Norway, Denmark and Scotland (Aasen *et al.*, 2003; Cembella, 2003, Gribble *et al.*, 2005).

Gymnodimines (Figure 60.12) containing a spiro centre and an imine function, are structurally related to spirolides. They are produced by dinoflagellates of *Gymnodinium* (*Karenia*) genus and accumulated in bivalves, such as oysters species (*Crassostrea gigas*, *Tiostrea chilensis*), greenshell (*Perna canaliculus*), scallops (*Pecten novaezelandiae*), also in tissues outside the digestive gland. These toxins were detected in New Zealand and other parts of the world, as for instance Tunisia (Seki *et al.*, 1995; Stewart *et al.*, 1997; Miles *et al.*, 2000; Stirling, 2001; MacKenzie *et al.*, 2002; FAO/IOC/WHO, 2005).

Pinnatoxins (Figure 60.13) consist of a 20-membered ring with 5,6-bicyclo, 6,7-azaspiro and 6,5,6-triketal moieties in their structure. They are accumulated in shellfish of the genus *Pinna*, common seafood in Japan and China, where their consumption caused human intoxications initially attributed to pinnatoxins (Kuramoto *et al.*, 2004). However, it was later shown that the poisoning was due to contamination by *Vibrio* species (FAO/IOC/WHO, 2005).

Other cyclic imines of algal origin are the cyclic polyethers prorocentrolide (Figure 60.14) and its analogue prorocentrolide B produced by dinoflagellates of the genus *Prorocentrum* (Torigoe *et al.*, 1988; Hu *et al.*, 1996).

## Pharmacokinetics/toxicokinetics

No data on absorption, metabolism, distribution and elimination of these cyclic imines are available.

## Mechanism of action

The mechanism of action of the cyclic imines is not completely known. Biological studies on spirolides suggest hippocampus and brain stem as their main toxicity targets with an action as muscarinic acetylcholine receptor antagonists and weak L-type transmembrane calcium channel activators (Gill *et al.*, 2003; Sleno *et al.*, 2004). The active pharmacophore of these compounds seems to be the cyclic imine moiety, since spirolides E and F, lacking of this structure, are inactive (Luckas *et al.*, 2005).

Gymnodimine seems to block nicotinic receptors at the neuromuscular junction (Munday *et al.*, 2004).

## Toxicity

### Human toxicity

No human poisoning syndrome due to cyclic imines was documented, so far. In Japan and China, seafood poisonings initially attributed to pinnatoxins were later shown to be caused by *Vibrio* species (FAO/IOC/WHO, 2005).

### Experimental toxicity

*Single administration*

After i.p. injection in mice, spirolides cause the death of animals within some minutes, preceded by neurotoxic symptoms as piloerection, abdominal spasms, hyperextension of the back and arching of the tail (Gill *et al.*, 2003; Ciminiello and Fattorusso, 2004). $LD_{50}$ in mice by i.p. was calculated to be about 40 μg/kg, the lethality being about 25 times higher than that after oral administration ($LD_{50}$ = 1 mg/kg) (Cembella *et al.*, 2002).

After i.p. injection of gymnodimine in mice, an $LD_{50}$ value of 96 μg/kg was determined for gymnodimine, which induced paralysis and respiratory distress (Munday *et al.*, 2004). After oral administration in mice by gavage, an $LD_{50}$ of 755 μg/kg was calculated, but no toxicity was seen if the toxin was ingested with food at a level corresponding to a dose of about 7500 μg/kg (Munday *et al.*, 2004).

The $LD_{50}$ of prorocentrolide by i.p. injection in mice was calculated to be 400 μg/kg (Torigoe *et al.*, 1988).

## Concluding remarks

Although cyclic imines are lethal in mice and contaminate shellfish in several parts of the world, no cases of adverse effects in humans due to these toxins were reported. Only pinnatoxins were associated to seafood poisonings, subsequently attributed to a contamination by *Vibrio* species (FAO/IOC/WHO, 2005). Therefore, no maximum tolerated level for these toxins was established, although their actual toxicity needs to be clarified.

The high i.p. toxicity of cyclic imines interferes with the MBA for the lipophilic toxins detection, giving positive results. Therefore, other methods are needed to quantify these toxins, such as the LC-MS methods, already set up for spirolides and gymnodimines analysis (FAO/IOC/WHO, 2005).

## REFERENCES


Aasen J, LeBlanc P, Hardstaff W, Hovgaard P, Burton IW, MacKinnon SL, Walter JA, Aune T, Quilliam MA (2003) Detection and identification of spirolides in Norwegian mussels and plankton. In *Proceedings of the Eighth Canadian Workshop on Harmful Marine Algae*, Bates SS (ed.). Fisheries and Oceans, Moncton, NB, Canada, pp. 5–7.

Aasen J, Samdal IA, Miles CO, Dahl E, Briggs LR, Aune T (2005a) Yessotoxins in Norwegian blue mussels (*Mytilus edulis*): uptake from *Protoceratium reticulatum*, metabolism and depuration. *Toxicon* **45**: 265–72.

Aasen J, MacKinnon SL, LeBlanc P, Walter JA, Hovgaard P, Aune T, Quilliam MA (2005b) Detection and identification of spirolides in Norwegian shellfish and plankton. *Chem Res Toxicol* **18**: 509–15.

Abraham WM, Bourdelais AJ, Ahmed A, Serebriakov I, Baden DG (2005) Effects of inhaled brevetoxins in allergic airways: toxin–allergen interactions and pharmacologic intervention. *Environ Health Perspect* **113**: 632–7.

Alfonso A, Román Y, Vieytes MR, Ofuji K, Satake M, Yasumoto T, Botana LM (2005) Azaspiracid-4 inhibits $Ca^{2+}$ entry by stored operated channels in human T lymphocytes. *Biochem Pharmacol* **69**: 1627–36.

Andrinolo D, Michea LF, Lagos N (1999) Toxic effects, pharmacokinetics and clearance of saxitoxin, a component of paralytic shellfish poison (PSP), in cats. *Toxicon* **37**: 447–64.

Andrinolo D, Iglesias V, Garcia C, Lagos N (2002) Toxicokinetics and toxicodynamics of gonyautoxins after an oral toxin dose in cats. *Toxicon* **40**: 699–709.

Aune T, Yndestad M (1993) Diarrhetic shellfish poisoning. In *Algal Toxins in Seafood and Drinking Water*, Falconer IR (ed.). Academic Press, London, pp. 87–104.

Aune T, Stabell OB, Nordstoga K, Tjøtta K (1998) Oral toxicity in mice of algal toxins from the diarrheic shellfish toxin (DST) complex and associated toxins. *J Nat Toxins* **7**: 141–58.

Aune T, Sørby R, Yasumoto T, Ramstad H, Landsverk T (2002). Comparison of oral and intraperitoneal toxicity of yessotoxin towards mice. *Toxicon* **40**: 77–82.

Baden DG, Adams DJ (2000) Brevetoxins: chemistry, mechanism of action, and methods of detection. In *Seafood and Freshwater Toxins: Pharmacology, Physiology, and Detection*, Botana LM (ed.). Marcel Dekker, New York, pp. 505–32.

Baden DG, Bourdelais AJ, Jacocks H, Michelliza S, Naar J (2005). Natural and derivative brevetoxins: historical background, multiplicity, and effects. *Environ Health Perspect* **113**: 621–5.

Bagnis RA, Legrand A-M (1987) Clinical features on 12,980 cases of ciguatera (fish poisoning) in French Polynesia. In Progress in Venom and Toxin Research. *Proceedings of the 1st Asia-Pacific Congress on Animal, Plant and Microbial Toxins*, Gopalakrishnakone P, Tan CK (eds). National University of Singapore, Singapore, pp. 372–84.

Benson JM, Tischler DL, Baden DG (1999) Uptake, tissue distribution, and excretion of brevetoxin 3 administered to rats by intratracheal instillation. *J Toxicol Environ Health A* **56**: 345–55.

Benson J, Hahn F, March T, McDonald J, Sopori M, Seagrave J, Gomez A, Bourdelais A, Naar J, Zaias J, Bossart G, Baden D (2004) Inhalation of brevetoxin 3 in rats exposed for 5 days. *J Toxicol Environ Health A* **67**: 1443–56.

Benson, J.M., Hahn, F.F., March, T.H., McDonald, J.D., Gomez, A.P., Sopori, M.J., Boudelais AJ, Naar J, Zaias J, Bossart GD, Baden DG (2005) Inhalation toxicity of brevetoxin 3 in rats exposed for twenty-two days. *Environ Health Perspect* **113**: 626–31.

Berven G, Sætre, F, Halvorsen K, and Seglen PO (2001) Effects of the diarrhetic shellfish toxin, okadaic acid, on cytoskeletal elements, viability and functionality of rat liver and intestinal cells. *Toxicon* **39**: 349–62.

Bialojan C, Takai A (1988) Inhibitory effect of a marine-sponge toxin, okadaic acid, on protein phosphatases. *Biochem J* **256**: 283–90.

Bianchi C, Fato R, Angelin A, Trombetti F, Ventrella V, Borgatti AR, Fattorusso E, Ciminiello P, Bernardi P, Lenaz G, Castelli GP (2004) Yessotoxin, a shellfish biotoxin, is a potent inducer of the permeability transition in isolated mitochondria and intact cells. *Biochim Biophys Acta* **1656**: 139–47.

Bottein Dechraoui MY, Wang Z, Turquet J, Chinain M, Darius T, Cruchet P, Radwan FF, Dickey RW, Ramsdell JS (2005) Biomonitoring of ciguatoxin exposure in mice using blood collection cards. *Toxicon* **46**: 243–51.

Bouaïcha N, Ammar M, Hennion MC, Sandra P (1997) A new method for determination of maitotoxin by capillary zone electrophoresis with ultraviolet detection. *Toxicon* **35**: 955–62.

Bowden B (2006) Yessotoxins – polycyclic ethers from dinoflagellates: relationship to diarrhetic shellfish toxins. *Toxin Rev* **25**: 137–57.

Burgess V, Shaw G (2001) Pectenotoxins – an issue for public health. A review of their comparative toxicology and metabolism. *Environ Int* **27**: 275–83.

Cattet M, Geraci JR (1993) Distribution and elimination of ingested brevetoxin (PbTx-3) in rats. *Toxicon* **31**: 1483–6.

Cembella A (2003) Chemical ecology of eukaryotic microalgae in marine ecosystems. *Phycologia* **42**: 420–47.

Cembella AD, Bauder AG, Lewis NI, Quilliam MA (2001) Association of the gonyaulacoid dinoflagellate *Alexandrium ostenfeldii* with spirolide toxins in size-fractionated plankton. *J Plan Res* **23**: 1413–19.

Cembella A, Bauder A, MacKinnon S, Quilliam M, Richard D, Walter J, Windust A (2002) Spirolides: emerging phycotoxins in plankton and shellfish from the North Atlantic. In *Proceeding of the Fourth International Conference on Molluscan Shellfish Safety*, Xunta de Galicia and IOC of UNESCO.

Cembella AD, Doucette G J, Garthwaite I (2003) *In vitro* assays for phycotoxins. In *Manual on Harmful Marine Microalgae*, Hallegraeff GM, Anderson DM, Cembella AD (eds). UNESCO, Saint-Berthevin, pp. 297–345.

Chae HD, Choi TS, Kim BM, Jung JH, Bang YJ, Shin DY (2005) Oocyte-based screening of cytokinesis inhibitors and identification of pectenotoxin-2 that induces Bim/Bax-mediated apoptosis in p53-deficient tumors. *Oncogene* **24**: 4813–19.

Ciminiello P, Fattorusso E (2004) Shellfish toxins – chemical studies on Northern Adriatic mussels. *Eur J Org Chem* **2004**: 2533–51.

Cohen P (1989) The structure and regulation of: protein phosphatases. *Annu Rev Biochem* **58**: 453–508.

Danaras AH, Norte M, Fernández JJ (2001) Toxic marine microalgae. *Toxicon* **39**: 1101–32.

Dechraoui MY, Naar J, Pauillac S, Legrand AM (1999) Ciguatoxins and brevetoxins, neurotoxic polyether compounds active on sodium channels. *Toxicon* **37**: 125–43.

de la Rosa L A, Alfonso A, Vilarino N, Vieytes MR, Botana LM (2001) Modulation of cytosolic calcium levels of human lymphocytes by yessotoxin, a novel marine phycotoxin. *Biochem Pharmacol* **61**: 827–33.

Dickey RW, Bobzin SC, Faulkner DJ, Bencsath FA, Andrzejewski D (1990) Identification of okadaic acid from a Caribbean dinoflagellate, *Prorocentrum concavum Toxicon* **28**: 371–7.

Doble A (2000) Pharmacology of domoic acid. In *Seafood and Freshwater Toxins: Pharmacology, Physiology, and Detection*, Botana LM (ed.). Marcel Dekker, New York, pp. 359–72.

Draisci R, Lucentini L, Mascioni A (2000) Pectenotoxins and yessotoxins: chemistry, toxicology, pharmacology, and analysis. In *Seafood and Freshwater Toxins: Pharmacology, Physiology and Detection*, Botana LM. (ed.). Marcel Dekker, New York, pp. 289–324.

EC (2002) Commission decision of 15 March 2002 laying down detailed rules for the implementation of Council Directive 91/492/EEC as regards the maximum levels and the methods of analysis of certain marine biotoxins in bivalve molluscs, echinoderms, tunicates and marine gastropods. (2002/225/EC). *Off J Eur Commun* **L75**: 62–4.

Escobar LI, Salvador C, Martinez M, Vaca L. (1998) Maitotoxin, a cationic channel activator. *Neurobiology* **6**: 59–74.

Estacion M (2000) Ciguatera toxins: mechanism of action and pharmacology of maitotoxin. In *Seafood and Freshwater Toxins: Pharmacology, Physiology and Detection*, Botana LM (ed.). Marcel Dekker, New York, pp. 473–503.

Fairey ER, Shuart NG, Busman M, Moeller PDR, Ramsdell JS (2001) Biomonitoring brevetoxin exposure in mammals using blood collection cards. *Environ Health Perspect* **109**: 717–20.

Falk M, Burton IW, Hu T, Walter JA, Wright JLC (2001) Assignment of the relative stereochemistry of the spirolides, macrocyclic toxins isolated from shellfish and from the cultured dinoflagellate *Alexandrium ostenfeldii*. *Tetrahedron* **57**: 8659–65.

FAO/IOC/WHO (2005) Available http://www.fao.org/es/ESN/food/risk_biotoxin_en.stm.

Farstad DJ, Chow T (2001) A case report and review of ciguatera poisoning. *Wilderness Environ Med* **12**: 263–9.

Fernández ML, Richard DJA, Cembella AD (2003) *In vivo* assays for phycotoxins. In *Manual on Harmful Marine Microalgae*, Hallegraeff GM, Anderson DM, Cembella AD (eds.). UNESCO, Saint-Berthevin, pp. 347–80.

Fessard V, Diogene G, Dubreuil A, Puiseux-Dao S (1994). Selection of cytotoxic responses to maitotoxin and okadaic acid and evaluation of toxicity of dinoflagellate extracts. *Nat Toxins* **2**: 322–8.

Fessard V, Grosse Y, Pfohl-Leszkowicz A, Puiseux-Dao S (1996) Okadaic acid treatment induces DNA adduct formation in BHK21 C13 fibroblasts and HESV keratinocytes. *Mutat Res* **361**: 133–41.

Fiorentini C, Matarrese P, Fattorossi A, Donelli G (1996) Okadaic acid induces changes in the organization of F-actin in intestinal cells. *Toxicon* **34**: 937–45.

Fujiki H, Suganuma M (1993) Tumor promotion by inhibitors of protein phosphataes 1 and 2A: the okadaic acid class of compounds. *Adv Cancer Res* **61**: 143–94.

Furey A, Moroney C, Magdalena AB, Saez MJF, Lehane M, James KJ (2003) Geographical, temporal, and species variation of the polyether toxins, azaspiracids, in shellfish. *Environ Sci Technol* **37**: 3078–84.

Gessner BD (2000) Neurotoxic Toxins. In *Seafood and Freshwater Toxins: Pharmacology, Physiology and Detection*, Botana LM (ed.). Marcel Dekker, New York, pp. 65–90.

Gessner BD, Bell P, Doucette GJ, Moczydlowski E, Poli MA, Van Dolah F, Hall S (1997). Hypertension and identification of toxin in human urine and serum following a cluster of mussel-associated paralytic shellfish poisoning outbreaks. *Toxicon* **35**: 711–22.

Gill S, Murphy M, Clausen J, Richard D, Quilliam M, MacKinnon S, LaBlanc P, Mueller R, Pulido O (2003) Neural injury biomarkers of novel shellfish toxins, spirolides: a pilot study using immunochemical and transcriptional analysis. *Neurotoxicology* **24**: 593–604.

Glaziou P, Legrand AM(1994) The epidemiology of ciguatera fish poisoning. *Toxicon* **32**: 863–73.

Gordon CJ, and Ramsdell JS (2005) Effects of marine algal toxins on thermoregulation in mice. *Neurotoxicol Teratol* **27**: 727–31.

Gribble KE, Keafer BA, Quilliam MA, Cembella AD, Kulis DM, Manahan A, Anderson DM (2005) Distribution and toxicity of *Alexandrium ostenfeldii* (Dinophyceae) in the Gulf of Maine, USA. *Deep-Sea Res II* **52**: 2745–63.

Gulland FM, Haulena MF, Fauquier D, Langlois G, Lander ME, Zabka T, Duerr R (2002) Domoic acid toxicity in Californian sea lions (*Zalophus californianus*): clinical signs, treatment and survival. *Vet Rec* **150**: 475–80.

Hallegraeff G M (2003) Harmful algal blooms: a global overview. In *Manual on Harmful Marine Microalgae*, Hallegraeff GM, Anderson DM, Cembella AD (eds). UNESCO, Saint-Berthevin, pp. 25–49.

Holmes MJ, Lewis RJ (1994) Purification and characterization of large and small maitotoxins from cultured *Gambierdiscus toxicus*. *Nat Toxins* **2**: 64–72.

How CK, Chern CH, Huang YC, Wang LM, Lee CH (2003) Tetrodotoxin poisoning. *Am J Emerg Med* **21**: 51–4.

Hu T, deFreitas ASW, Curtis JM, Oshima Y, Walter JA, Wright JLC (1996) Isolation and structure of prorocentrolide B, a fast-acting toxin from *Prorocentrum maculosum*. *J Nat Prod* **59**: 1010–14.

Hungerford JM (2006) Marine and freshwater toxins, Committee on natural toxins and food allergens. *J AOAC Int* **89**: 248–69.

Isbister GK, Kiernan MC (2005) Neurotoxic marine poisoning. *Lancet Neurol* **4**: 218–28.

Ishida H, Nozawa A, Nukaya H, Rhodes L, McNabb P, Holland PT, Tsuji K (2004) Confirmation of brevetoxin metabolism in cockle, *Austrovenus stutchburyi*, and greenshell mussel, *Perna canaliculus*, associated with New Zealand neurotoxic shellfish poisoning, by controlled exposure to *Karenia brevis* culture. *Toxicon* **43**: 701–12.

Ito E, Terao K (1994) Injury and recovery process of intestine caused by okadaic acid and related compounds. *Nat Toxins* **2**: 371–7.

Ito E, Satake M, Ofuji K, Kurita N, McMahon T, James K, Yasumoto T (2000) Multiple organ damage caused by a new toxin azaspiracid, isolated from mussels produced in Ireland. *Toxicon* **38**: 917–30.

Ito E, Satake M, Ofuji K, Higaschi M, Harigaya K, McMahon T, Yasumoto T (2002a) Chronic effects in mice caused by oral administration of sublethal doses of azaspiracid, a new marine toxin isolated from mussels. *Toxicon* **40**: 193–203.

Ito E, Yasumoto T, Akira T, Imanishi S, Harada K (2002b) Investigation of the distribution and excretion of okadaic acid in mice using immunostaining method. *Toxicon* **40**: 159–65.

Iverson F, Truelove J, Nera E, Tryphonas L, Campbell J, Lok E (1989) Domoic acid poisoning and mussel-associated intoxication; preliminary investigations into the response of mice and rats to toxic mussel extracts. *Food Chem Toxicol* **27**: 377–84.

James KJ, Sierra MD, Lehane M, Magdalena AB, Furey A (2003a) Detection of five new hydroxyl analogue of azaspiracids in shellfish using multiple tandem mass spectrometry. *Toxicon* **41**: 277–83.

James K, Moroney C, Roden C, Satake M, Yasumoto T, Lehane M, Furey A (2003b) Ubiquitous 'benign' alga emerges as the cause of shellfish contamination responsible for the human toxic sindrome, azaspiracid poisoning. *Toxicon* **41**: 145–51.

James KJ, Saez MJF, Furey A, Lehane M (2004) Azaspiracid poisoning, the food-borne illness associated with shellfish consumption. *Food Addit Contam* **9**: 879–92.

Jeffery B, Barlow T, Moizer K, Paul S, Boyle C (2004) Amnesic shellfish poison. *Food Chem Toxicol* **42**: 545–57.

Kao CY (1966) Tetrodotoxin, saxitoxin and their significance in the study of excitation phenomena. *Pharmacol Res* **18**: 997–1049.

Kemppainen BV, Reifenrath WG, Stafford RG, Mehta M (1991) Methods for *in vitro* skin absorption studies of a lipophilic toxin produced by red tide. *Toxicology* **66**: 1–17.

Khera KS, Whalen C, Angers G, Arnold DL (1994) Domoic acid: a teratology and homeostatic study in rats. *Bull Environ Contam Toxicol* **53**: 18–24.

Koike K, Horie Y, Suzuki T, Kobiyama A, Kurihara K, Takagi K, Kaga SN, Oshima Y (2006) *Protoceratium reticulatum* in northern Japan: environmental factors associated with seasonal occurrence and related contamination of yessotoxin in scallops. *J Plankton Res* **28**: 103–12.

Kotaki Y, Furio EF, Satake M, Lundholm N, Katayama T, Koike K, Fulgueras VP, Bajarias FA, Takata Y, Kobayashi K, Sato S, Fukuyo Y, Kodama M (2005) Production of isodomoid acids A and B as major toxin components of a pennate diatom *Nitzschia navis-varingica*. *Toxicon* **46**: 946–53.

Kuramoto M. Arimoto H, Uemura D (2004) Bioactive alkaloids from the sea: a review. *Mar Drugs* **1**: 39–54.

Lagos NW, Andrinolo D (2000) Paralytic shellfish poisoning (PSP): toxicology and kinetics. In *Seafood and Freashwater Toxins: Pharmacology, Physiology and Detection*, Botana LM (ed.). Marcel Dekker, New York, pp. 203–16.

Landsberg JH (2002) The effects of harmful algal blooms on aquatic organisms. *Rev Fish Sci* **10**: 113–390.

Lawrence JF, Niedzwiadek B, Menard C (2005) Quantitative determination of paralytic shellfish poisoning toxins in shellfish using prechromatographic oxidation and liquid chromatography with fluorescence detection: collaborative study. *J AOAC Int* **88**: 1714–32.

Le Hégarat L, Jacquin AG, Bazin E, Fessard V (2006) Genotoxicity of the marine toxin okadaic acid, in human Caco-2 cells and in mice gut cells. *Environ Toxicol* **21**: 55–64.

Lee J, Igarashi T, Fraga S, Dahl E, Hovgaad P, Yasumoto T (1989) Determination of diarrhetic shellfish toxins in various dinoflagellate species. *J Appl Phycol* **1**: 147–52.

Lehane L, Lewis RJ (2000) Ciguatera: recent advanced but the risk remains. *Int J Food Microbiol* **61**: 91–125.

Leira F, Alvarez C, Vieytes JM, Vieytes MR, Botana LM (2002a) Characterization of distinct apoptotic changes induced by okadaic acid and yessotoxin in the BE(2)-M17 neuroblastoma cell line. *Toxicol In Vitro* **16**: 23–31.

Leira F, Cabado AG, Vieytes MR, Roman Y, Alfonso A, Botana LM, Yasumoto T, Malaguti C, Rossini GP (2002b) Characterization of F-actin depolymerization as a major toxic event induced by pectenotoxin-6 in neuroblastoma cells. *Biochem Pharmacol* **63**: 1979–88.

Lewis RJ (2001) The changing face of ciguatera. *Toxicon* **39**: 97–106.

Lewis RJ, Holmes MJ (1993) Origin and transfer of toxins involved in ciguatera. *Comp Biochem Physiol* **106C**: 615–28.

Lewis RJ, Holmes MJ, Alewood PF, Jones A (1994) Ionspray mass spectrometry of ciguatoxin-1, maitotoxin-2 and -3, and related marine polyether toxins. *Nat. Toxins* **2**: 56–63.

Lewis RJ, Molgó J, Adams DJ (2000) Ciguatera toxins: pharmacology of toxins involved in ciguatera and related fish poisonings. In *Seafood and Freshwater Toxins: Pharmacolgy, Physiology and Detection*, Botana LM (ed.). Marcel Dekker, New York, pp. 419–48.

Llewellyn L (2006) Saxitoxin, a toxic marine natural product that targets a multitude of receptors. *Nat Prod Rep* **23**: 200–22.

Luckas B (1992) Phycotoxins in seafood – toxicological and chromatographic aspects. *J Chromatogr* **624**: 439–56.

Luckas B, Hummert C, Oshima Y (2003) Analytical methods for paralytic shellfish poisons. In *Manual on Harmful Marine Microalgae*, Hallegraeff GM, Anderson DM, Cembella AD (eds). UNESCO, Saint-Berthevin, pp. 191–209.

Luckas B, Dahlmann J, Erler K, Gerdts G, Wamund N, Hummert C, Hansen PD (2005) Overview of key phytoplankton toxins and their recent occurrence in the North and Baltic Seas. *Environ Toxicol* **20**: 1–17.

MacKenzie L, Holland P, McNabb P, Beuzenberg V, Selwood A, Suzuki T (2002) Complex toxin profiles in phytoplankton and

Greenshell mussels (*Perna canaliculus*), revealed by LC-MS/MS analysis. *Toxicon* **40**: 1321–30.

Magdalena AB, Lehane M, Moroney C, Furey A, James KJ (2003) Food safety implications of the distribution of azaspiracids in the tissue compartments of scallops (*Pecten maximus*) *Food Addit Contam* **20**: 154–60.

Malaguti C, Ciminiello P, Fattorusso E, Rossini GP (2002) Caspase activation and death induced by yessotoxin in HeLa cells. *Toxicol In Vitro* **16**: 357–63.

Matias WG, Creppy EE (1996a) Evidence for enterohepatic circulation of okadaic acid in mice. *Tox Subst Mech* **15**: 405–14.

Matias WG, Creppy EE (1996b) Transplacental passage of [$^3$H]-okadaic acid in pregnant mice measured by radioactivity and high-performance liquid chromatography. *Hum Exp Toxicol* **15**: 226–30.

Matias WG, Traore A, Creppy EE (1999) Variations in the distribution of okadaic acid in organs and biological fluids of mice related to diarrhoeic syndrome. *Hum Exp Toxicol* **18**: 345–50.

Miles CO, Wilkins AL, Stirling DJ, MacKenzie AL (2000) New analogue of gymnodimine from a *Gymnodinium* species. *J Agric Food Chem* **48**: 1373–6.

Miles CO, Wilkins AL, Samdal IA, Sandvik M, Petersen D, Quilliam MA, Naustvoll LJ, Rundberget T, Torgersen T, Hovgaard P, Jensen DJ, Cooney JM (2004) A novel pectenotoxin, PTX-12, in *Dinophysis* spp. and shellfish from Norway. *Chem Res Toxicol* **17**: 1423–33.

Miles CO, Wilkins AL, Hawkes AD, Jensen DJ, Selwood AI, Beuzenberg V, MacKenzie AL, Cooney JM, Holland P T (2006) Isolation and identification of pectenotoxin-13 and -14 from *Dinophysis acuta* in New Zealand. *Toxicon* **48**: 152–9.

Morales-Tlalpan V, Vaca, L (2002) Modulation of the maitotoxin response by intracellular and extracellular cations. *Toxicon* **40**: 493–500.

Mountfort DO, Suzuki T, Truman P (2001) Protein phosphatase inhibition assay adapted for determination of total DSP in contaminated mussels. *Toxicon* **39**: 383–90.

Munday R, Towers NR, MacKenzie L, Beuzenberg V, Holland PT, Miles, CO (2004) Acute toxicity of gymnodimine to mice. *Toxicon* **44**: 173–8.

Murata M, Kumagai M, Lee J S, Yasumoto T (1987) Isolation and structure of yessotoxin, a novel polyether compound implicated in diarrhetic shellfish poisoning. *Tetrahedron Lett* **28**: 5869–72.

Nakashima K, Arakawa O, Taniyama S, Nonaka M, Takatani T, Yamamori K, FuchiY, Noguchi T (2004) Occurrence of saxitoxins as a major toxin in the ovary of a marine puffer *Arothron firmamentum*. *Toxicon* **43**: 207–12.

Nijjar MS, Nijjar SS (2000) Ecobiology, clinical symptoms, and mode of action of domoic acid, an amnesic shellfish toxin. In *Seafood and Freshwater Toxins: Pharmacology, Physiology and Detection*, Botana LM (ed.). Marcel Dekker, New York, pp. 325–58.

Ofuji K, Satake M, McMahon T, Silke J, James JK, Naoki H, OshimaY, YasumotoT (1999) Two analogs of Azaspiracid isolated from mussels, *Myitlus edulis*, involved in human intoxication in Ireland. *Nat Toxins* **7**: 99–102.

Ofuji K, Satake M, McMahon T, James K, Naoki H, Oshima Y, Yasumoto T (2001) Structure of Azaspiracid Analogs, Azaspiracid-4 and Azaspiracid-5, causative toxins of Azaspiracid poisoning in Europe. *Biosci Biotechnol Biochem* **65**: 740–2.

Ogino H, Kumagai M, Yasumoto T (1997) Toxicologic evaluation of yessotoxin. *Nat Toxins* **5**: 255–9.

Pavela-Vrančič M, Meštrović V, Marasović I, Gillman M, Furey A, James K K (2001) The occurrence of 7-epi-pectenotoxin-2 seco acid in the coastal waters of the central Adriatic (Ka$$Ü tela Bay). *Toxicon* **39**: 771–9.

Paz B, Riobó P, Fernández ML, Fraga S, Franco JM (2004) Production and release of yessotoxin by the dinoflagellates *Protoceratium reticulatum*, and *Lingulodinium polyedrum* in culture. *Toxicon* **44**: 251–8.

Pearn J, Harvey P, De Ambrosis W, Lewis R, McKay R (1982) Ciguatera and pregnancy. *Med J Aust* **1**: 57–8.

Pearn J (2001) Neurology of ciguatera. *J. Neurol. Neurosurg. Psychiatry* **70**: 4–8.

Plakas, SM, Wang Z, El Said KR, Jester ELE, Granade HR, Flewelling, L, Scott, P, Dickey RW(2004) Brevetoxin metabolism and elimination in the Eastern oyster (*Crassostrea virginica*) after controlled exposures to *Karenia brevis*. *Toxicon* **44**: 677–85.

Poli MA, Templeton CB, Thompson WL, Hewetson FJ (1990) Distribution and elimination of brevetoxin PbTx-3 in rats. *Toxicon* **28**: 903–10.

Preston E, Hynie I (1991) Transfer constants for blood-brain barrier permeation of the neuroexcitatory shellfish toxin, domoic acid. *Can J Neurol Sci* **18**: 39–44.

Quilliam MA (2003) Chemical methods for lipophilic shellfish. In *Manual on Harmful Marine Microalgae*, Hallegraeff GM, Anderson DM, Cembella AD (eds.). UNESCO, Saint-Berthevin, pp. 211-65.

Radwan FFY, Wang Z, Ramsdell JS (2005) Identification of a rapid detoxification mechanism for brevetoxin in rats. *Toxicol Sci* **85**: 839–46.

Rhodes L, McNabb P, de Salas M, Briggs L, Beuzenberg V, Gladstone M (2006) Yessotoxin production by *Gonyaulax spinifera*. *Harmful Algae* **5**: 148–55.

Rogers RS, Rapoport H (1980) The p$K_a$'s of saxitoxin. *J Am Chem Soc* **102**: 7335–9.

Román Y, Alfonso A, Luozao MC, De la Rosa LA, Leira F, Vieytes JM, Vieytes MR, Ofuji K, Satake M, Yasumoto T, Botana LM (2002) Azaspiracid-1, a potent, nonapoptotic new phycotoxin with several cell targets. *Cell Signal* **14**: 703–16.

Ronzitti G, Callegari F, Malaguti C, Rossini GP (2004) Selective disription of the E-cadherin-catenin system by an algal toxin. *Brit J Cancer* **90**: 1100–7.

Sapse A, Rothchild R, Rhee K (2006) An *ab initio* study of the guanidinium groups in saxitoxin. *J Mol Model* **12**: 140–5.

Sasaki K, Takizawa A, Tubaro A, Sidari L, Della Loggia R, Yasumoto T (1999) Fluorometric analysis of pectenotoxin-2 in microalgal samples by high performance chromatography. *Nat Toxins* **7**: 241–6.

Satake M, MacKenzie L, Yasumoto T (1997) Identification of *Protoceratium reticulatum* as the biogenetic origin of yessotoxin. *Nat Toxins* **5**: 164–7.

Scallet AC (1995) Quantitative histological evaluation of neurotoxic hippocampal damage. *Ann NY Acad Sci* **765**: 303.

Schmued LC, Scallet AC, Slikker Jr W (1995) Domoic acid-induced neuronal degeneration in the primate forebrain revealed by degeneration specific histochemistry. *Brain Res* **695**: 64–70.

Seki T, Satake M, MacKenzie L, Kaspar HF, Yasumoto T (1995) Gymnodimine, a new marine toxin of unprecedented structure isolated from New Zealand oysters and the dinoflagellate. *Gymnodinium* sp. *Tetrahedron Lett* **36**: 7093–6.

Sleno L, Windust AJ, Volmer DA (2004) Structural study of spirolide marine toxins by mass spectrometry. Part I. Fragmentation pathways of 13-desmethyl spirolide C by collision-induced dissociation and infrared multiphoton dissociation mass spectrometry. *Anal Bioanal Chem* **378**: 969–76.

Stafford RG, Hines H (1995) Urinary elimination of saxitoxin after intravenous injection. *Toxicon* **33**: 1501–10.

Stewart M, Blunt JW, Munro MHG, Robinson WT, Hannah DJ (1997) The absolute stereochemistry of the New Zealand shellfish toxin gymnodimine. *Tetrahedron Lett* **38**: 4889–90.

Stirling D J (2001) Survey of historical New Zealand shellfish samples for accumulation of gymnodimine. *New Zeal J Mar Fresh* **35**: 851–7.

Suarez-Korsnes M, Hetland DL, Espenes A, Tranulis MA, Aune T (2006) Apoptotic events induced by yessotoxin in myoblast cell lines from rat and mouse. *Toxicol In Vitro* **20**: 1077–8.

Suganuma M, Tatematsu M, Yatsunami J, Yoshizawa S, Okabe S, Uemura D, Fujiki H (1992) An alternative theory of tissue specificity by tumor promotion of okadaic acid in glandular stomach of SD rats. *Carcinogenesis* **13**: 1841–5.
Suzuki CA, Hierlihy SL (1993) Renal clearance of domoic acid in the rat. *Food Chem Toxicol* **31**: 701–6.
Suzuki T, MacKenzie L, Stirling D, Adamson J (2001) Pectenotoxin-2 seco acid: a toxin converted from pectenotoxin-2 by the New Zealand greenshell mussel, *Perna canaliculus*. *Toxicon* **39**: 507–14.
Suzuki T, Walter JA, LeBlanc P, MacKinnon S, Miles CO, Wilkins AL, Munday R, Beuzenberg V, MacKenzie AL, Jensen DJ, Cooney JM, Quilliam, MA (2006) Identification of pectenotoxin-11 as 34S-hydroxypectenotoxin-2, a new pectenotoxin analogue in the toxic dinoflagellate *Dinophysis acuta* from New Zealand. *Chem Res Toxicol* **19**: 310-18.
Tachibana K, Scheuer PJ, Tsukitani Y, Kikuchi H, Van Engen D, Gopichand Y, Schmitz F (1981) Okadaic acid, a cytotoxic polyether from two marine sponges of the genus *Halichondria*. *J Am Chem Soc* **103**: 2469–71.
Terao K (2000) Ciguatera toxin: toxinology. In *Seafood and Freshwater Toxins: Pharmacology, Physiology and Detection*, Botana LM (ed.). Marcel Dekker, New York, pp. 449–72.
Terao K, Ito E, Yanagi T, Yasumoto T (1986) Histopathological studies on experimental marine toxin poisoning. I. Ultrastructural changes in the small intestine and liver of suckling mice induced by dinophysistoxin-1 and pectenotoxin-1. *Toxicon* **24**: 1141–51.
Terao K, Ito E, Oarada M, Murata M, Yasumoto T (1990) Histopathological studies on experimental marine toxin poisoning. 5. The effects in mice of yessotoxin isolated from *Patinopecten yessoensis* and of a desulfated derivative. *Toxicon* **28**: 1095–104.
Terao K, Ito E, Ohkusu M, Yasumoto T (1993) A comparative study of the effects of DSP-toxins on mice and rats. In *Toxic Phytoplankton Blooms in the Sea*, Smayda TJ, Shimizu Y (eds). Elsevier Science Publishers BV, Paris, pp. 581–6.
Tibbetts BM, Baden DG, BensonJM (2006) Uptake, tissue distribution, and excretion of brevetoxin-3 administered to mice by intratracheal instillation. *J Toxicol Environ Health A* **69**: 1325–35.
Todd ECD (1993) Domoic acid and Amnesic shellfish poisoning. A review. *J Food Prot* **56**: 69–86.
Torigoe K, Murata M, Yasumoto T (1988) Prorocentrolides, a toxic nitrogenous macrocycle from a marine dinoflagellate. *Prorocentrum lima*. *J Am Chem Soc* **110**: 7876–7.
Tripuraneni J, Koutsouris A, Pestic L, De Lanerolle P, Hecht G (1997) The toxin of Diarrheic Shellfish Poisoning, okadaic acid, increases intestinal epithelial paracellular permeability. *Gastroenterology* **112**: 100–8.
Truelove J, Iverson F (1994) Serum domoic acid clearance and clinical observations in the cynomolgus monkey and Sprague–Dawley rat following a single i.v. dose. *Bull Environ Contam Toxicol* **52**: 479–86.
Truelove J, Mueller R, Pulido O, Iverson F (1996) Subchronic toxicity study of domoic acid in the rat. *Food Chem Toxicol* **34**: 525–9.
Truelove J, Mueller R, Pulido O, Martin L, Fernie S, Iverson F (1997) 30-day oral toxicity study of domoic acid in cynomolgus monkeys: lack of overt toxicity at doses approaching the acute toxic dose. *Nat Toxins* **5**: 111–4.
Tubaro A, Sosa S, Carbonatto M, Altinier G, Vita F, Melato M, Satake, M, Yasumoto T (2003) Oral and intraperitoneal acute toxicity studies of yessotoxin and homoyessotoxins in mice. *Toxicon* **41**: 783–92.
Tubaro A, Sosa S, Altinier G, Soranzo MR, Satake M, Della loggia R, Yasumoto T (2004) Short-term oral toxicity of homoyessotoxins, yessotoxin and okadaic acid in mice. *Toxicon* **43**: 439–45.
Twiner MJ, Hess P, Bottein Decharaoui MY, McMahon T, Samons MS, Satake M, Yasumoto T, Ramsdell JS, Doucette GJ (2005) Cytotoxic and cytoskeletal effects of azaspiracid-1 on mammalian cell lines. *Toxicon* **45**: 891–900.
Vale P, de Sampayo MAM (2002) Pectenotoxin-2 seco acid, 7-epi-pectenotoxin-2 seco acid and pectenotoxin-2 in shellfish and plankton from Portugal. *Toxicon* **40**: 979–87.
van Apeldorn ME, van Egmond HP, Speijers GJA (2001) Neurotoxic shellfish poisoning: a review. RIVM Report 388802023. National Institute for Public Health and the Environment, Bilthoven, The Netherlands, pp. 1–70.
Van Dolah FM (2000) Diversity of marine and freshwater algal toxins. In*Seafood and Freshwater Toxins: Pharmacology, Physiology and Detection*, Botana LM (ed.). Marcel Dekker, New York, pp. 19–43.
Van Dolah FM, Finley EL, Haynes BL, Doucette GJ, Moeller PD, Ramsdell JS (1994) Development of rapid and sensitive high throughput pharmacologic assay for marine phycotoxins. *Nat Toxins* **2**: 189–96.
Wilkins AL, Rehmann N, Torgersen T, Rundberget T, Keogh M, Petersen D, Hess P, Miles CO (2006) Identification of fatty acid esters of pectenotoxin-2 seco acid in blue mussels (*Mytilus edulis*) from Ireland. *J Agric Food Chem* **54**: 2672–8.
Woofter R, Bottein Dechraoui M-Y, Garthwaite I, Towers NR, Gordon CJ, Córdova J, Ramsdell JS (2003) Measurement of brevetoxin levels by radioimmunoassay of blood collection cards after acute, long-term, and low-dose exposure in mice. *Environ Health Perspect* **111**: 1595–600.
Woofter R T, Spiess PC, Ramsdell J S (2005) Distribution of brevetoxin (PbTx-3) in mouse plasma: association with high-density lipoproteins. *Environ Health Perspect* **113**: 1491–6.
Wright JL, Bird CJ, de Freitas AS, Hampson D, McDonald J, Quilliam MA (1990) Chemistry, biology, and toxicology of domoic acid and its isomers. *Can Dis Wkly Rep* **16**(Suppl. 1E): 21–6.
Wu Z, Xie L, Xia G, Zhang J, Nie Y, Hu J, Wang S, Zhang R (2005) A new tetrodotoxin-producing actinomycete, *Nocardiopsis dassonvillei*, isolated from the ovaries of puffer fish *Fugu rubripes*. *Toxicon* **45**: 851–9.
Xu Q, Huang K, Gao L, Zhang H, Rong K (2003) Toxicity of tetrodotoxin towards mice and rabbits. *Wei Sheng Yan Jiu* **32**: 371–4.
Yasumoto T (1990) Diarrhetic shellfish poisoning and okadaic acid. *Jikken Igaku* **8**: 1243–7.
Yasumoto T (2000) Historic considerations regarding seafood safety. In *Seafood and Freshwater Toxins: Pharmacology, Physiology and Detection*, Botana LM (ed.). Marcel Dekker, New York, pp. 1–17.
Yasumoto T (2001) The chemistry and biological function of natural marine toxins. *Chem Rec* **1**: 228–42.
Yasumoto T, Satake M (1998) New toxins and their toxicological evaluations. In *Harmful Algae*, Reguera B, Blanco, J Fernández ML, Wyatt T (eds.). Xunta de Galicia and Intergovernmental Oceanographic Commission of UNESCO, Paris, pp. 461–4.
Yasumoto T, Oshima Y, Sugawara W, Fukuyo Y, Oguri H, Igarashi T, Fujita N (1980) Identification of *Dinophysis fortii* as the causative organism of diarrhetic shellfish poisoning. *Bull Jpn Soc Sci Fish* **46**: 1405–11.
Yasumoto T, Murata M, Oshima Y, Sano M, Matsumoto G K, Clardy J (1985) Diarrhetic shellfish toxins. *Tetrahedron* **41**: 128–131.
Yotsu-Yamashita M, Urabe D, Asai M, Nishikawa T, Isobe M (2003) Biological activity of 8,11-dideoxytetrodotoxin: lethality to mice and the inhibitory activity to cytotoxicity of ouabain and veratridine in mouse neuroblastoma cells, Neuro-2A. *Toxicon* **42**: 557–60.
Yu CF, Yu PHF, Chan PL, Yan Q, Wong PK (2004) Two novel species of tetrodotoxin-producing bacteria isolated from toxic marine puffer fishes. *Toxicon* **44**: 641–7.

# Part 11

## Bacterial Toxins

CHAPTER

# 61

# Botulinum neurotoxin

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## INTRODUCTION

Botulism, or "sausage poisoning", was reportedly first recognized in Germany around the late 1700s. However, it was not until the 1820s that Justinus Kerner systematically studied and described the fatal paralytic disease (*aka*, "Kerner's disease") associated with the ingestion of spoiled sausage (Jankovic and Brin, 1997; Ergbuth and Naumann, 1999). Kerner recognized that a poisonous substance isolated from the spoiled sausage was responsible for the clinical signs associated with botulism; however, he was unable to identify the origin of the deadly poison. With the subsequent introduction of anaerobic microbiological techniques in the late 1800s, the source of the poison was finally determined. In 1897, van Ermengem (1979) was able to identify the offending etiological agent as a bacterium, now known as *Clostridium botulinum* (*C. botulinum*), in spoiled ham. The deadly poison produced by the bacteria is now known as botulinum neurotoxin (BoNT), the most potent biological toxin ever encountered, with lethal doses as low as 0.03 ng/kg body weight depending on both toxin type and animal species (Gill, 1982). This chapter describes the toxicology of BoNT in relation to various animal species.

## BACKGROUND

BoNT is produced under anaerobic conditions by primarily, *C. botulinum*, a rod-shaped, spore-forming (subterminal) bacterium; however, other clostridial species such as *C. barati* and *C. butyricum* are also capable of producing the neurotoxin (Cato *et al.*, 1986; Hatheway, 1989; Schiavo *et al.*, 2000). Although often referred to collectively as a single toxin, there are actually seven immunologically distinct serotypes of BoNT, and they are designated alphabetically, A–G (Simpson, 1981). Serotypes A, B, C1, and D have been associated with outbreaks of botulism in our domestic animals, livestock, poultry, and wildlife; while serotypes A, B, E, and, rarely F, are known to cause disease in humans. When isolated from the bacterium, native BoNTs are found in complex with hemagglutinins and other non-hemagglutinin, non-toxic proteins (DasGupta and Sugiyama, 1972). These accessory proteins are thought to protect the toxin from harsh environmental conditions such as those found in the gut following ingestion of toxin-contaminated food products (Sharma and Singh, 1998). The molecular masses of these toxin complexes range between 300 and 500 kDa, depending on the toxin serotype. Although serologically distinct, the seven toxin serotypes share both structural and functional similarities (DasGupta and Sugiyama, 1972). When not complexed with other proteins, the active neurotoxin has a molecular mass of 150 kDa and exists as a polypeptide di-chain molecule. The di-chain consists of a heavy chain linked by a single disulfide bond to a light chain. The 100 kDa heavy chain is responsible for membrane targeting and cellular uptake, while the 50 kDa light chain mediates its intracellular action. The neurotoxin molecule can be further divided both structurally and functionally into three domains. The heavy chain contains both a binding domain and a translocation domain, while the smaller light chain contains a catalytically active domain.

## MECHANISM OF ACTION

BoNTs are potent zinc-dependent metalloproteases that exquisitely target acetylcholine (ACh) containing nerve terminals (Schiavo *et al.*, 1992b). Since BoNTs are too

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large to cross the intact blood brain barrier, their selective action on cholinergic terminals is generally limited to peripheral cholinergic systems (Ambache, 1949). The primary target site is the neuromuscular junction (NMJ), where they act within the motor nerve terminal to prevent the release of ACh, the primary neurotransmitter at this major synapse (Burgen *et al.*, 1949; Kao *et al.*, 1976). Intoxication by BoNT has been described as a multistage process (Simpson, 1980, 1989). In the first stage of intoxication, the toxins must bind selectively, via their heavy chains, to protein receptors (and gangliosides) located on the plasma membrane of the motor nerve terminal (Montecucco, 1986). The binding of the toxins to the nerve terminal membrane initiates the second stage of the intoxication process which is characterized by receptor-mediated uptake of the toxins into endosomes (e.g. endocytosis). The third stage of intoxication occurs within the endosome, where the disulfide linkage between the toxin heavy and light chains is reduced, and a subsequent drop in endosomal pH promotes a conformational change in the toxin molecule, allowing the light chain to escape across the endosomal membrane into the cytosol, possibly mediated by the translocation domain of the toxin (e.g. translocation). Following this translocation to the cytosol, the toxin light chain is free to act upon its intracellular target during the final stage of intoxication. During this last stage of the process, the neurotoxins enzymatically cleave one of three specific proteins found within the presynaptic terminal (Schiavo *et al.*, 1992a, b). These three proteins, SNAP-25, synaptobrevin, and syntaxin, known collectively as SNARE (soluble *n*-ethylmaleimide sensitive factor attachment protein receptor) proteins, are necessary for neurotransmitter release (Söllner *et al.*, 1993). Synaptobrevin, an integral membrane protein, is found on the synaptic vesicle, while SNAP-25, a membrane associated protein and syntaxin, another integral membrane protein, are both localized to the presynaptic terminal membrane. Under normal circumstances, these SNARE proteins interact to form a four helical "fusion" or "SNARE" complex that brings the ACh containing synaptic vesicles into close apposition with the terminal membrane. Fusion of the vesicular and terminal membranes and the subsequent release of ACh into the synaptic cleft are triggered by fast $Ca^{2+}$ signaling. The enzymatic cleavage of any one of the SNARE proteins by BoNT either destabilizes or prevents the formation of functional SNARE complexes, inhibiting vesicular fusion and neurotransmitter release (Schiavo *et al.*, 1992b). The catalytic active sites of the different BoNT serotypes vary slightly, giving each serotype both substrate and cleavage site specificity. Toxin serotypes A, C, and E cleave SNAP-25; serotypes B, D, F, and G cleave synaptobrevin, and serotype C cleaves syntaxin (Schiavo *et al.*, 1992a, 1993a, b, 1994; Blasi *et al.*, 1993a, b; Kalandakanond and Coffield, 2001).

## CLINICAL DIAGNOSIS AND TREATMENT

Clinically, botulism is recognized as a lower motor neuron disease resulting in progressive flaccid paralysis. Although deficits in somatic neuromuscular transmission are the most prominent effects, motor deficits in cranial nerve function, as well as the autonomic nervous system have also been reported (Sugiyama, 1980). With the exception of impaired vision (most likely related to disruption in autonomic function), neither altered sensation nor mentation has been specifically reported in botulism (Caya, 2001). In animals, paresis begins in the hind limbs and progresses cranially, often resulting in quadriplegia and recumbency. As in humans, death may result from respiratory muscle paralysis. The onset of clinical signs may be within hours or days of exposure, and is dependent on exposure conditions (dose and duration), as well as individual sensitivity. Susceptibility to the different serotypes varies across species. Botulism in large animals (herbivores) is commonly, but not exclusively, due to serotype B; while botulism in small animals (carnivores) and avian species is most commonly due to serotype C1. With the exception of serotype C1, the different toxin serotypes produce similar clinical disease. Serotype C1 toxin producing strains of *C. botulinum* bacteria may produce multiple exotoxins, including the C1 neurotoxin, and the C2 and C3 cytotoxins (Ohishi and Sakaguchi, 1982; Rubin *et al.*, 1988; Hauser *et al.*, 1993). The concurrent presence of these cytotoxins with C1 neurotoxin may account for the different clinical scenarios that have been reported in botulism associated with serotype C1 intoxication, including dysautonomias in horses and cats.

Botulism is most often acquired from the ingestion of preformed toxin in either spoiled vegetation or carrion-contaminated foodstuffs, although this may vary among species. In addition, two other forms of botulism are well recognized in veterinary medicine and include wound contamination with *C. botulinum*, or gastrointestinal (GI) colonization by *C. botulinum* (toxico-infection). The mainstay of therapy for all animal species with botulism is supportive care, although the specifications of this therapy may differ slightly between species. Antitoxin (equine origin) may be administered in certain instances; however, since the antitoxin is only protective against toxin in the general circulation, it must be given early in the disease course to be effective. Once the patient displays symptoms, antitoxin administration may be ineffective. Further, antitoxin administration can cause adverse effects. Antibiotic administration may be indicated to reduce the risk of secondary infections; however, aminoglycosides, tetracycline, and procaine penicillin should be avoided as these drugs potentiate neuromuscular weakness (L'Hommedieu *et al.*, 1979; Wang *et al.*, 1984). *C. botulinum*

is not sensitive to metronidazole, an antimicrobial used to treat anaerobic infections. Further, metronidazole has been associated with an increased risk of botulism in laboratory animals and workers exposed to the bacterium, possibly due to alterations in GI flora that permit clostridial growth (Wang and Sugiyama, 1984; Sweeney *et al.*, 1986; Whitlock, 2002).

## LABORATORY DIAGNOSIS

The diagnostic gold standard for botulism is the mouse bioassay (MBA). Although the MBA may have limited sensitivity, especially in horses, the test is highly specific. Samples of serum, vomitus, gastric contents, feces, wound tissues, and suspect food can be submitted to specific diagnostic laboratories for MBA analysis. All samples, with the exception of wound samples, should be kept at 4°C immediately following collection. Blood samples should be collected in red top tubes or serum separating tubes. Serum volumes between 10 and 15 ml should be collected as soon as clinical signs are detected; volumes less than 3 ml may yield inconclusive results. Whole blood should not be submitted as hemolysis may occur during shipment. For fecal samples, 20–50 g should be submitted. For constipated patients, 20 ml of rectal contents post-enema can be submitted; however, it is important to use as little fluid as possible in the enema to avoid over-dilution of samples. Suspect food should be left in its original container, and placed inside a sterile, unbreakable and well-labeled container. Empty food containers can also be submitted for testing. Environmental samples can also be tested with the MBA; submission of 50–100 g of soil and approximately 100 ml of water is recommended. All specimens should be placed in leak-proof containers marked "Medical Emergency, Biological Hazard, Refrigerate on Arrival." Specimens should be shipped with ice packs in order to maintain a temperature of 4°C. In cases of wound botulism, debrided tissues, exudates, and/or tissue swabs may be submitted. Wound specimens should be placed in anaerobic transport devices and shipped without refrigeration. For specific details on packaging and shipping, refer to the American Society of Microbiology Procedures for Transportation and Transfer of Biological Agents (www.asm.org). If samples cannot be shipped for several days, specimens should be frozen and then subsequently shipped on dry ice. Freezing samples may compromise the detection of *C. botulinum* bacteria; however, freezing will not affect toxin detection (CDC, 1998; ASM, 2006).

Only qualified diagnostic laboratories can perform both the MBA and neutralization tests to determine the presence, as well as the serotype of BoNT in submitted samples. During these tests, mice (20–30 g IRC strain) are injected IP with prepared samples of serum and/or other sample extracts. For the MBA, a group of mice is injected with sample only to determine the presence of the toxin. For the neutralization assay, a separate group of mice is injected with the sample combined with a serotype-specific antitoxin. In both cases, mice are observed for clinical signs of botulism including ruffled coats, abdominal breathing patterns, dyspnea, weakness, ataxia, respiratory failure, and ultimately death (CDC, 1998). *C. botulinum* bacteria can be isolated from anaerobic enrichment cultures of submitted samples. Following enrichment, culture supernatants are collected and tested similarly for the presence and serotype of toxin using the MBA and neutralization tests. In the former case, a positive MBA test is suggestive of the presence of *C. botulinum* bacteria in the sample (CDC, 1998). It should be emphasized that humans are extremely sensitive to certain serotypes of BoNT; therefore, both toxin and clostridial bacterial isolation should only be attempted by qualified immunized personnel in diagnostic laboratories approved by the Centers for Disease Control and Prevention (CDC).

Although the MBA is still the most widely utilized test for botulism, alternative diagnostic avenues are being explored in an effort to minimize the use of animals in toxicity testing. For example, enzyme-linked immunoabsorption (ELISA) techniques have been used to identify BoNT in both avian and bovine specimens (Sakaguchi *et al.*, 1987; Thomas, 1991; Rocke *et al.*, 1998; Trampel *et al.*, 2005). In one study comparing the capabilities of the ELISA and the MBA to detect BoNT/C and D in bovine serum and tissue samples, Thomas (1991) determined that the ELISA was not sensitive enough to replace the MBA. Further, a cross-reaction with *C. novyi* serotype A was reported. Similarly, Rocke *et al.* (1998) found that the MBA was more sensitive than the ELISA (immunostick method) in detecting BoNT/C in small serum volumes from wild birds with botulism. However, when larger sample volumes were tested, the sensitivity of the ELISA improved, and actually surpassed that of the MBA. When comparing these two methods, it should be noted that only the MBA detects biologically active toxin, while the ELISA detects both active and inactive forms as well as subunits of the toxin (Rocke *et al.*, 1998). The use of polyvalent antibodies to capture toxin antigen and the slight antigenic heterogenicity within serotypes may further complicate ELISA results. More recently, polymerase chain reaction (PCR) methods have also been used to detect the genes encoding BoNT. Trampel *et al.* (2005) used PCR to identify BoNT/C genes from cecal samples in caponized chickens. Further, Szabo *et al.* (1994) determined that PCR detection of the genes encoding BoNT/B following culture enrichment of equine serum, tissues, and fecal samples were comparable to results obtained by the MBA.

## SPECIES-SPECIFIC DISEASE

### Equine botulism

#### *Background*

Horses are thought to be among the most susceptible of species to intoxication by BoNT. The serotype most commonly reported to cause equine botulism in North America (greater than 80% of equine cases) is serotype B; however, serotypes A and C1 have also been associated with clinical disease in the horse (Whitlock *et al.*, 1989). Serotype B producing strains of *C. botulinum* are found ubiquitously in the soils of the northeastern and central United States, particularly in regions extending from Kentucky to the mid-Atlantic states. Conversely, serotype A producing strains are more prominent in the western states (California, Utah, Idaho, Oregon) and have also been reported in Ohio (Whitlock and Williams, 1999). Although intoxication with BoNT/C1 occurs with less frequency, cases have been reported in California, Florida, the New England States, and Canada (MacKay and Berkhoff, 1982; Kinde *et al.*, 1991; Whitlock, 1996).

Horses acquire botulism in one of three ways: (1) from the ingestion of preformed toxin in contaminated foodstuffs, (2) from the contamination of wounds with *C. botulinum*, and (3) from the colonization of the intestinal tract with *C. botulinum* bacteria (toxico-infection). The ingestion of preformed toxin, in either spoiled vegetation or carrion-contaminated foodstuffs, is the most common scenario for equine botulism. Contaminated feed sources such as alfalfa cubes, alfalfa hay, baled hay, wheat, oats, potatoes, bale silage, rye silage, grass clippings, oat chaff, and brewer's grains have all been purported sources of botulism in large animals (Whitlock and Buckley, 1997). Serotype B is more commonly associated with contamination of spoiled foodstuffs or moldy hay. Interestingly, the feeding of silage or hay stored in large plastic bags has been implicated as an increased risk factor for botulism. The damp, alkaline conditions of spoiled vegetation provide an optimal environment for clostridial growth, sporulation, and toxin production (Ricketts *et al.*, 1984; Broughton and Parsons, 1985; Haagsma *et al.*, 1990; Wichtel and Whitlock, 1991). For these reasons it is recommended that silage with a $pH > 4.5$ should not be fed to horses (Whitlock and Buckley, 1997; Galey, 2001).

Cases of equine botulism associated with the ingestion of carrion-laden foodstuffs are more often the result of intoxication with BoNT serotype C1 (Galey, 2001). A herd outbreak of BoNT/C1 intoxication in California was determined to be the result of the ingestion of preformed toxin in alfalfa cubes contaminated with rodent carcasses (Kinde *et al.*, 1991). Interestingly, another report of an outbreak of BoNT/C1 intoxication in horses revealed that birds may act as mechanical vectors, transporting toxin or bacterial spores from a rotting carcass to nearby horse farms (Schoenbaum *et al.*, 2000).

Wound botulism occurs from the contamination of a wound with the *Clostridium* bacteria. The anaerobic environment of the wound provides optimal conditions for *C. botulinum* growth and toxin production. The neurotoxin (most commonly serotype B) is produced within the wound and enters the peripheral circulation where it is distributed to the toxin's site of action, the NMJ. Distal limb wounds, castration sites, umbilical hernias, and injection site abscesses have all been associated with wound botulism (Swerczek, 1980a; Bernard *et al.*, 1987; Mitten *et al.*, 1994; Whitlock and Buckley, 1997).

"Shaker foal syndrome" is a form of toxico-infectious botulism that occurs in foals usually between 2 and 5 weeks of age (Rooney and Prickett, 1967; Vaala, 1991; Whitlock, 2002). The infection occurs most commonly in fast growing foals on high planes of nutrition. GI ulcerations and liver abscesses have been documented post-mortem in foals that succumbed to botulism (Swerzcek, 1980a, b). Thus, exposure to stress, high nutrient diets, or corticosteriod use may all play a role in the foal's susceptibility to toxico-infection. Such underlying conditions may lead to gastric ulcers, which then serve as a nidus for *C. botulinum* colonization. Further, as in human neonatal toxico-infection, the immature GI tract of foals may be more permissible to overgrowth by *C. botulinum*. Shaker foal syndrome is commonly associated with the production of toxin serotype B; however, a case of serotype C1 intoxication was documented in a foal in Florida (MacKay and Berkhoff, 1982). Serotype C1 producing clostridial strains have been identified in Florida soils, and in this particular case, it was concluded that the foal developed a toxico-infection after ingesting bacterial spores from the soil. Additionally, this foal was also diagnosed with sand colitis; an irritated GI mucosa likely increased this foal's susceptibility to toxico-infection.

Toxico-infection with BoNT serotype C1 has also been implicated as a causative agent of equine dysautonomia, also known as equine grass sickness (EGS). EGS is frequently diagnosed in Great Britain, and its occurrence correlates with the grazing season. EGS presents as an acute or subacute illness resulting in death (or euthanasia) within days of onset; however, classic neuromuscular symptoms of botulism are not observed in EGS. Rather, the primary symptoms associated with this syndrome include dysphagia, ileus, and weight loss. If the horse survives the subacute phase, a chronic form may persist. In addition, histological examination revealed neuronal degeneration in autonomic ganglia, the myenteric plexus, and the submucosal plexus (McGorum *et al.*, 2003). Such histological findings are not reported with classic botulism. Increased BoNT/C1 has been detected in ileal and fecal contents of horses diagnosed with EGS; however, it should be noted that in this same study, BoNT/C1 was also isolated from a small number of

control animals (Hunter *et al.*, 1999). Conversely, Garrett *et al.* (2002) demonstrated an increased number of bacterial colonies with a prominent number of clostridial species in the GI tract of horses with EGS, compared with healthy control horses. The possibility, however, that the increased colonization of the gut by clostridia was secondary to the ileus produced in EGS cannot be ruled out. McGorum *et al.* (2003) recently documented the occurrence of clinical and pathological signs associated with both EGS and toxico-infectious botulism in the same foal. One theory proposes that in addition to the blockade of acetylcholine release at cholinergic nerve terminals, the C1 neurotoxin also causes nerve cell degeneration. However, it is worth noting that serotype C1 producing clostridial strains may also produce the ADP-ribosylating cytotoxic toxins, C2 and C3. Interestingly, the C2 toxin has been associated with an increase in vascular permeability leading to the development of edema, congestion, and hemorrhage in *in vivo* models (Ohishi *et al.*, 1981; Ohishi and Sakaguchi, 1982; Simpson, 1982). It is plausible then these exotoxins may play a role in producing the clinical symptoms of EGS that deviate from those of classic botulism (Williamson and Neale, 1998; Cottrell *et al.*, 1999; Hunter *et al.*, 1999; Garrett *et al.*, 2002). To date, however, the exact cause of EGS and the potential role of a *C. botulinum* toxico-infection in its manifestation remain elusive.

### *Clinical signs*

The onset of clinical signs associated with equine botulism is variable and can occur anywhere between 12 h and 10 days post-exposure. The clinical presentation of poisoned horses may be gradual, acute, or peracute depending on exposure dose and duration, as well as on individual sensitivity to the toxin (Whitlock and Buckley, 1997). Adult horses that ingest low doses of toxin may show only mild dysphagia, and recover with minimal treatment; whereas, ingestion of large doses is associated with peracute illness and a grave prognosis. In peracute illness, muscle paralysis progresses rapidly and the animal is recumbent within 8–12 h; ultimately, paralysis of the respiratory muscles results in death.

With the exception of serotype C1 and EGS, clinical symptoms of equine botulism vary little between the different botulinum toxin serotypes (Whitlock, 1996; Whitlock and Buckley, 1997). Myasthenia and dysphagia are usually the first symptoms observed. Astute horse owners may first notice mild signs such as depression, exercise intolerance, and difficulty with grain consumption. Ataxia, gait stiffness, and muscle tremors (particularly in the triceps muscles) may be noted early in the course of disease. In addition, mydriasis, ptosis, and decreased pupillary light responses, as well as decreased palpebral reflexes are characteristic of early botulism. As the disease progresses, pupillary light responses diminish further. Periods of exercise may worsen paresis due to the reduction of acetylcholine release at the NMJ. As dysphagia and pharyngeal weakness progress, the swallowing of food becomes more difficult and secondary aspiration pneumonia may ensue. Horses may also have difficulty drinking as they tend to stand with their muzzles submerged in water troughs without swallowing (Whitlock, 1996; Galey, 2001; Whitlock, 2002). In horses infected experimentally with serotype C, there was a more pronounced mydriasis and an inability to lift the head; facial edema and inspiratory stridor resulted from low head carriage (Whitlock, 1996).

Initially, vital signs such as heart rate, respiration rate, body temperature, and capillary refill time are within normal limits. However, decreased borborygmi, ileus, colic, and constipation develop as botulism progresses. Diarrhea is often associated with serotype C, possibly in association with C2 toxin (Whitlock, 1996; Galey, 2001). Urine retention with resultant bladder distention often occurs, thereby increasing the risk of urinary tract infection. As the disease progresses, horses spend more time in sternal recumbency, and ultimately become laterally recumbent. Heart and respiratory rates may increase as recumbent horses struggle to stand. In late stages, dyspnea and other signs of respiratory distress may be observed. With serotype C intoxication, an exaggerated expiration and "prolonged abdominal lift" may be noted (Whitlock, 1996). In the final stages of botulism, horses are laterally recumbent, demonstrate significant respiratory difficulty and develop anoxia. As the anoxia progresses, horses may exhibit agonal paddling. At this point, the patient either dies due to respiratory failure or is euthanized (Whitlock and Buckley, 1997; Galey, 2001).

In the foal, botulism most commonly occurs between 2 and 5 weeks of age (Vaala, 1991). The first clinical signs usually observed are increased periods of recumbency and muscle tremors. Shortly after the foal rises, muscle tremors are evident and after brief periods of standing, the foal collapses from weakness. Recumbent foals appear to be bright and alert. Foals may dribble milk from their muzzles shortly following nursing due to dysphagia and pharyngeal muscle paresis. Thus, aspiration pneumonia is a common sequela in the foal. Constipation and ileus are also frequently observed. Other symptoms are similar to those observed in the adult horse (Whitlock, 2002).

### *Diagnosis*

A tentative diagnosis of botulism can be made following a comprehensive neurological assessment. Typically, abnormalities in palpebral reflexes, pupillary light responses, tail tone, tongue tone, prehension of food, dysphagia, and gate are detected with botulism. Both the tongue stress test and grain test are sensitive measures for botulism. In the tongue test, the examiner pulls the tongue laterally

through the interdental space. As the horse attempts to retract the tongue, muscular tone is assessed. Although this test is both subjective and variable, a flaccid tongue or weak retraction effort is suggestive of botulism. In the grain test, the consumption of 8 oz of grain is timed; typically, a healthy horse consumes 8 oz of grain in 2 min (Whitlock and Buckley, 1997). Difficulty in prehension, slow consumption, and a characteristic grain/saliva mixture hanging from the lips are indicative of botulism (Whitlock, 1996; Whitlock and Buckley, 1997). These examination findings may also be useful in the assessment of disease progression and treatment efficacy.

Abnormalities in routine diagnostic indicators (complete blood cell (CBC) count, blood chemistry, cerebral spinal fluid (CSF) analysis, and urinalysis) are not typically detected in early botulism, but do usually accompany other neurological diseases. Therefore, normal laboratory values in light of neurological deficits support the diagnosis of botulism. Differential diagnoses for botulism include infectious diseases such as equine protozoal myeloencephalitis, equine herpes virus-1, eastern and western equine encephalitis, rabies, guttural pouch mycosis, listeriosis; other toxicoses such as leukoencephalomalacia (moldy corn poisoning), ionophore poisoning (monesin, salinomycin, and narasin), yellow star thistle poisoning, yew poisoning, white snake root poisoning, and organochlorine poisoning; metabolic disorders such as equine motor neuron disease, azoturia, eclampsia, hypocalcemia, hyperkalemic periodic paralysis, white muscle disease; and pharyngeal ulceration (Whitlock and Buckley, 1997; Whitlock, 2002).

A tentative diagnosis of equine botulism may be confirmed by: (1) MBA detection of formed toxin in horse sera, GI contents, viscera, or wounds, (2) detection of *C. botulinum* spores or toxin in suspect foodstuffs in association with clinical signs, and (3) ELISA detection of serum antitoxin antibodies in unvaccinated horses with clinical signs (Whitlock, 1996). However, a definitive diagnosis of botulism is often difficult to achieve in the horse. There are no gross or pathognomonic histological lesions associated with botulism, and serum toxin levels in the horse are often too low to be detected by the MBA. Because the horse is more sensitive to BoNT than the mouse, the MBA is most valuable in early, peracute equine botulism, when higher concentrations of toxin may be present in the bloodstream (Kinde *et al.*, 1991; Whitlock and Buckley, 1997). In addition to serum, GI contents and liver samples can also be submitted for the MBA, although greater diagnostic success may be achieved through detection of BoNT in the foodstuff rather than within the patient (Galey, 2001). Fecal or tissue culture enrichment can be used to enhance bacterial spore numbers and toxin levels for greater detection. However, since spores may be present in the feces of healthy horses, direct detection of BoNT within the animal is a more reliable finding (Swerczek, 1980b; Whitlock, 1996; Whitlock and Buckley, 1997). Following a positive result from the MBA, the serotype can be identified using the mouse neutralization test.

### *Treatment*

Once botulism is suspected, the patient should be confined to the stall to prevent exertion. Polyvalent antiserum (antitoxin) should be given as soon as possible; the recommended dose for an adult horse is 70,000 IU (500 ml) and 30,000 IU (200 ml) for the foal. One dose usually provides passive immunity for approximately 60 days (Sprayberry and Carlson, 1997; Whitlock and Buckley, 1997). The use of parasympathomimetics should be avoided as these agents deplete acetylcholine stores and exacerbate paresis/paralysis (Galey, 2001). Antibiotic therapy is indicated in cases of wound botulism or secondary infections; however, as previously stated aminoglycosides, tetracycline, procaine penicillin, and metronidazole are contraindicated. Aminoglycosides block neurotransmission at the NMJ and will exacerbate muscle weakness and paralysis (Barsanti, 1990). Although Gram-positive anaerobes are sensitive to penicillin and metronidazole, administration of these drugs is controversial. These antimicrobials may cause more bacterial lysis, thus increasing the release of toxin (in the case of a toxico-infection) or they may promote *C. botulinum* colonization by altering the normal intestinal flora (Barsanti, 1990). Drugs such as the aminopyridines and guanidines should also be avoided as they will further deplete acetylcholine stores (Critchley, 1991).

Second only to antitoxin administration, supportive care is the mainstay of therapy for botulism. $H_2$ blockers and proton pump inhibitors may be indicated, especially for foals. Topical ophthalmic ointments should be used to prevent corneal abrasions and ulceration. Adult horses may need to be sedated with xylazine or diazepam to reduce anxiety and exertion. Patients should be muzzled between feedings to prevent aspiration pneumonia. Nutritional support should be provided to dysphagic patients. Alfalfa slurries with adequate amounts of water may be administered through a nasogastric tube to adult horses. Foals should receive milk replacer through a nasogastric tube, or parenteral nutrition if ileus is present. Patients should be maintained in sternal recumbency to prevent aspiration pneumonia and checked periodically for gastric reflux as ileus may lead to the accumulation of ingesta/fluid in the stomach. If gastric reflux is not present, some authors recommend that mineral oil be administered via a nasogastric tube to alleviate ileus and constipation; however, this should be done under close supervision due to the increased risk of aspiration in these patients. Recumbent patients should be turned frequently or suspended periodically by full body slings to prevent decubital ulcer formation, myopathies, and other complications of prolonged recumbency. Recumbent stallions

and geldings should be catheterized twice daily to empty the bladder and prevent pressure necrosis or cystitis (Whitlock and Buckley, 1997).

A tracheostomy should be performed in cases of botulism where horses show signs of upper airway obstruction as a result of paralysis of the nares or larynx. In more complicated cases, patients may require intravenous (IV) fluids to correct respiratory acidosis resulting from decreased ventilation. For foals in particular, arterial blood gases should be monitored frequently to determine the need for artificial ventilation. Intranasal oxygen insufflation and mechanical ventilation can be instituted in foals with poor arterial blood gas values and/or metabolic acidosis (Wilkins and Palmer, 2002, 2003). Unfortunately, mechanical ventilation is not practical in the adult horse.

The overall prognosis is favorable for horses that are exposed to low doses of the toxin, exhibit a slow disease progression (3–7 days), or display mild symptoms. Likewise, a grave prognosis is given to patients exposed to high doses of toxin, manifest a rapid onset of clinical disease, or become recumbent within 8–12 h (Whitlock and Buckley, 1997). Patients responsive to antitoxin therapy should be able to eat within 7–10 days post-treatment and regain full strength within a month. Recumbent foals are often able to stand within 7–10 days post-antitoxin administration. Although the prognosis for recumbent adult horses is poor, if the patient does not become distressed or show severe respiratory compromise, recovery may be achieved with extensive supportive care (Whitlock and Buckley, 1997). The most common complications associated with botulism are decubital ulcers and aspiration pneumonia; these problems can be resolved with supportive care and antimicrobial therapy.

### *Prevention*

Following recommended vaccination protocols, along with sound husbandry methods, reduces the occurrence of equine botulism. Forages should be examined for carrion, while pastures should be cleared of decaying vegetation and rotting animal carcasses. Appropriate wound management is also an important preventative measure (Galey *et al.*, 2000). To date, only serotype B toxoid vaccine is marketed for horses in the United States (Whitlock, 1996). The American Association for Equine Practitioners (2005) recommends vaccination only for horses in endemic areas. Adult horses in endemic areas should be vaccinated annually. Mares should be boostered 4–6 weeks prior to parturition to achieve adequate antitoxin immunoglobulin (Ig) levels in colostrum. Foals born to vaccinated mares should receive a series of three vaccinations, each 1 month apart, starting at 2–3 months of age. Foals born to unvaccinated mares should be vaccinated at 2, 4, and 8 weeks of age (Whitlock and Buckley, 1997; Galey, 2001).

## Avian botulism

### *Background*

Avian botulism, otherwise known as "limberneck" or "western bird disease", has been a significant problem worldwide in both domestic and wild fowl. The occurrence of avian botulism has been globally widespread, having been documented in as many as 17 countries and on every continent except Antarctica (Jensen and Price, 1987). The majority of the natural outbreaks of avian botulism have occurred in fowl (Lamana, 1987). Carnivorous, omnivorous, carrion-scavengers, and insectivorous birds, as well as aquatic bottom-feeding birds are all susceptible to botulism. In 1984, 117 avian species were determined to have been affected by the disease (Jensen and Price, 1987). Specifically, botulism has been reported in chickens, ducks, turkeys, pheasants, and ostriches (Jensen and Price, 1987; Allwright *et al.*, 1994). Although broiler outbreaks are not uncommon, botulism is a more significant problem for waterfowl, resulting in millions of deaths worldwide (Gross, 1984; Jensen and Price, 1987). Avian species are sensitive to serotypes A, B, C1, and E, although serotype C1 is most commonly associated with outbreaks (Smith, 1975; Gross, 1984; Dohms, 2003). Outbreaks of serotype C1 intoxication have been reported worldwide, while outbreaks of serotype A botulism have only been reported in western regions of North and South America; serotype B in the eastern United States, England, Europe, and China; and serotype E in the Great Lakes and North Sea (Gross, 1984). Interestingly, serotype A was found to be more toxic than serotype C1 when administered IV to chickens; however, when given orally, serotype C1 demonstrated greater toxicity.

The etiology of botulism among wild avian species and waterfowl differs from that observed in other animals. The process is a complex cycle involving environmental contamination, toxico-infection, bird die offs, bacterial proliferation in bird carcasses, and invertebrate vectors. *C. botulinum* often colonizes the intestinal tract and cecum of clinically normal birds, increasing the potential for toxico-infection in avian species (Dohms, 2003). Since these birds are already seeded with the bacteria, upon death, avian carcasses provide an excellent substrate for *C. botulinum* growth. The proliferating bacteria spread from the GI tract to other tissues, the carcass becomes flyblown and toxin accumulates in the fly larvae. Invertebrates concentrate the bacterium or toxin after feeding on contaminated carcasses; however, due to their neurophysiological differences, BoNT does not affect insects and aquatic invertebrates. Subsequently, birds ingest these animals and accumulate lethal amounts of BoNT. One gram of fly larvae may contain $1.8 \times 10^5$ mouse $LD_{50}$s, and ingestion of as little as eight fly larvae was sufficient to kill a pheasant (Gross, 1984). Bird and invertebrate die offs perpetuate botulism outbreaks by increasing the levels of

*C. botulinum* in soils, lakes, rivers, and estuaries (Gross, 1984; Lamana, 1987). Environmental factors such as shallow alkaline waters, warm seasons/summer months, and flooding of mudflats or dried out lakes may promote invertebrate die offs, further enhancing environmental levels of *C. botulinum* (Gross, 1984; Jensen and Price, 1987; Wobeser, 1987). As *C. botulinum* levels increase in the environment, the intestinal tracts of wild birds and waterfowl become seeded with the bacteria, and any cause of bird deaths can trigger an outbreak of botulism (Gross, 1984).

Contaminated feed, water, litter, carcasses, and insects may be associated with botulism in broilers. Often the source of BoNT cannot be identified and toxico-infection has been hypothesized to be the perpetuating factor (Dohms, 1987). *C. botulinum* has been isolated from the intestinal tract and cecum of healthy birds; further, the chicken body temperature (41°C) and cecal pH (7.4) are optimum for *C. botulinum* growth (Miyazaki and Sakaguchi, 1978; Trampel *et al.*, 2005). Most broiler outbreaks have occurred in chickens between 2 and 3 weeks of age; however, an outbreak in post-caponized chickens was documented in birds as old as 14 weeks (Page and Fletcher, 1974; Dohms *et al.*, 1981; Trampel *et al.*, 2005). Coprophagy has also been implicated as a causative factor in poultry outbreaks since both BoNT/C1 and *C. botulinum* are secreted in cecal droppings (Hyun and Sakaguchi, 1989). Broiler outbreaks are also more likely to occur in hot weather (Dohms, 1987).

Morbidity and mortality of avian botulism increase with the dose of BoNT ingested. The onset of clinical symptoms may be anywhere from a few hours to 2 days post-exposure (Gross, 1984; Dohms, 2003). The mortality rate in broilers has been reported to be as high as 27%, whereas thousands to millions of birds may have been lost as a result of outbreaks in waterfowl (Page and Fletcher, 1974; Dohms *et al.*, 1981; Jensen and Price, 1987). In fact, it had been suggested that botulism may have been the limiting factor of waterfowl population growth in predisposed areas of the United States (Jensen and Price, 1987).

### *Clinical signs*

As in other species, avian botulism is characterized by lower motor neuron deficits resulting in flaccid muscle paralysis. Paresis begins in the legs and progresses cranially to involve the wings, neck, and eyelids. Mildly affected birds may appear ataxic, reluctant to move, have a ruffled coat, and easily epilated feathers. The wings may droop and the neck become flaccid, hence the name "limberneck". Diarrhea is often noted in broilers. As the disease progresses, birds become recumbent. Neck muscles become paralyzed and birds eventually lie down with necks extended out, resting on the ground. Birds may appear comatose due to eyelid paralysis. Dyspnea may develop as paralysis progresses. Birds usually die from respiratory failure and dehydration. Broilers may succumb to hyperthermia as sick birds are smothered by others and the respiratory mucosal cooling mechanism is compromised (Page and Fletcher, 1974; Gross, 1984; Dohms, 1987).

### *Diagnosis*

The diagnosis of avian botulism is based on clinical signs, a lack of specific pathological changes, and the isolation of toxin from serum/tissues of clinically ill birds. Although no pathognomonic changes have been described, post-mortem hepatic and renal congestion along with signs of dehydration may be found (Page and Fletcher, 1974). The most definitive diagnosis of botulism is the isolation of BoNT from the sick bird. Ten milliliters of blood is the suggested minimum amount for the MBA; however, if necessary, equal aliquots of blood from individual sick birds may be pooled to accommodate volume requirements of the assay (Dohms, 1987). Following a positive result from the MBA, the serotype can be identified using the mouse neutralization test. Most outbreaks of avian botulism are due to BoNT/C1; therefore, antiserum for serotype C1 is usually tested first. One IU of antiserum/mouse typically neutralizes BoNT/C1 levels found in chickens suffering from botulism. BoNT/C1 and other serotypes can also be detected in bird serum using ELISA technology (Sakaguchi *et al.*, 1987; Rocke *et al.*, 1998; Trampel *et al.*, 2005). For small sample volumes, the MBA appears to be more sensitive; however, for larger serum samples, the ELISA sensitivity may be comparable to the MBA (Rocke *et al.*, 1998). Isolation of BoNT or *C. botulinum* from the bird intestines, cecum, or other tissues may aid in a diagnosis; however, these tests are less valuable as the bacterium can be isolated from the intestinal tract of healthy birds. Further, isolation of BoNT or the bacterium from carcass tissues is not definitive since *C. botulinum* may proliferate and spread from the intestinal tract to surrounding tissues of the carcass (Gross, 1984; Dohms, 2003). The MBA can be performed on intestinal, cecal, and crop flushes, or samples can be assayed for toxin or bacterium after culture enrichment (Dohms, 1987; Trampel *et al.*, 2005). PCR methods have been used to identify genes encoding BoNT/C light chain in cecal contents (Trampel *et al.*, 2005). In order to identify the source of contamination, feed, water, litter, carcasses, and insects should be assayed for toxin, or cultured to isolate the bacterium (Dohms, 1987). Both ELISA and the passive hemagglutination test can be performed to identify serum antibodies to BoNT. However, the levels of toxin that produce illness are usually insufficient to stimulate an immune response in chickens and ducks (Dohms, 1987; Dohms, 2003). Interestingly, antibody titers to several BoNT serotypes have been identified in healthy carrion-eating birds such as vultures and crows (Ohishi *et al.*, 1979).

Differential diagnoses for avian botulism in poultry include transient brain paralysis, coccidiostat toxicity, pesticide or other chemical toxicity, New Castle disease, Marek's disease, avian encephalomyelitis, avian reovirus, and musculoskeletal problems. Fowl cholera and chemical toxicity, lead poisoning in particular, are the common differentials for botulism in waterfowl (Rosen, 1971; Dohms, 1987, 2003; Smith, 1987). However, eyelid paresis and the lack of postmortem lesions are supportive of botulism as the diagnosis (Dohms, 2003).

### Treatment

When possible, clinically ill birds should be isolated and provided fresh water; once these measures are taken, birds often recover fully within a few days. Waterfowl should be herded to uncontaminated shores, and carcasses should be removed daily in poultry operations. Antitoxin therapy may be administered for valuable birds or zoo animals, but it is impractical for most production operations or wildlife (Dohms, 2003). Furthermore, antitoxin protection is transient and birds may again become susceptible to BoNT (Gross, 1984). In broiler outbreaks, antimicrobial therapy may be instituted through watering systems or feed. Administration of bacterin (100 g/ton of feed) or streptomycin (500–1000 g/ton of feed or 1 g/l of water for 3 days) was shown to decrease mortality rates in chickens (Schettler, 1979; Sato, 1987). Penicillin may also be administered, but a mixed efficacy has been reported with this treatment (Roberts and Aitken, 1973; Page and Fletcher, 1975; Dohms, 2003). Periodic use of chlortetracycline was reported to reduce botulism outbreaks on one poultry farm (Yamambe and Koga, 1982). Additives such as sodium selenite (6 g/1000 l of water for 5 days) and vitamins A, D3, and E may also reduce mortality (Schettler, 1979). Conversely, elevated iron levels in water or feed may promote the intestinal proliferation of *C. botulinum*; therefore, citric acid, an iron chelator, may be added to water as a preventative (Pecelunas *et al.*, 1999; Trampel *et al.*, 2005). Further, citric acid may lower the pH of the GI tract, inhibiting the growth of *C. botulinum* and promoting the growth of normal flora (Graham and Lund, 1986).

### Prevention

Immunization with the toxoid vaccine has been explored in broilers, pheasants, and ducks with mixed results (Shimizu and Kondo, 1978; Dohms *et al.*, 1981; Gross, 1984). Protection in broilers between 3 and 8 weeks of age was variable after vaccination at 1 and 14 days of age. Chickens are most susceptible to botulism between 2 and 8 weeks of age, and vaccinations to protect this group may be less efficacious due to interference from maternal antibody and immaturity of the immune system. Routine vaccination further increases production costs, and the toxoid may not provide adequate protection against the high doses of toxin obtained from maggot ingestion. Toxoid immunizations are also impractical for waterfowl (Gross, 1984; Dohms, 2003). Therefore, preventative measures to minimize outbreaks of avian botulism should be aimed at flock and environmental management in both production birds and waterfowl.

In broiler outbreaks, the goals are to limit further exposure and eliminate *C. botulinum* or BoNT from the environment. Unaffected birds should be moved to uncontaminated houses. Carcasses should either be incinerated or buried in a deep hole. Rodents should be eliminated from broiler houses as rodent carcasses may harbor *C. botulinum* (Yamambe and Koga, 1982). Chicken houses associated with outbreaks should be emptied and cleaned. All litter should be removed. Houses should be washed with high-pressure steam and cleaned with a detergent agent. A surface-active solution should be sprayed on the interior walls. The walls should then be disinfected with an organic iodine solution or an organic iodine and calcium hypochlorite solution. Twenty-four hours later, the interior walls should be sprayed with 10% formalin. Soil in contaminated areas may also be treated with calcium hypochlorite. Houses should also be sprayed with pesticides to limit flies (Schettler, 1979; Sato, 1987). Iron levels in feed and water sources should be monitored (Pecelunas *et al.*, 1999).

Prevention of waterfowl outbreaks is best achieved by reducing the potential for environmental contamination associated with the proliferation of *C. botulinum* in the carcasses of dead vertebrate and invertebrate animals. Carcasses should be collected and flocks should be herded away from shores associated with outbreaks. Pond management should maintain deep waters, steep banks, and smooth bottoms to prevent deaths of invertebrates and vertebrates. Routine flooding, which may lead to the death of terrestrial invertebrates, should be avoided in areas utilized by waterfowl. Water in wetland areas should be maintained as fresh as possible as oxygen depletion in shallow, stagnant waters leads to aquatic animal die offs. Any factors that may increase deaths in susceptible wetlands, such as overhead power lines, should be removed or avoided (Wobeser, 1987).

The possibility for transmission of botulism from birds to their predators may exist. Coincidence of avian outbreaks with botulism in omnivorous animals have been documented. For instance, Weiss *et al.* (1982) reported botulism in a fox and a weasel in association with a waterfowl outbreak. In addition, there have been several reports of canine botulism in hunting breeds (Barsanti *et al.*, 1978; Richmond *et al.*, 1978; Jensen and Price, 1987). Farrow *et al.* (1983) reported the occurrence of botulism in three dogs after the consumption of a rotten duck carcass. Outbreaks of botulism (BoNT/C and D) in cattle and sheep have been associated with the feeding of contaminated poultry litter in silage (Egyed, 1987; McLoughin *et al.*, 1988). No cases of human botulism resulting from the consumption or

handling of contaminated birds have been reported although both scenarios have likely occurred. The risk for the human acquisition of botulism from avian species appears to be limited. Although Smart *et al.* (1980) reported an outbreak of BoNT/C in non-human primates, humans do not appear susceptible to BoNT/C or D following oral exposure (Jensen and Price, 1987). Further, proper cooking of poultry should denature any toxin protein and eliminate the possibility of transmission through consumption.

## Bovine botulism

### *Background*

Cattle are susceptible to BoNT serotypes B, C1, and D, and acquire botulism most commonly from the ingestion of preformed toxin in spoiled silage, carrion-laden silage, or silage contaminated with poultry litter. As with horses, toxico-infectious and wound botulism are also potential routes of intoxication in cattle. All three toxin serotypes (B, C1, D) have been associated with clinical disease caused by the ingestion of spoiled or carrion-laden feedstuffs. Intoxication with BoNT/B is associated with the ingestion of poorly ensiled or spoiled silage, while BoNT/C1 is associated with the ingestion of carrion- or poultry-litter-laden feedstuffs. Although less frequent, BoNT/D has also been implicated with the ingestion of contaminated silage (Rings, 1987; Critchley, 1991). Interestingly, intoxication with BoNT/D has also been associated with the ingestion of bones by phosphorus-deficient cattle or cattle with pica. There are numerous studies documenting the association between outbreaks of bovine botulism and the ingestion of improperly ensiled silage or spoiled haylage contaminated with BoNT/B (Divers *et al.*, 1986; Yeruham *et al.*, 2003; Braun *et al.*, 2005). Wet hay or soil-contaminated hay, wrapped in plastic bags for storage, can provide the ideal moist anaerobic environment for *C. botulinum* growth (Braun *et al.*, 2005). Contamination of a total mixed ration (TMR) with a cat carcass was determined to be the source of a BoNT/C1 outbreak in a herd of adult Holstein dairy cattle in California (Galey *et al.*, 2000). The practice of feeding ensiled poultry litter to cattle has also been associated with outbreaks of serotype C1 botulism as documented in an Irish beef herd by McLoughin *et al.* (1988). Although serotype D is less commonly associated with food-borne botulism, an outbreak occurred on a Canadian feedlot following the feeding of a TMR containing spoiled bakery waste (Heider *et al.*, 2001). A separate outbreak of BoNT/D in a Holstein dairy herd occurred where the source was suspected, but not proven, to be contaminated haylage (Martin, 2003).

### *Clinical signs*

Bovine botulism usually occurs in the context of a herd outbreak. The classical signs of bovine botulism are similar to those observed in horses; however, cattle exhibit a more gradual progression of clinical signs, improving the prognosis and probability of recovery in cattle (Whitlock, 2002). Further, at least one study has reported that ruminal microbes degrade BoNT, decreasing the absorption of active toxin in cattle compared to horses (Allison *et al.*, 1976). The clinical course ranges anywhere from 2 to 30 days, depending on exposure dose and duration, and the administration of treatment. Early botulism may be confused with milk fever as generalized muscle weakness, increased ataxia, and muscle tremors may occur in both conditions. Cattle with botulism also exhibit depression, dysphagia, decreased tongue and jaw tone, hypersalivation, dehydration, decreased tail tone, decreased pupillary light responses, and mydriasis. Rumen contractions decrease and constipation may develop. Diarrhea and/or putrid smelling feces may also be noted. Cattle with botulism tend to spend significant amounts of time in sternal recumbency. At terminal stages of botulism, cattle are laterally recumbent, exhibit abdominal breathing patterns, and finally succumb from respiratory failure (Galey *et al.*, 2000; Braun *et al.*, 2005). Vital signs are often normal in early stages of botulism; however, as the disease progresses, increased heart and respiratory rates may be noted, while body temperature may decrease (Divers *et al.*, 1986; Galey *et al.*, 2000; Braun *et al.*, 2005).

Recently, a syndrome resembling equine dysautonomia has been described in German cattle and a link to BoNT has been proposed. These cattle may present with a subclinical to chronic "visceral" disease. Non-specific symptoms such as weight loss, decreased milk production, depression, alternating constipation and diarrhea, edema, laminitis, ataxia, retracted abdomen, emaciation, tachypnea, and unexpected death are associated with this syndrome (Böhnel *et al.*, 2001). In cattle exhibiting these symptoms, Böhnel and associates demonstrated the presence of both *C. botulinum* and BoNT in lower GI tract contents. Further, neither BoNT nor *C. botulinum* was isolated from asymptomatic herds. This study hypothesized that small levels of *C. botulinum* colonized the lower intestinal tract and created a low level, chronic exposure of BoNT. This low level of toxin may not reach the systemic circulation, and thus toxin may only disrupt nearby parasympathetic ganglionic innervation of the GI tract, altering intestinal function (Böhnel *et al.*, 2001).

In cases of bovine botulism, clinical pathology may reveal signs of dehydration such as increases in packed-cell volume and total plasma protein. Bicarbonate loss from excessive ptyalism may lead to a metabolic acidosis. Increases in muscle enzymes such as aspartate transaminase (AST) and creatinine kinase may be present due to muscle atrophy or trauma resulting from prolonged lateral recumbency. Electrolyte abnormalities and hyperglycemia may also be detected. One study documented indicators of renal failure in a herd poisoned by BoNT/B; increased

γ-glutamyl transpeptidase (GGT), urea, creatinine, and phosphorus were also detected (Heider *et al.*, 2001; Cobb *et al.*, 2002; Martin, 2003; Braun *et al.*, 2005).

As with other species, there are no definitive gross pathological or pathognomonic histological signs of botulism (Galey *et al.*, 2000). Aspiration pneumonia and pulmonary emphysema are the most frequent sequelae of botulism in cattle (Galey *et al.*, 2000; Heider *et al.*, 2001; Braun *et al.*, 2005). Other lesions such as gastric ulcerations, thickened intestinal mucosa, hepatic lipidosis, suppurative rumenitis, and renal failure have been documented in concurrence with botulism; however, these findings are not consistent in all cases of bovine botulism (Galey *et al.*, 2000; Yeruham *et al.*, 2003).

### *Diagnosis*

Botulism in cattle is usually a presumptive field diagnosis made on the basis of clinical signs and the ruling out of other diseases. Differential diagnoses include hypocalcemia, hypomagnesia, hypokalemia, hypophosphotemia, listeriosis, lead poisoning, polio-encephalomalacia, ionophore toxicity, nutritional or plant toxin induced myopathies, molds, organophosphate poisoning, and tick paralysis (Divers *et al.*, 1986; Heider *et al.*, 2001; Moeller *et al.*, 2003). Clinical diagnosis is usually made through the detection of neurological deficiencies in light of relatively unremarkable laboratory diagnostic findings. The neurological examination should assess cranial nerve responses, gait, posture, and attitude. Specifically, a tongue tone test, tongue stress test, and a jaw tone test should be performed. The tongue stress test is performed by placing a hand at the base of the cow's tongue and putting pressure on the tongue followed by an assessment of muscular tone. The tongue tone test is performed as in the horse. Cattle with botulism will exhibit weak tongue strength. The jaw test is performed by grasping the mandible near the symphysis and attempting to move the mandible laterally. This test assesses the strength of the masseter muscles. A "loose" jaw is suggestive of botulism (Whitlock, 2002).

A definitive diagnosis is made by identifying toxin in the patient's serum, ruminal fluid, or tissues. Identification of BoNT or *C. botulinum* in suspect feedstuffs previously consumed by clinically ill animals may further support a diagnosis. Isolation of BoNT from the rumen may prove difficult because the toxin is often diluted by rumen contents and/or degraded by ruminal microbes (Whitlock and Williams, 1999). Similar to botulism in other species, the MBA is the gold standard for a definitive diagnosis in cattle; however, as in horses, the MBA is often not sensitive enough to detect the low levels of toxin in the general circulation (Sakaguchi, 1983; Rocke, 1993; Galey *et al.*, 2000). The MBA may also be used to detect toxin in rumen contents, the liver and other organ tissues, milk, or feedstuffs. Due to the relatively slower progression of clinical signs in cattle, diagnostic samples are often obtained long after ingestion of toxin. Thus, the level of toxin in these samples may have fallen below the level of detection (Divers *et al.*, 1986). Specimens may also be cultured to isolate *C. botulinum*. An ELISA test has been developed to detect BoNT/C and D in cattle; however, this test is considered less sensitive than the MBA (Thomas, 1991). ELISA tests for the detection of antibodies to BoNT/C and D in cattle have also been developed (Gregory *et al.*, 1996).

### *Treatment*

Supportive care is the core of therapy for bovine botulism, and treatment should only be pursued in standing cattle. Affected cattle should be kept in confinement to minimize movement and exertion. Dehydration, electrolyte deficiencies, acid/base abnormalities, and glucose deficiencies should be managed with fluid therapy. Fluids can be administered orally (via an orogastric tube) or IV. Mineral oil or sodium sulfate can be administered with care as cathartics to treat ileus; however, magnesium sulfate should be avoided as it may potentiate muscle weakness (Divers *et al.*, 1986). Rumen transfaunation may also be performed. Alfalfa gruels may be administered via an orogastric tube to maintain caloric intake. Equine origin polyvalent antiserum may be administered to cattle. However, anti-toxin therapy may be less efficacious in cattle since most of the toxin will have been internalized into the neuron or degraded by the time the diagnosis is made. Antibiotics may be administered for secondary complications such as aspiration pneumonia; as in horses, those that produce muscle weakness should be avoided. Although toxoid vaccinations for serotypes B, C, and D are administered to cattle in other countries, there are no FDA approved vaccinations for cattle in the United States (www.vmtrc.ucdavis.edu).

### *Public health*

The Food Safety Act of the United States (1990) requires that meat or milk products be withheld from market for a minimum of 14 days after the onset of the last clinical case of botulism in an affected herd (Cobb *et al.*, 2002). However, the public health concern for transmission of BoNT through milk appears to be minimal. No cases of human botulism acquired from the consumption of meat or milk from botulism affected cattle have been reported; further, it does not appear that calves acquire botulism through nursing from affected cows. Only a single report to date has been able to detect BoNT in milk from a dairy cow affected with botulism (Böhnel *et al.*, 2005). In this report, BoNT/B was isolated in milk collected from one udder quarter that was simultaneously affected with mastitis. The toxin concentration in the milk was determined to be approximately $10^4$ mouse $LD_{50}$s. However, the milk did not test positive for *C. botulinum* bacteria. It is likely that the concurrent mastitis

infection enhanced the passage of the rather large toxin protein (150 kDa) across the normally protective blood:milk barrier by altering its permeability. This is supported by a much earlier report from Moberg and Sugiyama (1980), who isolated BoNT in milk using an infected rat model. Other studies have not been able to detect BoNT in milk from affected cows using either ELISA or MBA techniques (Galey *et al.*, 2000; Cobb *et al.*, 2002; Moeller *et al.*, 2003). Regardless, the pasteurization process would likely denature any toxin protein that was able to pass into milk, reducing the risk to the consumer. It should be noted, however, that the potential for BoNT contamination of milk may be more relevant in regions where unpasteurized milk is available for public consumption (Böhnel *et al.*, 2005).

## Canine and feline botulism

### *Background*

Although carnivores are thought to be more resistant to the development of botulism, cases of canine botulism have been documented in the United States, Great Britain, continental Europe, and Australia. Most reported cases of botulism in dogs result from the ingestion of BoNT/C1-contaminated carrion; however, a few cases of serotype D have been documented in Senegal (Barsanti, 1990, 2006). Barsanti *et al.* (1978) described an outbreak of type C1 botulism in a hunting colony of American foxhounds; however, the source of the toxin was not identified. Farrow *et al.* (1983) described type C1 botulism in three young Australian Cattle Dogs following the ingestion of rotting duck carcasses found around a local Sydney park. Canine botulism has also been associated with the ingestion of contaminated raw meat (Darke *et al.*, 1976).

Until recently, the only documented cases of feline botulism were experimentally induced; however, Elad *et al.* (2004) have described a natural outbreak of botulism in eight cats who ingested parts of a pelican carcass contaminated with BoNT/C1. Interestingly, BoNT/C1 botulism has also been reported in lions (Critchley, 1991).

### *Clinical signs*

The onset of canine botulism can occur within hours or as late as 6 days post-exposure. Severe cases are associated with an earlier onset of clinical signs. The course of the disease usually ranges from 12 to 24 days. In the clinical report of an outbreak of feline botulism, clinical symptoms were first noted 3 days post-ingestion of contaminated pelican muscle (Elad *et al.*, 2004). Although 50% of the exposed cats died, those that survived recovered significantly by 6 days post-intoxication.

Lower motor neuron dysfunction as well as to a lesser extent, cranial nerve, and autonomic nervous system deficits are observed in canine botulism. Paresis begins in the hind limbs and progresses cranially, ultimately resulting in flaccid muscle paralysis and quadriplegia. Interestingly, dogs with botulism maintain the ability to wag their tail. Tremors of the masseter and temporal muscles may be noticed. Muscle atrophy is variable throughout the course of the disease. Mydriasis, decreased pupillary light response, decreased palpebral reflexes, and decreased or weak vocalizations may occur. Hyperemic conjunctiva and decreased Shirmer tear tests may be noted. Heart rates and respiratory patterns are variable; however, as abdominal muscle tone diminishes, diaphragmatic breathing may be noted. Regurgitation, megaesophagus, urinary retention, and constipation are also observed. Secondary complications include aspiration pneumonia, bilateral keratoconjunctivitis sicca, and urinary tract infections. If paralysis progresses to the respiratory muscles, death may occur from respiratory failure; however, death may also result from progressive secondary pneumonia or urinary tract infections (Barsanti, 2006). If secondary complications do not arise, the prognosis for canine botulism is good. Recovery occurs in the reverse order from that of the onset of paralysis; cranial nerve function and motor function of the neck and limbs return first (Barsanti, 2006). In the one case study of natural botulism in cats, clinical signs were similar to those of dogs. Motor deficits and paresis were noted; however, cranial nerve reflexes were normal (Elad *et al.*, 2004). Depression, anorexia, mild dehydration, tachycardia, and urinary retention were also noted.

As with EGS in the horse, there has also been speculation of an association between feline dysautonomia ("Key–Gaskell's disease") and BoNT/C. Clinical signs for feline dysautonomia include depression, anorexia, vomiting, regurgitation, mydriasis, constipation, and urinary retention; however, the somatic lower motor neuron paralysis characteristic of classical botulism is not observed. Histological evidence of neuronal degeneration in autonomic ganglia confirms the diagnosis of dysautonomia. Interestingly, BoNT/C was detected in feces, ileal contents, and foodstuffs of cats displaying symptoms of dysautonomia (Nunn *et al.*, 2004). Further, affected cats had higher levels of anti-BoNT/C and *C. botulinum* surface antigen IgA in their feces when compared to control cats. Additional studies are warranted to determine the potential role of BoNT/C in feline dysautonomia.

### *Diagnosis*

With the exception of dehydration or secondary infection, the CBC count, blood chemistry, urinalysis and CSF analysis are usually within normal limits in canine and feline botulism. Thoracic radiographs may reveal a megaesophagus and aspiration pneumonia. Electromyographic (EMG) findings may indicate lower motor neuron disease in clinically ill animals. Decreases in the amplitudes of compound

muscle action potentials and motor unit potentials are often detected. Furthermore, fibrillation potentials and decreases in nerve conduction velocity may also be detected (Barsanti *et al.*, 1978; van Nes *et al.*, 1986; Braund, 1994; Barsanti, 2006). In order to make a definitive diagnosis, toxin must be identified in serum, vomitus or gastric contents, feces, or food samples from animals showing clinical signs. The gold standard MBA appears to have adequate sensitivity for the detection of toxin in canine and feline biological samples or in carrion (Barsanti, 1978, 2006; Elad *et al.*, 2004). It should be noted that the isolation of *C. botulinum* bacteria through cultures of feces, GI contents, or viscera is not a definitive diagnosis, as this bacterium can be isolated from the GI tract and viscera of healthy dogs.

Differential diagnoses for canine botulism should include tick paralysis, polyradiculoneuritis (coonhound paralysis), myasthenia gravis, coral snake envenomization, and the dumb form of rabies (Chrisman, 1991). Both the lower motor neuron deficits and EMG findings are similar to those of tick paralysis and polyradiculoneuritis; however, due to its action on cholinergic terminals, botulism also causes cranial nerve and autonomic deficits (Oliver *et al.*, 1997; Coleman, 1998; Barsanti, 2006). The nature of botulism outbreaks to affect multiple animals further differentiates the disease from other causes of lower motor neuron dysfunction.

### *Treatment*

Treatment of canine botulism mainly consists of supportive care (Barsanti, 1990; Critchley, 1991). If the ingestion of toxin-contaminated food has been recent, gastric lavage, cathartics, and enemas may be used to decrease toxin absorption from the GI tract. However, as in other species, magnesium sulfate should be avoided. Supplemental fluids should be administered as needed to maintain hydration. Nutritional support via orogastric or parenteral administration may also be needed. Animals should be monitored for aspiration pneumonia due to megaesophagus and decreased gag reflexes. If constipation develops, enemas and stool softeners may be administered. Manual expression of the bladder may be required to decrease the occurrence of urinary tract infections. Topical ophthalmic ointments should be used to prevent corneal ulcers, which may result from diminished palpebral tone and tear production. Adequate bedding and frequent repositioning are necessary to prevent the development of decubital ulcers. In cases where respiration is compromised, mechanical ventilation may be necessary. Antimicrobial therapy may be needed for secondary infections; however, as in other species aminoglycosides, tetracycline, procaine penicillin, metronidazole, aminopyridines, and guanidines should be avoided.

Administration of the equine antitoxin in small animals is controversial. By the time clinical signs are noted, antitoxin is likely to be ineffective as most of the toxin is already bound to the nerve cell or has translocated into the neuron. Only the trivalent antitoxin vaccine for serotypes A, B, and E is available in the United States; this antitoxin is less useful for dogs and cats, which are usually affected by serotype C (Barsanti, 2006). However, the heptavalent antitoxin is available in other countries (Byrne and Smith, 2000; Arnon *et al.*, 2001). Since adverse reactions to antitoxin may occur, and patients with mild disease often recover with supportive care alone, antitoxin administration is usually reserved for severe cases (Chrisman, 1991).

### *Prevention*

Limiting exposure of small animals to carrion, and fully cooking all meat products to be fed to companion animals are simple measures to prevent botulism in these species. Heating foodstuffs to 80°C for 30 min or 100°C for 10 min denatures the toxin protein. Although a toxoid vaccine is available for other species, no such vaccine exists for dogs or cats.

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## REFERENCES

Allison MJ, Maloy SE, Matson RR (1976) Inactivation of *Clostridium botulinum* toxin by ruminal microbes from cattle and sheep. *Appl Environ Microbiol* **32**: 685–8.

Allwright DM, Wilson M, van Rensburg WJJ (1994) Botulism in ostriches (*Struthio camelus*). *Avian Pathol* **23**: 183–6.

Ambache N (1949) The peripheral action of *C. botulinum* toxin. *J Physiol (London)* **108**: 121–41.

American Association of Equine Practitioners (2005) *AAEP Guidelines for Vaccination in Horses*. http://www.aaep.org/pdfs/AAEP_vacc_guide.pdf.

American Society for Microbiology (2006) Botulinum toxin. In *Sentinel Laboratory Guidelines for Suspected Agents of Bioterrorism*. ASM, Washington, DG, February.

American Society for Microbiology. www.asm.org/ASM/files/BotulismFinalVersion73003.pdf

Arnon SS, Schechter R, Inglesby TV, Henderson DA, Bartlett JG, Ascher MS, Eitzen E, Fine AD, Hauer J, Layton M, Lillibridge S, Osterholm MT, O'Toole T, Parker G, Perl TM, Russell PK, Swerdlow DL, Tonat K (2001) Consensus statement: botulinum toxin as a biological weapon: medical and public health management. *J Am Med Assoc* **25**(8): 1059–70.

Barsanti JA (1990) Botulism. In *Infectious Disease of the Dog and Cat*, 1st edn, Greene CE (ed.). A.B. Saunders, Philadelphia, PA, pp. 515–20.

Barsanti JA (2006) Botulism. In *Infectious Disease of the Dog and Cat*, 3rd edn, Greene CE (ed.). Elsevier Saunders, Philadelphia, PA, pp. 389–94.

Barsanti JA, Walser M, Hatheway CL, Bowen JM, Crowell W (1978) Type C botulism in American foxhounds. *J Am Vet Med Assoc* **172**(7): 809–13.

Bernard W, Divers TJ, Whitlock RH (1987) Botulism as a sequel to open castration in a horse. *J Am Vet Med Assoc* **191**(1): 73–4.

Blasi J, Chapman ER, Link E, Binz T, Yamasaki S, De Camilli P, Sudhof TC, Niemann H, Jahn R (1993a) Botulinum neurotoxin A selectively cleaves the synaptic protein SNAP-25. *Nature (London)* **365**: 160–3.

Blasi J, Chapman ER, Yamasaki S, Binz T, Niemann H, Jahn R (1993b) Botulinum neurotoxin C1 blocks neurotransmitter release by means of cleaving HPC-1/syntaxin. *EMBO J* **12**: 4821–8.

Böhnel H, Schwagerick B, Gessler F (2001) Visceral botulism – a new form of bovine *Clostridium botulinum* toxication. *J Vet Med A Physiol Pathol Clin Med* **48**(6): 373–83.

Böhnel H, Neufeld B, Gessler F (2005) Botulinum neurotoxin type B in milk from a cow affected by visceral botulism. *Vet J* **169**: 124–5.

Braun U, Feige K, Schweizer G, Pospischil A (2005) Clinical findings and treatment of 30 cattle with botulism. *Vet Rec* **156**: 438–41.

Braund KG (1994) *Clinical Syndromes in Veterinary Neurology*. Mosby, St. Louis, MO, pp. 86, 271–2.

Broughton J, Parsons L (1985) Botulism in horses fed big bale silage. *Vet Rec* **117**: 674.

Burgen ASV, Dickens F, Zatman LJ (1949) The action of botulinum toxin on the neuromuscular junction. *J Physiol* **109**: 10–24.

Byrne MP, Smith LA (2000) Development of vaccines for prevention of botulism. *Biochimie* **82**: 955–66.

Cato EP, George WL, Finegold SM (1986) Genus *Clostridium*. In *Bergey's Manual of Systemic Bacteriology*, vol. 2, Sneath PHA *et al.* (eds). Williams and Wilkins, Baltimore, MD, pp. 1141–200.

Caya JG (2001) *Clostridium botulinum* and the ophthalmologist: a review of botulism, including biological warfare ramifications of botulinum toxin. *Surv Ophthalmol* **46**(1): 25–34

CDC (1998) Botulism in the United States, 1899–1996. In *Handbook for Epidemiologists, Clinicians, and Laboratory Workers*. Center for Disease Control and Prevention, National Center for Infectious Diseases, Division of Bacterial and Mycotic Diseases.

Chrisman CL (1991) *Problems in Small Animal Neurology*. Lea and Febiger, Philadelphia PA.

Cobb CP, Hogg RA, Challoner DJ, Brett MM, Livesey CT, Sharpe RT, Jones TO (2002) Suspected botulism in diary cows and its implication for the safety of human food. *Vet Rec* **150**: 5–8.

Coleman ES (1998) Clostridial neurotoxins: tetanus and botulism. *Compend Contin Educ Pract Vet* **20**: 1089–97.

Cottrell DF, McGorum BC, Pearson GT (1999) The neurology and enterology of equine grass sickness: a review of basic mechanisms. *Neurogastroenterol Motil* **11**: 79–92.

Critchley EM (1991) A comparison of human and animal botulism: a review. *J Roy Soc Med* **84**: 295–8.

Darke PGG, Roberts TA, Smart JL, Bradshaw PR (1976) Suspected botulism in foxhounds. *Vet Rec* **99**: 98–9.

DasGupta BR, Sugiyama HA (1972) A common subunit structure in *Clostridium botulinum* type A, B, and E toxins. *Biochem Biophys Res Commun* **48**: 108–12.

Divers TJ, Bartholomew RC, Messick JB, Whitlock RH (1986) *Clostridium botulinum* type B toxicosis in a herd of cattle and a group of mules. *J Am Vet Med Assoc* **188**(4): 382–6.

Dohms JE (1987) Laboratory investigation of botulism in poultry. In *Avian Botulism: An International Perspective*, Eklund MW, Dowell Jr VR (eds). Charles C Thomas, Springfield, IL, pp. 295–314.

Dohms JE (2003) Botulism. In *Diseases of Poultry*, Saif YM *et al.* (eds). Iowa State Press (Blackwell), Ames, IA, pp. 785–91.

Dohms JE, Allen PH, Rosenberger JK (1981) Cases of type C botulism in broiler chickens. *Avian Dis* **26**(1): 204–10.

Egyed MN (1987) Outbreaks of botulism in ruminants associated with ingestion of feed containing poultry waste. In *Avian Botulism: An International Perspective*, Eklund MW, Dowell Jr VR (eds). Charles C Thomas, Springfield, IL, pp. 371–80.

Elad D, Yas-Natan E, Aroch I, Shamir MH, Kleinbart S, Hadash D, Chaffer M, Greenberg K, Shlosberg A (2004) Natural *Clostridium botulism* type C toxicosis in a group of cats. *J Clin Microbiol* **42**(11): 5406–8.

Ergbuth FJ, Naumann M (1999) Historical aspects of botulinum toxin: Justinus Kerner (1786–1862) and "sausage poison". *Neurology* **53**: 1850–3.

Farrow BRH, Murrell WG, Revington ML, Stewart BJ, Zuber RM (1983) Type C botulism in young dogs. *Aust Vet J* **60**(12): 374–7.

Galey FD (2001) Botulism in the horse. *Vet Clin North Am Equine Pract* **17**(3): 579–88.

Galey FD, Terr R, Walker R, Adaska J, Etchebarne MA, Puschner B, Fisher E, Whitlock RH, Rocke T, Willoughby D, Tor E (2000) Type C botulism in diary cattle from feed contaminated with a dead cat. *J Vet Diagn Invest* **12**: 204–9.

Garrett LA, Brown R, Poxton IR (2002) A comparative of the intestinal microbiota of healthy horses and those suffering from equine grass sickness. *Vet Microbiol* **87**: 81–8.

Gill DM (1982) Bacterial toxins: a table of lethal amounts. *Microbiol Rev* **46**: 86–94.

Graham AF, Lund BM (1986) The effect of citric acid on growth in proteolytic strains of *Clostridium botulinum*. *J Appl Bacteriol* **61**: 39–49.

Gregory AR, Ellis TM, Jubb TF, Nickels RJ, Cousins DV (1996) Use of enzyme-linked immunoassays for antibody to types C and D botulinum toxins for investigations of botulism in cattle. *Aust Vet J* **73**(2): 55–61.

Gross WB (1984) Miscellaneous bacterial diseases: botulism. In *Diseases of Poultry*, Hofstad MS *et al*. (ed.). Iowa State University Press, Ames, IA, pp. 257–9.

Haagsma J, Haesebrouck R, Devriese L, Bertels G (1990) An outbreak of botulism type B in horses. *Vet Rec* **127**: 206.

Hatheway CL (1989) Bacterial sources of clostridial neurotoxins. In *Botulinum Neurotoxin and Tetanus Toxin,* Simpson LL (ed.). Academic Press, New York, pp. 3–24.

Hauser D, Gibert M, Eklund MW, Boquet P, Popoff M (1993) Comparative analysis of C3 and botulinum neurotoxin genes and their environment in *Clostridium botulinum* types C and D. *J Bact* **175**: 7260–8.

Heider LC, McClure JT, Leger ER (2001) Presumptive diagnosis of *Clostridium botulinum* type D intoxication in a herd of feedlot cattle. *Can Vet J* **42**: 210–2.

Hunter LC, Miller JK, Poxton IR (1999) The association of *Clostridium botulinum* type C with equine grass sickness: a toxicoinfection? *Equine Vet J* **31**(6): 492–9.

Hyun SH, Sakaguchi G (1989) Implication of coprophagy in pathogenesis of chicken botulism. *Nippon Juigaku Zasshi* **51**(3): 582–6.

Jankovic J, Brin MF (1997) Botulinum toxin: Historical perspective and potential new indications. *Muscle Nerve* (Suppl. 6): S129–45.

Jensen WI, Price JI (1987) The global importance of type C botulism in wild birds. In *Avian Botulism: An International Perspective*, Eklund MW, Dowell Jr VR (eds). Charles C Thomas, Springfield, IL, pp. 33–54.

Kalandakanond S, Coffield JA (2001) Cleavage of SNAP-25 by botulinum toxin A requires receptor-mediated endocytosis, pH-dependent translocation and zinc. *J Pharmacol Exp Ther* **296**: 980–6.

Kao I, Drachman DB, Price DL (1976) Botulinum toxin: mechanism of presynaptic blockade. *Science* **192**: 1256–8.

Kinde H, Betty RL, Ardans A, Galey FD, Daft BM, Walker RL, Eklund MW, Byrd JW (1991) *Clostridium botulinum* type C intoxication associated with consumption of processed alfalfa hay cubes in horses. *J Am Vet Med Assoc* **199**(6): 742–6.

Lamana C (1987) The scope of the avian botulism problem. In *Avian Botulism: An International Perspective*, Eklund MW, Dowell Jr VR (eds). Charles C Thomas, Springfield, IL, pp. 5–11.

L'Hommedieu C, Stough R, Brown L, Kettrick R, Polin R (1979) Potentiation of neuromuscular weakness in infant botulism by aminoglycosides. *Pediatr Pharmacol Ther* **95**: 1065–70.

MacKay RJ, Berkhoff GA (1982) Type C toxicoinfectious botulism in a foal. *J Am Vet Med Assoc* **180**(2): 163–4.

Martin S (2003) *Clostridium botulinum* type D intoxication in a diary herd in Ontario. *Can Vet J* **44**: 493–5.

McGorum BC, Kyles KW, Prince D, Hahn CN, Mayhem IG (2003) Clinicopathological features consistent with both botulism and grass sickness in a foal. *Vet Rec* **152**: 334–6.

McLoughin MF, McIlroy SG, Neill SD (1988) A major outbreak of botulism in cattle being fed ensiled poultry litter. *Vet Rec* **122**: 579–81.

Mitten LA, Hinchcliff KW, Holcombe SJ, Reed SM (1994) Mechanical ventilation and management of botulism secondary to an injection abscess in an adult horse. *Equine Vet J* **26**: 420–3.

Miyazaki S, Sakaguchi G (1978) Experimental botulism in chickens: the cecum as the site of production and absorption of botulinum toxin. *Jpn J Med Sci Biol* **31**: 1–15.

Moberg LJ, Sugiyama H (1980) The rat as an animal model for infant botulism. *Infect Immun* **29**(2): 819–21.

Moeller RB, Puschner B, Walker RL, Rocke T, Galey FD, Cullor JS, Adrans AA (2003) Determination of the median toxic dose of type C botulinum toxin in lactating dairy cows. *J Vet Diagn Invest* **15**: 523–6.

Montecucco C (1986) How do tetanus and botulinum toxins bind to neuronal membranes? *Trend Biochem Sci* **11**: 314–7.

Nunn F, Cave TA, Knottenbelt C, Poxton IR (2004) Association between Key–Gaskell syndrome and infection by *Clostridium botulinum* type C/D. *Vet Rec* **155**: 111–5.

Ohishi I, Sakaguchi G (1982) Production of C2 toxin by *Clostridium botulinum* types C and D as determined by its vascular permeability activity. *Infect Immun* **35**: 1–4.

Ohishi I, Sakaguchi G, Riemann H, Behmer D, Hurvell B (1979) Antibodies to *Clostridium botulinum* toxins in free-living birds and mammals. *J Wildl Dis* **15**: 3–10.

Ohishi I, Iwasaki M, Sakaguchi G (1981) Vascular permeability activity of botulinum C2 toxin elicited by cooperation of two dissimilar protein components. *Infect Immun* **31**: 890–5.

Oliver JE, Lorenz MD, Kornegay JN (1997) *Handbook of Veterinary Neurology.* W.B. Saunders, Philadelphia, PA, pp. 188–90.

Page RK, Fletcher OJ (1975) An outbreak of type C botulism in three week old broilers. *Avian Dis* **19**(1): 192–5.

Pecelunas KS, Wages DP, Helm JD (1999) Botulism in chickens associated with elevated iron levels. *Avian Dis* **43**: 783–787.

Richmond RN, Hatheway CL, Kaufmann AF (1978) Type C botulism in a dog. *J Am Vet Med Assoc* **173**(2): 202–3.

Ricketts SW, Greet TR, Glyn PJ, Ginnett CD, McAllister EP, McCaig J, Skinner PH, Webbon PM, Frape DL, Smith GR (1984) Thirteen cases of botulism in horses fed big bale silage. *Equine Vet J* **16**: 515–8.

Rings DM (1987) Bacterial meningitis and diseases caused by bacterial toxins. *Vet Clin North Am* **3**: 85–97.

Roberts TA, Aitken ID (1973) Botulism in birds and mammals in Great Britain and an assessment of toxicity of *Clostridium botulinum* type C in domestic fowl. In *Spore Research*, Barkder AN, *et al.* (eds). Academic Press, London, pp. 1–9.

Rocke TE (1993) *Clostridium botulinum*. In *Pathogenesis of Bacterial Infections*, 2nd edn, Gyles CL, Theoen CO (eds). Iowa State University Press, Ames, IA, pp. 86–96.

Rocke TE, Smith SR, Nashold SW (1998) Preliminary evaluation of a simple *in vitro* test for the diagnosis of type C botulism in wild birds. *J Wildl Dis* **34**(4): 744–51.

Rooney JP, Prickett ME (1967) Shaker foal syndrome. *Mod Vet Pract* **48**: 44–5.

Rosen MN (1971) Botulism. In *Infectious and Parasitic Diseases of Wild Birds*, Davis JW, *et al.* (eds). Iowa State University Press, Ames, IA, pp. 100–117.

Rubin EJ, Gill DM, Boquet P, Popoff MR (1988) Functional modification of a 21 kilodalton G protein when ADP-ribosylated by exoenzyme C3 of *Clostridium botulinum*. *Mol Cell Biol* **8**: 418–26.

Sakaguchi G (1983) *Clostridium botulinum* toxins. *Pharmacol Ther* **19**: 165–94.

Sakaguchi G, Sakaguchi S, Kurazono H, Kamata Y, Kosaki S (1987) Persistence of specific antigenic protein in the serum of chickens given intravenously botulinum toxin type B, C, D, E, or F. *FEMS Microbiol Lett* **43**: 355–9.

Sato S (1987) Control of botulism in poultry flocks. In *Avian Botulism: An International Perspective*, Eklund MW, Dowell Jr VR (eds). Charles C Thomas, Springfield, IL, pp. 349–56.

Schettler CH (1979) *Clostridium botulinum* type C toxin infection in broiler farms in North West Germany. *Berl Munch Tierarztl Wscr* **92**: 50–7.

Schiavo G, Benfenati F, Poulain B, Rossetto O, Polverino de Laureto P, DasGupta BR, Montecucco C (1992a) Tetanus and botulinum-B neurotoxins block neurotransmitter release by proteolytic cleavage of synaptobrevin. *Nature (London)* **359**: 832–5.

Schiavo G, Rossetto O, Santucci A, DasGupta BR, Montecucco C (1992b) Botulinum neurotoxins are zinc proteins. *J Biol Chem* **267**: 23479–83.

Schiavo G, Rossetto O, Catsicas S, Delaureto PP, DasGupta BR, Benefenati F, Montecucco CL (1993a) Identification of the nerve terminal targets of botulinum neurotoxin serotype-A, serotype-D, and serotype-E. *J Biol Chem* **68**: 23784–87.

Schiavo G, Shone DD, Rossetto O, Alexander FCG, Montecucco CL (1993b) Botulinum neurotoxin serotype-F is a zinc endopeptidase specific for VAMP/synaptobrevin. *J Biol Chem* **268**: 11516–19.

Schiavo G, Malizio C, Trimble WS, Polverino de Laureto, P, Milan G, Sugiyama H, Johnson EA, Montecucco C (1994) Botulinum G neurotoxin cleaves VAMP/synaptobrevin at a single Ala-Ala peptide bond. *J Biol Chem* **269**: 20213–16.

Schiavo G, Matteoli M, Montecucco C (2000) Neurotoxins affecting neuroexocytosis. *Physiol Rev* **80**(2): 717–66.

Schoenbaum MA, Hall SM, Glock RD, Grant K, Jenny AL, Schiefer TJ, Sciglibaglio P, Whitlock RH (2000) An outbreak of type C botulism in 12 horses and a mule. *J Am Vet Med Assoc* **217**(3): 365–8.

Sharma SK, Singh BR (1998) Hemagglutinin binding mediated protection of botulinum neurotoxin from proteolysis. *J Nat Toxins* **7**(3): 239–53.

Shimizu T, Kondo H (1978) Preparation and evaluation of botulinal type C toxoid for immunization of pheasants. In *Avian Botulism: An International Perspective*, Eklund MW, Dowell Jr VR (eds). Charles C Thomas, Springfield, IL, pp. 357–69.

Simpson LL (1980) Kinetic studies on the interaction between botulinum toxin type A and the cholinergic neuromuscular junction. *J Pharmacol Exp Ther* **212**: 16–21.

Simpson LL (1981) The origin, structure, and pharmacological activity of botulinum toxin. *Pharmacol Rev* **33**: 155–88.

Simpson LL (1982) A comparison of the pharmacological properties of *Clostridium botulinum* type C1 and C2 toxins. *J Pharmacol Exp Ther* **223**: 695–701.

Simpson LL (1989) Peripheral actions of the botulinum toxins In *Botulinum Neurotoxin and Tetanus Toxin*, Simpson LL (ed.). Academic Press, New York, pp. 153–78.

Smart JL, Robert TA, McCullagh KG, Lucke VM, Pearson H (1980) An outbreak of type C botulism in captive monkeys. *Vet Rec* **107**: 445–6.

Smith GR (1987) Botulism in water birds and its relation to comparative medicine. In *Avian Botulism: An International Perspective*, Eklund MW, Dowell Jr VR (eds). Charles C Thomas, Springfield, IL, pp. 73–86.

Smith LDS (1975) *The Pathogenic Anaerobic Bacteria*. Charles C Thomas, Springfield, IL.

Söllner T, Bennett MK, Whiteheart S, Scheller RH, Rothman JE (1993) A protein assembly–disassembly pathway *in vitro* that may correspond to sequential steps of synaptic vesicle docking, activation, and fusion. *Cell* **75**: 409–18.

Sprayberry KA, Carlson GP (1997) Review of equine botulism. *AAEP Proc* **43**: 379–81.

Sugiyama H (1980) *Clostridium botulinum* neurotoxin. *Microbiol Rev* **44**(3): 419–48.

Sweeney RW, Sweeney CR, Soma LR, Woodward CB, Charlton CA (1986) Pharmacokinetics of metronidazole given to horses by intravenous and oral routes. *Am J Vet Res* **47**: 1726–9.

Swerczek TW (1980a) Toxicoinfectious botulism in foals and adult horses. *J Am Vet Med Assoc* **176**(3): 217–20.

Swerczek TW (1980b) Experimentally induced toxicoinfectious botulism in horses and foals. *Am J Vet Res* **41**(3): 348–50.

Szabo EA, Pemberton JM, Gibson AM, Thomas RJ, Pascoe RR, Desmarchelier PM (1994) Application of PCR to a clinical and environmental investigation of a case of equine botulism. *J Clin Microbiol* **32**(8): 1986–91.

Thomas RJ (1991) Detection of *Clostridium botulinum* types C and D toxin by ELISA. *Aus Vet J* **68**(3): 111–3.

Trampel DW, Smith SR, Rocke TE (2005) Toxicoinfectious botulism in commercial caponized chickens. *Avian Dis* **49**: 301–303.

University of California Davis, UC Davis. www.vmtrc.ucdavis.edu/dfs/botu/botulism.pdf.

Vaala WE (1991) Diagnosis and treatment of *Clostridium botulinum* infection in foals: a review of 53 cases. *Proc 9th Am Coll Vet Med Forum* **9**: 379–81.

van Ermengem E (1979) Uber einem neuen anaeroben Bacillus und seine Beziehungen zum Botulismus. *Z Hyg Infektionskranskh* 1897; 26(1–56): 701–19 (English transl). A new anaerobic bacillus and its relation to botulism. *Rev Infect Dis* **1**: 701–9.

van Nes JJ, van der Most, van Spijk D (1986) Electrophysiologic evidence of peripheral nerve dysfunction in six dogs with botulism type C. *Res Vet Sci* **40**: 372–6.

Wang Y, Sugiyama H (1984) Botulism in metronidazole-treated conventional adult mice challenged orogastrically with spores of *Clostridium botulinum* type A or B. *Infect Immun* **46**: 715–9.

Wang Y, Burr DH, Korthals GJ, SugiyamaH (1984) Acute toxicity of aminoglycoside antibiotics as an aid in detecting botulism. *Appl Environ Microbiol* **48**: 951–5.

Weiss HE, Wacker R, Dalchow W (1982) Botulismus als ursache eines Massensterbens bei Wassergoveln. *Teirarztl Umschau* **37**: 842–6.

Whitlock RH (1996) Botulism, type C: experimental and field cases in horses. *Equine Pract* **18**(10): 11–7.

Whitlock RH (2002) Botulism (shaker foals; forage poisoning). In *Large Animal Internal Medicine*, 3rd edn, Smith BP (ed.). Mosby, St. Louis, MO, pp. 1003–8.

Whitlock RH, Buckley C (1997) Botulism. *Vet Clin North Am Equine Pract* **13**(1): 107–28.

Whitlock RH, Williams JM (1999) Botulism toxicosis in cattle. *Bovine Proc* **32**: 44–53.

Whitlock RH, Buckley C, Messick J (1989) Investigation of herd outbreaks of botulism in cattle and horses. *Proc Am Assoc Vet Lab Diagn* **40**: 38.

Wichtel JJ, Whitlock RH (1991) Botulism associated with feeding alfalfa hay to horses. *J Am Vet Med Assoc* **199**: 471–2.

Wilkins PA, Palmer JE (2002) Botulism in foals: a survivable disease. *AAEP Proc* **48**: 124–6.

Wilkins PA, Palmer JE (2003) Mechanical ventilation in foals with botulism: 9 cases (1989–2002). *J Vet Intern Med* **17**: 708–12.

Williamson LC, Neale EA (1998) Syntaxin and 25-kDa synaptosomal-associated protein: differential effects of botulinum neurotoxins C1 and A on neuronal survival. *J Neurosci Res* **52**(5): 569–83.

Wobeser GA (1987) Control of botulism in wild birds. In *Avian Botulism: An International Perspective*, Eklund MW, Dowell Jr VR (eds). Charles C Thomas, Springfield, IL, pp. 339–48.

Yamambe K, Koga (1982) An outbreak of botulism by *Clostridium botulinum* type C in broiler chickens. *J Jpn Soc Poult Dis* **18**: 21–4.

Yeruham I, Elad D, Avidar Y, Grinberg K, Tiomkin D, Monbaz A (2003) Outbreak of botulism type B in a dairy cattle herd: clinical and epidemiological aspects. *Vet Rec* **153**(9): 270–2.

C H A P T E R

# 62

# Enterotoxins

*Larry J. Thompson*

## INTRODUCTION

The term food poisoning can be used to describe the result of eating pathogenic organisms or toxins in contaminated food. The list of causative agents for food-borne illness can be quite lengthy including infectious agents (e.g. bacteria, viruses and parasites), natural toxins (e.g. bacterial toxins, mycotoxins, shellfish poisons and plant poisons) and other contaminants (Mead *et al.*, 1999). Veterinarians and owners often use the term garbage intoxication or garbage poisoning to describe the situation where animals, especially dogs, eat discarded or otherwise poorly preserved foodstuffs and subsequently develop a syndrome composed mainly of severe vomiting and diarrhea. This chapter is focused on the effects of enterotoxins, which are defined as those bacterial exotoxins that are specific for the intestinal tract causing vomiting, diarrhea and abdominal pain. Although many organisms produce enterotoxins (Vaishnavi, 1996; Fasano, 2002) those produced by *Staphylococcus aureus* and *Bacillus cereus* will be stressed in this chapter. Botulism is covered separately in the previous chapter. In contrast to enterotoxin, the term endotoxin is used to describe a cell-associated bacterial toxin, usually a lipopolysaccharide complex that is found on the outer membrane of Gram-negative bacteria. Endotoxins essentially remain associated with the cell wall until the destruction of the bacteria by autolysis, external lysis or phagocytic digestion. While all animals can be affected by endotoxin, the horse is especially prone to disease complications due to endotoxemia (Werners *et al.*, 2005).

## BACKGROUND

This chapter is focused on the most common situations associated with enterotoxin exposures in veterinary medicine, namely dogs ingesting garbage, carrion or other spoiled foodstuffs. Dogs readily consume a wide variety of such material and owners often are unaware when their animal has had access to or has consumed foodstuffs of doubtful origin. Many owners have the mistaken opinion that dogs (and other animals) are not susceptible to food poisoning and thus may feed their pets foods that humans would rather not ingest. Discarded foodstuffs are often high in proteins and carbohydrates and serve as excellent substrates for the rapid growth of bacterial, often with enterotoxin release. Instances of garbage intoxication increase in warm weather and around major holidays when increased food is prepared and discarded. Under conditions of warm temperatures and adequate moisture, these discarded foodstuffs can have an almost explosive growth of bacteria, especially *S. aureus* and *B. cereus*, which are the most common causes of enterotoxin related food poisonings in the human. While a great deal of time and energy has been expended by public health officials in the pursuit of the origin and control of food poisonings in humans, there is a paucity of specific information concerning animals.

## PHARMACOKINETICS AND MECHANISM OF ACTION

*S. aureus* is a facultative anaerobic Gram-positive coccus that may be single, paired or in a grape-like cluster. *S. aureus* does not form spores and thus contamination may be avoided by proper heat treatment of food to kill the bacteria. *S. aureus* is able to grow in a wide range of temperatures (7–48.5°C) with an optimum range from 30°C to 37°C. *S. aureus* can also grow over a wide pH range (4.2–9.3) and can tolerate sodium chloride concentrations of up to 15%. These characteristics allow it to grow in a wide variety of foodstuffs and in situations of discarded food. *S. aureus* is an important infective pathogen as well and can easily be found in the nostrils and on the skin of most mammals (Le Loir *et al.*, 2003). At least 14 different staphylococcal

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enterotoxin (SE) types have been found and they are best described as short proteins secreted by the cell into the growth matrix (Balaban and Rasooly, 2000). All SE types are water soluble, very heat resistant and resist most proteolytic enzymes, such as trypsin and pepsin, which make it possible for them to travel through the digestive tract to their site of action. Thus all SE types resist the conditions that could easily destroy the bacteria that produced them. SEs are thought to have a direct effect on the intestinal epithelium as well as on the vagus nerve to cause stimulation of the emetic center as well as increasing peristalsis. Foods that are frequently associated with staphylococcal food poisoning include meat and meat products; poultry and egg products; salads such as egg, tuna, chicken, potato and macaroni; bakery products such as cream-filled pastries; and milk and other dairy products. An illustration of a proverbial high-risk food would be the potato salad at the summer picnic left out for several hours without refrigeration.

*B. cereus* is in the family Bacillaceae which are all Gram-positive rod-shaped bacteria which form endospores. The family has two main divisions: the anaerobic spore-forming bacteria of the genus *Clostridium* and the aerobic or facultatively anaerobic spore-forming bacteria of the genus *Bacillus*. *B. cereus* is a primary inhabitant of soils and contaminates almost all agricultural products and is routinely involved in the contamination and spoilage of food products. *B. cereus* can also be involved in wound, eye or systemic infections. *B. cereus* food poisoning is generally described as having two types of illness caused by different metabolites. The diarrheal type of illness is caused by one or several heat-labile high molecular weight proteins, while the vomiting (emetic) type of illness is believed to be caused by a low molecular weight, heat-stable peptide which has been named cereulide (Schoeni and Wong, 2005). In the human, the diarrheal syndrome is thought to be the consequence of a food-borne infection with enterotoxic *B. cereus* following the observation that the diarrheal enterotoxins are produced during the vegetative growth of *B. cereus* in the small intestine. *B. cereus* food poisoning has been classically associated with fried rice and other cooked rice dishes because the bacteria is frequently present in uncooked rice and heat-resistant spores may survive cooking. If cooked rice is subsequently held at room temperature, vegetative forms multiply, and a heat-stable toxin is produced that can survive brief heating, such as stir frying. *B. cereus* food poisoning can also be associated with meat- or vegetable-containing foods after cooking where the food was held above room temperature for a prolonged period of time.

## TOXICITY

The symptoms of staphylococcal food poisoning occur in humans when as little as 100 ng of enterotoxin is ingested (Bennett, 2005). There is a paucity of information on the dose of individual enterotoxins which will cause clinical signs in common domestic animals. The first and most common clinical sign in dogs is vomiting, which usually occurs within 2–3 h following ingestion. This can often suffice to remove enough contaminated food from the gastrointestinal tract to prevent the development of more severe clinical signs. However, vomiting can be protracted and lead to fluid and electrolyte abnormalities. Diarrhea can often develop within 2–48 h following ingestion of contaminated food and can be severe, sometimes bloody. The combination of both vomiting and diarrhea in the affected animal can quickly lead to profound fluid and electrolyte abnormalities. The animal may exhibit tenderness to the abdomen or the stomach and intestinal tract can be distended with gas (Coppock and Mostrom, 1986).

The emetic form of *B. cereus* food poisoning is characterized in the human by an acute attack of nausea and vomiting. This occurs within 1–5 h after consumption of contaminated food and diarrhea is not a common feature in this type of illness. In the human, the diarrheal syndrome is characterized by abdominal pain, abdominal cramping and diarrhea that often can be very watery. There is an incubation period of 4–16 h and clinical signs can persist for 12–24 h. Nausea may accompany the diarrhea but vomiting rarely occurs.

## TREATMENT

It is important to remember three factors when a food-borne problem is included in the differential diagnosis. These factors include the presenting clinical signs, the possible exposure to foodstuffs not normally included in the animal's diet, and the interval between exposure and onset of clinical problems. Many of these may not be known at the time of initial presentation but should be explored as the case is developed.

In general, treatment of garbage intoxication in animals should be directed at correcting the fluid and electrolyte abnormalities along with considerations of acid/base balance. As mentioned above, non-complicated cases will often resolve within hours of presentation with only supportive care. However, because the clinical signs can be similar to more serious conditions, a more thorough diagnostic workup (e.g. survey abdominal radiographs) and close observation should always be considered. Since cases most often present following several vomiting episodes, the use of emetics is usually not indicated. In the case of protracted vomiting, the judicious use of antiemetics should be considered. Administration of activated charcoal is not required for known garbage intoxications but many clinicians will use it as part of a general approach to these

cases. Activated charcoal should not be given to dehydrated animals unless fluid administration is also initiated. Antibiotics are not indicated in uncomplicated cases of garbage intoxication.

## CONCLUDING REMARKS

This chapter has reviewed garbage intoxication with emphasis on preformed enterotoxins as the causative agents. The great number of other types of food-borne illnesses, including infectious agents and toxins, will require other diagnostic and treatment considerations.

## REFERENCES


Balaban N, Rasooly A (2000) Staphylococcal enterotoxins. *Int J Food Microbiol* **61**: 1–10.

Bennett RW (2005) Staphylococcal enterotoxin and its rapid identification in foods by enzyme-linked immunosorbent assay-based methodology. *J Food Protect* **68**: 1264–70.

Coppock RW, Mostrom MS (1986) Intoxication due to contaminated garbage, food, and water. In *Current Veterinary Therapy IX Small Animal Practice*, Kirk RW (ed.). W.B. Saunders Co., Philadelphia, PA, pp. 221–5.

Fasano A (2002) Toxins and the gut: role in human disease. *Gut* **50**: 9–14.

Le Loir Y, Baron F, Gautier M (2003) Staphylococcus aureus and food poisoning. *Genetic Molec Res*, **2**: 63–76.

Mead PS, Slutsker L, Dietz V, McCaig LF, Bresee JS, Shapiro C, Griffin PM, Tauxe RV (1999) Food-related illness and death in the United States. *Emerg Inf Dis* **5**: 607–25.

Schoeni JL, Wong ACL (2005) Bacillus cereus food poisoning and its toxins. *J Food Protect* **68**: 636–48.

Vaishnavi C (1996) Bacterial enterotoxins. *Trop Gastroenterol* **17**: 160–4.

Werners AH, Bull S, Fink-Gremmels J (2005) Endotoxaemia: a review with implications for the horse. *Equine Vet J* **37**: 371–83.

# Part 12

# Poisonous and Venomous Organisms

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C H A P T E R

# 63

# Caterpillars and mare reproductive loss syndrome

*Manu M. Sebastian, William Bernard, and Lenn R. Harrison*

## INTRODUCTION

During the spring of 2001, horse farms in and around Central Kentucky experienced an epidemic of early fetal and late fetal loss (EFL/LFL) which, together with smaller numbers of fibrinous pericarditis (FP) and unilateral uveitis (UU), are now known as the mare reproductive loss syndrome (MRLS). The EFL and LFL were identified during the last week of April, peaked on May 5th and declined rapidly thereafter. FP and UU were identified during the same time period (Kane and Kilby, 2001; Harrison, 2001; Powell, 2001). The same pattern of abortion, but in lesser numbers, was observed in 2002.

Four epidemiological investigations were conducted to identify risk factors associated with MRLS (Cohen *et al*., 2003a, b; Dwyer *et al*., 2003; Seahorn *et al*., 2003). Risk factors identified included exposure to moderate to high concentrations of eastern tent caterpillars (*Malacosoma americanum*, ETCs) (Figure 63.1) in the pastures, the presence of cherry trees, ⩾50 broodmares/farm, feeding hay in pasture, greater than usual amounts of white clover in pastures, abortion during a previous pregnancy, being fed on pastures exclusively during the 4-week period prior to abortion, access to pasture after midnight during the 4-week period prior to abortion, drinking from a water trough, or not, access to water buckets or automatic water taps, etc. Risk factors associated with FP included being from a farm with mares and foals affected by MRLS, exposure to ETC in or around the pastures, younger age, shorter duration of residence in Kentucky, being fed hay grown outside Kentucky, lack of access to pond water and a lack of direct contact with cattle.

**FIGURE 63.1** Late instar ETCs in nest. This figure is reproduced in color in the color plate section.

Approximately 14,980 foals of all breeds were lost due to MRLS in 2001. Smaller numbers of clinically similar fetal losses, abortions and still births were reported from Southern Ohio, West Virginia and Tennessee during the same time period, indicating that MRLS was not limited to Kentucky. A lesser incidence of MRLS was recorded in Central Kentucky in 2002, and few cases were reported in 2003. At least 17 breeds of horses were affected. The abortions were heavily concentrated in Central Kentucky but aborted equine fetuses from 32 counties in Kentucky were submitted to the University of Kentucky diagnostic laboratory for necropsy. The specific number of LFL and EFL abortions in the field was not recorded.

The economic loss due to MRLS in 2001 was estimated at $336 million. The combined losses from MRLS in 2001 and 2002 were estimated at approximately $500 million. MRLS was therefore one of the most economically devastating

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acute diseases to strike livestock industry. The syndrome of foal deaths drew substantial media attention, resulting in national and international news reports (Paulick, 2001).

## CLINICAL FINDINGS

### Early fetal loss

Mares undergoing EFL showed few systemic clinical signs. Some mares were presented with a sero-sanguineous or purulent vulvar discharge, and some with fetal membranes protruding from their vulva with fetuses located either in the vagina or vulva. A proportion of mares (approximately <15%) reportedly exhibited mild colic signs, abdominal straining or low-grade fevers (101.0–101.5°F) 1–3 days before EFL.

In most EFL cases rectal palpation was apparently normal. The typical ultrasound presentation of an EFL/MRLS loss was a dead fetus (i.e. no heartbeat), surrounded by echogenic allantoic fluid and a more echogenic amniotic fluid. In cases where the fetus was not dead, ultrasound examination generally showed a fetus with slow heart rate and slow movements, suspended in an amniotic fluid which was echogenic, either "cloudy" or "flocculent". Some mares aborted markedly autolyzed fetuses, while some retained the fetus in the vaginal canal. Most mares which had EFL pregnancies as identified by ultrasound expelled the fetus within 2 days to 2 weeks. Majority of EFL cases occurred between days 40 and 80 of gestation, with few cases up to 140 days. It was also noted that mares less than 30 days pregnancy were apparently less susceptible to MRLS.

Culture of aspirates of allantoic fluid collected from three EFL mares in 2001 grew alpha *Streptococcus* bacteria in two cases and *Escherichia coli* in one case. Complete blood counts and blood chemistry panel evaluation from these mares did not show any significant abnormalities. Microscopic examination of uterine biopsies from EFL mares within 7 days of abortion showed moderate to severe inflammation (Riddle and LeBlanc, 2003).

### Late fetal loss

LFL losses were those observed in the last trimester of gestation. The majority of LFL mares exhibited few signs of impending foaling or abortion. Clinical features associated with LFL included explosive parturition, dystocia, foaling while standing, premature placental separation ("red bag delivery") stillbirth, foals born weak and agalactic mares. Clinical signs associated with foals born weak were consistent with asphyxia and many required resuscitation. Many of the weak foals were admitted to local equine veterinary hospitals and survived for up to 4 days. Blood cultures were rarely positive for bacteria. The most frequently isolated bacteria were alpha *Streptococcus* and *Actinobacillus* spp. These foals were dehydrated, hypothermic, and tachycardic with irregular respiration. Leukopenia, hypoglycemia and acidosis were also consistent findings. Bilateral hyphema was observed in many of these foals at birth (Byars and Seahorn, 2002).

## FIBRINOUS PERICARDITIS

Thirty-eight cases of FP were admitted to two large Central Kentucky referral veterinary clinics. The pericarditis cases occurred in horses of all ages, breeds and sexes, and were not restricted to pregnant mares. The clinical presentation of the pericarditis cases included ultrasonographic evidence of effusive FP, tachycardia, muffled heart sounds, hyperfibrinogenemia, lethargy, tachypnea, jugular pulse distention and pleural effusion and/or ascitis. Analysis of fluid removed from the pericardial sac of most cases demonstrated a low white cell count and high protein content. This fluid was mostly sterile exudates, but in cases where bacteria grew, they were similar to those observed in EFL/LFL abortions. Less than 15 confirmed cases of pericarditis were recorded in 2002 and two cases were reported associated with MRLS in 2003 (Bolin *et al.*, 2004).

## UNILATERAL UVEITIS

During MRLS in 2001, 40 UU cases were observed in foals, yearlings and adult horses of all breeds, ages and sexes. The condition was unilateral in all cases. The onset of clinical signs was peracute, with profound exudative opthalmitis occurring within the first 12-h period. Consistent changes included corneal edema and exudates in the anterior and posterior chambers. The anterior chamber exudates were tan yellow, presumably proteinaceous and often accompanied by hemorrhage from the surface of the iris. A markedly turbid yellow vitreous was visible through a mid-range pupil, and this was confirmed by ultrasound. Affected eyes appeared refractive to treatment and blindness ensued, followed by various degrees of global atrophy. No eyes were enucleated for histopathological examination. The culture and cytology results were negative ($n = 1$). Fewer cases were reported in 2002. One colt affected by MRLS in 2001 UU won the Illinois Derby and raced in the 2004 Kentucky Derby (Latimer, 2002).

Three cases of *Actinobacillus* meningoencephalitis (age >4 years), caused by the same organism associated with the EFL/LFFL/FP cases, were diagnosed on necropsy during

MRLS in 2001. No cases of *Actinobacillus* meningoencephalitis were observed during MRLS in 2002 in Central Kentucky (Sebastian *et al.*, 2002a).

## PATHOLOGICAL FINDINGS

The EFL fetoplacental units were moderately to markedly autolyzed at the time of submission, which limited pathological evaluation and related testing. Most fetoplacental units showed moderate neutrophilic infiltrates in the placental membranes, and the amniotic and allantoic fluids. The overall finding of EFL was a bacterial placentitis. Severe autolysis precluded location of the inflammation to specific anatomical regions of the placenta, in contrast to the LFL histopathological findings. The same organism isolated from LFL was isolated from the EFL aborted fetuses and no funisitis was observed in these.

The majority of LFL fetuses were between 10 months of gestation and term. Gross lesions included pale brown placenta, in sharp contrast to its normal dark reddish brown color, with a thick, dull, edematous yellowish umbilical cord, hemorrhage in the eyes, marked placental thickening, placental edema and intact cervical star ("red bag"). In some fetuses the lungs were edematous with fluid oozing from cut sections.

Characteristic histopathological changes associated with LFL included placentitis, funisitis and perinatal pneumonia. The alveoli contained free bacterial colonies with and without inflammatory response and numerous free squamous epithelial cells. Funisitis (inflammation of umbilical cord) was a characteristic lesion of LFL/MRLS and was mostly confined to the amniotic segment of the umbilical cord. Umbilical cord lesions were generally confined to the surface and consisted of mild to dense infiltration of inflammatory cells, mostly neutrophils admixed with bacterial colonies. Microscopic lesions observed in the placenta were largely confined to the celomic space in the subchorionic stroma, and they were mostly perivascular. The MRLS placentitis was distinguishable from the ascending placentitis by the pattern of distribution of inflammatory cells. No single lesion was pathognomonic for MRLS/LFL. The diagnosis depended on overall evaluation of the case and consideration of its history (Williams *et al.*, 2002; Cohen *et al.*, 2003c).

Standard bacteriological isolation procedure yielded up to 10 different bacterial species from lung, stomach fluid, allantochorion and placental fluid of LFL 2001 cases. Nonbeta hemolytic streptococci (51%) and/or *Actinobacillus* spp. (13 %) comprised the majority of the isolates fetuses (Donahue *et al.*, 2002).

The gross findings of FP cases included enlarged heart with large quantities of sero-fibrinous fluid in the pericardial cavity, in some cases up to 3 l of fluid. Histopathological findings consisted of the visceral pericardium covered with fibrin, admixed with neutrophils and lesser numbers of lymphocytes. In many cases the inflammation extended beneath the epicardium into the myocardium forming a thick uniform layer of fibrinous/fibrinopurulent exudate made up of neutrophils and lymphocytes admixed with proliferating fibroblasts and large quantities of fibrin. While *Actinobacillus* spp. was isolated from 31% of these cases, all other attempts showed no bacterial growth, suggesting that an unidentified factor, not simply a bacterial infection, was responsible for the condition. A number of pericarditis patients failed to survive in spite of treatment and were submitted for necropsy examination. *Actinobacillus* spp. was isolated from a total of 10 nontreated horses of 32 cases submitted for necropsy during MRLS in 2001 (Bolin *et al.*, 2004).

No eye tissue from uveitis cases was submitted for histopathological examination and hence the histopathological findings in this condition are unknown. In the three cases of encephalitis that occurred during the same period, *Actinobacillus* spp. were isolated from the brain and other organs and the isolates were similar to the EFL/LFL cases. Detailed virological and serological examinations were conducted on the fetal and placental samples and no viral agents were identified, thus ruling out a primary infectious cause.

## INVESTIGATION OF ETIOLOGICAL AGENTS

A variety of possible causes were considered as etiological agents. A primary infectious cause was ruled out based on the absence of specific clinical symptoms in aborting mares. The point source onset of the syndrome suggested a noninfectious cause and investigations were directed toward an environmental toxin.

Fetal fluid samples were negative for nitrate/nitrite which ruled out its toxicity. Pasture samples collected during this period showed less than toxic levels of nitrate/nitrite. The aborting mares did not show prolonged gestations and the placentas from MRLS mares showed marked inflammation. These findings and preliminary testing conducted in the University of Kentucky ruled out ergot alkaloid/fescue toxicosis. Detailed examinations for phytoestrogens and other mycotoxins were conducted during the first few weeks of the outbreak and were ruled out.

Epidemiological investigations identified both spatial and temporal association between the presence of ETC and MRLS abortions. Preliminary field investigations confirmed these time and place associations between dispersing ETC and MRLS. These findings led to a search for an

ETC or its habitat associated toxin. Cyanide associated with the black cherry tree ETC biological system was the only significant toxin identified. This investigation was supported by positive tests for low concentrations (about 500 ppb) of cyanide in some aborted fetal heart tissue. Extensive studies were conducted in pregnant mares by exposing them with variable cyanide doses which were not lethal to mares and none of the studies could induce abortion. The blood cyanide concentration in MRLS aborted mares was not different from the concentration in control mares used in experimental studies further supporting the fact that cyanide may not be directly involved in MRLS.

Prunasin is the primary organic cyanide in cherry tree leaves, the principal food of ETC and it is digested/metabolized to mandelonitrile. Mandelonitrile, which spontaneously hydrolyzes to yield cyanide, is also present in the regurgitated materials of ETC and is used by the ETC as a defensive chemical against predators (Fitzgerald, 2002). Based on this scientific evidence, pregnant mares were challenged with mandelonitrile. Mandelonitrile was administered at a dose of 2 mg/kg/horse in feed, BID, for 14 days to six late-term pregnant mares. Each administration yielded a sharp increase in the plasma concentration of cyanide, peaking at about 600 ng/ml, followed by an 8–12 h increase in plasma thiocyanate. Mild diarrhea was observed in the treated mares. No abortions were observed in any treated mare. No pathological changes suggestive of EFL/LFL were observed, and the role of free cyanide or its precursors in MRLS was eventually discounted (Harkins *et al.*, 2002).

The possibility that MRLS could be due to inhalation of mandelonitrile/cyanide was also investigated. Four early-term pregnant mares were exposed to mandelonitrile vapor for 6 h for 3 consecutive days. Two mares were administered 2–50 mg mandelonitrile/day and another two were administered 2–500 mg mandelonitrile/day. Two hours after the last challenge, fetal pulse rates increased by 25% above pre-challenge values in three of the four exposed mares. The control mares remained normal. Forty-eight hours after the first observation, fetal pulse rates returned to normal, while the amniotic turbidity scores increased by one grade. The control mare values remained unchanged. Total serum progestagens and ECG values remained within normal limits. Fetal heartbeat and amniotic turbidity scores returned to baseline values and no fetal losses were observed. These experiments indicated that mandelonitrile by itself is not a toxic compound responsible for MRLS.

## EXPERIMENTAL STUDIES WITH CATERPILLARS

Experiments specifically designed to replicate the natural exposure of mares to ETC were carried out in spring of 2002. Pregnant mares were kept in experimental plots and exposed to live ETC or their frass (feces). Mares were exposed to ETC for 6 h each day for a maximum of 10 days or until they aborted. Pregnant mares exposed to ETC and frass aborted, however no abortions occurred in the frass-only group. The fetoplacental units recovered showed no significant gross findings, but bacteria similar to natural cases of MRLS were cultured from most of the fetal/placental tissues. Based on estimates of caterpillar concentrations in the treatment fields it was estimated that an average caterpillar concentration of 11.3–27.5 larvae/25 $cm^2$ was likely sufficient to cause MRLS abortion. This experiment established that ETC exposure will cause EFL/MRLS abortions (Webb *et al.*, 2004).

In another experiment mares (<102 days of pregnancy) were challenged with ETC and frass. This experiment comprised five control mares (administered saline), five mares administered 2.5 g/day of frass (by gastric tube) that had been stored at −80ºC and five mares administered 50 g/day homogenized ETC, by nasogastric tube. All three groups were treated for 10 days. All mares were confined in stalls and fed with hay from Nevada and treated for a period of 10 days. Four of the five mares treated with ETCs aborted during days 8–13 of the experiment. This study confirmed the relationship of ETC exposure to fetal losses in early stages of gestation, consistent with MRLS and ruled out involvement of Kentucky hay or pastures (Bernard *et al.*, 2004).

The LFL was reproduced by administration of homogenized ETC from Michigan to late-term pregnant mares. Six mares were administered 50 g/day of ETCs by nasogastric intubation for 9 days, the ETC having been fed fresh Kentucky Black cherry leaves. All mares exposed to ETC aborted and none of the control mares aborted. All placentas had intact cervical stars. No abnormal clinical signs, blood chemistry panel changes or blood cultures were recorded throughout the entire experiment. This experiment confirmed that ETC is abortigenic for mares in the late term of pregnancy and also demonstrated that ETCs from a region other than Central Kentucky are capable of causing MRLS (Sebastian *et al.*, 2002b).

Later on, the potency of different ETC body components to induce abortion was investigated. The integument of 100 g of ETC was separated from their digestive tracts and administered each separated fraction to six mares (early- to mid-gestation stage) each for 5 days. Two of the five mares administered the ETC integument fraction aborted, while the five mares administered the digestive tract contents did not abort. The results of this experiment suggested that the ETC abortifacient property is associated with the ETC integument, and not the digestive tract fraction. This experiment did not identify the factors/structures of the ETC integument that is abortigenic.

In an experiment to determine the abortifacient potential in different body components of ETC, pregnant mares were exposed to selected anatomical components/fractions

and filtrates of ETC. This experiment consisted of seven groups of five early pregnant mares, and the ETC used in the experiments was preserved at −80ºC. Group 1, the positive controls, was fed 50 g ETC homogenate mixed in sweet feed. Group 2, the negative controls, was fed saline mixed sweet feed. Groups 3–6 were fed specific parts, extracts, filtrates and retentates of ETC. For this experiment, ETC was dissected into three distinct body parts, exoskeleton, gut and internal contents. All the three components were fed to pregnant mares in early stages of gestation. Another group of early-term mares were fed filtrates of a phosphate buffered saline homogenate of ETC that had been filtered (<45 μm) and the retentate from this preparation was fed to two groups of mares in early stages of gestation. A positive and a negative control groups were also maintained in the experiment. All five mares in the positive control group aborted. Three of five mares in the group fed with exoskeleton aborted and one mare in the group fed the retentate aborted. No other groups were affected. This experiment confirmed that the abortifacient potential is associated with ETC exoskeleton. Filtration of the homogenate appears to have removed the abortifacient component, and it is worth noting that the homogenization/filtration process virtually completely, but not quite completely, inactivated the abortigenic factor (Webb *et al.*, 2004).

The ability of frozen ETCs, autoclaved ETC and gypsy moth caterpillars (GMC), a hairy caterpillar, to induce MRLS was investigated in detail. Three of five mares fed frozen ETCs (maintained at −80ºC) aborted; none of the five mares administered ETC that were autoclaved before administration aborted. One of the four GMC treated mares aborted; however, the abortion was considered atypical for MRLS and as such excluded from the analysis. This experiment showed that the abortifacient is not lost by freezing, being stable for at least 4 months at −80 ºC, but that the abortigenic factor is inactivated by autoclaving. The failure of GMC to cause MRLS abortion indicates that GMC which have many hairs (setae) similar to ETC do not have abortigenic factor or potential to induce abortion in mares (Webb *et al.*, 2004).

Investigations were undertaken to determine if the closely related forest tent caterpillars (*Malacosoma distria*, FTC) would produce MRLS. A group of early-term mares were challenged with 100 g of FTC homogenate/mare for 5 days (the FTC was raised/hatched out in laboratory), by stomach tube. A second group of early-term mares served as controls. There were no abortions in the FTC challenge group or the controls. This experiment suggests that the abortifacient responsible for MRLS is not uniformly present in all *Malacosoma* or tent caterpillar species. FTC also have setae/hairs of caterpillar and hence it indicate that setae as such may not induce abortions.

Experiments were conducted to rule out the role of bacteria associated with ETC. The ETCs were sterilized with alcohol by blending with 95% ethanol in a mechanical blender for 5 min and allowed to sit at room temperature for 2 h. This homogenate was cultured to assure the absence of viable bacteria A 50 g dose was administered to each mare for 10 consecutive days via a nasogastric tube. In Group 2 mares, saline replaced the ethanol, while the negative control mares received 200 ml of 10% ethanol. Two of the five mares that received the ethanol/ETC homogenate aborted. These abortions were considered typical of MRLS. No abortions were observed in the ethanol sham control groups. This experiment suggested that the causative agent is not an ETC associated bacteria. Although the ethanol treatment was bactericidal, it may not have been potent enough to destroy viruses and the possibility of a viral agent as the cause of MRLS was not excluded. In another experiment to rule out the role of virus and bacteria associated with ETC, homogenate of ETC prepared in normal saline was exposed to 30 kGy cobalt irradiation, a level demonstrated to destroy/inactivate marker bacteria and viruses while not significantly altering the activity of enzymatic or chemical markers. All six mares administered 100 g fresh frozen ETCs by stomach tube aborted within 32–120 h after the onset of treatment. Three of the six mares administered with 100 g irradiated ETCs aborted. These fetuses had gross and histopathological changes similar to those observed in the natural MRLS cases. *Actinobacillus* spp. and *Streptococcus* spp. were isolated from these fetoplacental units. This was the first experiment in which the typical MRLS funisitis has been demonstrated in experiment, funisitis being a typical and to some extent defining lesion in many field MRLS cases. The results of this experiment demonstrated that frozen ETC from 2003 preserved at −80ºC will induce abortion in late gestation mares. This experiment ruled out the role of naturally occurring virus or bacteria in ETC. The abortions that occurred as a result of exposure to the irradiated ETCs were remarkably similar to the experiments of 2001 and 2002. Irradiation markedly reduced the abortifacient capabilities of the 2003 ETC but did not eliminate all together their capability to induce abortion (Sebastian *et al.*, 2003a).

## ANIMAL MODELS

Attempts to produce a laboratory model of MRLS using mice were not successful. Various preparations of fresh and frozen ETC, ETC homogenates, ETC frass and ETC setal (hair) fragments were evaluated using 5–12 day pregnant mice. No significant number of abortions was observed in the mouse studies (Sebastian *et al.*, 2002c). The overall findings of the experiments suggest that there may be a species susceptibility for ETC abortifacient property. Experimental trials in rats by feeding finely chopped ETC mixed in feed to 4 days pregnant rats through the end of gestation did

not result in statistically significant number of abortions, but setal granulomas (granulomas with hair/setae cross sections in the center) were observed in the gastrointestinal tract of some animals. The abortifacient capability of ETC was investigated in pregnant pigs. Two of five gilts fed ETCs aborted their litters, while none of the controls aborted. This experiment showed that species other than equines can be affected by the ETC-related abortifacient, although this experiment was not repeated. A study was conducted in pregnant goats (>100 days gestation) with a dose of 50 g/ETC/day (same dose as in horse experiment) for 10 days by oral dosing and none of the goats aborted, but setal granulomas were observed in the gastrointestinal tract of all animals in the test group. Hence the studies in goats and rats suggest that setae as itself may not induce abortions in multiple species of animals and abortifacient property of ETC is species specific.

## UNIQUE FEATURES

The unique features of MRLS were the absence of premonitory clinical signs, limited distribution pattern of lesions, pathological lesions confined to pericardium, eye and fetoplacental unit and the bacteriological findings. Consistent with the absence of clinical signs, no abnormalities in clinical chemistry panels or complete blood counts were documented in the natural or experimental cases. Also blood cultures did not demonstrate a bacteremia in field or experimental cases of MRLS.

The histopathological studies conducted on LFL fetuses and placenta show no indications suggestive of the two well-characterized routes of entry of pathogenic organisms into the fetal membranes, either ascending via the cervix or hematogenous. Ascending infection via the cervix is considered unlikely by virtue of the lack of inflammation where the placenta contacts the cervix. The intact cervical star of placentas of aborted fetuses in late-term experimental studies supports this argument. The hematogenous route of infection also does not fit the inflammatory pattern as there is minimal inflammation on the chorionic surface of allantochorion. In LFL MRLS, the microscopic lesions observed in the allantochorion are generally confined to the celomic space in the subchorionic stroma and very closely associated with blood vessels suggesting a primary vascular involvement (Sebastian and Harrison, 2005). In the LFL fetuses submitted to the LDDC no single pathological finding was identified as being specific for MRLS, although a characteristic finding was funisitis. Funisitis, inflammation of the umbilical cord, has not previously been reported in the veterinary literature as a primary lesion, although inflammatory cell infiltrates of the umbilical cord have been reported. The specific pathogenesis of the characteristic funisitis of MRLS is unknown.

Prior to the identification of MRLS, it was very rare for the bacterial species *Actinobacillus* spp. and *Streptococcus* spp. to be associated with an abortion or placentitis. Recently it was discovered the MRLS-like *Streptococcus* spp. were found in the alimentary tract of mares with a greater concentration in the upper part of the tract, the tongue, tonsillar area and esophagus. Comparison of isolates from different mares demonstrates differences suggesting that the source of fetal and placental infection in each mare is derived from its own bacterial population.

One case of pericarditis was observed in a mare which aborted as part of EFL. Pericarditis and uveitis together have not been identified in any mares which aborted during the MRLS period. Prior to 2001, pericarditis had been an uncommon diagnosis in the equine population of Central Kentucky. The incidence of pericarditis during MRLS in 2001 was 30-fold (an average of seven cases per year in years before 2001) above the mean local prevalence, therefore warranting the inclusion of this condition in the case definition of MRLS.

The occurrence of UU was not reported prior to MRLS in any species of animals as an outbreak. UU was not diagnosed in any individual mare affected by EFL or LFL. Additionally, neither pericarditis nor UU was reproduced in any of the ETC exposure/administration experiments. It is a unique condition and the pathogenesis is unknown.

## OTHER OBSERVATIONS AND PATHOGENESIS

The EFL/MRLS affected short-term fertility but not long-term fertility. The LFL/MRLS did not affect either short- or long-term fertility. MRLS did not have a chronic or negative impact on fertility. Furthermore, it has been reported that the 2001 MRLS affected mares bred in 2002 conceived and maintained their pregnancies at rates consistent with prior years. There is no substantial evidence that MRLS had a long-term effect on fertility.

Blood samples collected before and 7 days after oral administration of irradiated and non-irradiated ETC were tested for lymphocyte subpopulation distribution, lymphocyte proliferation, phagocytosis and oxidative burst capacity, opsonic capacity of serum, and serum immunoglobulin isotype levels. No definitive evidence of an immunosuppressive condition was observed in experimental studies (Flaminio *et al.*, 2005).

In a retrospective study conducted to evaluate the EFL on four equine farms in Central Kentucky, one farm had

fewer fetal losses suggesting that management or environmental factors may have an effect. The study also ruled out the association of any specific sire used for mating in the stud farm (Morehead *et al.*, 2002).

The time course of the abortions observed after administration of different doses of ETC to late-term mares well fitted in an unusual statistical model called accelerated failure time analysis. Review of the experimental data and the statistical analysis showed that ETC induced abortions are characterized by a dose-dependent lag time, followed by initiation of the abortions, which occur at a rate that is also dose dependent. Based on this study, it was estimated that during MRLS in 2001 mares had an initial exposure to about 5 g ETC/day, increasing to about 30 g/day at the peak of the outbreak (Sebastian *et al.*, 2003b).

An abortion storm similar to MRLS is believed to have occurred previously in Kentucky during the spring of 1981 and 1982, and there was anecdotal evidence of an ETC population explosion during the same period in Central Kentucky. No detailed epidemiological studies were conducted during that episode and hence the risk factors were not recorded. Recently MRLS was reported from Florida in spring of 2006 and many ETC were observed in the pasture where aborted mares were maintained which strongly supports the role of ETC in MRLS.

The intact cervical stars and the light brown/yellow appearance of the allantochorions in the late-term pregnancy mare experiments suggest that placental detachment happened *in utero*. It is well known that placental detachment leads to hypoxia, resulting in fetal death and abortion. So this experiment suggests that the abortifacient component of ETC may be acting on the uteroplacental interface leading to placental detachment, which may be the probable pathogenesis or at least a component of the mechanism of the disease.

Currently two hypotheses are being investigated: (1) A breach in the gastrointestinal mucosal barrier allows the entry of bacteria (isolated from the MRLS cases) into the bloodstream, crosses the uterus and reaches the fetus and its membranes resulting in the death of the fetus. The specific mechanism by which the bacteria can induce abortion is unknown. (2) The second hypothesis is that MRLS is caused by an unidentified toxin carried and/or deposited in the environment by the ETC that is either toxic to the placenta or the fetus. The results of experiments with irradiated ETC support the toxin hypothesis, as the abortions in irradiation ETC exposed group could culture the same bacterial organisms from the fetuses as well as reproduce the funisitis which has not been reproduced in experimental settings. The statistical analysis of the results of experiments defining the correlation between abortion time and dose supports the hypothesis that MRLS is caused by a toxin (Sebastian *et al.*, 2003b; 2006).

## TREATMENT AND PREVENTION

Many therapeutic approaches to EFL were attempted. Sulfonamide–trimethoprim combinations and other broad-spectrum antibiotics were administered to combat the bacterial infections. In EFL abortions, mares were lavaged (uterine) and treated with antibiotics based on culture and sensitivity. In the subsequent heat most of the mares had normal uterine cytological findings. No bacteria were grown from the uterus. No significant pathological changes were observed in the uterine biopsies performed on 10 mares, 1–2 months after their 2001 abortions. Therapy in foals born with MRLS included supportive care with fluids, resuscitation, and antibiotics, but the majority of these foals did not survive.

In FP cases the pericardial fluid was removed/drained and antibiotic treatments (Ceftiofur sodium, Ampicillin) were administered to horses admitted to hospitals. Some horses recovered after treatment with antibiotics. Several pericarditis cases were reported during the same time period in 2002; however, the numbers admitted to veterinary hospitals were markedly fewer. Attempted treatments in UU included systemic and topical antibiotics, non-steroidal anti-inflammatory drugs (NSAID and corticosteroid), atropine, tissue plasminogen activator, mycotoxin binders and cyclosporine.

Domperidone was administered to treat possible ergotoxin involvement, mycotoxin binders were administered to eliminate possible mycotoxins, non-steroidal anti-inflammatory agent such as Flunixin meglumine to reduce inflammatory responses and Pentoxifylline to improve the blood supply to the fetus, all with no obvious therapeutic effect. Prevention of ETC included cutting down cherry trees, spraying of ETC nests with insecticides and muzzling of mares when out in pasture. These preventive measures reduced the incidence of MRLS in subsequent year, in farms which adopted the ETC control measures (Riddle and LeBlanc, 2003).

## CONCLUSION

The epidemiological findings identified exposure to ETC as a risk factor for MRLS. The experiments with ETC came up with the following findings: exposure of live ETC to pregnant mares can induce abortion, administration of ETC homogenate to early- and late-term pregnant mares can induce abortion, ETCs but not the frass cause MRLS although the dose administered was not similar, integument/skin of ETC may be the anatomical component with the abortifacient property, hairy caterpillar and gypsy moth which have hairs similar to ETC do not induce MRLS abortions, laboratory raised FTC do not induce abortions,

autoclaving destroys abortifacient property in ETC but is conserved at −80°C, irradiation partially reduces the abortifacient potential of ETC and MRLS is not caused by the direct effect of virus or bacteria in ETC. In the studies using irradiated ETC, the bacteria isolated from the aborted fetuses and their membranes did not originate from the ETC. Results of that experiment strongly suggest that an ETC-resident virus is not the cause of MRLS and the abortifacient appears to be a factor other than bacteria and virus in the ETC. The placentitis observed in LFL/MRLS appears to be different from typical ascending placentitis. The specific pathogenesis of MRLS is still unknown. Elimination of ETC in horse pastures and avoiding direct exposure to ETC appear to prevent MRLS.

## REFERENCES


Bernard WV, LeBlanc MM, Webb BA, *et al.* (2004) Evaluation of early fetal loss induced by gavage with eastern tent caterpillars in pregnant mares. *J Am Vet Med Assoc* **225**: 717–21.

Bolin DC, Donahue M, Vickers ML, *et al*. (2004) Microbiologic and pathologic findings in an epidemic of equine pericarditis. *J Vet Diagn Invest* **17**: 38–44.

Byars TD, Seahorn TL (2002) Clinical observations of mare reproductive loss syndrome in critical care mares and foals. *Proceedings First Workshop on Mare Reproductive Loss Syndrome*, University of Kentucky, Lexington, KY, USA, 15–16.

Cohen ND, Carey VJ, Donahue JG, *et al.* (2003a) Case-control study of late-term abortions associated with mare reproductive loss syndrome in Central Kentucky. *J Am Vet Med Assoc* **222**: 199–209.

Cohen ND, Donahue JG, Carey VJ, *et al.* (2003b) Case-control study of early-term abortions (early fetal losses) associated with mare reproductive loss syndrome in Central Kentucky. *J Am Vet Med Assoc* **222**: 210–17.

Cohen ND, Carey VJ, Donahue JG, *et al.* (2003c) Descriptive epidemiology of late-term abortions associated with the mare reproductive syndrome loss in Central Kentucky. *J Vet Diagn Invest* **15**: 295–7.

Donahue JM, Sells S, Giles RC, *et al.* (2002) Bacteria associated with mare reproductive loss syndrome. *Proceedings First Workshop on Mare Reproductive Loss Syndrome*, University of Kentucky, Lexington, KY, USA, 27–9.

Dwyer RM, Lindsey PG, Traub-Dargatz JL, *et al.* (2003) Case–control study of factors associated with excessive proportions of early fetal losses associated with mare reproductive loss syndrome in Central Kentucky during 2001. *J Am Vet Med Assoc* **222**: 613–19.

Fitzgerald TD (2002) The biology of tent caterpillars as it relates to mare reproductive loss syndrome. *Proceedings First Workshop on Mare Reproductive Loss Syndrome*, University of Kentucky, Lexington, KY, USA, 84–7.

Flaminio JF, Nydam DV, Sebastian MM, *et al.* (2005) The Mare Reproductive Loss Syndrome (MRLS) and the eastern tent caterpillar: immunological testing of aborting mares. *Int J Appl Res Vet Med* **3**: 207–16.

Harkins JD, Dirikolu L, Sebastian M, *et al.* (2002) Cherry trees, plant cyanogens, caterpillars, and the Mare Reproductive Loss Syndrome (MRLS): toxicological evaluation of a working hypothesis. *Proceedings of the First Workshop on Mare Reproductive Loss Syndrome*, University of Kentucky, Lexington, KY, USA, 68–74.

Harrison LR (2001) Kentucky abortion storm and related conditions (2001). *Proc US Anim Health Assoc* **105**: 227–9.

Kane E, Kilby E (2001) Death in the bluegrass: an epidemic of lost pregnancies, dead foals and sick horses strikes Central Kentucky and beyond, challenging researchers and veterinarians to identify the cause and staunch the unprecedented loss of equine lives. *Equus* **287**: 60–8.

Latimer C (2002) Endopthalmitis syndrome: Spring 2001, 2002. *Proceedings of the First Workshop on Mare Reproductive Loss Syndrome*, University of Kentucky, Lexington, KY, USA, 26–8.

Morehead JP, Blanchard TL, Thompson JA, *et al.* (2002) Evaluation of early fetal losses on four equine farms in Central Kentucky: 73 cases (2001). *J Am Vet Med Assoc* **220**: 1828–30.

Paulick R (2001) MRLS Kentucky economic impact: $336 million. *The Horse* **18**(12): 15.

Powell DG (2001) Mare reproductive loss syndrome (MRLS). *Equine Dis Q* **9**(4): 5–7.

Riddle WT, LeBlanc MM (2003) Update on mare reproductive loss syndrome. *Proceedings Society for Theriogenology and Symposium*, Columbus, OH, USA, 85–99.

Seahorn JL, Slovis N, Reimer J (2003) Case-control study of factors associated with fibrinous pericarditis among horses in Central Kentucky during spring 2001. *J Am Vet Med Assoc* **223**: 832–8.

Sebastian M, Harrison LR (2005) Placental pathology in late term abortions of 2002. Mare reproductive loss syndrome. *Vet Pathol* **42**: 717.

Sebastian M, Bernard W, Fitzgerald T (2006) Mare Reproductive Loss Syndrome. *Compendium on Continuing Education for the Practicing Veterinarian* **1**: 20–33

Sebastian M, Giles R, Donahue J, *et al.* (2002a) Encephalitis due to *Actinobacillus* species in three adult horses. *Vet Pathol* **39**: 630.

Sebastian M, Williams D, Harrison L, *et al.* (2002b). Clinical and pathological features of experimentally induced MRLS late-term abortion with eastern tent caterpillar. *Proceedings of the First Workshop on Mare Reproductive Loss Syndrome*, University of Kentucky, Lexington, KY, USA, 80–1.

Sebastian M, Harkins JD, Jackson C, *et al.* (2002c) Preliminary evaluation of a mouse model of MRLS. *Proceedings of the First Workshop on Mare Reproductive Loss Syndrome*, University of Kentucky, Lexington, KY, USA, 51–3.

Sebastian M, Bernard W, Harrison L, *et al.* (2003a) Experimental induction of mare reproductive loss syndrome with irradiated eastern tent caterpillar to assess whether the primary pathogen in mare reproductive loss syndrome is a toxic molecule or a microorganism. *Proceedings of the Workshop on The Equine Placenta*, University of Kentucky, Lexington, KY, USA, 27–8.

Sebastian M, Gantz MG, Tobin T, *et al.* (2003b) The mare reproductive syndrome and the eastern tent caterpillars: a toxicokinetic/statistical analysis with clinical, epidemiologic, and mechanistic implications. *Vet Therap* **4**: 324–39.

Webb BA, Barney WE, Dahlman DL, *et al.* (2004). Eastern tent caterpillars (*Malacosoma americanum*) cause mare reproductive loss syndrome. *J Inst Physiol* **50**: 185–93.

Williams NM, Bolin DC, Donahue JM, *et al.* (2002) Gross and histopathological correlates of MRLS. *Proceedings of the First Workshop on Mare Reproductive Loss Syndrome*, University of Kentucky, Lexington, KY, USA, 29.

C H A P T E R

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# Terrestrial zootoxins

*Sharon M. Gwaltney-Brant, Eric K. Dunayer, and Hany Y. Youssef*

## INTRODUCTION

The Animal Kingdom is populated by a vast variety of creatures whose main focus in life is to live to see tomorrow. To this end, many animals have developed chemical means of defense and/or food procurement. Every phylum within the Animal Kingdom contains species that produce poisons or venoms. *Poisons* are compounds produced in non-specialized tissues as secondary products of metabolism that accumulate in the host animal or that accumulate in predators following ingestion of prey. *Poisonous* animals, therefore, lack means of actively delivering these chemical compounds to others – exposure generally requires oral contact (rarely dermal) in order for another animal to become poisoned. In contrast, *venoms* are produced in specialized tissues or glands, and *venomous* animals have developed a variety of *venom apparatuses* (stingers, teeth, etc.) to deliver their venom to target animals, a process termed *envenomation*.

Most venoms and poisons are not composed of a single chemical substance, but are mixtures of a variety of chemical compounds that often act synergistically to produce their toxic effect. Typical constituents might include peptides, amines, serotonin, quinones, polypeptides, and enzymes (Russell, 2001). These compounds are collectively termed *toxins* (the science of study of toxins is termed *toxinology*), and toxins produced by members of the Animal Kingdom are collectively termed *zootoxins*.

Not every exposure to a poisonous or venomous animal will necessarily result in a toxicosis (Meier, 1995). Variations in size, age, sex, season, toxin composition, and geographic location can result in zootoxins with relatively more or less toxicity. Similarly, the animal exposed to the zootoxin may be more or less susceptible to toxicosis based on its age, weight, sex, and degree of exposure.

Compared to other means of injury or illness in animals, envenomation or poisoning from zootoxins are relatively rare causes for presentation of domestic animals to their veterinarians, due largely to the protection afforded by animal owners. Wildlife, on the other hand, probably face potential exposure to zootoxins on a nearly daily basis. Bites and stings from arthropods and snakes certainly can occur in any species, and the potential for oral exposure to animals such as poisonous toads, snakes, or insects will vary with the region and environment.

In managing known or suspected zootoxicoses, veterinarians should be mindful to "treat the patient, not the poison." Patient assessment and stabilization, along with supportive care, are critical in managing these patients. Although antivenins are available for treatment of some types of envenomation, financial and availability issues may limit their use in many instances.

## ARTHROPODA

## ARANEAE: SPIDERS

### Introduction

There are at least 30,000 species of spiders distributed throughout the world (Goddard, 2003). Spiders are characterized by a two-segmented body: a head/thorax (prosoma or cephalothorax) and the abdomen (opisthosoma). They have eight segmented legs (Lucas and Meier, 1995b). Venom is stored in two glands located in the cephalothorax and empties through fangs (chelicerae) located at the rostral end of the prosoma (Lucas and Meier, 1995b). With the exception of spiders in the family Ulobiridae (found

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in Australia), all spiders are capable of inflicting an envenomating bite via fangs. Most spider envenomations, however, are likely to cause few signs other than local swelling and pain. Anaphylaxis and other allergic reactions to venom components are possible (Goddard, 2003). While there is disagreement in literature, it appears that fewer than 100 spider species can inflict a bite of medical significance (Lucas and Meier, 1995b).

## WIDOW SPIDERS (*LATRODECTUS* SPP.)

### Background

Spiders of this genus are found throughout the world. The spiders are identified by a red, yellow, or orange hourglass-shaped marking on the ventral abdomen of the female. Males and immature females are brown; the immature females do not have the hourglass marking. The fangs of the male are considered too short to inflict an envenomating bite to humans or other mammals (Lucas and Meier, 1995b). In the United States, there are five major species of widow spiders (Goddard, 2003):

1. *L. mactans* (black widow spider): These spiders are found throughout the United States but especially in the Southern United States. They prefer dark places, especially under debris such as leaf litter or cardboard.
2. *L. hesperus* (Western black widow spider): Common black widow of the Western United States.
3. *L. variolus* (Northern widow spider): Common in Northern United States; the hourglass mark is not joined.
4. *L. bishopi* (red widow spider, red-legged widow spider): Found in palmetto fronds of sandy, scrub-pine region of Central and Southern Florida. They are brightly colored.
5. *L. geometricus* (brown widow spider): The hourglass is orange in this species. They are an introduced species to the United States found mainly in Florida.

Widow spiders are found mostly outside of living spaces, preferring dark secluded area such as piles of debris, and densely growing plants. Inside houses, they prefer dark, rarely disturbed spaces, and will often settle under appliances and cabinets. Adult spiders are most active in the warm months and will die during the colder months if not in a heated space. They build webs to ensnare prey. In general, widow spiders are shy and do not bite larger animals unless provoked (Goddard, 2003).

### Mechanism of action

Widow-spider venom is a complex mixture of about six neuroactive proteins; there are also some proteolytic enzymes. The principle toxin for mammals is $\alpha$-latrotoxin, a polypeptide that causes a large release and then depletion of acetylcholine and norepinephrine at postganglionic sympathetic synapses (Lucas and Meier, 1995b).

### Toxicity

The venom of widow spiders is extremely potent. For instance, the $LD_{50}$ for whole venom of *L. mactans tredecimguttas* (European black widow) in guinea pigs is 0.0075 mg/kg and for mice is 0.9 mg/kg (White *et al.*, 1995).

The syndrome caused by widow venom is called lactrodectism. Following a widow-spider bite, the venom is taken up by lymphatics and then enters the bloodstream (White *et al.*, 1995). Clinically, there may be short-lived, localized pain at the bite site. Then, in about 30–120 min, myalgia and muscle cramps near the site of envenomation will begin. The pain begins to spread to the large muscles groups of the legs, thorax, back, and abdomen, peaking in about 2–3 h. Mild to moderate hypertension and tachycardia are common. In most cases, signs resolve in 48–72 h but signs such as weakness, fatigue, and insomnia may persist for weeks to months (White *et al.*, 1995; Goddard, 2003).

Cats are very sensitive to the effects of widow venom. In one study, 20 of 22 cats died after widow-spider bites with average survival time of 115 h. Paralysis occurs early in the course; severe pain is evidenced by howling and other vocalizations. Hypersalivation, restlessness, vomiting, and diarrhea were common. Muscle tremors, cramping, ataxia, and inability to stand may precede an atonic paralysis. Cheyne–Stokes respiratory pattern may develop prior to death (Peterson and McNalley, 2006a).

### Treatment

Treatment is largely symptomatic with control of pain by opioids and use of muscle relaxants such as diazepam and methocarbamol to control the muscle rigidity. Calcium gluconate, which was once used for treatment of widow envenomation, is no longer recommended as it was found to be less effective at controlling pain than opioids and muscle relaxants (Clarke *et al.*, 1992; Peterson and McNalley, 2006a). An equine-origin antivenin (Lycovac®, available in the United States from Merck and Co.) has been used in humans. It is generally administered to high-risk patients (the very young or old) or in those whose signs are progressing despite other medical care. The antivenin is extremely effective; in one study, all 58 patients receiving the agent had resolution of signs within 30 min (Clarke *et al.*, 1992). In one case report (Twedt *et al.*, 1999), a cat with suspected lactrodectism was treated with antivenom about 26 h after signs began. The cat rapidly recovered neurologic function.

## RECLUSE OR VIOLIN SPIDERS (*LOXESCELES* SPP.)

### Background

While there are at least 50 species of *Loxesceles*, few are capable of inflicting a medically significant envenomation known as loxoscelism. In the United States, where several *Loxesceles* species occur, the brown recluse spider (*L. reclusa*) is considered the only one of medical importance. *L. reclusa* is found principally in Missouri, Arkansas, Oklahoma, Kansas, and Tennessee, but its range extends from the Southern Gulf states and Southern Atlantic states through Indiana and Illinois. Other species of *Loxesceles* are found principally in the Southwest US and California. In South America, *L. laeta* and *L. gaucho* have been associated with severe loxoscelism in Brazil, Chile, Argentina, Peru, and Uruguay (White *et al.*, 1995). (*L. laeta* now appears to have been introduced into the Los Angeles, California area (Goddard, 2003).) While species of *Loxesceles* may occur in other regions of the world, significant morbidity and mortality has not been associated with these spiders (White *et al.*, 1995). Brown recluse spiders (*L. reclusa*) are nocturnal, non-aggressive spiders. They are recognizable by the "fiddle"-shaped marking on the dorsal surface of the cephalothorax.

### Mechanism of action

The venom of the recluse spider contains several necrotizing enzymes including hyaluronidase, esterases, and alkaline phosphatases. Sphingomyelinase D, a phospholipase, appears to be the most important component; it binds to cell membranes and causes migration and activation of neutrophils in the area of the envenomation. Additionally, the venom inactivates serum hemolytic complement leading to intravascular coagulation, occlusion of small capillaries, and tissue necrosis; systemic depletion of clotting factors (VII, IX, XI, and XII) can also occur (White *et al.*, 1995; Goddard, 2003; Peterson and McNalley, 2006b). Platelet activation can also be seen (Peterson and McNalley, 2006b). Additionally, in the presence of calcium and C-reactive protein, sphingomyelinase D can cause hemolysis. Platelet activation can be seen. Finally, lipases can cause free lipids in the blood which may act as inflammatory mediators and/or cause embolization (Peterson and McNalley, 2006b).

### Toxicity

In one study (Denny *et al.*, 1964), dogs injected with brown recluse venom intravenously developed thrombocytopenia, absence of reticulocytes, and evidence of hemolysis with decreased hematocrit. Necropsy showed widespread petechial and ecchymotic hemorrhage, dehydration, and hypocellular bone marrow with depression of the erythroid and platelet cell lines. Another study (Futrell *et al.*, 1979) of *in vitro* hemolysis found that human and pig erythrocytes were far more susceptible to lysis from brown recluse venom than those of dogs.

Initially, the bite may produce little pain or local reaction. Approximately 3–8 hours after envenomation, the site becomes red, swollen, and tender; this is a so-called "bull's-eye" lesion. A vesicle may form and be replaced by a black scab or eschar. Tissue around the bite may slough leaving a 1–25 cm ulcer. Distribution may occur in areas dependent to the bite due to gravity. Healing is slow and may take months, often leaving a large scar (Goddard, 2003). Diagnosing a brown recluse bite may be difficult if the bite is not witnessed. In many cases, a brown recluse bite is "blamed" for necrotic lesions due to other causes (Mullen, 2002b). Systemic signs, though uncommon, can be seen with a brown recluse bite; they may develop 48–72 h after exposure. Hemolysis with anemia and hematuria, tachycardia, pyrexia, myalgia, vomiting, dyspnea, DIC, and coma have been reported but are rare (Goddard, 2003).

### Treatment

For the necrotic lesion, local wound care including chemical debridment with Burrows solution (aluminum acetate) or hydrogen peroxide and bandaging should be performed. Pruritus may be controlled with diphenhydramine (2.2 mg/kg q8h). Antibiotics, especially if infection is evident, should be administered. Analgesics for pain (NSAIDs for mild, opioids for severe) may be required. Surgical excision, used in the past, is no longer recommended (Peterson and McNalley, 2006b).

Dapsone (4,4'-diaminodiphenylsulfone) may be helpful in limiting the severity of the necrotic lesion as it inhibits neutrophil migration. In dogs, a dose of 1 mg/kg/day for 14 days has been used experimentally. Dapsone can cause methemoglobinemia as well as hemolysis in individuals with G6PD deficiency (Peterson and McNalley, 2006b).

Antivenins are available for South American *Loxesceles* species. Experimental antivenins for *L. reclusa* have been developed. It has shown to be effective if given within 4 h of envenomation. However, this antivenin is not commercially available at this time (Mullen, 2002b).

## HOBO SPIDERS (*TEGENARIA AGRETIS*)

### Background

Hobo spiders are native to Europe but were introduced in the Pacific Northwest in the 1930s. They occur mainly in

Washington, Oregon, and Idaho but their range includes Central Utah through Western Canada to the Alaskan Panhandle. In the 1980s, the appearance of necrotic spider bites in the Pacific Northwest was initially blamed on *L. reclusa* but later the bites were correctly attributed *T. agretis* (Goddard, 2003).

Hobo spiders are poor climbers and build a funnel web at ground levels or in basements. Males will leave their web at night in search of females and may enter houses while doing so. Males are more venomous than females and are more likely to bite (Mullen, 2002a, b; Goddard, 2003).

### Toxicity

In most cases, the bite is initially painless. Within 30 min, a localized expanding area of erythema may occur; the lesion may eventually reach 15 cm in diameter. Then, in about 15–35 h, the area ruptures and there is a serous discharge from the wound. The wound may require 3 years to heal especially if it is in fatty tissue (Goddard, 2003).

In humans, systemic signs may occur in about 45% of bites and about one-third of these may require hospitalization. Signs may include headache (which can last for days), nausea, weakness, and vision changes. The signs may progress to vomiting (often intractable), watery diarrhea, and bone marrow destruction resulting in anemia, pancytopenia, and thrombocytopenia. Fatalities are rare (Mullen, 2002b).

### Treatment

Treatment of the Hobo spider bite is supportive and symptomatic.

## FUNNEL WEB SPIDERS (*ATRAX* AND *HADRONYCHE* SPP.)

### Background

There are approximately 35 species of funnel web spiders. They are all found in Australia, principally distributed along the Southeast coastal region. While all may potentially dangerous to mammals, the Sydney funnel web spider (*A. robustus*) is considered the most dangerous as this is the only species in which human fatalities have been recorded (White *et al.*, 1995). *A. robustus* is found within a 160 km (100 miles) from the center of Sydney. They are large aggressive black spiders (Sutherland and Tibballs, 2001).

### Mechanism of action

The toxic component of the funnel web spider's venom is the neurotoxin, robustoxin, a 42 amino acid protein. The toxin binds to the pre-synaptic neuron and both inhibit central nervous system (CNS) mediated neurotransmitter release while increasing spontaneous release of neurotransmitters; the toxin affects both the autonomic nervous system and skeletal muscles. Other species of funnel web spiders contains structurally similar neurotoxins which are less potent than robustoxin (White *et al.*, 1995).

### Toxicity

There is a wide variation of reaction in different species of animals; the sex of the spider also effects toxicity of the bite. For instance, rats, rabbits, and cats are unaffected by the bite of a female spider while 20% of mice and guinea pigs died after the bite of a female. When mice or guinea pigs are bitten by a male spider, most die. The male funnel web spider's venom appears to be six times more potent than the females based on minimum lethal dose determinations. However, in dogs and cats, male funnel web spider bites cause mild transient effects. Humans and other primates appear to be extremely sensitive to the venom of this spider (Sutherland and Tibballs, 2001).

## TARANTULAS

Tarantulas are large, ground dwelling spiders. During mating season, males wander in search of females and may be more aggressive at these times. In most cases, the bite of the tarantula causes little more than localized pain that develops slowly but usually resolves within 30 min. There are about a dozen genera of tarantulas that may deliver a potentially life-threatening bite. These spiders occur in the tropical regions of South America, Africa, and Australia. Their venom contains a neurotoxin; there may also be a necrotoxin and hemolytic toxin. Following envenomation, muscle spasms, edema, hemoglobinuria, jaundice, and circulatory shock may develop (Mullen, 2002b).

Tarantulas of the family Theraphosidae (bird-eating or whistling spiders) are often kept as pets in Australia and Asia. In one Australian study (Isbister *et al.*, 2003), the bite of these spiders was found to cause only local effects in humans. However, seven dogs bitten by spiders of the genera *Phlogiellus* and *Selenocosmia* died rapidly, often within 2 h of the envenomation. (In two cases, the dog and their owners were bitten by the same spider.) The spiders are also considered highly poisonous for cats. The exact cause of death was not discussed but, based on experimental data, apnea and cardiac arrhythmias may occur.

Species of tarantula living in the United States are not capable of delivering serious envenomation. However, many of these species possess urticating hairs on their abdomen; the hairs possess spines and barbs which can penetrate skin. When threatened, these tarantulas can stroke

their abdomen and flick the urticating hairs at their attacker. The hairs can cause severe inflammation of the skin, eyes, mouth, and respiratory tract. The effect is purely mechanical as there is no toxin associated with the hairs. Urticaria, edema, and vasodilation may occur in the skin. In the eyes, swelling of the lids and corneal abrasions are common (Mullen, 2002b). Dogs or cats attempting to ingest tarantulas may gag or vomit.

## SCORPIONES: SCORPIONS

### Background

Scorpions are arachnids with two body divisions: the combined head and thorax referred to as the prosoma or cephalothorax and the abdomen or opisthosoma. They possess large paired claws or pincers known as pedipalps and four pairs of legs. At the caudal portion of the abdomen, scorpions have a tail that terminates in the telson – a barbed appendage that houses two venom glands that exit via a stinger at the end of the telson. Scorpions are nocturnal hunters; they may spend the day in burrows or hiding under rocks or vegetation. They may also hide in blankets, shoes, and clothing; this a common way for humans to exposed to them (Keegan, 1980).

There are about 1400 species of scorpions occurring on all continents except Antarctica (Goddard, 2003). Most occur in tropical or temperate regions (Lucas and Meier, 1995a). Table 64.1 lists locations and species of scorpions capable of inflicting life-threatening envenomations in humans. The main genera of scorpions found in North America are the bark scorpions (*Centruroides* spp.). They are found primarily in Central America, Mexico, Southern through Southwest United States (Mullen and Stockwell, 2002). In the United States, only *Centruroides exilicauda* (Arizona bark scorpion) is considered capable of inflicting a life-threatening sting (Goddard, 2003).

**TABLE 64.1 Scorpions of medical importance: species and location**

| Location | Species |
|---|---|
| India | *Buthotus tamulus* |
| Mexico and Central America | *Centruroides* spp. |
| Middle East | *Androctonus* spp.<br>*Buthus* spp.<br>*Hemiscorpion lepturus*<br>*Leiurus quinquestriatus*<br>*Mesobuthus gibbosus* |
| South Africa | *Parabuthus* spp.<br>*Buthus minax* |
| South America | *Tityus* spp.<br>*Centruroides gracilis* |
| United States | *Centruroides sculpturatus* |
| West Indies (Trinidad) | *Tityus trinitatis* |

Adapted from Lucas and Meier (1995a) and Keegan (1980).

### Mechanism of action

All scorpions can deliver an envenomating sting (Keegan, 1980). Scorpion venom components vary greatly between genera and may even differ based on geographic location within species (Mullen and Stockwell, 2002). The venom consists of a mixture of low molecular weight polypeptides. At least two potent neurotoxins have been identified: $\alpha$-scorpion toxin found in *Androctonus*, *Leiurus*, and *Buthus* spp. and $\beta$-scorpion toxin found in *Centruroides* spp. Both toxins can be found in the venom of *Tityus* spp. These venoms block the voltage-sensitive sodium and potassium channels in nerves (Mullen and Stockwell, 2002).

### Toxicity

Scorpion stings cause instant, sharp pain at the site of envenomation. Some stings will cause localized pain that resolves over hours. Localized edema and pruritus are common. Regional lymph nodes may enlarge, there may be an allergic reaction characterized by swelling of the eyelids, tongue, and vomiting. Sloughing of the skin at the site of envenomation can also occur (Mullen and Stockwell, 2002). Signs usually resolve within 24 h (Keegan, 1980). Systemically, signs can vary but generally include numbness of face, myalgia, tachycardia or bradycardia, respiratory depression, and seizures; however, there is a lack of evidence that scorpion envenomation in dogs and cats is a serious concern (Mullen and Stockwell, 2002).

### Treatment

The treatment of scorpion stings, in most cases, consists of analgesics and local wound care. Systemic signs are treated symptomatically with control of hypertension, heart rate changes, and neurologic signs. Some antivenoms in the United States are produced locally; however, since these are not FDA approved they cannot legally be transported to another state (Goddard, 2003). In addition, their use in veterinary patients is considered controversial (Dalefield and Oehme, 2006).

## IXODIDA: TICKS

### Background

Ticks are well known as being vectors for a large number of human and animal diseases, with the first reports of tick

paralysis originating in Australia in 1890 and British Columbia in 1912 (Sonenshine *et al.*, 2002). Worldwide, 43 species of ticks from nine different genera have been associated with tick paralysis: *Amblyomma*, *Argas*, *Dermacentor*, *Haemaphysalis*, *Hyalomma*, *Ixodes*, *Ornithodoros*, *Otobius*, and *Rhipicephalus* (Dipeolu, 1976; Fowler, 1993). Tick paralysis has been reported in North America, Europe, Africa, Australia, and the former Soviet Union.

### Mechanism of action

The exact mechanism(s) of action of tick toxins is(are) not well known, but in most tick species it is suspected that the toxin interferes with the synthesis and/or release of acetylcholine at the neuromuscular junctions, resulting in lower motor neuron paresis and paralysis very similar to that produced by botulinum toxin (Fowler, 1993; Grattan-Smith *et al.*, 1997). The Australian tick, *Ixodes holocyclus*, toxin may differ, as it appears to have more of an effect on central nerve centers rather than peripheral (Fowler, 1993).

### Toxicity

Tick paralysis has been reported in a large variety of animal species, including dogs, cats, cattle, sheep, goats, llamas, poultry, wild antelope, bison, foxes, wolves, mice, ground hogs, black-tailed deer, and several species of wild birds (Fowler, 1993; Beyer and Grossman, 1997; Sonenshine *et al.*, 2002). In the North America, most cases of tick paralysis in livestock occur in the Pacific Northwest due to *Dermacentor andersoni*, while most cases in dogs are due to *D. variabilis* (Fowler, 1993; Sonenshine *et al.*, 2002). *D. occidentalis* occasionally causes tick paralysis in cattle, ponies, and deer but not dogs (Sonenshine *et al.*, 2002). In the Southeastern United States, *Ixodes brunneus* has been associated with tick paralysis in wild passerine birds (Luttrell *et al.*, 1996). In Australia, tick paralysis is caused by *I. hyolocyclus*, while *I. rubicundus* and *Rhipecephalus evertsi* are the primary species responsible for tick paralysis in Africa (Sonenshine *et al.*, 2002). Tick paralysis in Europe and Asia has been reported due to bites from *Hyalomma punctata* and *I. ricinus.*

Tick paralysis has occurred following the bite of as few as one tick and heavily infested animals may succumb quickly (Fowler, 1993). Clinical signs include an ascending ataxia that progresses to paresis and flaccid paralysis. Early in the intoxication, animals remain bright, alert, and able to eat and drink if properly supported. Eventually, paralysis of the respiratory muscles leads to respiratory failure and death. Paralysis produced by *I. holocyclus*, the Australian tick, generally occurs more rapidly and tends to persist following removal of the tick.

### Treatment

The main goal of treatment is to remove the ticks and provide supportive care (especially respiratory support) until recovery occurs. Recovery can occur quite rapidly following complete removal of the ticks, or it may take a few days (Fowler, 1993). The use of topical insecticides may aid in the removal of ticks, and can be especially helpful in cases where numerous ticks are embedded. Heavily coated animals may need to be shaved in order to ensure that all embedded ticks are found and removed. Removal of embedded ticks should be performed carefully to avoid expressing additional toxin into the wound or leaving the head embedded in the skin. Forceps may be used to grasp the tick as close to the skin as possible and gentle traction should be used to remove the tick. In most cases where ticks are removed before bulbar paralysis has occurred, the prognosis for full recovery is very good. A short-term immunity develops following recovery from tick paralysis. In Australia, a polyclonal dog antiserum has been developed to treat tick paralysis, but it is only effective early in the stages of paralysis (Masina and Broady, 1999).

## MYRIAPODA: CENTIPEDES AND MILLIPEDES

Centipedes and millipedes are distantly related to lobsters, crayfish, and shrimp. These arthropods are widely distributed throughout the world and vary, and are characterized by a long, flat, multi-segmented body with one (centipede) or two (millipede) legs emerging from each body segment. Although most centipedes and millipedes are less than 1.5 in. long, centipedes from the genus *Scolopendra* may grow to be 4–6 in. long. All centipedes have a pair of modified front legs (forcipules) that serve as fangs and that are connected to venomous glands directly under the head (Norris, 2004). Larger centipedes have the ability to inflict painful bites resulting in local swelling, erythema and lymphangitis (Stewart, 1985). Additionally the legs of *Scolopendra* spp. are tipped with sharp claws that are capable of penetrating skin, and toxin produced at the attachment point of each leg may drop into these wounds, causing inflammation and irritation.

Centipede venom has been poorly studied but components identified in various centipedes include serotonin, a cytolysin, a hemolysin, and a vesicating agent (Deng *et al.*, 1997; Norris, 2004). In most cases systemic toxicosis is not expected, although local necrosis may occur (Russell, 1996); a single case report exists of a human fatality from the bite of *Scolopendra subspinipes* (Norris, 2004). Signs generally subside within 48 h and rarely require more than symptomatic care (e.g. analgesics). Millipedes do not bite, but can emit irritating and foul-smelling secretions from repugnatorial glands; some species can spray these fluids over

distances of several inches. These secretions are irritating to mucosal surfaces, particularly the eyes, and corticosteroids have been recommended to decrease the inflammatory response (Russell, 1996).

# INSECTS

## Introduction

Insect and insect-related problems are common in domestic and wild animals. Insects such as lice, fleas, deer flies, horse flies, sand flies, mosquitoes, black flies, biting midges, and sand flies may cause severe annoyance to animals because of biting behavior. Members of several groups of insects can inject venom when they bite or sting, most notably bees, wasps, and ants. Bites or stings from insects such as lice, fleas, bees, ants, wasps, mosquitoes, and chiggers may cause direct effects from the venoms or may result in allergic host reactions resulting from over-responsive host immune systems. Contact allergies may occur when certain beetles or caterpillars touch the skin. Other insects, such as blister beetles and certain caterpillars, produce toxins that can cause adverse reactions when they are touched or ingested (Durden and Mullen, 2002). Respiratory allergies can result from inhaling allergic air-borne particles from cockroaches, fleas, or other arthropods (Durden and Mullen, 2002). Some insects invade the body tissues of their host. Various degrees of invasion may occur, ranging from subcutaneous infestations to invasion of organs such as the lungs and intestine (Durden and Mullen, 2002). Table 64.2 provides a list of nine orders of insects that are of particular interest to veterinary entomologists.

# COLEOPTERA: MELOIDAE (BLISTER BEETLES)

## Background

More than 300,000 species of beetles have been described, representing 30–40% of all known insects. About 25,000 species of beetles occur in the United States and Canada (Krinsky, 2002). Fewer than 100 species of beetles worldwide are known to be of public health or veterinary importance. Most of these are in the suborder Polyphaga. The species that have the greatest impact on the health of human and domestic animals are in the following families: Melidae (blister beetles), Oedemeridae (false blister beetles), Staphylinidae (rove beetles), Tenebrionidae (darkling beetles), Dermestidae (larder beetles), and Scarabaeidae (scarab or dung beetles) (Krinsky, 2002).

Most blister beetles are in the family Melidae, although some are in the Staphylinidae family. Blister beetles are elongate, soft-bodied specimens in which the pronotum (section between head and wings) is narrower than the head or wings. Two of the common blister beetle species are potato beetles, one with orange and black longitudinal stripes and one black with gray wing margins (Goddard, 2003). Members of the genus *Mele* are called oil beetles because they exude an oily substance from their legs when disturbed. Oil beetles are approximately 20–25 mm long and black, with no hind wings, giving the appearance that their wings are too short (Goddard, 2003).

Species that pose problems in the United States include the striped blister beetle (*Epicauta vittata*), the black blister beetle (*E. pennsylvanica*) (Figure 64.1), the margined blister beetle (*E. pestifera*), and the three-striped blister beetle (*E. lemniscata*), as well as *E. fabricii*, *E. occidentalis*, and *E. temexa*. Of these the most common species associated with

**TABLE 64.2 Insects of veterinary importance**

| Order | Common names | Clinical significance |
|---|---|---|
| Coleoptera | Beetles | Toxic beetles (e.g. blister beetle); vectors for pathogenic organisms; intermediate host for nematodes, flukes, cestodes, acanthocephalans |
| Dictyoptera | Cockroaches | Intermediate hosts nematodes, flukes, cestodes, acanthocephalans; vectors for pathogenic organisms |
| Diptera | Flies, mosquitoes | Vectors for pathogenic organisms; myiasis; decreased production due to annoyance or anemia from biting/sucking insects |
| Hemiptera | True bugs | Transmission of infectious agents (e.g. trypanosomiasis); decreased production due to anemia from blood-sucking bugs |
| Hymenoptera | Wasps, hornets, ants, and bees | Envenomation, allergy from bites or stings; poisonous insects (e.g. sawfly larvae) |
| Lepidoptera | Moths and butterflies | Urticaria, irritation, ulceration from urticating hairs; poisonous insects (e.g. monarch butterfly) |
| Phasmida | Stick insects and leaf insects | Irritation from secretions of walkingsticks |
| Phthiraptera | Lice | Decreased production due to annoyance or anemia; vector for pathogenic organisms |
| Siphonaptera | Fleas | Decreased production due to annoyance or anemia; vector for pathogenic organisms |

**FIGURE 64.1** An adult striped blister beetle (*E. vittata*). This figure is reproduced in color in the color plate section. (Photo courtesy of James E. Appleby, University of Illinois.)

**FIGURE 64.2** An adult blister beetle (*Epicauta*), with hemolymph droplets containing cantharidin. This figure is reproduced in color in the color plate section. (Photo courtesy of Dr. Maria Eisner, Cornell University.)

toxicosis are *E. occidentalis* and *E. temexa* (Stair and Plumlee, 2004).

## Mechanism of action

The toxic principle in melioid beetles, cantharidin, has the formula $C_{10}H_{12}O_4$ and is a bicyclic terpenoid (Goddard, 2003; Stair and Plumlee, 2004). The concentration of cantharidin present in beetles varies from 1% to 5% of the dry weight of the beetle. Males always have a higher concentration because the male produces the cantharidin and transfers a quantity to the female during copulation (Krinsky, 2002). Cantharidin is present in the hemolymph as well as in the clear, yellow secretion that is exuded at the joints of the legs of these beetles by reflex bleeding (Figure 64.2).

The mechanism of action of cantharidin may involve interference with enzyme systems responsible for active transport across mitochondrial membranes, resulting in membrane disruption and permeability changes. Mitochondrial damage results in cellular disruption, acantholysis and vesicle formation. Cantharidin also has been shown to inhibit protein phosphatase 2A, resulting in disruption of signal transduction and cell metabolism (Stair and Plumlee, 2004).

Purified, crystalline cantharidin is a potent vesicating agent that is readily absorbed through the gastrointestinal mucosa and, to a lesser degree, the skin, and is eliminated unchanged through the kidneys (Krinsky, 2002).

## Toxicity

Adult blister beetles feed on flowering foliage, especially blooming alfalfa (*Medicago sativa*). Blister beetles in alfalfa fields contain enough cantharidin to provide lethal doses to livestock that feed on forage when it is used as hay. Modern methods of hay harvesting, especially crimping, decrease the opportunity of blister beetles to vacate the foliage before it is incorporated into hay, thereby increasing the likelihood of blister beetles being trapped in the hay (Stair and Plumlee, 2004). Cantharidin released from crushed adult blister beetles may contaminate hay without visual evidence of insect parts. Cantharidin is very stable and may persist in hay for long periods of time. Because in the United States alfalfa is most widely grown in the South, cantharidin toxicosis is most commonly found in the Southern states; however, due to interstate transport of alfalfa hay across the country, cantharidin toxicosis could potentially occur in any state.

Horses are the species in which cantharidin toxicosis is most commonly reported. However, the following hosts have also been poisoned in natural or experimental situations: cattle, sheep, goats, rabbits, rats, hedgehogs, dogs, and emus. Poisoning in human beings has been noted for decades and is usually the result of either improper medicinal use of cantharides or malicious poisoning (Krinsky, 2002). Horses are considered to be particularly susceptible to the effects of cantharidin (Stair and Plumlee, 2004). The estimated lethal dosage of cantharidin for the horse is approximately 0.5–1 mg/kg, and as little as 4 g of dried beetles may be lethal to a horse (Krinsky, 2002). The oral experimental lethal dosage of crystalline cantharidin for dogs and cats was 1.0–1.5 mg/kg and 20 mg/kg for rabbits. It is estimated that the lethal dosage for a human being is less than 1.0 mg/kg.

Cantharidin produces an intense, direct irritant effect on the skin and mucous membranes of the esophagus, stomach, and intestines. Once absorbed, the toxin may affect many different organs. Excretion is via the kidneys, resulting in transfer of the irritant effect to the urinary tract, particularly the bladder and urethra (Krinsky, 2002).

Clinical signs will vary with the dose ingested. Massive doses may cause shock and death within 4 h (Krinsky, 2002). Smaller doses may cause gastroenteritis, nephrosis, cystitis, and/or urethritis; thus, signs may include anorexia, soft feces, mucoid to bloody feces, intestinal atony, colic, dysuria (frequent, painful urination, or oliguria to anuria), and hematuria. The body temperature may elevate to 106°F (41.1°C). Other signs observed include depression, weakness, muscle rigidity, collapse, prostration, dehydration, and sweating (Krinsky, 2002). Animals frequently become dyspneic and rales may be detected on auscultation due to pulmonary edema. Myocarditis may initiate cardiovascular signs including tachycardia, congested mucous membranes, and decreased capillary refill time. Synchronous diaphragmatic flutter and muscle fasciculations have been reported in horses and are thought to be a result of hypocalcemia (Stair and Plumlee, 2004). Ulceration of the oral mucosa membranes may be observed and animals may be seen dipping their muzzles into water without drinking (Krinsky, 2002). Diarrhea may be observed in animals that live for a few days. The course of the disease may be as short as 4 h, with massive dose ingestion, to 5 days in lethal poisoning. In horses, the mortality rate is approximately 50%, with horses surviving more than 1 week having a favorable prognosis (Krinsky, 2002).

In both field cases and experimental cantharidin poisoning, there are elevations in serum protein and packed cell volume caused by dehydration and shock. The damaged gastrointestinal mucosa allows rapid invasion of enteric bacteria, resulting in bacteremia and leukocytosis. There may be mild elevation in serum urea nitrogen. Profound hypocalcemia (5.9 mg/dl, normal 12.8 $\pm$ 1.2) and hypomagnesemia (0.7–1.8 mg/dl, normal 2.5 $\pm$ 0.3) have been reported. Specific gravity of urine is low in the early stages of the disease, and erythrocytes are usually present in the urine, yielding a positive occult blood reaction.

Gross lesions may be minimal with massive dose ingestion. In more protracted cases, oral ulcers, vesication, and desquamation of patches of the distal esophagus, erosions, and ulceration of the gastrointestinal tract, mucus in the renal pelvis, and renal cortical hemorrhages may be seen. Hyperemia and hemorrhages are seen in the urethra and bladder mucosa. Ventricular myocarditis, pulmonary edema, petechial hemorrhages of serosal surfaces, hepatomegaly, and splenomegaly may also be present.

The initial microscopic lesion is acantholysis of mucosa of the gastrointestinal tract, epithelium of the urinary tract, and endothelium of vessels (Krinsky, 2002). Other microscopic lesions include myocarditis, renal tubular nephrosis, and degenerative changes in the kidneys and digestive tract.

Cantharidin may be detected in urine, tissue (kidney and blood), gastrointestinal contents, and the dried beetles themselves by high-pressure liquid chromatography (HPLC), or gas chromatography/mass spectrometry (GC–MS). Cantharidin is excreted rapidly and may not be present in detectable amounts after 4–5 days following ingestion (Krinsky, 2002).

### Treatment

There is no specific treatment. The administration of either activated charcoal or mineral oil (but not together) via a gastric tube may aid animals that have consumed a small dose or are in the early stages of poisoning. General supportive therapy should include correction of fluid loss and electrolyte imbalances, particularly hypocalcemia and hypomagnesemia (Krinsky, 2002).

Broad-spectrum antimicrobial therapy may be necessary to counter secondary bacterial invasion from the gastrointestinal tract. Aminoglycoside antibiotics and others that are potentially nephrotoxic or that are excreted via the kidney should be avoided, as nephrosis is a potential effect of cantharidin.

## COLEOPTERA: FIREFLIES

### Background

Fireflies of the genus *Photinus* have been reported to cause death when fed to captive reptiles and amphibians (Knight *et al.*, 1999).

### Mechanism of action

Lucibufagins, steroidal pyrones structurally related to cardiotoxic bufodienolides of toads, have been isolated from the bodies of *Photinus*. It is suspected that lucibufagins have cardiotoxic effects similar to bufodienolides, i.e. they inhibit sodium–potassium ATPase activity in the myocardial cell membrane in a mechanism similar to digitalis (Brubacher *et al.*, 1999).

### Toxicity

It is likely that the lucibufagins protect the fireflies from predation from birds and spiders (Knight *et al.*, 1999). Reported cases of firefly toxicosis have occurred when captive reptiles and amphibians were fed the fireflies by their owners. As few as one firefly has caused death in lizards. Toxicosis has been reported in bearded dragons (*Pogona* spp.), an African chameleon (*Chamaleleo* spp.), White's tree frogs (*Litoria caerulea*), and a *Lacerta derjugini* lizard.

Within 5–90 min of being fed fireflies, affected lizards displayed head shaking, oral gaping, dyspnea, skin color change to black, and regurgitation, with death occurring as rapidly as 15 min postingestion (Knight *et al.*, 1999).

No gross lesions were reported in animals presented for postmortem.

### Treatment

Because of the rapid onset of signs and rapid progression to death, no veterinary care could be instituted in the reported cases (Knight *et al.*, 1999). Treatment for affected animals would include management of any cardiac arrhythmias and general supportive care. As the lucibufagins are similar in structure to bufodienolides, in theory one would expect that digoxin-specific Fab fragments (e.g. Digibind®) might be of benefit in treating animals with firefly toxicosis (Brubacher *et al.*, 1999). However, given the rapid development and progression of clinical signs following ingestion of fireflies, the prognosis in most cases will be poor.

## COLEOPTERA: OTHER TOXIC BEETLES

Ingestion of rove beetles, *Paederus fuscipes* (Staphylinidae), has resulted in toxicosis in horses and cattle in Southeast Asia (Krinsky, 2002). Pederin, a toxin more potent than *Latrodectus* spider venom, can cause severe vesicular damage to the mucosa of the alimentary tract. Ingestion of the rose chafer (*Macrodactylus subspinosus*), a member of the Scarabaeidae, has been associated with deaths in chickens, ducklings, goslings, and young turkeys in North America, although modern confinement poultry production operations have largely reduced the risk of toxicosis.

In Michigan, a dog that ingested large numbers of Asian ladybeetles (*Harmonia axyridis*) developed severe shock, cyanosis, rapid pulses, and lateral recumbency (Hoenerhoff *et al.*, 2002). The dog subsequently died, and on postmortem, large numbers of partially digested and intact Asian ladybeetles were detected in the small and large intestines associated with severe hemorrhage, thickening and ulceration of the jejunum and ascending colon. There was a pungent odor to the gastrointestinal contents that mirrored the foul-smelling fluid that Asian ladybeetles can produce when disturbed. Asian ladybeetles were introduced as a biologic control agent and are spreading rapidly throughout the United States.

## HYMENOPTERA: BEES, WASPS, AND HORNETS

### Background

Bee and wasp stings caused approximately 23 human deaths per year in the United States during the 1950s (Akre and Reed, 2002). During that same period, approximately 13 individuals per year died of snakebite. No statistics are available for fatal bee or wasp stings in animals. Over 20,000 species of bees are distributed throughout the world (Fowler, 1993). The honeybee, *Apis mellifera*, is one of two domesticated insect species (the other is the silk worm, *Bombyx mori*). Native honeybees in tropical Southeast Asia were the source of the domestic honeybee. When early American colonists imported European strains of the honeybee to the Virginia Colonies, the bees became known as European honeybees. Africanized honeybees (*A. mellifera scutellata*) are a subspecies of the common honeybee native to Africa that has spread from South and Central America up into South Texas, New Mexico, Arizona, Nevada, California, and Puerto Rico (Akre and Reed, 2002).

### Mechanism of action

There are numerous variations in the venom apparatus of members of Hymenoptera (Fowler, 1993). The stinger is a modification of the ovipositor apparatus and is found only in female bees and wasps. Venom secreted from specialized cells in the acid glands is transported to the venom sac reservoir via small tubules. One-way valves in the bulb of the venom apparatus control the flow of venom during envenomation. At the time of venom injection, the alkaline gland contributes a secretion that enhances the toxicity of the venom. The stinger of the honeybee is covered with retrograde barbs that cause the stinger to remain impaled in thick-skinned victims. When this occurs and the bee attempts to withdraw, the entire stinger apparatus is pulled from the bee, resulting in death of the honeybee.

Honeybee venoms are complex mixtures of proteins, peptides, and small organic molecules (Akre and Reed, 2002). Phospholipases and hyalouronidases present in the venom account for the majority allergic responses to bee venoms in humans and likely other animals as well. Phospholipase A2 is one of the most lethal peptides in honeybee venom (Schmidt, 1995). Mellitin is a membrane disruptive compound that increases the susceptibility of cell membranes to the damage caused by phospholipases within the venom (Akre and Reed, 2002). Mellitin also can cause pain, trigger hemolysis, increase capillary blood flow, increase cell permeability, and enhance spread of venom constituents within tissue. Mellitin, in combination with phospholipase and a mast cell degranulating peptide, triggers the release of histamine and serotonin. In mice, mellitin was found to be the primary lethal component of honeybee venom (Schmidt, 1995). Apamin is a neurotoxin that blocks calcium-activated potassium channels and has been associated with transient peripheral nerve effects in humans after bee stings (Saravanan *et al.*, 2004). In cats, bee venom can cause contraction of bronchiolar muscles.

Like honeybees, vespid wasps (including yellow jackets and hornets) produce venoms containing peptides, enzymes and amines designed to trigger pain (Akre and Reed, 2002). The primary pain-inducing substances are kinins; however, other compounds present in vespid venom, such as serotonin, histamine, tyramine, catecholamines, and acetylcholine, can contribute to the pain as well as local vasoactivity. Several of the constituents of vespid venom can act as allergens and trigger allergic reactions. Some vespid venoms contain neurotoxins or alarm pheromones that alert the swarm to an intruder.

## Toxicity

Honeybees can inflict only a single sting, but an animal attacked by a swarm or hive of bees may sustain multiple stings and the cumulative envenomation may be lethal. The Africanized honeybee (*A. mellifera scutellata*) presents a special case. Although its venom is no more toxic than that of the domestic honeybee, the aggressive behavior of the African honeybee increases the likelihood of multiple stings occurring by swarms of these bees (Akre and Reed, 2002). Wasps and hornets either lack a barbed stinger or the barbs are small and do not prevent withdrawal of the stinger. Therefore, a single wasp may inflict multiple stings. Also, because many wasps are highly social, multiple stings are commonplace.

A sting by a single bee or vespid rarely causes more than a transient, painful prick in animals, in contrast to humans, where sensitive individuals may die peracutely from a single sting. However, death following a single bee sting has been reported in a dog (Fowler, 1993). Deaths following attacks on livestock by Africanized bees have been reported, although the majority of interactions between livestock and bees or wasps result in only local reactions. Yellow jacket envenomation leading to skin lesions and death has been reported in a group of pigs (Fowler, 1993).

Local reactions to single bee, hornet, or wasp stings consist of swollen, edematous, and erythematous plaques at the site of the sting (Fowler, 1993). Most small animal patients present with facial, periorbital, and/or aural edema. Honeybee stingers will occasionally be located embedded in the area, and a small abscess may form at the site. In the Middle East, the German wasp, *Vespula germanica*, has been observed to injure the teats and udders of dairy cattle, causing lesions which can lead to mastitis. Apparently the wasps served as a vector in spreading *S. dysgalactiae* infection in the herds (Yeruham *et al.*, 1998).

Multiple stings may produce numerous wheals, urticaria, and in severe cases may cause severe systemic responses as direct toxic effects of the venom. Systemic effects reported in dogs following multiple stings from bees or vespids include prostration, convulsions, CNS depression, shock, hyperthermia, bloody diarrhea, bloody vomiting, leukocytosis, intravascular hemolysis, disseminated intravascular coagulopathy, and elevations of blood urea nitrogen (BUN), and alanine transaminase (ALT), suggesting renal and hepatic involvement (Wysoke *et al.*, 1990; Cowell *et al.*, 1991; Fowler, 1993). Hepatic injury in cats has also been reported following exposure to hornet venom. Acute lung injury similar to the human acute respiratory distress syndrome (ARDS) developed in a dog following envenomation by over 100 bees (Walker *et al.*, 2005).

The classic anaphylactoid response to bee and vespid venoms that is not uncommon in humans has not been documented in livestock, but anaphylaxis in dogs due to bee stings has been reported (Akre and Reed, 2002). Immune-mediated hemolytic anemia secondary to bee envenomation developed in two dogs (Noble and Armstrong, 1999). Clinical signs included lethargy, hematuria, ataxia, and seizures, and one dog died. Clinicopathologic data included non-regenerative anemia, spherocytosis, and positive results for Coomb's test, and occult hematuria. Treatment included oral administration of corticosteroids at immunosuppressive dosages and supportive care. The surviving dog initially responded to corticosteroids, but hemolysis recurred as the dosage was tapered. Hemolysis resolved with prolonged administration of corticosteroids.

## Treatment

Ideally, when an insect has stung an animal, identification of the insect should be attempted. Most often, stings occur in areas that are free of hair or have short hair. For bee stings, the site should be examined to determine if any part of the stinger remains. Retained stingers should be scraped away from the injection site; grasping the stinger with forceps may result in more venom being expressed into the injection site. Cold compresses may be used as first aid to relieve pain and swelling. Antihistamines and corticosteroids are thought to have questionable value once lesions have developed, but are not contraindicated (Fowler, 1993). Patients should be monitored for development of systemic or anaphylactic reactions, which need to be treated promptly and aggressively. Epinephrine may be used in cases where anaphylaxis is suspected. In cases of true systemic toxicosis, aggressive intravenous fluid therapy is recommended using balanced fluid solutions. Other treatments that have been recommended include corticosteroids, antihistamines, oxygen for dyspnea, and diazepam as needed for convulsions (Fowler, 1993). Renal and hepatic function should be monitored in animals that develop systemic reactions. In most cases exposure to single stings from bees and vespids is rarely fatal.

# HYMENOPTERA: ANTS

## Background

There are more than 10,000 species of ants, some of which bite, some of which sting, and others that both bite and sting. Some ants that lack a sting have the ability to spray formic acid, which can cause local irritation to the victim if it gets in the eyes or wounds produced by the ant's mandibles.

Multiple stings from venomous ants have resulted in the death of pets, livestock, and wild animals in North, Central, and South America (Fowler, 1993). The red imported fire ant, *Solenopsis invicta*, is the most important species in terms of envenomation of animals. This ant was native to Brazil but entered the United States in the 1940s, where it has become a serious pest in the Southeast as far West as Texas.

## Mechanism of action

Ant venoms, like bee venoms, are complex mixtures of compounds that can induce a variety of adverse effects in animals. Fire ant venoms consist largely of alkaloids with less than 1% proteinaceous component (Akre and Reed, 2002). The alkaloids consist of solenopsins (methyl-*n*-alkylpiperidines) and a piperidine which cause dermal necrosis when injected in skin. These alkaloids have cytotoxic, hemolytic, fungicidal, insecticidal, and bactericidal properties.

## Toxicity

Animals most likely to be severely affected by fire ants tend to be those that are unable to move away from a colony and would include those that are neonatal, juvenile, or disabled. Attacks of fire ants resulting in deaths of newborn game animals such as rabbits and deer have been reported (Akre and Reed, 2002). Nesting bird chicks and newly hatched quail and poultry have been killed (and often eaten) by fire ants (Fowler, 1993).

Clinical effects of fire ant envenomation include intense pain at the site of the sting. In humans, within minutes of a sting there is formation of an urticarial wheal, which progresses to a vesicle then a pustule within 24 h (Fowler, 1993). Within a few days, the pustule will rupture, resulting in a crusting lesion. Dogs do not appear to develop the pustules described in humans, instead they develop erythematous pruritic papules that generally resolve within 24 h (Rakich *et al*., 1993). There are no reports of anaphylaxis in animals secondary to fire ant stings (Akre and Reed, 2002). Multiple stings may result in systemic signs similar to those of multiple bee or wasp stings.

## Treatment

Single bites from fire ants generally require no treatment. Multiple envenomations resulting in severe systemic reactions or anaphylaxis should be managed similarly to systemic reactions to bee stings (i.e. fluids, corticosteroids, supportive care).

# HYMENOPTERA: SAWFLIES

## Background

Sawflies are primitive hymenopterans belonging to the suborder Symphyta and are widely distributed around the world. Sawfly poisoning has been reported in livestock in Denmark by *Arge pullata*, in Australia by *Lophotoma* spp., and in Uruguay by *Perreyia flavipes* (Fowler, 1993; Thamsborg *et al*., 1996; Dutra *et al*., 1997).

## Mechanism of action

Sawfly larvae harbor D-amino acid containing peptides that are considered the toxic principle in sawfly larvae poisoning, which results in hepatic, and occasionally renal, degeneration, and necrosis (Oelrichs *et al*., 1977). Lophyrotomin, an octapeptide structurally similar to cyclic peptide hepatotoxins from *Amanita* mushrooms (Thamsborg *et al*., 1996), is the major toxin found in the Australian and Danish sawfly larvae (Oelrichs *et al*., 1977). The bile acid transport system is important in the hepatocellular uptake of lophyrotomin, which causes a periportal hemorrhagic necrosis of the liver (Oelrichs *et al*., 2001; Chong *et al*., 2002). Pergidin, a heptapeptide containing a phosphoseryl residue, is the major toxin found in the South American sawfly. In contrast to lophyrotomin, pergidin causes a periacinar coagulative necrosis in the liver (Oelrichs *et al*., 2001).

## Toxicity

In natural outbreaks, deaths have occurred in livestock, primarily sheep and cattle, when large numbers of sawfly larvae have been ingested. In Australia, outbreaks have occurred in Queensland, where large forests of silver-leaf iron bark trees (*Eucalyptus melanophloia*) provide sustenance for *Lophorotoma interrupta* (Dutra *et al*., 1997). Large piles of larvae accumulate on the ground under the trees and are ingested by cattle and sheep. In Denmark, a similar outbreak in sheep and goats involving the sawfly *Arge pullata* occurred associated with birch trees (*Betula pendula*) heavily infested with larvae. Outbreaks of sawfly

poisoning of cattle, sheep, and pigs in Uruguay were associated with masses of *Perreyia flavipes* crawling through grass in orderly columns. In Uruguay, sheep were reported to be less frequently affected than cattle. Mortality rates on affected farms have been reported to range from 1.3% to 28% (Dutra *et al.*, 1997).

Minimum lethal dosages of lophyrotomin and pergidin when dosed intraperitoneally in C57/B16 male mice were 8 and 32 mg/kg, respectively (Oelrichs *et al.*, 2001). Administration of intact *P. flavipes* larvae to pigs, calves, and lambs resulted in mortality (Dutra *et al.*, 1997). Deaths occurred in pigs and calves at dosages of 40 g of larvae per kilogram body weight. Sheep administered 10, 20, and 40 g of larvae per kilogram body weight died at 68, 43, and 14 h, respectively. One calf administered 9 g of larvae per kilogram body weight developed diarrhea and became depressed, anorexic, and ataxic, but recovered 10 days following dosing. The toxicity of live and dead larvae is equivalent.

Livestock ingesting sawfly larvae may be simply found dead without prodromal signs (Dutra *et al.*, 1997). Clinically, affected cattle may develop weakness, muscle tremors, depression, agitation, aggression, stupor, and death within 2–7 days. Icterus and mild photosensitization were reported in some cattle that survived. In sheep and goats, clinical signs included depression, anorexia, ataxia, recumbency, and death (Thamsborg *et al.*, 1987).

Clinical laboratory abnormalities associated with sawfly larvae poisoning include elevations in liver enzymes aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, and gamma glutamyltransferase (Thamsborg *et al.*, 1996).

Massive hepatic necrosis was the primary postmortem lesion found in cattle and sheep, while renal tubular degeneration was occasionally identified in sheep (Thamsborg *et al.*, 1987; Dutra *et al*. 1997). Lesions secondary to hepatic failure included widespread hemorrhage, icterus, and photosensitization (Dutra *et al.*, 1997).

### Treatment

Management of sawfly larvae poisoning would include removing animals from the source of the larvae and providing symptomatic and supportive care. Administration of silymarin (44–58 mg/kg IV) in combination with penicillin (200,000 IU/kg IV) and glucose (20 g/lamb) at 7 and 24 h following dosing with sawfly larvae prevented clinical signs of toxicosis and significant liver enzyme changes in experimental lambs (Thamsborg *et al.*, 1996). Lambs receiving larvae along with glucose and penicillin without silymarin developed a milder course of clinical illness and lower liver enzyme changes than untreated control lambs administered sawfly larvae.

## LEPIDOPTERA: BUTTERFLIES AND MOTHS

Caterpillars of many species of moths and butterflies may possess hairs and spines on their surfaces that can trigger urticarial reactions in humans and animals (Mullen, 2002a). Necrosis and partial sloughing of the tongue was reported in three dogs in Israel following exposure to the pine processionary moth (*Thaumetopoea wilkinsoni*); early signs included vomiting, swelling of the tongue, dyspnea, hyperthermia, tachypnea, cyanosis, lingual edema, labial angioedema, ptyalism, bilateral submandibular lymphadenopathy, and conjunctivitis (Bruchim, 2005). All dogs survived following symptomatic care. Caterpillars of monarch butterflies that feed on milkweed plants may accumulate the cardiotoxic glycosides from the plant into their bodies (Fowler, 1993). Upon metamorphosis into monarch butterflies, the glycosides are localized to the wings, and predatory animals such as birds, lizards, and small mammals may be poisoned if they ingest the monarchs.

## PHASMATODEA: WALKINGSTICKS

Ulcerative keratitis has been reported when dogs encountered the two-striped walkingstick (*Anisomorpha buprestoides*). These insects are distributed through Florida and the Gulf coastal plain west to Texas and possess defensive glands that produce a lachrymogenic and irritation substance. When annoyed, the walkingstick will spray this secretion at its harasser (Dziezyc, 1992).

# AMPHIBIA: TOADS

### Introduction

Poisonous amphibians include frogs, salamanders, and toads, but only toads have been associated with toxicosis in domestic animals. Poisonous frogs belong to the Dendrobatidae family and are popular aquarium pets due to their vivid, gem-like coloration. Poisonous frogs have been found to have nearly 500 different bioactive alkaloids in their skin secretions, some of which have been used for food procurement by Central and South American aboriginals who live in areas that these frogs inhabit (Daly, 1980; Daly *et al.*, 2002; Takada *et al.*, 2005). Most of these alkaloids are not thought to be synthesized *de novo* by the frog, but originate from insects that make up the frogs' diets; the alkaloids are absorbed from the insects and sequestered in the glands produce the skin secretions (Daly, 1995a, b; Donnelly, 2002; Saporito *et al.*, 2004). Captive dendrobatids tend to lack these toxic alkaloids in their skin

secretions, most likely due to the difference in diets. The remaining discussion will focus on poisonous toads.

## Background

Members of the genus *Bufo* are found all over the world and all are considered to produce zootoxins capable of causing clinical effects in animals. However, only the larger toads, specifically *B. marinus*, *B. alvarius*, *B. regularis*, and *B. blombergi*, are considered to produce sufficient poison to cause serious toxicosis (Fowler, 1993). *B. marinus* is found in Florida, Texas, Hawaii, the Caribbean, Central and South America, Fiji, Australia, the Philippines, and the Marianas. *B. alvarius* is found in the Imperial Valley of California and the Colorado River basin between Arizona and California. *B. blombergi* is found only in Colombia and *B. regularis* is found in Ethiopia (Fowler, 1993).

Toads possess mucous glands, which are widely distributed over the skin and produce a slimy secretion which keeps the skin moist and lubricated. The mucus secretions may aid in defense through their objectionable odor and/or taste to predators. Toads produce their toxic secretions from granular glands, modified mucous glands, throughout the head, shoulders, and dorsolateral areas of their skin. The more toxic toad species possess a parotid gland, which is an aggregation of granular glands located caudal and lateral to the ear (Fowler, 1993).

## Toxicokinetics

Dogs are the species most commonly involved in toad toxicosis. Mouthing of toads stimulates release of toxins from the parotid gland into the mouth, with absorption occurring across the mucous membranes in the mouth (Roder, 2004). Toad secretions contain a variety of compounds including bufogenins, bufotoxins, and bufotenines.

## Mechanism of action

Bufogenins inhibit sodium–potassium ATPase activity in a manner similar to cardiac glycosides such as digitalis, ultimately causing increased intracellular calcium in myocardial cells which results in cardiac arrhythmias (Eubig, 2001). Bufotoxins are bufogenins conjugated with suberyl arginine, and they have a similar mechanism of action as bufogenins. Bufotenines are indolalkylamines such as serotonin and 5-hydroxytryptophan; in combination with catecholamines present in *Bufo* toxin, these agents may be responsible for many of the neurologic and gastrointestinal effects of *Bufo* toxins (Eubig, 2001).

## Toxicity

The relative potency of *Bufo* toxins varies with species of toad, geographic location, and size of the toad (Fowler, 1993). Smaller patients may show more severe signs when exposed to toad secretions (Eubig, 2001). *Bufo* toads in Florida appear to be more toxic than those in Hawaii and Texas, based on mortality rates of dogs exposed to the toads (Roder, 2004). One hundred milligrams of crude *Bufo* toxin is said to be toxic for dogs weighing 9–14 kg.

Clinical effects of toad poisoning include hypersalivation, anxiety, vomiting, which can occur almost immediately following exposure, and death may occur as rapidly as 15 min following exposure (Eubig, 2001). Other signs including hyperemic mucous membranes, recumbency, collapse, and tachypnea may also be present. Neurologic effects are common and include convulsions, ataxia, nystagmus, stupor, or coma (Fowler, 1993; Roder, 2004). A variety of cardiac arrhythmias have been reported, including bradycardia, sinus tachycardia, and sinus arrhythmias (Eubig, 2001). Pulmonary edema, hyperthermia, and hyperkalemia have also been reported (Fowler, 1993).

## Treatment

On-the-spot decontamination of the oral cavity by copious water lavage is recommended in cases where no signs beyond ptyalism and gagging have occurred (Eubig, 2001). Patients displaying more severe signs should be transported to a veterinary facility for stabilization prior to oral lavage. If an entire toad has been ingested, emesis under veterinary supervision is indicated for dogs where no signs beyond hypersalivation have occurred. Endoscopic or surgical removal of the toad from the stomach may be required in cases where signs have developed, but it is important to stabilize the patient prior to attempts to remove the toad. Alternatively, multiple doses of activated charcoal with a cathartic may be used when entire toads are ingested (Eubig, 2001).

Patients displaying severe signs of toxicosis should be treated symptomatically and aggressively. Seizures may be managed with diazepam or a barbiturate. Intravenous fluid therapy is essential to aid in cardiovascular support. Arrhythmias should be managed as they develop. Bradycardia may be treated using atropine, while propranolol or esmolol may be used to treat tachycardia (Eubig, 2001). Correction of potassium imbalances should be performed as needed. Animals experiencing severe hyperkalemia, severe neurologic signs, or severe arrhythmias unresponsive to therapy may be treated with digoxin-specific antigen-binding fragments (digoxin immune Fab), however the high cost of this product often makes its use in veterinary medicine unfeasible (Eubig, 2001).

The prognosis for patients exposed to small toads and/or showing mild clinical signs is good. Animals developing advanced neurologic or cardiac signs have a more guarded prognosis.

# REPTILIA

Venomous reptiles come from the order *Squamata*, which includes the lizards and the snakes. Outbreaks of poisonings from consumption of the flesh of certain turtles and tortoises have occasionally been reported in humans, but the incidence is fairly low; reports in the veterinary literature of chelonitoxism are lacking.

## SNAKES

### Introduction

Of the 3500+ species of snakes in the world, approximately 400 are venomous (Russell, 2001). Venomous snakes are widely distributed around the world, with the exception of certain islands, such as Hawaii, Ireland, and New Zealand (Fowler, 1993). Venomous snakes come from the families Colubridae, Crotalidae, Elapidae, Hydrophiidae, Laticaudidae, and Viperidae; only members of the Crotalidae and Elapidae are represented in the Western hemisphere.

## CROTALIDS

### Background

The crotalids are also known as pit vipers, so named for the indented, heat-sensing pits located between the nostrils and eyes. Other features of this family include elliptical pupils, triangular-shaped heads, retractable and hollow front fangs, and a single row of subcaudal scales distal to the anal plate (Peterson, 2004). Rattlesnakes have special keratin "rattles" on the ends of their tails and are members of the genera *Crotalus* and *Sistrus*. Other crotalids native to North America include water moccasins, also known as cottonmouths (*Agkistrodon picivorus*) and copperheads (*A. contortrix*).

At least 29 subspecies of rattlesnakes are found throughout the United States, while copperheads and water moccasins are distributed through Eastern and Central United States. The approximate distributions of some of the more common species are illustrated in Figure 64.3. Because of their tendency to associate near areas of human habituation, copperheads are responsible for the majority of human (and likely animal) snakebites in the United States. However, the majority of animal and human deaths in the United States are attributed to rattlesnakes, due to their more potent venoms. It is estimated that anywhere from 150,000 to 300,000 animals are bitten every year by pit vipers within the United States (Peterson, 2004; Wallis, 2005).

### Toxicokinetics

Pit vipers inject their venom by rotating their retractable fangs downward and forward in a stabbing motion (Peterson, 2004). Contraction of muscles in the venom glands then forces the venom through the hollow fangs and into the tissues of the victim. Snakes can control the amount of venom delivered to the victim by regulating the muscular contraction of the venom glands.

### Mechanism of action

Crotalid venoms are complex combinations of enzymes, cytotoxins, neurotoxins, cardiotoxins, hemolysins, coagulants/anticoagulants, lipids, nucleosides, nucleotides, organic acids, and cations such as zinc (Fowler, 1993; Peterson, 2004). Most venoms contain a minimum of 10 of these different components. In North American rattlesnakes, three general venom types have been defined (Peterson, 2004). "Classic" diamondback venom causes marked tissue destruction, coagulopathy, and hypotension. Mojave A venom causes little tissue destruction or coagulopathy but causes severe neurotoxicosis. The third venom class contains components of both classic and neurotoxic venoms.

Hyaluronidase ("spreading factor") and other enzymes break down collagen and other connective tissues, allowing for rapid penetration of venom components throughout the victim's tissues. Low molecular weight myotoxins open sodium channels in the muscle cell membrane, leading to myocyte necrosis. Phospholipase A stimulates hypercontraction of myocyte membranes, resulting in myofibril rupture (Peterson, 2004). Alteration of blood coagulation may lead to either hyper- or hypocoagulation through either direct effects on clotting factors or induction of hyperfibrinolysis, resulting in dissolution of clots as they are forming. Some diamondback rattlesnake venoms contain cardiotoxic agents (myocardial depressant factors) that cause profound hypotension unresponsive to fluid therapy. Neurotoxic components bind the pre-synaptic nerve membrane, inhibiting neurotransmitter release, and causing paralysis (Fowler, 1993).

### Toxicity

Dogs are the species most commonly bitten by pit vipers. In a study of prairie rattlesnake bites in dogs, most bites

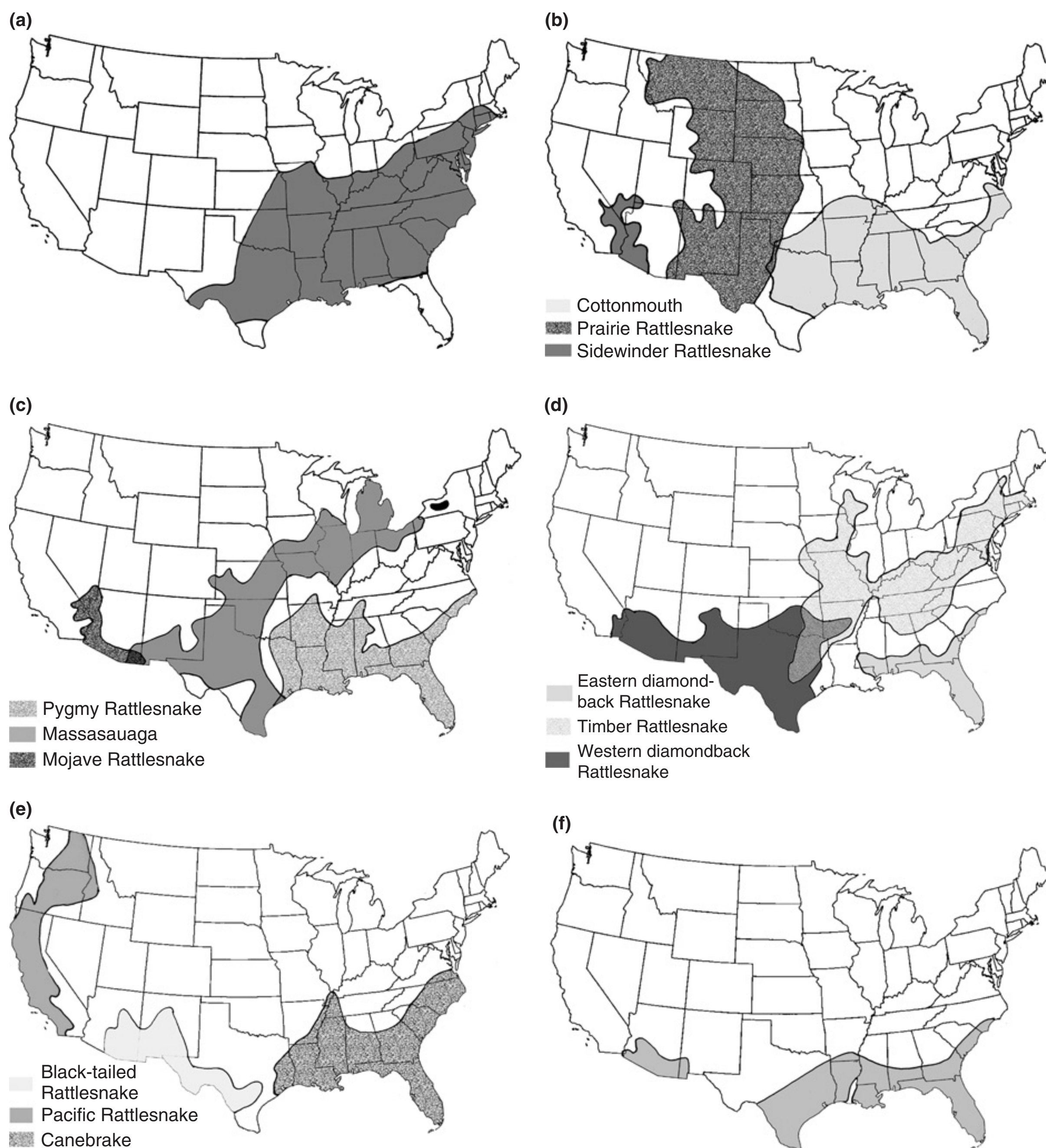


**FIGURE 64.3** Distribution of venomous snakes in the United States. (a) Distribution of copperheads (*Agkistrodon contortrix*). (b) Distribution of cottonmouths (*Agkistrodon picivorus*), prairie rattlesnakes (*Crotalus viridis*), and sidewinder rattlesnakes (*Crotalus cerastes*). (c) Distribution of pygmy rattlesnakes (*Sistrurus miliarius*), massasauga rattlesnake (*Sistrurus catenatus*), and Mojave rattlesnake (*Crotalus scutulatus*). (d) Distribution of the Eastern diamondback rattlesnake (*Crotalus adamanteus*), timber rattlesnake (*Crotalus horridus*), and Western diamondback rattlesnake (*Crotalus atrox*). (e) Distribution of the black-tailed rattlesnake (*Crotalus molossus*), Pacific rattlesnake (*Crotalus atrox*), and canebrake rattlesnake (*Crotalus horridus atricaudatus*). (f) Distribution of coral snakes (*Micruroides euryoxanthus*, *Micrurus fulvius*). (Adapted from Fowler, *Veterinary Zootoxicology*, CRC Press, and Professional Pest Control Products website, http://www.pestproducts.com/snakeinfo.htm)

occurred between May and September during the late afternoon (Hackett *et al.*, 2002). Most bites involved young dogs (median age 3.7 years) and were located on the head.

Dogs are most commonly bitten on the front legs and head, horses are most commonly bitten on the muzzle, and cattle are more commonly bitten on the tongue or muzzle (Peterson, 2004). Cats are considered more resistant to

pit viper venom, but cats are often presented in more severe clinical conditions due to their small body size, tendency to be struck on the torso, and, often, delay in obtaining veterinary care when they run and hide following a bite. Bites to the thoracic wall allow for more rapid and complete venom absorption, while bites to the tongue are essentially equivalent to intravenous envenomations.

The toxicity of any given crotalid bite will depend on both victim and snake factors. The species of the victim is an important factor as some species of animals have a degree of natural immunity to snake venoms. Additionally, the victim's size, pre-existing health, interval until medical care is obtained, and site of the bite are victim-related factors that can influence the severity of the snakebite. Snake factors that affect toxicity include species, size and age of the snake, as well as the time of year, time since the snake's last bite, and whether the bite was defensive or offensive. "Dry" bites, those in which no venom is injected, may occur in up to 25% of snakebites (Peterson, 2004). Snakes that have recently fed may be depleted of venom, and inject less (Fowler, 1993). Older snakes may have more potent venom, and larger snakes may harbor larger volumes of venom to deliver.

Analysis of venoms from a variety of North American pit vipers indicates that the ranking of relative toxicity of venoms, from highest to lowest is Mojave A rattlesnake (*Crotalus scutulatus*), Eastern diamondback (*C. adamanteus*), Western diamonback (*C. atrox*), timber rattlesnake (*C. horridus*), cottonmouth (*Agkistrodon picivorus*), and copperhead (*A. contortrix*).

## Clinical effects

In most cases of snakebite, the initial signs are usually local pain and swelling, followed by petechiation, ecchymosis, and discoloration of the skin in the region of the bite. Swelling and/or the haircoat of the victim may mask bite wounds. Bites from snake species that possess only neurotoxic venom may show little local swelling. Several hours may elapse before more significant clinical signs develop. Hypotension, shock, tachycardia or other arrhythmia, vomiting, diarrhea, shallow respiration, lethargy, nausea, obtundation, muscle fasciculations, ptyalism, and pain and enlargement of regional lymph nodes may occur (Peterson, 2004). Local tissue necrosis may be severe, especially in areas with minimal muscle mass. Local swelling from bites to the head or front legs in dogs may result in regional circulatory alterations that reduce venom uptake (Peterson, 2004). Horses or llamas bitten on the muzzle may succumb to asphyxiation due to occlusion of the nares due to tissue swelling. Horses develop pitting edema that frequently progresses to involve the entire head and neck or limb, but tissue necrosis is uncommon (Fowler, 1993). Hemolysis, rhabdomyolysis, thrombocytopenia, and coagulopathy may occur.

## Treatment

Historically, a variety of first aid measures have been described for managing snakebites in humans and animals, including suction, electric shock, ice packs, and tourniquets (Bush, 2004; Peterson, 2004). However, most of these measures are of questionable value, and they waste precious time that could be used to transport the patient to a veterinary medical facility. Initial first aid should be focused on keeping the animal quiet (exertion will hasten the spread of venom factors into the tissues) and attempting to keep the bitten area below heart level.

The haircoat around the bite area should be clipped in order to fully visualize the bite. Use of a magic marker to delineate the margins of local edema and/or circumferential measurements above, at and below the bite site will allow objective evaluation of the progression of local tissue involvement (Fowler, 1993; Peterson, 2004). Vital signs, including blood pressure should be monitored closely for the first several hours; all patients should be monitored for a minimum of 24 h before being released (Fowler, 1993). Tracheostomy may be required in cases where severe swelling in the head or neck region results in respiratory compromise or in cases of obligate nasal breathers (e.g. horses, llamas) that are bitten on the muzzle. Intravenous crystalloid therapy is recommended to manage hypotension or hypovolemia. Coagulopathy and hemolysis should be managed using blood or platelet transfusions. Conditions such as muscle tremors, seizures, cardiac arrhythmias, and pulmonary edema should be managed as they develop using standard medical therapies. Corticosteroid use is controversial in snakebites, with some indicating that corticosteroids have no place in management of snakebite (Peterson, 2004), while others suggest that judicious use of corticosteroids may be of benefit (Fowler, 1993). In horses, topical application of dimethyl sulfoxide to the bite site should be avoided as it enhances systemic absorption of venom (Peterson, 2004). Diphenhydramine may be useful for its sedative effect. Broad-spectrum antibiotics are advocated by some to aid in prevention of infection (Peterson, 2004), and horses should receive tetanus antitoxin or toxoid (Fowler, 1993).

The use of intravenous antivenin in crotalid snakebites can result in the reversal of potentially life-threatening problems such as coagulopathy, thrombocytopenia, and paralysis (Peterson, 2004). Antivenin cannot reverse tissue necrosis or secondary effects such as renal damage. One study on prairie rattlesnake bites in dogs suggested that antivenin administration was not significantly associated with clinical outcome, and the value of its use in

prairie rattlesnake envenomation was questioned (Hackett *et al.*, 2002).

Equine-origin polyvalent antivenin has historically been used to manage snakebites in animals. More recently, polyvalent immune Fab fragments (CroFab, Protherics) have become available to treat snakebites in humans and animals. Advantages of the crotalid Fab antivenin over the equine-origin antivenin include decreased antigenicity (and therefore decreased potential for allergic reaction), more rapid elimination, increased stability, and more rapid reconstitution (Gwaltney-Brant and Rumbeiha, 2002). The crotalid Fab antivenin has been shown to be effective in management of canine snakebite (Peterson, 2004). Administration of either antivenin should be begun as early as possible and patients should be closely monitored for signs of anaphylaxis during antivenin administration. Studies in dogs have shown that single vial doses of crotalid Fab antivenin were sufficient to manage most cases of canine snakebite envenomation. Coagulation parameters should be monitored, and additional vials of antivenin should be administered if deterioration is noted.

The prognosis for recovery from snake envenomation is dependent on the type of snake involved, the severity of the envenomation, and the rapidity and aggressiveness of veterinary intervention.

Recently a rattlesnake vaccine (Crotalus Atrox Toxoid, Red Rock Biologics) has been developed and marketed with the aim of reducing the morbidity and mortality of dogs due to crotalid snakebites (Wallis, 2005). The vaccine is designed to elicit an immune response to the major protein fractions of *Crotalus atrox* (Western diamondback rattlesnake) that may also cross-react to the major protein fractions of some other rattlesnake venoms. The vaccine does not induce neutralizing antibodies against Mojave neurotoxin. The vaccine is administered as two injections (three for dogs exceeding 100 pounds of body weight) spaced 4 weeks apart. Titers peak in 4–6 weeks and decline after about 6 months, and twice-yearly vaccination is recommended for dogs that have potential to be exposed to rattlesnakes year round. The manufacturer emphasizes that the vaccine does not eliminate the need to seek veterinary care in the event that a venomous snake bites a vaccinated dog. In clinical trials, vaccinated dogs experiencing mild envenomations from subsequent snakebites frequently had their swelling receding within hours of the bite, compared to unvaccinated dogs, which may have swelling progress and persist for 1–2 days following a mild envenomation. Vaccinated dogs that are severely envenomated may still require treatment with antivenom, although the amount of antivenom required may be reduced in vaccinated dogs (Wallis, 2005). The vaccine has only recently been on the market and additional time and study will be required to determine its overall efficacy and value.

## ELAPIDS

### Background

In North America, there are two species of venomous elapid snakes: the Sonoran coral snake (*Micruroides euryoxanthus*) and several subspecies of *Micrurus fulvius*, including the Texas coral snake (*M. f. tenere*), the Eastern coral snake (*M. f. fulvius*) and the South Florida coral snake (*M. f. barbouri*) (Peterson, 2004). The Sonoran coral snake is found in Central and Southeastern Arizona and Southwestern New Mexico. The Texas coral snake is found from Eastern and South-central Texas north into Louisiana and Southern Arkansas. The Eastern coral snake is present from Eastern North Carolina south to Central Florida and west through Alabama and Mississippi. The South Florida coral snake is located in Southern Florida.

North American coral snakes tend to be shy, nonaggressive, and nocturnal, making interactions between them and domestic animals less common than pit vipers (Peterson, 2004). These snakes are brightly colored, with alternating bands of black, red and yellow, and they have small heads and round pupils. The venom delivery apparatus of coral snakes includes short, fixed (non-hinged) front fangs that are partially covered by a membrane (Fowler, 1993). During the bite, the membrane is pushed away and the venom duct empties at the base of the fang, bathing the fang with venom that runs down grooves in the fang. During the bite, coral snakes hold onto the victim and chew, delivering additional venom to the wound.

### Toxicokinetics

Like crotalid venom, coral snake venom is composed of a variety to compounds, mostly small polypeptides and enzymes. Neurologic signs following envenomation may be delayed in onset for up to 12 h, and the duration of effects is prolonged (Peterson, 2004). Total clearance of venom from the body may take up to 14 days.

### Mechanism of action

Neurotoxic peptides in coral snake venom cause a nondepolarizing postsynaptic neuromuscular blockade similar to the effects of curare (Peterson, 2004). Binding of neurotoxins to postsynaptic receptors appears to be irreversible. Enzymes within the venom can cause local tissue necrosis, myoglobinemia in cats, and hemolysis in dogs.

### Toxicity

As with crotalid envenomations, the severity of the bite is related to the size of the victim and the amount of venom

delivered. The amount of venom injected is related to the duration of the bite, intensity of chewing and reason for the bite (offensive versus defensive). Clinical signs vary with the species of the victim. In cats, the signs are primarily neurologic including progressive ascending flaccid paralysis, decreased nociperception, CNS depression, and diminished spinal reflexes (Peterson, 2004). Hypotension, respiratory depression, anisocoria, myoglobinemia, and hypothermia may have also been described in cats. In dogs, depression of the CNS, decreased spinal reflexes, muscle weakness, and respiratory depression may occur. Vomiting, hypersalivation, hypotension, dyspnea, dysphagia, muscle fasciculation, tachycardia, and hemolysis have also been reported in dogs. Potential complications include dysphagia leading to aspiration pneumonia. Death is due to respiratory paralysis.

### Treatment

Management of coral snake envenomations in animals should entail immediate transport for veterinary care. Because onset of clinical signs may be delayed up to 12 h, patients that have been bitten should be closely monitored for a minimum of 24 h. Baseline serum chemistry and complete blood count values should be obtained, and respiratory function should be closely monitored. Ventilatory support may be required if clinical signs progress to the point where respiration is compromised; ventilation may be required for 72 h or more (Peterson, 2004). Administration of specific *Micrurus* antivenin should be considered if neurologic signs begin to develop; early administration is recommended as the antivenin is considered poorly effective at displacing venom components bound to receptor sites (Peterson, 2004). As with crotalid antivenin, anaphylaxis is a potential complication of *Micrurus* antivenin administration. Broad-spectrum antibiotics and symptomatic wound care may be indicated. Recovery periods of 7–10 days have been reported in cats envenomated by coral snakes (Chrisman *et al.*, 1996).

The prognosis for coal snake envenomations in animals is generally good provided prompt and aggressive veterinary care is obtained.

## LIZARDS

### Background

Venomous lizards are only found in North and Central America and are members of the genus *Heloderma*. *H. suspectum* and *H. cinctum* are commonly referred to as Gila monsters, while *H. horridum* is known as the Mexican beaded lizard. The Gila monsters are found in Arizona and parts of Utah, New Mexico, Nevada, and Southern California, while the Mexican beaded lizard is found in Mexico, from Sonora down the Pacific Coast into Guatemala (Cantrell, 2003; Peterson, 2004).

Venomous lizards are large and heavily bodied, with blunt, rounded tails, powerful jaws, and short legs with clawed, hand-like feet. The colorful, bead-like scales form a reticular pattern of dark brown to black on a yellow, orange, pink, and/or cream background. Gila monsters, which can reach 55 cm in length, are smaller than Mexican beaded lizards, which can grow up to 1 m in length and weigh up to 2 kg (Cantrell, 2003; Peterson, 2004).

*Heloderma* spp. possess venom glands in the lower jaw at the base of the teeth, and venom is delivered through grooves in the teeth via capillary action as the lizard masticates. These lizards are generally considered to be docile, but when provoked can be aggressive biters that can hang on tenaciously to their victim, often requiring prying the jaws open to release the victim (Cantrell, 2003). The teeth are loosely attached and brittle, and may break off into the wound. The venom is considered a defensive weapon rather than one for procuring food (Peterson, 2004).

### Mechanism of action

*Heloderma* venom is composed of a complex mixture of proteins and enzymes, many of which are similar to those found in snake venoms, including hyaluronidase, phospholipase $A_2$, serotonin, and a variety of enzymes (Cantrell, 2003). Hyaluronidase ("spreading factor") catalyzes the cleavage of internal glycoside bonds of acid mucoglycosides, resulting in decreased viscosity of hyaluronic acid, which in turn increases tissue permeability and allows deeper penetration of venom into tissue. Phospholipase $A_2$ uncouples oxidative phosphorylation which inhibits cellular respiration, causes cell membrane destruction, and inhibits platelet aggregation (Huang and Chiang, 1994; Peterson, 2004). Various proteolytic enzymes result in local tissue damage as well as aid in the spread of venom through the tissue. In addition, *Heloderma* venoms contain several unique components that have a variety of clinical effects; these are summarized in Table 64.3. Gilatoxin is considered to be the major lethal factor in *Heloderma* venom (Fowler, 1993).

### Toxicity

The toxicity of *Heloderma* venoms is dependent on the amount of venom delivered to tissues, which in turn is dependent on the duration and severity of the bite. Dogs are the species most likely to have a significant encounter *Heloderma* spp. due to their inquisitive nature and tendency

**TABLE 64.3 Contents and effects of various *Heloderma* spp. venom components**

| Component | Effects | Reference |
|---|---|---|
| Gilatoxin | "Lethal factor", kallikrein-like activity, pain, hypotension | Fowler (1993); Utaisincharoen *et al.* (1993) |
| Helodermin | Vasodilation, hypotension | Grundemar and Hogestatt (1990) |
| Helospectin I and II | Vasodilation, hypotension | Grundemar and Hogestatt (1990) |
| Helothermine | Lethargy, paresis, hypothermia | Peterson (2004) |
| Hyaluronidase | "Spreading factor," degrades connective tissue | Peterson (2004) |
| Phospholipase $A_2$ | Inhibition of platelet aggregations | Huang and Chiang (1994) |

to harass wildlife that they encounter. Although rare, *Heloderma* bites to dogs and cats have occurred, and death in at least one dog has been reported (Fowler, 1993; Peterson, 2004).

The clinical effects of *Heloderma* envenomation include intense local pain, edema, and hemorrhage at the site of the wound (which may contain fractured teeth). Regional lymphangitis and local ecchymoses may occur, although tissue necrosis is not common. Systemic signs described in humans include weakness, dizziness, tinnitus, muscle fasciculations, hypotension, and tachycardia. In dogs and cats, signs may include tachypnea, vomiting, polyuria, salivation, and lacrimation. Aphonia has been reported in cats (Peterson, 2004). Hypotension, tachycardia, and respiratory distress have been reported in dogs and cats injected with boluses of *Heloderma* venom, although reports of these conditions in natural exposures are lacking.

### Treatment

Management of *Heloderma* bites in small animals includes initial first aid followed by assessment and monitoring. The first course of action is often disengaging a lizard that is still attached to the victim, as these lizards may hold on tenaciously once they bite. Removal should be attempted as quickly as possible because the amount of venom delivered is proportional to the duration of the bite. Suggested means of removal of the lizard include applying a flame from a match or cigarette lighter to the underjaw of the lizard, prying the jaws open with metal bar, or killing the lizard by incising the jugular vein with a knife. Attempts to pull the lizard off the victim or to strike the lizard in the head may enlarge or deepen the wounds at the site of the bite (Fowler, 1993). Once the lizard is removed, a veterinarian should evaluate the patient. The bite site should be irrigated with 2% lidocaine and the wound probed with a 25-gauge needle to detect any embedded tooth fragments (Peterson, 2006). Diazepam, 0.1–0.5 mg/kg IM may be helpful in sedating agitated or highly painful animals. Analgesics should be administered as needed, and the animal should be monitored for several hours for the development of hypotension or shock; if either develops, appropriate medical treatment including intravenous fluid therapy is indicated. Broad-spectrum antibiotics should be administered to prevent infection from the myriad of potentially pathogenic bacteria that have been reported to frequent the mouth of reptiles (Peterson, 2006). General wound care should be instituted and the wound should be examined daily for evidence of infection. Envenomation of pets by *Heloderma* spp. is rare, and in most cases a favorable outcome can be expected provided prompt and appropriate veterinary care is obtained.

## AVES

Two genera of passerine birds native to New Guinea, *Pitohui* and *Ifrita*, have poisonous secretions on their feathers, most likely as means of defense against predators (Dumbacher *et al.*, 1992; Weldon, 2000; Dumbacher and Fleischer, 2001). The toxins are present in highest concentrations in the contour feathers of the belly, breast, and legs, with lesser amounts on the head, back, tail, and wing feathers (Weldon, 2000). Several steroidal alkaloids have been isolated from the feathers of these birds, including batrachotoxin and homobatrachotoxin as well as derivatives of these compounds; similar compounds are present on the skin of poison dart frogs (Dendrobatidae) from Central and South America (Daly, 1995b).

The origin of the toxins found in *Pitohui* ("rubbish bird") and *Ifrita* ("bitter bird") is thought to be through accumulation from insects ingested by the birds, rather than by *de novo* synthesis (Dumbacher *et al.*, 2004). High levels of batrachotoxins have been found in beetles of the genus *Choresine* (family Melyridae) that serve as part of the diet of *Pitohui* species. The beetles themselves are unable to synthesize steroidal skeletons and likely accumulate the base steroids from plant phytosterols that they ingest. The beetles then convert the steroids to the steroidal alkaloids and accumulate them within their bodies.

Batrachotoxins act through the opening of sodium channels in nerve and muscle cells, resulting in depolarization and paralysis (Dumbacher *et al.*, 2004). However, exposure of humans to the feathers of *Pitohui* and *Ifrita* species primarily result in respiratory irritation, allergic-type responses, burning sensations of the eyes, skin, and oral mucosa (Dumbacher *et al.*, 2000).

## MAMMALIA

Only a handful of mammals are poisonous or venomous, and toxicosis from exposure to these animals is uncommon. Poisonous mammals include certain carnivores (e.g. polar bears) and pinnipeds (e.g. walruses, seals), whose livers contain high levels of vitamin A; ingestion of these livers may result in acute or chronic hypervitaminosis A (Cleland and Southcott, 1969; Fishman, 2002). Additionally, the meat of marine mammals such as whales may accumulate high levels of organotins, mercury, and other agents that can pose a toxicologic hazard if ingested in sufficient quantity (Endo *et al.*, 2005). Impairment in cell-mediated immunity was found in sled dogs from Greenland that were fed the blubber of minke whales (*Balaenoptera acutorostrata*) (Sonne *et al.*, 2006).

Venomous mammals belong to the orders Monotremata and Insectivora (Fowler, 1993). Venomous Monotremes are the platypus and echidna, of which only the platypus is considered to be of toxicologic significance, as the venom gland in the echidna is non-functional. The male platypus possesses a curved, grooved, conical, sharp spur on the medial aspect of the tarsus. The spur is normally kept retracted against the leg, but when stimulated, muscles cause the spur to project perpendicularly to the leg. The venom gland is kidney shaped and located on the medial aspect of the thigh, and venom is first transported to a reservoir near the spur then is moved through a duct to the spur. Envenomation occurs when the platypus kicks the spur into the victim. Although poorly studied, components identified in platypus venom include a protein similar to natiuretic peptide, a defensin-like peptide, a hyaluronidase, 5-hydroxytryptophan, and histamine (Hodgson, 1997; Torres *et al.*, 2006). Based on reports of human envenomation by platypus, expected signs include intense pain and numbness at the site of the wound and local swelling that may progress proximally (Fowler, 1993). Regional lymph nodes may become enlarged and painful. Humans have reported a feeling of faintness. No reports of human or animal fatality from platypus exist. There are infrequent anecdotal reports of dogs being envenomated by platypus.

Venomous insectivores include various shrews and solenodons, and possibly some moles and hedgehogs (Dufton, 1992). The European hedgehog (*Erinaceus europaes*) has been noted to mix its frothy saliva with toad toxins and smear this mixture over their forward spines. Some moles will store "paralyzed" worms and slugs away, the paralysis thought to be due to paralytic toxins within the mole's saliva. Studying the American short-tailed shrew (*Blarina brevicauda*), the Haitian solenodon (*Solenodon paradoxus*), the European water shrew (*Neomys fodiens*), and the Mediterranean shrew (*N. anomalous*) have obtained more definitive evidence of presence of venoms. These mammals possess three pairs of salivary glands: parotid, retrolingual, and submaxillary. The submaxillary glands are thought to contribute to the bulk of the venom in these species. The Haitian solenodon has grooved incisors that direct the venom to the wound, while the shrews have teeth with concave inner surfaces that may play a similar role as grooves.

Envenomation is achieved by biting and chewing the victim, accompanied by copious salivation. Insectivore venoms contain a variety of peptides and enzymes, and recently a compound isolated from *Blarina* venom has been shown to have kallikrein-like activity (Kita *et al.*, 2005). The venom of *Blarina* appears to be the most toxic, with that of *N. fodiens*, *N. anamalous*, and *Solenodon* being 1/3, 1/6, and 1/20 as toxic, respectively (Dufton, 1992). There is considerable species variation in sensitivity to the venom in shrew and solenodon venom, with voles and rabbits being most sensitive to the effects, and mice, cats, and humans being relatively more resistant (Dufton, 1992). In most natural exposures, the primary effects of shrew and solenodon venoms are related to neurotoxicity. Mice bitten by *Blarina* rapidly develop depression and immobility. When submaxillary gland extracts are injected into experimental animals, depression, irregular respiration, dyspnea, ptosis, rear limb paralysis, convulsions, and death occur. Intravenous injection of 7 mg/kg of *Blarina* toxin extract in a cat resulted at decreases in respiratory and heart rates with concomitant decrease in blood pressure. These effects resolved within 15 min but returned with subsequent doses of 2.5 and 7 mg/kg. Apparent recovery following the final dose was followed by acute respiratory depression and cardiac arrhythmia, and the cat subsequently died. In humans, symptoms of bites from shrews or solenodons result in localized burning sensation surrounding the lower jaw tooth puncture sites and localized swelling. The burning sensation may persist for several days. Systemic toxicosis has not been reported in natural exposures to the bites of shrews or solenodons in humans or domestic animals.

## CONCLUSION

Every phylum of the Animal Kingdom contains animals capable of producing toxic effects, either through envenomation or poisoning. However, only a small number of these animals are sufficiently toxic to be of importance to veterinary clinicians. Arachnids, insects, toads, and snakes cause the most clinically significant problems for domestic and wild animals. Although in some cases specific antidotes (i.e. antivenins) exist for exposures to venomous animals, availability and cost of these antidotes often make their use in veterinary medicine unfeasible. Therefore,

most cases of zootoxicosis in animals will be managed with symptomatic and supportive care.

## REFERENCES


Akre RD Reed HC (2002) Ants, wasps, and bees (Hymenoptera). In *Medical and Veterinary Entomology*, Mullen G, Durden L (eds). Academic Press, New York, pp. 383–410.

Beyer A, Grossman M (1997) Tick paralysis in a red wolf. *J Wildl Dis* **33**: 900–2.

Brubacher JR, Lachmanen D, Ravikumar PR, Hoffman RS (1999) Efficacy of digoxin specific Fab fragments (Digibind) in the treatment of toad venom poisoning. *Toxicon* **37**: 931–42.

Bruchim Y, Ranen E, Saragusty J, Aroch I (2005) Severe tongue necrosis associated with pine processionary moth (*Thaumetopoea wilkinsoni*) ingestion in three dogs. *Toxicon* **45**: 443–7.

Bush SP (2004) Snakebite suction devices don't remove venom: they just suck. *Ann Emerg Med* **43**: 187–8.

Cantrell FL (2003) Envenomation by the Mexican beaded lizard: a case report. *J Toxicol Clin Toxicol* **41**: 241–4.

Chrisman CL, Hopkins AL, Ford SL, Meeks JC (1996) Acute, flaccid quadriplegia in three cats with suspected coral snake envenomation. *J Am Anim Hosp Assoc* **32**: 343–9.

Chong MW, Wong BS, Lam PK, Shaw GR, Seawright AA (2002) Toxicity and uptake mechanism of cylindrospermopsin and lophyrotomin in primary rat hepatocytes. *Toxicon* **40**: 205–11.

Clarke RF, Wethern-Kestner S, Vance MV, Gerkin R (1992) Clinical presentation and treatment of black widow spider envenomation: a review of 163 cases. *Ann Emerg Med* **7**: 782–7.

Cleland JB, Southcott RV (1969) Illnesses following the eating of seal liver in Australian waters. *Med J Aust* **1**: 760–3.

Cowell AK, Cowell RL, Tyler RD, Nieves MA (1991) Severe systemic reactions to Hymenoptera stings in three dogs. *J Am Vet Med Assoc* **198**: 1014–6.

Dalefield RR, Oehme FW (2006) Antidotes for specific poisons. In *Small Animal Toxicology*, 2nd edn, Peterson ME, Talcott PA (eds). Saunders Elsevier, St. Louis, MO, pp. 459–74.

Daly JW (1980) Levels of batrachotoxin and lack of sensitivity to it action in poison-dart frogs (*Phyllobates*). *Science* **208**: 1383–5.

Daly JW (1995a) Alkaloids from frog skins: selective probes for ion channels and nicotinic receptors. *Braz J Med Biol Res* **28**: 1033–42.

Daly JW (1995b) The chemistry of poisons in amphibian skin. *Proc Natl Acad Sci USA* **92**: 9–13.

Daly JW, Kaneko T, Wilhalm J, Garraffo HM, Spande TF, Espinosa A, Davidson WR, Nettles VF, Hayes LE, Howerth EW, Couvillion CE (1992) Diseases diagnosed in gray foxes (*Urocyon cinereoargenteus*) from the Southeastern United States. *J Wildl Dis* **28**: 28–33.

Daly JW, Kaneko T, Wilhalm J, Garraffo HM, Spande TF, Espinosa A, Donnelly MA (2002) Bioactive alkaloids of frog skin: combinatorial bioprospecting reveals that pumiliotoxins have an arthropod source. *Proc Natl Acad Sci USA* **99**: 13996–14001.

Deng F, Fang H, Wang K (1997) Hemolysis of *Scolopendra* toxins. *Zhong Yao Cai* **20**: 36–7.

Denny WF, Dillaha CJ, Morgan PN (1964) Hemotoxic effect of *Loxesceles reclusus* venom: *in vivo* and *in vitro* studies. *J Lab Clin Med* **64**: 291–8.

Dipeolu OO (1976) Tick paralysis in a sheep caused by nymphs of *Amblyomma variegatum*. A preliminary report. *Z Parasitenkd* **10**: 293–5.

Donnelly MA (2002) Bioactive alkaloids of frog skin: combinatorial bioprospecting reveals that pumiliotoxins have an arthropod source. *Proc Natl Acad Sci USA* **99**: 13996–4001.

Dufton MJ (1992) Venomous mammals. *Pharmac Ther* **53**: 199–215.

Dumbacher JP, Fleischer RC (2001) Phylogenetic evidence for colour pattern convergence in toxic pitohuis: mullerian mimicry in birds? *Proc Biol Sci* **268**: 1971–6.

Dumbacher JP, Beehler BM, Spande TF, Garraffo HM, Daly JW (1992) Homobatrachotoxin in the genus *Pitohui*: chemical defense in birds? *Science* **258**: 799–801.

Dumbacher JP, Spande TF, Daly JW (2000) Batrachotoxin alkaloids from passerine birds: a second toxic bird genus (*Ifrita kowaldi*) from New Guinea. *Proc Natl Acad Sci USA* **97**: 12970–5.

Dumbacher JP, Wako A, Derrickson SR, Samuelson A, Spande TF, Daly J W (2004) Melyrid beetles (*Choresine*): a putative source for the batrachotoxin alkaloids found in poison-dart frogs and toxic passerine birds. *Proc Natl Acad Sci USA* **101**: 15857–60.

Durden LA, Mullen GR (2002) Introduction. In *Medical and Veterinary Entomology*, Mullen G, Durden L (eds). Academic Press, New York, pp. 1–8.

Dutra F, Riet-Correa F, Mendez MC, Paiva N (1997) Poisoning of cattle and sheep in Uruguay by sawfly (*Perreyia flavipes*) larvae. *Vet Hum Toxicol* **39**: 281–6.

Dziezyc J (1992) Insect defensive spray-induced keratitis in a dog. *J Am Vet Med Assoc* **200**: 1019.

Endo T, Hotta Y, Haraguchi K, Sakata M (2005) Distribution and toxicity of mercury in rats after oral administration of mercury-contaminated whale red meat marketed for human consumption. *Chemosphere* **61**: 1069–73.

Eubig PA (2001) *Bufo* species toxicosis: big toad, big problem. *Vet Med* **96**: 594–9.

Fishman RA (2002) Polar bear liver, vitamin A, aquaporins, and pseudotumor cerebri. *Ann Neurol* **52**: 531–3.

Fowler ME (1993) *Veterinary Zootoxicology*. CRC Press, Boca Raton, FL.

Futrell JM, Morgan BB, Morgan PN (1979) An *in vitro* model for studying hemolysis associated with venom from the brown recluse spider (*Loxesceles reclusa*). *Toxicon* **17**: 355–62.

Goddard J (2003) *Physician's Guide to Arthropods of Medical Importance*, 4th edn. CRC Press, New York.

Grattan-Smith PJ, Morris JG, Johnston HM, Yiannikas C, Malik R, Russell R, Ouvrier RA (1997) Clinical and neurophysiological features of tick paralysis. *Brain* **120**: 1975–87.

Grundemar L, Hogestatt ED (1990) Vascular effects of helodermin, helospectin I and helospectin II: a comparison with vasoactive intestinal peptide. *Br J Pharmacol* **99**: 526–8.

Gwaltney-Brant SM, Rumbeiha WK (2002) Newer antidotal therapies. *Vet Clin Small Anim* **32**: 323–39.

Hackett TB, Wingfield WE, Mazzaferro EM, Benedetti JS (2002) Clinical findings associated with prairie rattlesnake bites in dogs: 100 cases (1989–1998). *J Am Vet Med Assoc* **220**: 1675–80.

Hoenerhoff M, Rumbeiha WK, Patterson JS, Thorpe T (2002) Small intestinal hemorrhage associated with *Harmonia axyridis* (Asian ladybeetle) ingestion in a dog. *Proceedings of the American Association of Veterinary Laboratory Diagnosticians 45th Annual Conference*, St. Louis, MO, p. 36.

Hodgson WC (1997) Pharmacological action of Australian animal venoms. *Clin Exp Pharmacol Physiol* **24**: 10–7.

Huang TF, Chiang HS (1994). Effect on human platelet aggregation of phospholipase A2 purified from *Heloderma horridum* (beaded lizard) venom. *Biochem Biophys Acta* **1211**: 61–8.

Isbister GK, Seymour JE, Gray MR, Raven RJ (2003) Bite by spiders of the family Theraphosidae in humans and canines. *Toxicon* **41**: 519–24.

Keegan HL (1980) *Scorpions of Medical Importance*. University Press of Mississippi, Jackson, MS.

Kita M, Okumura Y, Ohdachi SD, Oba Y, Yoshikuni M, Nakamura Y, Kido H, Uemura D (2005) Purification and characterisation of blarinasin, a new tissue kallikrein-like protease from the short-tailed shrew *Blarina brevicauda*: comparative studies with blarina toxin. *Biol Chem* **386**: 177–82.

Knight M, Glor R, Smedley SR, Gonz·les, Adler K, Eisner T (1999) Firefly toxicosis in lizards. *J Chem Ecol* **62**: 378–80.

Krinsky WL (2002) Beetles (*Coleoptera*). In *Medical and Veterinary Entomology*, Mullen G, Durden L (eds). Academic Press, New York, pp. 87–100.

Lucas SM, Meier J (1995a) Biology and distribution of scorpions of medical importance. In *Handbook of Clinical Toxicology of Animal Venoms and Poisons*, Meier J, White J (eds). CRC Press, New York, pp. 239–58.

Lucas SM, Meier J (1995b) Biology and distribution of spiders of medical importance. In *Handbook of Clinical Toxicology of Animal Venoms and Poisons*, Meier J, White J (eds). CRC Press, New York, pp. 205–20.

Luttrell MP, Creekmore LH, Mertins JW (1996) Avian tick paralysis caused by *Ixodes brunneus* in the Southeastern United States. *J Wildl Dis* **32**: 133–6.

Masina S, Broady KW (1999) Tick paralysis: development of a vaccine. *Int J Parasitol* **29**: 535–41.

Meier J (1995) Venomous and poisonous animals – a biologist's view. In *Handbook of Clinical Toxicology of Animal Venoms and Poisons*, Meier J, White J (eds). CRC Press, Boca Raton, FL, pp. 1–8.

Mullen GR (2002a) Moths and butterflies (*Lepidoptera*). In *Medical and Veterinary Entomology*, Mullen G, Durden L (eds). Academic Press, New York, pp. 363–81.

Mullen GR (2002b) Spiders (*Araneae*). In *Medical and Veterinary Entomology*, Mullen G, Durden L (eds). Academic Press, New York, pp. 427–48.

Mullen GR, Stockwell SA (2002) Scorpions (*Scorpiones*). In *Medical and Veterinary Entomology*, Mullen G, Durden L (eds). Academic Press, New York, pp. 411–23.

Noble SJ, Armstrong PJ (1999) Bee sting envenomation resulting in secondary immune-mediated hemolytic anemia in two dogs. *J Am Vet Med Assoc* **214**: 1021, 1026–7.

Norris R (2004) Centipede Envenomations. Emedicine website, http://www.emedicine.com/EMERG/topic89.htm

Oelrichs PB, Vallely PJ, Macleod JK, Cable J, Kiely DE, Summons RE (1977) Lophyrotomin, a new toxic octapeptide from the larvae of sawfly, *Lophyrotoma interrupta*. *Lloydia* **40**: 209–14.

Oelrichs PB, MacLeod JK, Seawright AA, Moore MR, Ng JC, Dutra F, Riet-Correa F, Mendez MC, Thamsborg SM (1999) Unique toxic peptides isolated from sawfly larvae in three continents. *Toxicon* **37**: 537–44.

Oelrichs PB, MacLeod JK, Seawright AA, Grace PB (2001) Isolation and identification of the toxic peptides from *Lophyrotoma zonalis* (Pergidae) sawfly larvae. *Toxicon* **39**: 1933–6.

Peterson ME (2004) Reptiles. In *Clinical Veterinary Toxicology*, Plumlee KH (ed.). Mosby, St. Louis, MO, pp. 104–11.

Peterson ME (2006) Poisonous lizards. In *Small Animal Toxicology*, 2nd edn, Peterson ME, Talcott PA (eds). Saunders, St. Louis, MO, pp. 812–16.

Peterson ME, McNalley J (2006a) Spider envenomation: black widow. In *Small Animal Toxicology*, 2nd edn, Peterson ME, Talcott PA (eds). Saunders Elsevier, St. Louis, MO, pp. 1063–69.

Peterson ME, McNalley J (2006b) Spider envenomation: brown recluse. In *Small Animal Toxicology*, 2nd edn, Peterson ME, Talcott PA (eds). Saunders Elsevier, St. Louis, MO, pp. 1070–75.

Rakich PM, Latimer KS, Mispagel ME, Steffens WL (1993) Clinical and histologic characterization of cutaneous reactions to stings of the imported fire ant (*Solenopsis invicta*). *Vet Pathol* **30**: 555–9.

Roder JD (2004) Toads. In *Clinical Veterinary Toxicology*, Plumlee KH (ed.). Mosby, St. Louis, MO, p. 113.

Russell FE (1996) Toxic effects of animal toxins In *Casarett and Doull's Toxicology: The Basic Science of Poisons*, 5th edn, Klassen CD (ed.). McGraw-Hill, New York, pp. 801–39.

Russell FE (2001) Toxic effects of terrestrial animal venoms and poisons. In *Casarett and Doull's Toxicology: The Basic Science of Poisons*, 6th edn, Klassen CD (ed.). McGraw-Hill, New York, pp. 945–64.

Saporito RA, Garraffo HM, Donnelly MA, Edwards AL, Longino JT, Daly JW (2004) Formicine ants: an arthropod source for the pumiliotoxin alkaloids of dendrobatid poison frogs. *Proc Natl Acad Sci USA* **101**: 8045–50.

Saravanan R, King R, White J (2004) Transient claw hand owing to a bee sting. A report of two cases. *J Bone Joint Surg Br* **86**: 404–5.

Schmidt JO (1995) Toxinology of venoms from the honeybee genus *Apis*. *Toxicon* **33**: 917–27.

Sonenshine DE, Lane RS, Nicholson WL (2002) Ticks (*Ixodida*). In *Medical and Veterinary Entomology*, Mullen G, Durden L (eds). Academic Press, New York, pp. 517–58.

Sonne C, Dietz R, Larsen HJ, Loft KE, Kirkegaard M, Letcher RJ, Shahmiri S, Moller P (2006) Impairment of cellular immunity in West Greenland sledge dogs (*Canis familiaris)* dietary exposed to polluted minke whale (*Balaenoptera acutorostrata*) blubber. *Environ Sci Technol* **40**: 2056–62.

Stair EL, Plumlee KH (2004) Blister beetles. In *Clinical Veterinary Toxicology*, Plumlee KH (ed.). Mosby, St. Louis, MO, pp. 101–3.

Stewart JW (1985) Centipedes and millipedes. *Texas Agricultural Extension Service Bulletin L-1747*. Texas A&M University System, http://insects.tamu.edu/extension/bulletins/l-1747.html

Sutherland SK, Tibballs J (2001) *Australian Animal Toxins: The Creatures, Their Toxins and Care of the Poisoned Patient.* Oxford University Press, Oxford.

Takada W, Sakata T, Shimano S, Enami Y, Mori N, Nishida R, Kuwahara Y (2005) Scheloribatid mites as the source of pumiliotoxins in dendrobatid frogs. *J Chem Ecol* **31**: 2403–15.

Thamsborg SM, Jorgensen RJ, Brummerstedt E (1987) Sawfly poisoning in sheep and goats. *Vet Rec* **121**: 253–5.

Thamsborg SM, Jorgensen RJ, Brummerstedt E, Bjerregard J (1996) Putative effect of silymarin on sawfly (*Arge pullata*)-induced hepatotoxicosis in sheep. *Vet Hum Toxicol* **38**: 89–91.

Torres AM, Tsampazi M, Tsampazi C, Kennet EC, Belov K, Geraghty DP, Bansal PS, Alewood PF, Kuchel PW (2006) Mammalian L-to-D-amino-acid-residue isomerase from platypus venom. *FEBS Lett* **580**: 1587–91.

Twedt DC, Cuddon PA, Horn TW (1999) Black widow spider envenomation in a cat. *J Vet Int Med* **13**: 613–6.

Utaisincharoen P, Mackessy SP, Miller R, Al, Tu AT (1993) Complete primary structure and biochemical properties of gilatoxin, a serine protease with kallikrein-like and angiotensin-degrading activities. *J Biol Chem* **268**: 21973–5.

Walker T, Tidwell AS, Rozanski EA, DeLaforcade A, Hoffman AM (2005) Imaging diagnosis: acute lung injury following massive bee envenomation in a dog. *Vet Radiol Ultrasound* **46**: 300–3.

Wallis DM (2005) Rattlesnake vaccine to prevent envenomation toxicity in dogs. *Proceedings of the 77th Annual Western Veterinary Conference*, Las Vegas.

Weldon PJ (2000) Avian chemical defense: toxic birds not of a feather. *Proc Natl Acad Sci USA* **97**: 12948–9.

White JW, Cardoso JL, Fan HW (1995) Clinical toxicology of spider bites. In *Handbook of Clinical Toxicology of Animal Venoms and Poisons*, Meier J, White J (eds). CRC Press, New York, pp. 259–329.

Wysoke JM, Bland VDBP, Marshall C (1990) Bee sting-induced haemolysis, spherocytosis and neural dysfunction in three dogs. *J South Afr Vet Assoc* **61**: 29–32.

Yeruham I, Braverman Y, Schwimmer A (1998) Wasps are the cause of an increasing mastitis problem in dairy cattle in Israel. *Vet Q* **20**: 111–4.

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# Part 13

## Estrogenic Toxicants

C H A P T E R

# 65

# Chemical-induced estrogenicity

*Stephen H. Safe, Shaheen Khan, Fei Wu, and Xiangrong Li*

## INTRODUCTION

17β-estradiol (E2) and related steroidal hormones play an important role in multiple physiological processes. However, these hormones are risk factors for hormone-dependent diseases including breast and endometrial cancer. Inappropriate exposures (high or low) to estrogens can also lead to adverse health effects. The identification of estrogenic compounds in the environment, coupled with human exposures to these compounds has generated public, regulatory and scientific concern regarding their potential hormonal toxicity to humans and wildlife. This chapter is focused on chemical-induced estrogenicity and the potential toxicological impacts of these compounds.

## ESTROGENS PLAY A ROLE IN NORMAL PHYSIOLOGICAL FUNCTION AND DISEASE

### Background

Estrogens regulate the development and function of the female reproductive system and their effects are mediated through estrogen receptor $\alpha$ (ER$\alpha$) and ER$\beta$. However, estrogens are by no means only female hormones. In both males and females, estrogens play crucial physiological roles, including sex differentiation, bone development and maintenance, central nervous system and cardiovascular function (Martinez-Vargas *et al.*, 1975; Ansar Ahmed *et al.*, 1989; Maggi *et al.*, 1989; Smith *et al.*, 1994; Brosnihan *et al.*, 1997; McEwen and Alves, 1999). The ER is important for mammary gland development and ER$\alpha$ knockout ($\alpha$ERKO) mice are viable but sterile and are estrogen-insensitive in several estrogen target organs, including mammary glands, reproductive tracts and gonads. The well-recognized estrogenic responses, such as uterine weight increase and vaginal cornification, are all absent in $\alpha$ERKO mice (Lubahn *et al.*, 1993; Korach, 1994). In $\alpha$ERKO mice, mammary glands undergo normal development in the prenatal period, but fail to develop terminal end buds and are severely undertropic during adulthood (Lubahn *et al.*, 1993; Korach, 1994). No significant ovarian abnormalities were detected in neonatal $\alpha$ERKO mice, but ovaries in the mature $\alpha$ERKO mice have disrupted ovulation and luteinization and develop multiple hemorrhagic cysts (Schomberg *et al.*, 1999). Surprisingly, adult male $\alpha$ERKO mice are also infertile because of impaired spermatogenesis (Eddy *et al.*, 1996). Similar results were observed in male mice with disrupted aromatase, an enzyme essential for estrogen synthesis (Robertson *et al.*, 1999). In contrast to the extensive reproductive tract abnormalities in $\alpha$ERKO mice, ER$\beta$ knockout ($\beta$ERKO) mice are fertile and have no significant abnormalities except reduced fertility (Korach, 1994; Krege *et al.*, 1998).

### Estrogens and women's health

Women have significantly lower risk of cardiovascular disease than men and this has been putatively attributed to the beneficial effects of estrogen on cardiovascular function (Nathan and Chaudhuri, 1997). Estrogen decreases low-density lipoprotein (LDL) level and increases high-density lipoprotein (HDL) levels (Bush, 1990; Knopp *et al.*, 1996). Estrogen exhibits vasodilatory effects and protects against vascular injury (Pare *et al.*, 2002) and also induces COX-2-dependent upregulation of atheroprotective prostacyclin $PGI_2$ (Egan *et al.*, 2004). In epidemiological studies, estrogen has been linked with improved cardiovascular functions (Grodstein *et al.*, 1996). Bilateral ovariectomy before

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menopause elevates the risk for coronary heart disease (CHD) and the increased risk is eliminated by estrogen-replacement therapy (Colditz *et al.*, 1987). However, a large clinical trial conducted by Women's Health Initiative has reported contradictory results on the effects of estrogens since women taking hormone replacement therapies (HRT) have an increased risk for heart diseases and stroke (Rossouw *et al.*, 2002). The relationships between estrogen and cardiovascular diseases require further assessment.

Estrogen is also important for bone development and maintenance. Before and during puberty, estrogen stimulates bone growth in both males and females and may be the major driving force of pubertal growth spurt (Cutler, 1997). In adulthood, estrogen is crucial for maintaining bone density. A young adult male with homozygous disrupted ER$\alpha$ gene has normal masculinization, but incomplete epiphyseal closure, tall stature and decreased bone density (Sudhir *et al.*, 1997). In premenopausal women, estrogen exposure is strongly associated with bone mass (Armamento-Villareal *et al.*, 1992). The deficiency of estrogen in postmenopausal women leads to an increased risk of osteoporosis and increased estrogen intake can alleviate bone loss (Ettinger *et al.*, 1985). The Women's Health Initiative also confirmed that HRT protect against hip fracture in postmenopausal women (Rossouw *et al.*, 2002).

Estrogen also plays important roles in sexual differentiation in the brain and reproductive behavior (Breedlove, 1992; Bakker *et al.*, 2003) and sustained estrogen treatment improves learning ability, memory and fine motor skills in animal models (O'Neal *et al.*, 1996; Lacreuse and Herndon, 2003). However, results of the Women's Health Initiative show that the use of HRT can lead to increased risks for heart disease and stroke. There is concern that these estrogens may induce some adverse neurological responses (Rossouw *et al.*, 2002; Shumaker *et al.*, 2003; Espeland *et al.*, 2004). The most well-characterized adverse responses of endogenous and exogenous estrogens is associated with their effects on both breast and endometrial cancer in women (Bernstein, 2002; Hilakivi-Clarke, 2002). The potential effects of synthetic estrogenic compounds on breast cancer risk stems for extensive research and epidemiological studies on this disease. In addition there is also concern that fetal or early postnatal exposure of males to estrogens will affect the male reproductive tract functions in adults (Skakkebaek *et al.*, 2001).

## ESTROGENS AS A RISK FACTOR FOR BREAST CANCER

### Genetic factors

There are several different risk factors for breast cancer and there is a strong interplay of genetic and environmental factors in the initiation and progression of this disease (Martin and Weber, 2000). Breast cancer risk increases with age and 77% of women diagnosed with breast cancer are older than 50. Risk factors can be broadly classified as genetic, hormonal, environmental and other lifestyle factors. Five to ten percent of all breast cancers are associated with the inheritance of mutations in one of the two major breast cancer susceptibility genes, BRCA1 and BRCA2 (Struewing *et al.*, 1996; Peto *et al.*, 1999). There is an 80% chance of developing breast cancer during a lifetime in women with an inherited BRCA1 or BRCA2 mutation (Rebbeck *et al.*, 1996; Nathanson *et al.*, 2001). BRCA1 and BRCA2 are tumor suppressor genes and their proteins have been implicated in a multitude of different processes including DNA repair and recombination, cell cycle control and transcription (Venkitaraman, 2002). Several other genetic mutation also contribute to the risk in breast cancer and these include genes such as p53 which also enhance risk for multiple cancers.

### Hormonal factors

Several studies have shown that prolonged exposure to the hormone estrogen increases the risk of breast cancer. Epidemiological studies have established a strong link between higher risk of breast cancer and reproductive factors that increase the overall number of menstrual cycles such as early menarche (before age 12), late menopause (after age 55), age of women at first birth (over 30–35) and nulliparity (Russo *et al.*, 1992; Talamini *et al.*, 1996). Breast cancer risk is lower in women with multiple pregnancies and women with a pregnancy prior to age 24 (Lambe *et al.*, 1996; Meeske *et al.*, 2004). Women who are above 30–35 years of age at first birth are at higher risk compared to nulliparous women (Albrektsen *et al.*, 1994; Rosner *et al.*, 1994). The protective effects of pregnancy against breast cancer is explained by the induction of complete differentiation of the breast that may markedly reduce the carcinogen susceptibility of the fully differentiated mammary gland to due, at least in part, to the decreased proliferative activity of parous epithelium (Russo *et al.*, 2000). Another hypothesis is that the decreased risk may also be due to the altered hormonal environment during pregnancy, and these include specific molecular changes induced by estrogen and progesterone and decreased circulating growth hormone (Sivaraman and Medina, 2002; Russo *et al.*, 2005). Breastfeeding is also protective against breast cancer and this may be due to the reduction of total number of ovulatory menstrual cycles and consequently cumulative ovarian hormone exposure (Enger *et al.*, 1997; Lipworth *et al.*, 2000; Russo *et al.*, 2000; Lee *et al.*, 2003). Recent studies have shown that the use of HRT and oral contraceptives for long time periods can also increase the risk of developing breast cancer (Althuis *et al.*, 2003; Ewertz *et al.*, 2005).

In some studies women exposed to diethylstilbestrol (DES) were also found to be at slightly increased breast cancer risk (Titus-Ernstoff *et al.*, 2001; Sasco *et al.*, 2003).

### Lifestyle and dietary risk factors

Ionizing radiation is the most well-characterized environmental risk factor for breast cancer. Radiation-induced breast cancer risk depends on the various factors including age at exposure (highest before age 30 years), the status of hormone levels, parity and other genetic disorders (Coyle, 2004). A number of reports showed that patients who received radiation therapy for Hodgkin's disease, breast cancer and other illnesses, had an increased risk of breast cancer and women in Japan exposed to atomic bomb radiation also have a high rate of breast cancer (Hancock *et al.*, 1993; Goodman *et al.*, 1997; Land *et al.*, 2003). Other risk factors include solar radiation, light and chemicals. Solar radiation creates an active form of vitamin D that may lower the risk of breast cancer and studies show that women who work at nights are at higher breast cancer risk. This may be due to decreased vitamin D synthesis and suppression of normal nocturnal production of melatonin by the pineal gland, increases the estrogen release by the ovaries thereby disrupting circadian patterns (Garland *et al.*, 1990; Davis *et al.*, 2001; Schernhammer *et al.*, 2001; Grant, 2002).

Various lifestyle factors such as diet, exercise, smoking and alcohol consumption are related to an increased risk of developing breast cancer (Key *et al.*, 2003). Confirmation of the risk of dietary fat intake and breast cancer has not been substantiated in large epidemiology studies (Velie *et al.*, 2000; Smith-Warner *et al.*, 2001), however a dietary pattern of high fiber and low fat intakes is associated with a lower risk of breast cancer in postmenopausal women (Baghurst and Rohan, 1994; Mattisson *et al.*, 2004; Saadatian-Elahi *et al.*, 2004). In some studies protective effects of some vegetable fats, vitamin E, selenium and other antioxidants have been observed (Gerber *et al.*, 2003; Gaudet *et al.*, 2004). Decreased ovarian hormone levels decreased the risk of breast cancer in populations in Asia and is related to their high consumption of soya products containing significant amount of the isoflavones, daidzein and genistein, that act as weak estrogens (Mezzetti *et al.*, 1998; Lu *et al.*, 2000).

## ESTROGENS AS RISK FACTORS FOR MALE REPRODUCTIVE TRACT PROBLEMS

Although estrogens are female steroid hormones and influence normal physiology and disease in women, there has been increasing concern on the potential adverse effect of estrogenic compounds on development of the male reproductive tract (Sharpe and Skakkebaek, 1993). Support for the hypothesis that "environmental estrogens" (xenoestrogens) and other endocrine disrupting compounds are responsible for an increase in male reproductive tract problems is derived from laboratory animal, wildlife and human studies. For example, there are numerous reports of feminized fish in river systems, particularly those in the United Kingdom and this has been linked to their exposures to endogenous estrogens (17$\beta$-estradiol/estrone), synthetic contraceptives (17-ethinylestradiol) and industrial by-products such as alkylphenols (Desbrow *et al.*, 1998; Routledge *et al.*, 1998). There is some evidence that nonylphenol and alkylphenols may contribute to estrogenization of fish near sewage outflows.

It has also been suggested that the major concern regarding environmental/dietary estrogens is *in utero* or early postnatal exposures during the important initial periods of male reproductive tract development (Skakkebaek *et al.*, 2001). The potent estrogenic drug DES was extensively used by pregnant women with disastrous consequences for their male and female offspring (Giusti *et al.*, 1995). Females exposed *in utero* develop a high incidence of a rare vaginal adenocarcinoma whereas male offsprings developed a range of responses including deformed genitalia. The effects by DES form one of the bases of the endocrine disruption hypothesis and the opinion that a testicular dysgenesis syndrome which includes low sperm counts, increased hypospadias and cryptorchidism and testicular cancer my be linked to *in utero* exposure to estrogenic compounds and other endocrine disrupting compounds (Skakkeback *et al.*, 2001). This opinion and hypothesis have been challenged (Safe, 2000; Handelsman, 2001). However, it has become increasingly clear that humans are exposed to a wide range of xenoestrogens and phytoestrogens which constitute the major subclass of endocrine disruptors of concern. The following section describes several different structural classes of synthetic estrogenic compounds, phytoestrogens and estrogens/antiestrogens such as DES and tamoxifen that have been used as pharmaceutical agents. In addition the mechanism of estrogen action and implications for risk assessment of these compounds will also be discussed.

## ESTROGENIC CHEMICALS OF CONCERN

The hypothesis that environmental/dietary estrogens may play a role in male reproductive tract problems and the increased incidence of breast cancer is controversial and the significance of these compounds on human health is not

resolved. Nevertheless this resulted in legislation in the United States requiring the Environmental Protection Agency to develop testing procedures for examining industrial compounds for their activity as estrogens/antiestrogens, androgens/antiandrogens and thyroid hormone mimics. Initial screening studies for estrogens used the MCF-7 cell proliferation assay (E-screen) (Sonnenschein and Soto, 1998) and this was complemented by development of receptor binding and transactivation studies in various cell lines. All of these assays have advantages and disadvantages and for the most part give complementary results on the estrogenic activity of individual compounds.

Steroidal and non-steroidal estrogens and antiestrogens have been developed as pharmacologic agents and Figure 65.1 illustrates the structures of 17$\beta$-estradiol, the endogenous hormone, DES and three clinically used antiestrogens, tamoxifen, ICI 182780 (fulvestrant) and raloxifene (Jordan, 2003a, b). It is well known that phytoestrogenic compounds in the diet also constitute a major source of exposure to estrogens and these compounds are present in fruits, nuts and vegetables and are particularly high in many soya products which are enriched in isoflavonoids (Havsteen, 2002). Figure 65.2 shows the structures of genistein, a major isoflavonoid in soy, equol a genistein metabolite and naringenin a flavonoid which is found in grapefruit. Genistein and equol can be detected in human serum and urine along with the estrogenic lignans enterodiol and enterolactone and these compounds are used as biomarkers of exposure to dietary phytoestrogenic compounds. Resveratrol is an estrogenic polyphenolic stilbene analog found in grapes and wine.

The list of synthetic estrogenic compounds continues to grow as testing of current and new synthetic industrial chemicals progresses and expands. A major class of xenoestrogens of concern are the alkyl phenols such as nonylphenol which are widely used in industry as ethoxylates (Soto *et al.*, 1991). These compounds are surfactants and

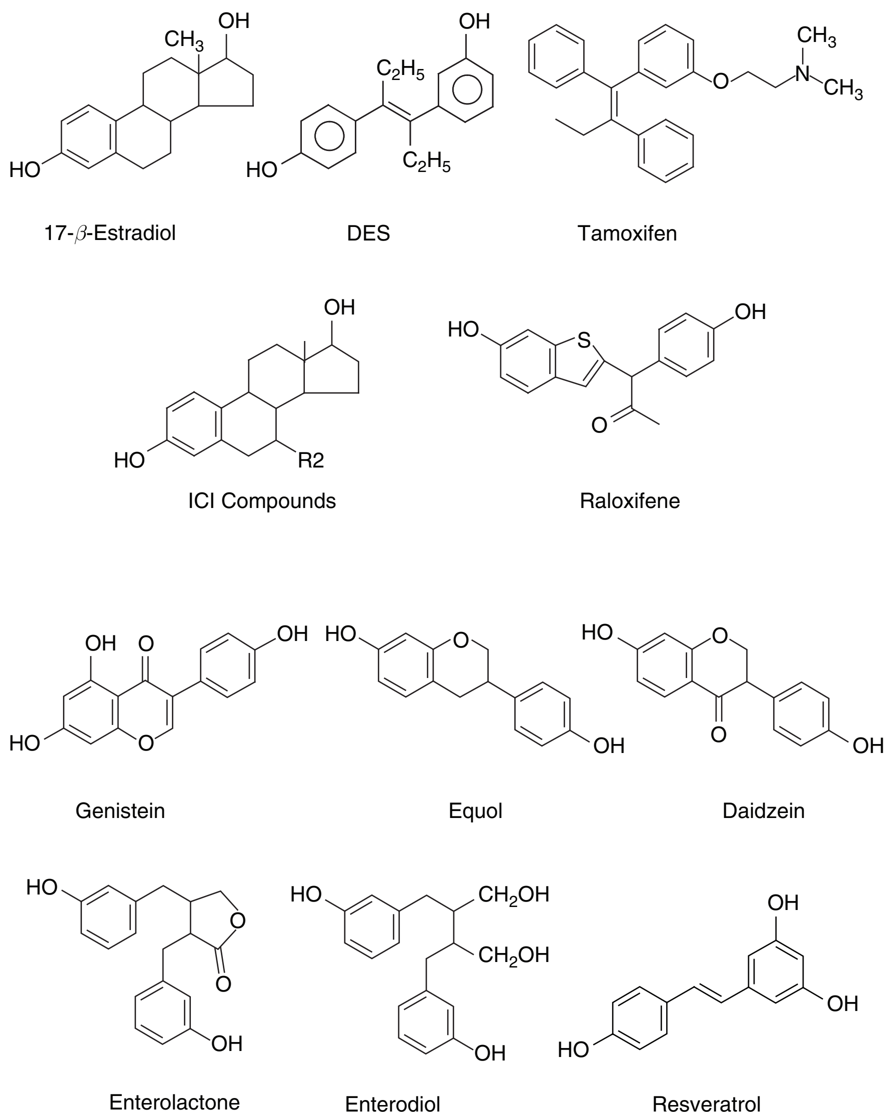


**FIGURE 65.1** Structures of 17$\beta$-estradiol, DES and the clinically used SERMs tamoxifen, ICI compounds (R = $C_{14}H_{24}F_5OS$, fulvestrant), and raloxifene.

**FIGURE 65.2** Structures of the phytoestrogenic flavonoids (genistein, equol and daidzein), lignans (enterolactone and enterodiol) and resveratrol.

are used in detergents, paints, herbicide/pesticide formulation and plastics. Alkyl hydroxybenzoates (parabens) (Figure 65.3) used as preservative agents and cosmetic UV filters such as 4-methylbenzylidene camphor that are used in homecare products also exhibit estrogenic activity (Schlumpf *et al.*, 2004; Schreurs *et al.*, 2005). Bisphenol A (BPA) is perhaps one of the most controversial xenoestrogens since it is extensively used in plastic production and plastic lines in which trace levels of BPA have leached directly into food products (Krishnan *et al.*, 1993). This estrogenic compound has also been identified in the environment. Other estrogens of concern include polychlorinated biphenyls (PCBs) and hydroxy-PCBs which have been identified in the environment and in humans; the pesticide metabolite 1,1-bis(*p*-chlorophenyl)-2,2-dichloroethylene (DDE) and kepone (Kutz *et al.*, 1991; Bergman *et al.*, 1994). These compounds are persistent environmental contaminants and kepone-induced estrogenic poisoning in a group of exposed production workers. The compounds illustrated in Figure 65.4 exhibit relatively weak binding to the ER and induce reporter gene expression in transactivation assays in cell culture systems and some of these compounds exhibit uterotrophic effects in female rodents. The important toxicological question concerning these chemical estrogens is whether they differ only in potency which is governed by their intrinsic estrogenicity and bioavailability based on their metabolism and pharmacokinetics. On the other hand, since the estrogenic activity of these compounds is receptor dependent (ER), is the estrogenic activity of structurally diverse xenoestrogens more unique and not necessarily governed by intrinsic ER binding affinities and pharmacodynamic factors?

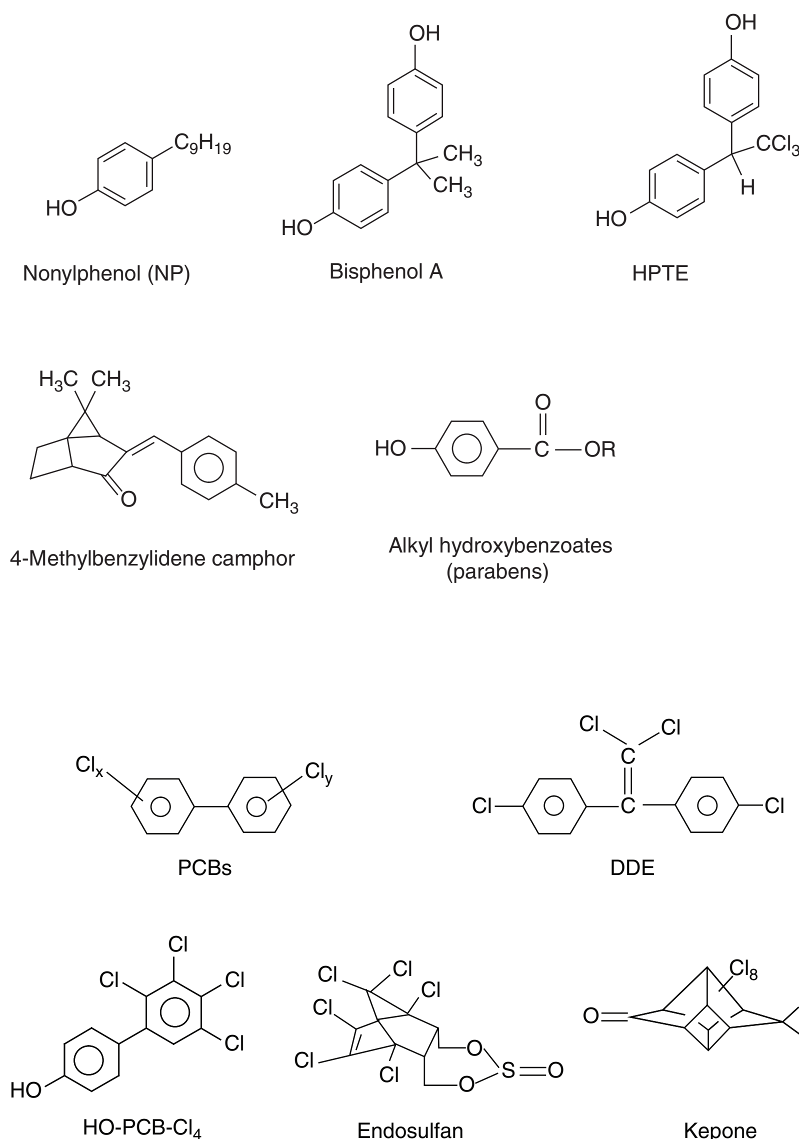


**FIGURE 65.3** Structures of the aromatic xenoestrogens nonylphenol, bisphenol A, HPTE, 4-methylbenzylidene camphor and parabens.

**FIGURE 65.4** Structures of the organochlorine xenoestrogens PCBs, DDE, hydroxyl-PCB, endosulfan and kepone.

# MECHANISMS OF ESTROGEN-INDUCED TRANSACTIVATION

## ER$\alpha$ and ER$\beta$ domain structure

The ER is a member of the nuclear receptor superfamily of transcription factors which exhibit modular structures and similar mechanisms of action. For example, the transactivation function of ER is mediated by two separate but not mutually exclusive transactivation domains namely, ligand-independent activation function (AF) 1 (within the A/B) and ligand-dependent AF-2 (within the ligand binding domain, LBD) (Tora *et al.*, 1989) (Figure 65.5). The A/B domain is the least conserved region between ER$\alpha$ and ER$\beta$ with only 30% similarity at the amino acid level. The activity of AF-1 in ER$\beta$ is negligible on estrogen-responsive element (ERE) reporter constructs compared to the AF-1 of ER$\alpha$ in several different cell lines (Cowley and Parker, 1999).

ER$\alpha$ and ER$\beta$ also exhibit distinctive responses to the synthetic antiestrogens such as tamoxifen and raloxifene which act as partial ER agonists for ER$\alpha$ and as pure ER antagonists for ER$\beta$ (Barkhem *et al.*, 1998). The functional differences between the respective A/B regions of ER$\alpha$ and ER$\beta$ may explain their differences in ligand-dependent activation (Matthews and Gustafsson, 2003). The AF-1 region of ER$\alpha$ interacts with different transcriptional regulators and co-activators that affect ligand-independent transactivation. The activity of AF-1 is also regulated through kinase-dependent phosphorylation and the individual pathways involved vary with cell and promoter context (Tzukerman *et al.*, 1994). In most cell lines, both AFs act synergistically to attain maximum transcriptional activity while in other cells only one AF may be activated (Benecke *et al.*, 2000).

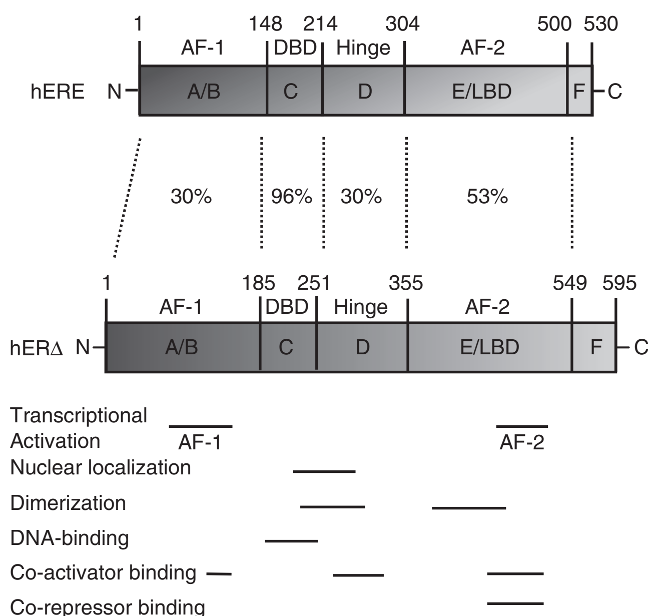


**FIGURE 65.5** Structural domains of human ER$\alpha$ and ER$\beta$. The percent identity between the individual domains at the amino acid level is indicated (modified from Pearce and Jordan (2004) and Koehler *et al.* (2005)).

The DNA binding domain (DBD) (region C) is highly conserved between ER$\alpha$ and ER$\beta$ and exhibits 96% identity. This domain contains two zinc finger (CI and CII) as observed for other nuclear receptors. The DBD of both ER$\alpha$ and ER$\beta$ bind with high affinity to EREs (Mosselman *et al.*, 1996; Pettersson *et al.*, 1997). The minimal consensus ERE sequence is a palindromic inverted repeat (IR): 5′-GGTCAnnnTGACC-3′. Extension of the length of the ERE palindrome, e.g. 5′-CAGGTCAnnnTGACCTG-3′, forming a 17-bp palindromic IR, and the sequences immediately flanking the ERE are important for determining ER binding affinities for this motif (Klinge, 2001). The hinge region or D domain is a 40–50 aa sequence separating the DBD and LBD and is not well conserved between the two receptors. The C-terminal E/F region encompasses the LBD, a co-regulator binding surface, a dimerization domain, another nuclear localization signal and AF-2 (Nilsson *et al.*, 2001). Significant homology between the two receptors exists in the E/F region and both proteins display essentially the same binding affinity for E2 and many other estrogenic compounds (Kuiper *et al.*, 1997). However, the two receptors differ in their binding affinities for only a few ligands including antiestrogens and phytoestrogens. For example, the phytoestrogen geneistein binds with approximately a 30-fold higher affinity for ER$\beta$ than ER$\alpha$ (Barkhem *et al.*, 1998).

## X-ray crystallographic studies

Crystallographic studies with the LBDs of ER$\alpha$ and ER$\beta$ revealed that both ER$\alpha$ and ER$\beta$ share a similar overall architecture. The AF-2 interaction surface is composed of amino acids in helix 3, 4, 5 and 12 and the position of helix 12 is altered upon ligand binding. Amino acids within helices 3, 5 and 11 are important for ligand binding since mutation of these residues significantly decrease the binding affinity for E2 (Wurtz *et al.*, 1998). Deletion and mutation analysis have revealed that ER dimerization is mediated through helices 7–10 (Fawell *et al.*, 1990; Lees *et al.*, 1990). Crystal structures of the ER-LBD complexed with E2, DES, the antiestrogens raloxifene and tamoxifen, and the pure antiestrogen ICI 164,384, an analog of fulvestrant (ICI 182,780), have been determined (Brzozowski *et al.*, 1997; Shiau *et al.*, 1998; Pike *et al.*, 1999; Pike *et al.*, 2001). These structures reveal critical information regarding the agonist and antagonist activity of various ligands and showed that the structure of helix 12 with AF-2 is sensitive to ligand binding. For example, when the ER$\alpha$ LBD is complexed

with agonists such as E2, helix 12 is repositioned over the ligand binding pocket and it generates a functional AF-2 that interacts with LXXL motifs of co-activators. In contrast, binding of antagonists such as raloxifene or tamoxifen with ERα-LBD results in displacement of helix 12 from its agonist positioning and this helix is repositioned into the hydrophobic groove formed by helices 3, 4 and 5. This disrupts formation of the co-activator interaction surface (Shiau *et al.*, 1998; Pike *et al.*, 1999) and the ligand-dependent effects of helix 12 positioning is dependent on the agonistic or antagonistic activity of various ER ligands (Brzozowski *et al.*, 1997; Pike *et al.*, 2001).

# XENOESTROGENS AND PHYTOESTROGENS AS SELECTIVE ER MODULATORS

## Complexity of estrogenic activity

Results of X-ray crystallographic analysis clearly demonstrates that both ER agonists differentially bind the ER and induce compound-specific changes in the bound complex. Although tamoxifen and E2 induce distinct conformations of the ER and exhibit antiestrogenic and estrogenic activity in breast cancer, these compounds both induce ER-dependent activity in the uterus and prolonged treatment with tamoxifen increases the risk for endometrial cancer (Bernstein *et al.*, 2002). Tamoxifen is also an ER agonist in the bone and vascular system and there are tissue- and species-specific ER agonist or antagonist activities in animal models where tamoxifen is an ER antagonist in chicks, a partial ER agonist/antagonist in rats and an ER agonist in several short-term assays in mice (MacGregor and Jordan, 1998). Studies on tamoxifen and other ER agonists has led to the development of the acronym selective ER modulators (SERMs) which exhibit a complex pharmacology and induce tissue-specific ER agonist or antagonist activities. These structure-dependent differences are related to differential activation of estrogen-responsive genes/pathways and due to several factors (Katzenellenbogen *et al.*, 1996; Smith and O'Malley, 2004), including:

a the structure of the estrogenic compound;
b tissue-specific expression of ER subtype (ERα and ERβ) or variant;
c tissue-specific expression of critical co-activators and other co-regulatory proteins
d promoter context and chromatin state which is dependent on histone methylation or acetylation, promoter methylation, and expression of critical modulating *trans*-acting factors.

## Development of bioassays for clinically relevant SERMs

SERMs were primarily developed for treatment of hormone-dependent diseases or conditions and define the increasing complexity of ER action. If xenoestrogens and phytoestrogens are SERMs, then their estrogenic and antiestrogenic activities will also be tissue-specific and their role in causation or protection from hormone-dependent problems will depend on the structure of the individual compound, the amount of exposure (assuming a threshold) and the time of exposure where critical modifications of hormone-responsiveness are induced. The structurally diverse SERMs, E2, tamoxifen, raloxifene and ICI 164,384 have unique *in vivo* biologies and were used as model compounds to develop an *in vitro* bioassay that would distinguish between these compounds (Tzukerman *et al.*, 1994; McDonnell *et al.*, 1995). This bioassay utilizes the modular structure of ERα and ERβ in which the various domains (E–F) exhibit both separable and overlapping functions which govern their interactions with other co-regulatory proteins and promoter DNA. AF-1 and AF-2 are located in the A/B and E domains, respectively, and are particularly important for this assay system. Results in Table 65.1 show that these four compounds differentially induce transactivation in human hepatoma HepG2 cells transfected with the E2-responsive pC3 construct (human complement C3 promoter linked to the luciferase gene) and expression plasmids for wild-type ERα, ERα-AF-2 in which the AF-1 domain has been deleted, or ERα-AF-1 in which the critical amino acids in AF-2 have been mutated (aa D538N, E542Q and D545N). E2 induces transactivation in HepG2 cells transfected with wild-type/variant ERα; in contrast raloxifene activates ERα-AF-1, tamoxifen activates ERα and ERα-AF-1, and ICI 164,384 does not induce or inhibits transactivation.

## Xenoestrogens and phytoestrogens as SERMs

This assay has been used for distinguishing between different structural classes of xenoestrogens and phytoestrogens including BPA (2′,4′,6′-tricholo-4-biphenylol ($Cl_3$–PCB–OH), 2′,3′,4′,5′-tetrachloro-4-biphenylol ($Cl_4$–PCB–OH), *p*-*t*-octylphenol (OP), *o*-nonylphenol (NP), naringenin, kepone, resveratrol and 2,2-bis(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE). E2, ICI 182,780 and 4-hydroxytamoxifen were used as positive controls (Gould *et al.*, 1998; Yoon *et al.*, 2000, 2001). Results obtained in several cell lines using the E2-responsive complement pC3-luc construct or a construct ($pERE_3$) containing three estrogen-responsive elements (EREs) linked to luciferase demonstrate that xenoestrogens/phytoestrogens also differentially induce transactivation.

**TABLE 65.1 The SERMs tamoxifen, raloxifene, ICI 164384 and E2 differentially activate ERα and also exhibit unique *in vivo* biologies (Tzukerman *et al.*, 1994)**

| SERM | ERα | | | | ERα-AF-1 | | | | ERα-AF-2 | | | *In vivo* ER activity | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | A/B | C/D | E | F | A/B | C/D | E*** | F | C/D | E | F | Bone | Breast | Uterus |
| E2 | | +++[a] | | | | +++ | | | | +++ | | Ag[b] | Ag | Ag |
| Tamoxifen | | + | | | | + | | | | ND | | Ag | Ant | Ag |
| Raloxifene | | ND | | | | + | | | | ND | | Ag | Ant | – |
| ICI 164,384 | | ND | | | | ND | | | | ND | | – | Ant | Ant |

[a]E2 induced maximal responses in all assays (+++), and responses >40% (++) or <40%(+) of that observed for E2 are indicated. ND indicates no significant induction or inhibition of activity.
[b]Ag and Ant indicate ER agonist and antagonist, respectively; – indicates no agonist activity.

**TABLE 65.2 Different structural classes of xenoestrogens, phytoestrogens, E2 and 4-hydroxytamoxifen differentially induce transactivation in HepG2 and U2 cells transfected with $pERE_3$, wild-type and variant forms of ERα (Yoon *et al.*, 2001)**

| | ERα | | ERα-AF-1 | | ERα-AF-2 | |
|---|---|---|---|---|---|---|
| | A/B C/D | E F | A/B C/D | E*** F | C/D E | F |
| | HepG2 | U2 | HepG2 | U2 | HepG2 | U2 |
| 17β-estradiol | +++* | +++ | +++ | +++ | +++ | +++ |
| 4-Hydroxytamoxifen | + | ND | ND | ND | ND | ND |
| NP | + | + | ++ | ND | ++ | ND |
| OP | + | + | + | + | ND | ND |
| $HO–PCB–Cl_4$ | ++ | ++ | + | ND | ++ | +++ |
| $HO–PCB–Cl_3$ | ++ | ++ | + | ++ | +++ | +++ |
| HPTE | +++ | + | + | ND | +++ | ++ |
| BPA | ++ | ++ | + | ++ | ++ | +++ |
| Kepone | ND | ND | + | ND | + | ND |
| Naringenin | ND | ND | ND | ND | ND | ND |
| Resveratrol | ND | ND | ND | ND | ND | ND |

* E2 induced a maximal response (+++) in all assays. A significant induction response of >40% (++) or <40% (+) of that observed for E2, and no significant induction (ND) are indicated. ICI 182,780 gave an ND for all responses. The doses used were 10 nM and 1 μM for E2 and 4′-hydroxytamoxifen, respectively, 100 μM for BPA, and 10 μM for the remaining compounds.

Results summarized in Table 65.2 compare the maximal induced responses observed for these compounds using human hepatoma HepG2 and human U2 osteogenic cancer cell lines transfected with $pERE_3$ and ERα, ERα-AF-1 or ERα-AF-2 (Yoon *et al.*, 2001). Even among structurally related compounds such as the hydroxy-PCBs, alkylphenols and bisphenolics HPTE and BPA, there were some significant differences in their induction of transactivation. Moreover, using a similar approach in HepG2, U2 and MDA-MB-231 cancer cell lines transfected with a pC3-luc construct similar differences were observed (Yoon *et al.*, 2000). For example, the two bisphenolic compounds HPTE and BPA exhibit a similar pattern of transactivation in many assays except that in U2 cells BPA but not HPTE induces transactivation in cells transfected with ERα-AF-1. It was also apparent from other *in vivo* and *in vitro* studies that BPA and HPTE exhibit difference in their estrogenic activities. For example, HPTE was a more potent estrogen than BPA in the female rat uterus; however in combination with E2, lower doses of BPA inhibited E2-induced uterine progesterone receptor (PR) binding and peroxidase activity (Gould *et al.*, 1998). HPTE versus BPA also exhibited other differences in HepG2 cells where both HPTE and BPA are ERα agonists, whereas HPTE is an ERβ and androgen receptor (AR) antagonist and BPA is an ERβ agonist and did not affect AR in this cell line (Gaido *et al.*, 1999). These activities can also vary in other cell contexts but clearly demonstrate significant *in vitro* and *in vivo* biological differences between HPTE and BPA, suggesting that structurally diverse estrogenic compounds are SERMs.

Several other *in vitro* studies have demonstrated significant differences among different structural classes of estrogenic compounds and the planar phytoestrogens coumestrol and genistein. For example, an ERα mutant (D351G) is activated by E2 and DES but not by 4′-hydroxytamoxifen or the non-planar HPTE and related compound (Jordan *et al.*, 2001). It was also reported that isoflavones, daidzein, biochanin and genistein are ERβ-selective and this is due to their preferential induction of co-activator interactions with AF-2 of ERβ compared to AF-2 of ERα (An *et al.*, 2001). Other reports also show that activation of gene expression in *in vitro* assays by structurally diverse xenoestrogens/phytoestrogens depends on the promoter context

(i.e. different consensus versus non-consensus EREs) and on co-activator interactions (Hall *et al.*, 2002; Mueller *et al.*, 2004). Recent studies in the laboratory have also demonstrated structure-dependent activation of non-genomic kinase pathways by xenoestrogens/phytoestrogens (Li and Safe, 2006). Moreover it has also been shown that some estrogenic compounds activate kinases via ER-independent pathways by directly binding to a G-protein coupled receptor (GPR30) (Thomas *et al.*, 2005; Revankar *et al.*, 2005). The structure dependence on this ER-dependent pathway has yet to be determined. These results clearly imply that xenoestrogens/phytoestrogens are SERMs.

## XENOESTROGENS AND PHYTOESTROGENS AS SERMS AND IMPLICATIONS FOR RISK ASSESSMENT

Xenoestrogens and phytoestrogens differentially activate wild-type and variant ER$\alpha$ *in vitro* suggesting that these compounds are SERMs. This implies that their estrogenic activity and potency cannot be determined by simple ER binding or gene expression assays. Thus, in order to fully understand the estrogenic or antiestrogenic activities of phytoestrogens and xenoestrogens studies will have to focus on their tissue-specific impacts at various critical periods of exposure. Risk assessment of these compounds will be complex and dependent on all the variables indicated in a previous section. Moreover, since individual SERMs exhibit unique biologies, the overall impact of mixtures of these compounds may not be additive. These factors highlight the challenges faced by scientists and regulators in addressing the health risks and benefits of estrogenic compounds.

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## REFERENCES

Albrektsen G, Heuch I, Tretli S, Kvale G (1994) Breast cancer incidence before age 55 in relation to parity and age at first and last births: a prospective study of one million Norwegian women. *Epidemiology* **5**: 604–11.

Althuis MD, Brogan DR, Coates RJ, Daling JR, Gammon MD, Malone KE, Schoenberg JB, Brinton LA (2003) Hormonal content and potency of oral contraceptives and breast cancer risk among young women. *Br J Cancer* **88**: 50–7.

An J, Tzagarakis-Foster C, Scharschmidt TC, Lomri N, Leitman DC (2001) Estrogen receptor $\beta$-selective transcriptional activity and recruitment of coregulators by phytoestrogens. *J Biol Chem* **276**: 17808–14.

Ansar Ahmed S, Dauphinee MJ, Montoya AI, Talal N (1989) Estrogen induces normal murine CD5 + B cells to produce autoantibodies. *J Immunol* **142**: 2647–53.

Armamento-Villareal R, Villareal DT, Avioli LV, Civitelli R (1992) Estrogen status and heredity are major determinants of premenopausal bone mass. *J Clin Invest* **90**: 2464–71.

Baghurst PA, Rohan TE (1994) High-fiber diets and reduced risk of breast cancer. *Int J Cancer* **56**: 173–6.

Bakker J, Honda S, Harada N, Balthazart J (2003) The aromatase knockout (ArkO) mouse provides new evidence that estrogens are required for the development of the female brain. *Ann NY Acad Sci* **1007**: 251–62.

Barkhem T, Carlsson B, Nilsson Y, Enmark E, Gustafsson J, Nilsson S (1998) Differential response of estrogen receptor $\alpha$ and estrogen receptor $\beta$ to partial estrogen agonists/antagonists. *Mol Pharmacol* **54**: 105–12.

Benecke A, Chambon P, Gronemeyer H (2000) Synergy between estrogen receptor $\alpha$ activation functions AF1 and AF2 mediated by transcription intermediary factor TIF2. *EMBO Rep* **1**: 151–7.

Bergman A, Klasson-Wehler E, Kuroki H (1994) Selective retention of hydroxylated PCB metabolites in blood. *Environ Health Perspect* **102**: 464–9.

Bernstein L (2002) Epidemiology of endocrine-related risk factors for breast cancer. *J Mammary Gland Biol Neoplasia* **7**: 3–15.

Breedlove SM (1992) Sexual dimorphism in the vertebrate nervous system. *J Neurosci* **12**: 4133–42.

Brosnihan KB, Li P, Ganten D, Ferrario CM (1997) Estrogen protects transgenic hypertensive rats by shifting the vasoconstrictor-vasodilator balance or RAS. *Am J Physiol* **273**: R1908–15.

Brzozowski AM, Pike AC, Dauter Z, Hubbard RE, Bonn T, Engstrom O, Ohman L, Greene GL, Gustafsson JA, Carlquist M (1997) Molecular basis of agonism and antagonism in the oestrogen receptor. *Nature* **389**: 753–8.

Bush TL (1990) The epidemiology of cardiovascular disease in postmenopausal women. *Ann NY Acad Sci* **592**: 271–3.

Colditz GA, Willet WC, Stampfer MJ, Rosner B, Speizer FE, Hennekens CH (1987) Menopause and the risk of coronary heart disease in women. *N Engl J Med* **316**: 1105–10.

Cowley SM, Parker MG (1999) A comparison of transcriptional activation by ER$\alpha$ and ER$\beta$. *J Steroid Biochem Mol Biol* **69**: 165–75.

Coyle YM (2004) The effect of environment on breast cancer risk. *Breast Cancer Res Treat* **84**: 273–88.

Cutler Jr GB (1997) The role of estrogen in bone growth and maturation during childhood and adolescence. *J Steroid Biochem Mol Biol* **61**: 141–4.

Davis S, Mirick DK, Stevens RG (2001) Night shift work, light at night, and risk of breast cancer. *J Natl Cancer Inst* **93**: 1557–62.

Desbrow C, Routledge EJ, Brighty GC, Sumpter JP, Waldrock M (1998) Identification of estrogenic chemicals in STW effluent. 1. Chemical fractionation and *in vitro* biological screening. *Environ Sci Technol* **32**: 1549–58.

Eddy EM, Washburn TF, Bunch DO, Gouldring EH, Gladen BC, Lubahn DB, Korach KS (1996) Targeted disruption of the estrogen receptor gene in male mice causes alteration of spermatogenesis and infertility. *Endocrinology* **137**: 4796–805.

Egan KM, Lawson JA, Fries S, Koller B, Radar DJ, Smyth EM, Fitzgerald GA (2004) COX-2 derived prostacyclin confers atheroprotection on female mice. *Science* **18**: 18.

Enger SM, Ross RK, Henderson B, Bernstein L (1997) Breastfeeding history, pregnancy experience and risk of breast cancer. *Br J Cancer* **76**: 118–23.

Espeland MA, Rapp SR, Shumaker SA, Brunner R, Manson JE, Sherwin BB, Hsia J, Margolis KL, Hogan PE, Wallace R, Dailey M, Freeman R, Hays J (2004) Conjugated equine estrogens and global cognitive function in postmenopausal women. Women's Health Initiative Study. *J Am Med Assoc* **291**: 2959–68.

Ettinger B, Genant HK, Cann CE (1985) Long-term estrogen replacement therapy prevents bone loss and fractures. *Ann Intern Med* **102**: 319–24.

Ewertz M, Mellemkjaer L, Poulsen AH, Friis S, Sorensen HT, Pedersen L, McLaughlin JK, Olsen JH (2005) Hormone use for menopausal symptoms and risk of breast cancer. A Danish cohort study. *Br J Cancer* **92**: 1293–7.

Fawell SE, Lees JA, White R, Parker MG (1990) Characterization and colocalization of steroid binding and dimerization activities in the mouse estrogen receptor. *Cell* **60**: 953–62.

Gaido KW, Leonard LS, Maness SC, Galluzzo JM, McDonnell DP, Saville B, Safe S (1999) Differential interaction of the methoxychlor metabolite HPTE with estrogen receptors $\alpha$ and $\beta$. *Endocrinology* **140**: 5746–53.

Garland FC, Garland CF, Gorham ED, Young JF (1990) Geographic variation in breast cancer mortality in the United States: a hypothesis involving exposure to solar radiation. *Prev Med* **19**: 614–22.

Gaudet MM, Britton JA, Kabat GC, Steck-Scott S, Eng SM, Teitelbaum SL, Terry MB, Neugut AI, Gammon MD (2004) Fruits, vegetables, and micronutrients in relation to breast cancer modified by menopause and hormone receptor status. *Cancer Epidemiol Biomar Prev* **13**: 1485–94.

Gerber B, Muller H, Reimer T, Krause A, Friese K (2003) Nutrition and lifestyle factors on the risk of developing breast cancer. *Breast Cancer Res Treat* **79**: 265–76.

Giusti RM, Iwamoto K, Hatch EE (1995) Diethylstilbestrol revisited: a review of the long-term health effects. *Ann Intern Med* **122**: 778–88.

Goodman MT, Cologne JB, Moriwaki H, Vaeth M, Mabuchi K (1997) Risk factors for primary breast cancer in Japan: 8-year follow-up of atomic bomb survivors. *Prev Med* **26**: 144–53.

Gould JC, Leonard LS, Maness SC, Wagner BL, Conner K, Zacharewski T, Safe S, McDonnell DP, Gaido KS (1998) Bisphenol A interacts with the estrogen receptor $\alpha$ in a distinct manner from estradiol. *Mol Cell Endocrinol* **142**: 203–14.

Grant WB (2002) An ecologic study of dietary and solar ultraviolet-B links to breast carcinoma mortality rates. *Cancer* **94**: 272–81.

Grodstein F, Stampler MJ, Manson JE, Coldwitz GA, Willett WC, Rosner B, Speizer FE, Hennekens CH (1996) Postmenopausal estrogen and progestin use and the risk of cardiovascular disease. *N Engl J Med* **335**: 453–61.

Hall JM, McDonnell DP, Korach KS (2002) Allosteric regulation of estrogen receptor structure, function, and coactivator recruitment by different estrogen response elements. *Mol Endocrinol* **16**: 469–86.

Hancock SL, Tucker MA, Hoppe RT (1993) Breast cancer after treatment of Hodgkin's disease. *J Natl Cancer Inst* **85**: 25–31.

Handelsman DJ (2001) Estrogens and falling sperm counts. *Reprod Fertil Dev* **13**: 317–24.

Havsteen BH (2002) The biochemistry and medical significance of flavonoids. *Pharmacol Ther* **96**: 67.

Hilakivi-Clarke L (2000) Estrogens, BRCA1, and breast cancer. *Cancer Res* **60**: 4993–5001.

Jordan VC (2003a) Antiestrogens and selective estrogen receptor modulators as multifunctional medicines. 2. Clinical considerations and new agents. *J Med Chem* **46**: 1081–111.

Jordan VC (2003b) Antiestrogens and selective estrogen receptor modulators as multifunctional medicines. 1. Receptor interactions. *J Med Chem* **46**: 883–908.

Jordan VC, Schafer JM, Levenson AS, Liu H, Pease KM, Simons LA, Zapf JW (2001) Molecular classification of estrogens. *Cancer Res* **61**: 6619–23.

Katzenellenbogen JA, O'Malley BW, Katzenellenbogen BS (1996) Tripartite steroid hormone receptor pharmacology – interaction with multiple effector sites as a basis for the cell- and promoter-specific action of these hormones. *Mol Endocrinol* **10**: 119–31.

Key TJ, Allen NE, Spencer EA, Travis RC (2003) Nutrition and breast cancer. *Breast* **12**: 412–16.

Klinge CM (2001) Estrogen receptor interaction with estrogen response elements. *Nucleic Acid Res* **29**: 2905–19.

Knopp RH, Zhu X, Bonet B, Bagatell C (1996) Effects of sex hormones on lipoproteins, clotting, and the arterial wall. *Semin Reprod Endocrinol* **14**: 15–27.

Koehler KF, Helguero LA, Haldosen KA, Warner M, Gustafsson JA (2005) Reflections on the discovery and significance of estrogen receptor $\beta$. *Endocr Rev* **26**: 465–78.

Korach KS (1994) Insights from the study of animals lacking functional estrogen receptor. *Science* **266**: 1524–7.

Krege JH, Hodgin JB, Couse JF, Enmark E, Warner M, Mahler JF, Sar M, Korach KS, Gustafsson JA, Smithies O (1998) Generation and reproductive phenotypes of mice lacking estrogen receptor $\beta$. *Proc Natl Acad Sci USA* **95**: 15677–82.

Krishnan AV, Stathis P, Permuth SF, Tokes L, Feldman D (1993) Bisphenol-A: an estrogenic substance is released from polycarbonate flasks during autoclaving. *Endocrinology* **132**: 2279–86.

Kuiper GG, Carlsson B, Grandien K, Enmark E, Haggblad J, Nilsson S, Gustafsson JA (1997) Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors $\alpha$ and $\beta$. *Endocrinology* **138**: 863–70.

Kutz FW, Wood PH, Bottimore DP (1991) Organochlorine pesticides and polychlorinated biphenyls in human adipose tissue. *Rev Environ Contam Toxicol* **120**: 1–82.

Lacreuse A, Herndon JG (2003) Effects of estrodiol and aging on the fine manual performance in female rhesus monkeys. *Horm Behav* **43**: 359–66.

Lambe M, Hsieh CC, Chan HW, Ekbom A, Trichopoulos D, Adami HO (1996) Parity, age at first and last birth, and risk of breast cancer: a population-based study in Sweden. *Breast Cancer Res Treat* **38**: 305–11.

Land CE, Tokunaga M, Koyama K, Soda M, Preston DL, Nishimori I, Tokuoka S (2003) Incidence of female breast cancer among atomic bomb survivors, Hiroshima and Nagasaki, 1950–1990. *Radiat Res* **160**: 707–17.

Lee SY, Kim MT, Kim SW, Song MS, Yoon SJ (2003) Effect of lifetime lactation on breast cancer risk: a Korean women's cohort study. *Int J Cancer* **105**: 390–3.

Lees JA, Fawell SE, White R, Parker MG (1990) A 22-amino-acid peptide restores DNA-binding activity to dimerization-defective mutants of the estrogen receptor. *Mol Cell Biol* **10**: 5529–31.

Li X, Safe S (2006) Activation of kinase pathways in MCF-7 cells by 17$\beta$-estrodiol and structurally-diverse estrogenic compounds. *J Steroid Biochem Mol Biol* **98**: 122–32.

Lipworth L, Bailey LR, Trichopoulos D (2000) History of breast-feeding in relation to breast cancer risk: a review of the epidemiologic literature. *J Natl Cancer Inst* **92**: 302–12.

Lu LJ, Anderson KE, Grady JJ, Kohen F, Nagamani M (2000) Decreased ovarian hormones during a soya diet: implications for breast cancer prevention. *Cancer Res* **60**: 4112–21.

Lubahn DB, Moyer JS, Golding TS, Couse JF, Korach KS, Smithies O (1993) Alteration of reproductive function but not prenatal sexual

development after insertional disruption of the mouse estrogen receptor gene. *Proc Natl Acad Sci USA* **90**: 11162–6.

MacGregor JI, Jordan VC (1998) Basic guide to the mechanisms of antiestrogen action. *Pharmacol Rev* **50**: 151–96.

Maggi A, Pellegrini S, Bettini E (1989) Estrogen-induced proteins in rat hypothalamus. *Eur J Pharmacol* **172**: 357–62.

Martin AM, Weber BL (2000) Genetic and hormonal risk factors in breast cancer. *J Natl Cancer Inst* **92**: 1126–35.

Martinez-Vargas MC, Gibson DB, Sar M, Stumpf WE (1975) Estrogen target sites in the brain of the chick embryo. *Science* **190**: 1307–8.

Matthews J, Gustafsson JA (2003) Estrogen signaling: a subtle balance between ER$\alpha$ and ER$\beta$. *Mol Interv* **3**: 281–92.

Mattisson I, Wirfalt E, Johansson U, Gullberg B, Olsson H, Berglund G (2004) Intakes of plant foods, fibre and fat and risk of breast cancer – a prospective study in the Malmo Diet and Cancer cohort. *Br J Cancer* **90**: 122–7.

McDonnell DP, Clemm DL, Hermann T, Goldman ME, Pike JW (1995) Analysis of estrogen receptor function *in vitro* reveals three distinct classes of antiestrogens. *Mol Endocrinol* **9**: 659–69.

McEwen BS, Alves SE (1999) Estrogen actions in the central nervous system. *Endocr Rev* **20**: 279–307.

Meeske K, Press M, Patel A, Bernstein L (2004) Impact of reproductive factors and lactation on breast carcinoma *in situ* risk. *Int J Cancer* **110**: 102–9.

Mezzetti M, La Vecchia C, Decarli A, Boyle P, Talamini R, Franceschi S (1998) Population attributable risk for breast cancer: diet, nutrition, and physical exercise. *J Natl Cancer Inst* **90**: 389–94.

Mosselman S, Polman J, Dijkema R (1996) ER$\beta$: identification and characterization of a novel human estrogen receptor. *FEBS Lett* **392**: 49–53.

Mueller SO, Simon S, Chae K, Metzler M, Korach KS (2004) Phytoestrogens and their human metabolites show distinct agonistic and antagonistic properties on estrogen receptor alpha (ER$\alpha$) and ER$\beta$ in human cells. *Toxicol Sci* **80**: 14–25.

Nathan L, Chaudhuri G (1997) Estrogens and atherosclerosis. *Annu Rev Pharmacol Toxicol* **37**: 477–515.

Nathanson KL, Wooster R, Weber BL (2001) Breast cancer genetics: what we know and what we need. *Nat Med* **7**: 552–6.

Nilsson S, Makela S, Treuter E, Tujague M, Thomsen J, Andersson G, Enmark E, Pettersson K, Warner M, Gustafsson JA (2001) Mechanisms of estrogen action. *Physiol Rev* **81**: 1535–65.

O'Neal MF, Means LW, Poole MC, Hamm RJ (1996) Estrogen affects performance of ovariectomized rats in a two-choice water-escape working memory task. *Psychoneuroendocrinology* **21**: 51–65.

Pare G, Krust A, Karas RH, Duport S, Aronovitz M, Chambon P, Mendelsohn ME (2002) Estrogen receptor $\alpha$ mediates the protective effects of estrogen against vascular injury. *Circ Res* **90**: 1087–92.

Pearce ST, Jordan VC (2004) The biological role of estrogen receptors $\alpha$ and $\beta$ in cancer. *Crit Rev Oncol Hematol* **50**: 3–22.

Peto J, Collins N, Barfoot R, Seal S, Warren W, Rahman N, Easton DF, Evans C, Deacon J, Stratton MR (1999) Prevalence of BRCA1 and BRCA2 gene mutations in patients with early-onset breast cancer. *J Natl Cancer Inst* **91**: 943–9.

Pettersson K, Grandien K, Kuiper GG, Gustafsson JA (1997) Mouse estrogen receptor $\beta$ forms estrogen response element-binding heterodimers with estrogen receptor $\alpha$. *Mol Endocrinol* **11**: 1486–96.

Pike AC, Brzozowski AM, Hubbard RE, Bonn T, Thorsell AG, Engstrom O, Ljunggren J, Gustafsson JA, Carlquist M (1999) Structure of the ligand-binding domain of oestrogen receptor $\beta$ in the presence of a partial agonist and a full antiestrogen. *EMBO J* **18**: 4608–18.

Pike AC, Brzozowski AM, Walton J, Hubbard RE, Thorsall AG, Li YL, Gustafsson JA, Carlquist M (2001) Structural insights into the mode of action of a pure antiestrogen. *Structure* **9**: 145–53.

Rebbeck TR, Couch FJ, Kant J, Calzone K, DeShano M, Peng Y, Chen K, Garber JE, Weber BL (1996) Genetic heterogeneity in hereditary breast cancer: role of BRCA1 and BRCA2. *Am J Hum Genet* **59**: 547–53.

Revankar CM, Cimino DF, Sklar LA, Arterburn JB, Prossnitz ER (2005) A transmembrane intracellular estrogen receptor mediates rapid cell signaling. *Science* **307**: 1625–30.

Robertson KM, O'Donnell L, Jones ME, Meachem SJ, Boon WC, Fisher CR, Graves KH, McLachlan RI, Simpson ER (1999) Impairment of spermatogenesis in mice lacking a functional aromatase (cyp 19) gene. *Proc Natl Acad Sci USA* **96**: 7986–91.

Rosner B, Colditz GA, Willett WC (1994) Reproductive risk factors in a prospective study of breast cancer: the Nurses' Health Study. *Am J Epidemiol* **139**: 819–35.

Rossouw JE, Anderson GL, Prentice RL, LaCroix AZ, Kooperberg C, Stefanick ML, Jackson RD, Beresford SA, Howard BV, Johnson KC, Kotchen JM, Ockene J (2002) Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principle results from the Women's Health Initiative randomized controlled trial. *J Am Med Assoc* **288**: 321–33.

Routledge EJ, Sheahan D, Desbrow C, Brighty GC, Waldock M, Sumpter JP (1998) Identification of estrogenic chemicals in STW effluent. 2. *In vivo* responses in trout and roach. *Environ Sci Technol* **32**: 1559–65.

Russo J, Hu YF, Yang X, Russo IH (2000) Developmental, cellular, and molecular basis of human breast cancer. *J Natl Cancer Inst Monogr* **27**: 17–37.

Russo J, Mailo D, Hu YF, Balogh G, Sheriff F, Russo IH (2005) Breast differentiation and its implication in cancer prevention. *Clin Cancer Res* **11**: 931s–6s.

Russo J, Rivera R, Russo IH (1992) Influence of age and parity on the development of the human breast. *Breast Cancer Res Treat* **23**: 211–18.

Saadatian-Elahi M, Norat T, Goudable J, Riboli E (2004) Biomarkers of dietary fatty acid intake and the risk of breast cancer: a meta-analysis. *Int J Cancer* **111**: 584–91.

Safe S (2000) Endocrine disrupters and human health – is there a problem: an update. *Environ Health Perspect* **110**(S6): 487–93.

Sasco AJ, Kaaks R, Little RE (2003) Breast cancer: occurrence, risk factors and hormone metabolism. *Expert Rev Anticancer Ther* **3**: 546–62.

Schernhammer ES, Laden F, Speizer FE, Willett WC, Hunter DJ, Kawachi I, Colditz GA (2001) Rotating night shifts and risk of breast cancer in women participating in the nurses' health study. *J Natl Cancer Inst* **93**: 1563–8.

Schlumpf M, Schmid P, Durrer S, Conscience M, Maerkel K, Henseler M, Gruetter M, Herzog I, Reolon S, Ceccatelli R, Faass O, Stutz E, Jarry H, Wuttke W, Lichtensteiger W (2004) Endocrine activity and developmental toxicity of cosmetic UV filters-an update. *Toxicology* **205**: 113–22.

Schomberg DW, Couse JF, Mukherjee A, Lubahn DB, Sar M, Mayo KE, Korach KS (1999) Targeted disruption of the estrogen receptor $\alpha$ gene in female mice: characterization of ovarian responses and phenotype in the adult. *Endocrinology* **140**: 2733–44.

Schreurs RH, Sonneveld E, Jansen JH, Seinen W, van der Burg B (2005) Interaction of polycyclic masks and UV filters with the estrogen receptor (ER), androgen receptor (AR), and progesterone receptor (PR) in reporter gene bioassays. *Toxicol Sci* **83**: 264–72.

Sharpe RM, Skakkebaek NE (1993) Are oestrogens involved in falling sperm counts and disorders of the male reproductive tract? *Lancet* **341**: 1392–5.

Shiau AK, Barstad D, Loria PM, Cheng L, Kushner PJ, Agard DA, Greene GL (1998) The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. *Cell* **95**: 927–37.

Shumaker SA, Legault C, Rapp SR, Thal L, Wallace RB, Ockene JK, Hendrix SL, Jones III BN, Assaf AR, Jackson RD, Kotchen JM, Wassertheil-Smoller S, Wactawski-Wende J (2003) Estrogen plus progestin and the incidence of dementia and mild cognitive impairment in postmenopausal women: the Women's Health Initiative Memory Study: a randomized controlled trial. *J Am Med Assoc* **289**: 2651–62.

Sivaraman L, Medina D (2002) Hormone-induced protection against breast cancer. *J Mammary Gland Biol Neoplasia* **7**: 77–92.

Skakkebaek NE, Rajpert-De ME, Main KM (2001) Testicular dysgenesis syndrome: an increasingly common developmental disorder with environmental aspects. *Hum Reprod* **16**: 972–8.

Smith CL, O'Malley BW (2004) Coregulator function: a key to understanding tissue specificity of selected receptor modulators. *Endocr Rev* **25**: 45–71.

Smith EP, Boyd J, Frank GR, Takahashi H, Cohen RM, Specker B, Williams TC, Lubahn DB, Korach KS (1994) Estrogen resistance caused by a mutation in the estrogen-receptor gene in a man. *N Engl J Med* **331**: 1056–61.

Smith-Warner SA, Spiegelman D, Adami HO, Beeson WL, van den Brandt PA, Folsom AR, Fraser GE, Freudenheim JL, Goldbohm RA, Graham S, Kushi LH, Miller AB, Rohan TE, Speizer FE, Toniolo P, Willett WC, Wolk A, Zeleniuch-Jacquotte A, Hunter DJ (2001) Types of dietary fat and breast cancer: a pooled analysis of cohort studies. *Int J Cancer* **92**: 767–74.

Sonnenschein C, Soto AM (1998) An updated review of environmental estrogen and androgen mimics and antagonists. *J Steroid Biochem Mol Biol* **65**: 143–50.

Soto AM, Justicia H, Wray JW, Sommenschein C (1991) *p*-Nonylphenol: an estrogenic xenobiotic released from "modified" polystyrene. *Environ Health Perspect* **92**: 167–73.

Struewing JP, Tarone RE, Brody LC, Li FP, Boice Jr JD (1996) BRCA1 mutations in young women with breast cancer. *Lancet* **347**: 1493.

Sudhir K, Chou TM, Chatterjee K, Smith EP, Williams TC, Kane JP, Malloy MJ, Korach KS, Rubanyi GM (1997) Premature coronary artery disease associated with a disruptive mutation in the estrogen receptor gene in a man. *Circulation* **96**: 3774–7.

Talamini R, Franceschi S, La Vecchia C, Negri E, Borsa L, Montella M, Falcini F, Conti E, Rossi C (1996) The role of reproductive and menstrual factors in cancer of the breast before and after menopause. *Eur J Cancer* **32A**: 303–10.

Thomas P, Pang Y, Filardo EJ, Dong J (2005) Identity of an estrogen membrane receptor coupled to a G protein in human breast cancer cells. *Endocrinology* **146**: 624–32.

Titus-Ernstoff L, Hatch EE, Hoover RN, Palmer J, Greenberg ER, Ricker W, Kaufman R, Noller K, Herbst AL, Colton T, Hartge P (2001) Long-term cancer risk in women given diethylstilbestrol (DES) during pregnancy. *Br J Cancer* **84**: 126–33.

Tora L, White J, Brou C, Tasset D, Webster N, Scheer E, Chambon P (1989) The human estrogen receptor has two independent nonacidic transcriptional activation functions. *Cell* **59**: 477–87.

Tzukerman MT, Etsy A, Santiso-Mere D, Danielian P, Parker MG, Stein RG, Pike JW, McDonnell DP (1994) Human estrogen receptor transactivational capacity is determined by both cellular and promoter content and mediated by two functionally distinct intramolecular regions. *Mol Endocrinol* **8**: 21–30.

Velie E, Kulldorff M, Schairer C, Block G, Albanes D, Schatzkin A (2000) Dietary fat, fat subtypes, and breast cancer in postmenopausal women: a prospective cohort study. *J Natl Cancer Inst* **92**: 833–9.

Venkitaraman AR (2002) Cancer susceptibility and the functions of BRCA1 and BRCA2. *Cell* **108**: 171–82.

Wurtz JM, Egner U, Heinrich N, Moras D, Mueller-Fahrnow A (1998) Three-dimensional models of estrogen receptor ligand binding domain complexes, based on related crystal structures and mutational and structure-activity relationship data. *J Med Chem* **41**: 1803–14.

Yoon K, Pallaroni L, Ramamoorthy K, Gaido K, Safe S (2000) Ligand structure-dependent differences in activation of estrogen receptor $\alpha$ in human HepG2 liver and U2 osteogenic cancer cell lines. *Mol Cell Endocrinol* **162**: 211–20.

Yoon K, Pallaroni L, Stoner M, Gaido K, Safe S (2001) Differential activation of wild-type and variant forms of estrogen receptor $\alpha$ by synthetic and natural estrogenic compounds using a promoter containing three tandem estrogen-responsive elements. *J Steroid Biochem Mol Biol* **78**: 25–32.

# Part 14

## Poisonous Plants

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C H A P T E R

# 66

# Important poisonous plants of the United States

*Kip E. Panter, D.R. Gardner, S.T. Lee, J.A. Pfister, M.H. Ralphs, B.L. Stegelmeier, and L.F. James*

## INTRODUCTION

Poisonous plants and the secondary compounds they produce cause large economic losses to the livestock industries throughout the world. Using 1989 figures, it was determined that poisonous plants cause losses of over $340 million annually to the livestock industry in the 17 western states of the United States (Nielsen *et al.*, 1988; Nielsen and James, 1992). This cost only considered death losses and specific reproductive losses in cattle and sheep and only the 17 western states were included. Less obvious costs such as lost grazing opportunities, additional feed costs, increased health care costs, management changes, increased culling costs, lost weight gains, delayed or failed reproduction, and the emotional stress accompanying many poisonous plant cases were not included in the Nielsen and James analysis. When one considers these other costs, inflation, current animal values and when all pastures and ranges in the United States are factored in, the economic cost of poisonous plants to the livestock industry is very large. Additionally, an often ignored cost is the lost biodiversity frequently resulting from invasive species, many of which are poisonous. These invasive and poisonous species are often aggressive invaders and reduce optimum utilization of private, federal and state managed forest, range and pasture lands. This aspect alone has far reaching implications, not only for livestock producers, but also for many other segments of society.

A frequently asked question is, why do animals consume plants which may harm them or their offsprings? In some cases it is a matter of survival. For example, in the arid livestock-producing regions of the world, such as the western United States, regions of South Africa, Australia, China, and others, browsing or grazing animals may have limited access to high-quality forage at certain times of the year and are forced to survive by grazing some poisonous species. In other instances, hay or forages harvested in areas where poisonous plants are abundant may be contaminated with a high percentage of poisonous plants and when animals are fed contaminated hay, they may be poisoned. Poisonous plant problems are often exacerbated during periods of below normal rainfall when the abundance of grasses is reduced. Frequently, the animal's diet shifts during the season as grasses and palatable forbs mature and senesce, e.g. the consumption of some poisonous plants such as lupines, locoweeds, or larkspurs, which stay green longer into the season, may increase as the season progresses. In other instances poisoning occurs early in the season before grasses begin to grow but poisonous plants have emerged and are usually very toxic. Poisoning by plants only occurs when animals eat too much too fast or graze it over a prolonged period of time. Therefore, management strategies to control these factors can minimize losses from poisonous plants.

At the Poisonous Plant Research Laboratory, poisonous plant problems are attacked using a multi-disciplinary approach. Veterinary medicine, chemistry, toxicology, pathology, animal and plant physiology, and range management are all key in the approach to reduce losses to the livestock industry. The plants in this chapter are some of the most important poisonous plants in the United States and considerable research has been conducted to reduce their incidence of poisoning.

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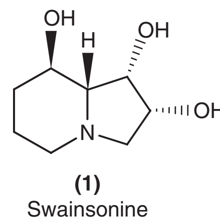
This chapter is not intended to be all inclusive but focuses on some of the most economically important and geographically widespread poisonous plants to livestock producers in the United States. There are excellent comprehensive texts available with a more general and broad coverage of specific poisonous plants (Kingsbury, 1964; Keeler *et al*., 1978 (ISOPP1); Seawright *et al*., 1985 (ISOPP2); James *et al*., 1992 (ISOPP3); Colegate and Dorling, 1994 (ISOPP4); Cheeke, 1998; Garland and Barr, 1998 (ISOPP5); Burrows and Tyrl, 2001; Knight and Walter, 2001; Acamovic *et al*., 2004 (ISOPP6); Panter *et al*., 2007 (ISOPP7)). The ISOPP series, 1–7, 1978–2006 (*Proceedings of the International Symposium on Poisonous Plants*) provides a worldwide view of poisonous plant research and is currently in its seventh publication in 2006.

## *ASTRAGALUS* AND *OXYTROPIS* SPECIES (LOCOWEEDS, NITRO AND SELENIUM SPECIES)

### Locoweeds

Of all the poisonous plants in the United States the *Astragalus* and *Oxytropis* genera cause the most losses to the livestock industry in the Western States (James *et al*., 1981). The locoweeds are those species of the *Astragalus* and *Oxytropis* genera that contain the "loco" toxin (swainsonine) and induce the classic neurological and pathological signs of "locoism." Swainsonine is also present in other *Astragalus* species not usually considered locoweeds such as some selenium and nitro-containing *Astragalus*. There are three toxic syndromes associated with these species: (1) locoism, caused by the indolizidine alkaloid swainsonine **(1)** (24 species); (2) nitrotoxins (356 taxa); and (3) species that accumulate selenium at high levels (22 species).




#### *Description and ecology*

These species are members of the Fabaceae (Leguminosae) or pea family. This family is distinguished by its distinct papilionaceous flower (butterfly-like) having a single large banner petal, two side petals or wings, and two lower petals fused together to form a keel. Flowers are leguminous (pea-like), few to many, in axillary racemes, may be blue, purple, yellow, or white in color; fruit is a legume pod of various shapes, sizes and surfaces among the species containing one to many kidney-shaped seeds. The distinguishing feature in *Oxytropis* is the porrect beak on the keel petal, *Astragalus* have a blunt keel petal (Figure 66.1). Some *Astragalus* species have extensions of the keel forming points, but they are not turned upward as in *Oxytropis*. In addition, *Oxytropis* species are acaulescent (without a stem, all leaves basal) with leafless flowering stalks originating from the crown, whereas leaflets of *Astragalus* species grow from the multi-branched stems with the flowering head on top (Figure 66.1). Leaflets are opposite pinnately compound. Pod shape, size, and chambers are very diverse, and are a principal means of distinguishing species (Figure 66.1).

*Astragalus* is a very large and complex genus, with 354 species and 198 varieties of *Astragalus* (552 taxa) in the

**FIGURE 66.1** Comparison of *Astragalus* and *Oxytropis*. *Astragalus mollissimus* (purple loco) is on the left and *Oxytropis lambertii* is on the right.

United States and Canada (Welsh *et al*., 2007). *Oxytropis* is much smaller with 22 species and 35 varieties (57 taxa).

Barneby (1952, 1964) earlier described and classified 368 species and 184 varieties of *Astragalus* and 35 species of *Oxytropis* in North America. Irregardless, the *Astragalus* genera is the largest of the Leguminosae family. Species and variety identification are difficult even for trained botanists. Because of obvious similarities between *Oxytropis* and *Astragalus* some botanists treat them together as *Astragalus*. Certainly, from a toxicology perspective, both contain swainsonine and induce the same condition in livestock, therefore both genera will be treated the same in this discussion.

### *Distribution and ecology*

The *Astragalus* and *Oxytropis* are worldwide in their distribution and toxic effects on livestock. Table 66. 1 lists those species in the western United States suspected of field cases of poisoning or which have been shown by chemical analysis to contain swainsonine (Marsh, 1909, 1924; Barneby, 1952, 1964; Molyneux and James, 1991; Molyneux *et al*., 1991).

Species of *Astragalus* and *Oxytropis* occur in every major plant community. However, livestock poisoning is erratic, due to the cyclic nature of the locoweed populations. Locoweeds have different survival strategies which allows perpetuation of the species through long-term climatic cycles and short-term weather conditions (Ralphs *et al*., 2003). Climate controls the establishment and growth of these plants by the amount and timing of precipitation. The three main survival strategies include the following:

1 Annual plants avoid drought by seed-dormancy through dry cycles, and germinate in years when sufficient moisture is available (winter annuals such as *A. wootonii* and *A. emoryanus*).
2 Biennial or short-lived perennial plants exhibit opportunistic survival strategies by relying on both timely and adequate moisture for germination, growth, flowering, and seed set. Seeds germinate in fall following autumn rains, persist over winter and flower in spring. If sufficient moisture is available, they will remain for 2–3 years until the next drought occurs (*A. mollissimus, A. lentiginosus, A. pubentissimus*).

**TABLE 66.1 Locoweeds (*Astragalus* and *Oxytropis*) species, habitat, and distribution (Ralphs *et al*., 2003) and *Astragalus* species containing nitrotoxins or accumulating selenium (Welsh *et al*., 2007)**

| Species | Common name | Habitat | Distribution |
|---|---|---|---|
| *A. allochrous* | Rattleweed | Desert grassland | AZ, NM |
| *A. asymmertricus* | Horse loco | Annual grasslands | CA |
| *A.* bisulcatus[a] | Two-grooved milkvetch | Limestone, shale, high in Se | MT, ND, WY, CO, NM, Ut |
| *A. didymocarpus* | | Creosote deserts | CA, AZ, NV |
| *A. drummondii*[a] | Drummond milkvetch | Prairies, sage, oak, P/J | MT, WY, CO, NM, UT |
| *A. emoryanus*[b] | Red stem peavine | Creosote, Mesquite, P/J | NM, TX |
| *A. humistratus* | Ground cover milkvetch | P/J woodlands | AZ, NM |
| *A. lentiginosus* | Spotted locoweed | Salt-desert shrub, sage, P/J | AZ, UT, NV, ID |
| *A. lonchocarpus* | Great rushy milkvetch | P/J woodlands | CO, UT, AZ, NV |
| *A. missouriensis* | Missouri milkvetch | Short-grass prairies | Canada to TX |
| *A. mollissimus* | Woolly loco | Short-grass prairies | CO, KA, OK, TX, NM |
| *A. nothoxys* | Beaked milkvetch | Oakbrush, P/J woodlands | AZ |
| *A. oxyphysus* | Diablo loco | Desert grasslands | CA |
| *A. praelongus*[a] | Stinking milkvetch | Sandstone, shale high in Se | UT, NM, AZ |
| *A. pubentissimus* | Green River milkvetch | Salt-desert shrub | CO, WY, UT |
| *A. purshii* | Pursh loco | Sagebrush, P/J woodlands | WY, MT, ID, NV |
| *A. pycnostachyus* | Brine milkvetch | Salt marshes and beaches | CA |
| *A. tephrodes* | Ashen milkvetch | Oakbrush, P/J woodlands | AZ, NM |
| *A. thurberi* | Thurber milkvetch | Creosote, Oak, P/J woodlands | AZ, NM |
| *A. wootonii* | Garbancillo | Creosote desert | AZ, NM, TX |
| *O. besseyi* | Red loco | Gravely hill tops | MT, WY |
| *O. campestris* | Yellow loco | Prairies, mountains meadows | MT, Canada |
| *O. lambertii* | Lambert locoweed | Short and mid-grass prairies | MT, ND, SD, WY, CO, NM |
| *O. sericea* | White locoweed | Rocky soils, foothills and mountains | MT. SD, WY, CO, NM, UT |
| **Nitrotoxins** | | | |
| *A. atropubescens* | Kelsey milkvetch | Gravelly benches | ID, MT |
| *A. canadensis* | Canada milkvetch | Introduced | Throughout US |
| *A. convallarius* | Rushy milkvetch | Sagebrush | ID, NV, UT, WY |
| *A. emoryanus* | Red-stem peavine | Short-grass prairies | AZ, NM, TX |

(*Continued*)

**TABLE 66.1 (Continued)**

| Species | Common name | Habitat | Distribution |
|---|---|---|---|
| *A. falcatus* | | Introduced species | |
| *A. miser* | Timber milkvetch | | |
| var *serotinus* | Columbia milkvetch | Rough fescue grasslands | BC Canada |
| var *hylophilus* | Yellowstone milkvetch | Sagebrush/timber | WY, MT |
| var *oblongifolius* | Wasatch milkvetch | Mountains sagebrush | UT, CO |
| *A. pterocarpus* | Winged milkvetch | Sagebrush | CA, NV |
| *A. tetrapterus* | Four-winged milkvetch | Sagebrush | CA, NV |
| *A. toanus*[c] | Toano milkvetch | Salt-desert shrub | Great Basin |
| *A. whitneyi* | | Sierra Mountains | CA, NV |
| **Selenium accumulators** | | | |
| *A. albulus*[c] | Cibola milkvetch | Salt-desert shrub | Northwestern NM, AZ |
| *A. beathiii*[c] | Beath milkvetch | Badlands | AZ, NV, UT |
| *A. bisulcatus* | Two-grooved milkvetch | Sagebrush | MT, WY, ND, SD, CO,UT, NM |
| *A. crotalariae*[c] | Rattle box milkvetch | Desert | AZ, CA |
| *A. cutleri*[c] | | | |
| *A. debequaeus*[c] | | | |
| *A. eastwoodiae*[c] | Eastwood milkvetch | Badlands | Four Corners |
| *A. flavus* | Yellow milkvetch | Alkaline soils | Colorado plateau |
| *A. grayii*[c] | Grays milkvetch | Badlands | WY, MT |
| *A. moencoppenis* | Moenkopi milkvetch | Badlands | UT, AZ |
| *A. nelsonianus*[c] | | Alkaline flats | WY |
| *A. oocalycis*[c] | | Sagebrush | CO, NM |
| *A. osterhouti* | Osterhout milkvetch | Clay hills | Grand Company, CO |
| *A. pattersoni* | Patterson milkvetch | Oakbrush | Rocky Mountains |
| *A pectinatus* | Tiny-leaved milkvetch | Northern prairies | MT, ND, WY, CO, KA |
| *A. praelongus* | Stinking milkvetch | Badlands | Four Corners |
| *A. racemosus* | Alkali milkvetch | Badlands | Plains states |
| *A. sabolosus* | Cisco milkvetch | Badlands | Eastern UT |
| *A.* saurinus[c] | Dinosaur milkvetch | Badlands | Eastern UT |
| *A. sophoroides*[c] | Painted desert milkvetch | | AZ |
| A. *toanus*[c] | Toano milkvetch | Salt-desert shrub | Great Basin |

There are many varieties, especially of *A. lentiginosus, A. mollissimus*, and *O. sericea*, that have been referred to as separate species in the past.
Species taken from Marsh (1909), Molyneux *et al*. (1991), Smith *et al*. (1992), and Fox *et al*. (1998).
[a]Also contains selenium.
[b]Also contains nitrotoxins.
[c]Minor species.

3 Long-lived perennial plants exhibit a stress-tolerant survival strategy. They have deep taproots that can access deep-stored water. They grow where moisture is more abundant and more regularly available. The plants flower and produce seeds for many years following initial establishment, although they too may die out during extended droughts (*O. sericea, O. lambertii*).

The seed bank in the soil supports these cycles. The seeds have hard coats and remain viable for many years, thus providing an ecological advantage to exploit environmental conditions and maintain the "boom and bust" population cycles (Ralphs and Cronin, 1987). Livestock poisonings follow these cycles, often in catastrophic proportions.

### *Toxicology*

Locoweed poisoning was one of the first poisonous plant problems recognized by stockmen and reported as early as 1873 (Vasey, in Marsh, 1909). Extensive stock losses in the western United States eventually led to the establishment of a field station in Hugo, Colorado in 1905 to study the problem. Research from 1905 to 1907 determined that certain species of the *Astragalus* and *Oxytropis* genera were responsible and this research was published in 1909 as a USDA bulletin titled "The locoweed disease of the plains" by C.D. Marsh.

There are numerous effects of locoweed on animals but the classic syndrome from which the term "locoism" came is one of neurological dysfunction. The disease is a chronic one developing after weeks of ingesting locoweeds and beginning with depression, dull appearing eyes, incoordination progressing to aberrant behavior including aggression, staggering, solitary behavior, emaciation, and ending in death if continued consumption is allowed. Other problems associated with locoweed ingestion include reproductive failure, abortion, birth defects, weight loss, and enhanced susceptibility to brisket disease at high elevations (James *et al*., 1981).

Locoweed poisoning affects all animals but because of the transient nature of the poisoning, animals removed from the locoweed early in the toxicosis will recover most of their function and may be productive animals. In the final stages of locoism, central nervous system tissue shows swelling of axonal hillocks (meganeurites) and growth of new dendrites and synapses (Walkley and Siegel, 1989). This altered synaptic formation in nervous tissue in severely affected animals is permanent and may be the cause of some irreversible neurological signs. Because of neurological dysfunction and apparent permanence of some lesions in the nervous system, horses are believed to be unpredictable and therefore unsafe to use for riding yet may remain reproductively sound once they have recovered from the poisoning (James *et al.*, 1969).

### Toxin

The toxin in locoweeds is the indolizidine alkaloid swainsonine **(1)**. First isolated from the Australian plant *Swainsona canescens* (Colegate *et al.*, 1979), swainsonine and its *N*-oxide were isolated and identified soon thereafter from the locoweeds (Molyneux and James, 1982).

Remarkably, C.D. Marsh (1909) made the connection between the clinical and pathological syndrome of locoweed poisoning and a similar condition reported in Australian livestock called "peastruck." Confirmation feeding trials in New South Wales with Darling pea (*Swainsona* spp.) were reported a few years before Marsh reported the details of locoweed poisoning (Martin, 1897, in Marsh, 1909). Ironically, the toxin (swainsonine) was isolated and characterized from *Swainsona canescens* and reported in Australia in 1979 (Colegate *et al.*, 1979), and subsequently identified and isolated from locoweeds (*Astragalus* and *Oxytropis*) in the United States and reported in 1982 (Molyneux and James, 1982).

The isolation and characterization of swainsonine from *Swainsona canescens* resulted from investigations by a multidisciplinary research team in Australia whose members were experienced in various aspects of lysosomal storage disease in domestic animals (Dorling *et al.*, 1989). This investigation concentrated on the details of the pathogenesis of the toxicosis enabling the research team to elucidate the mechanism of action which provided the bioassay method for toxin isolation and identification. A review of the historical aspects can be found in Dorling *et al.* (1989). In essence the locoism syndrome is a lysosomal storage disease in which $\alpha$-mannosidase is inhibited resulting in prevention of hydrolysis of mannose-rich oligosaccharides in cells and accumulation of these oligosaccharides resulting in cellular dysfunction. Most organ systems are affected but some are more susceptible such as glandular and neurological tissues.

Recent research has shown that inhibition of $\alpha$-mannosidase is relatively transient and quickly reversible once animals stop eating locoweed (Stegelmeier *et al.*, 1994). Blood serum clearance of swainsonine is rapid (half-life 20 h) thus the effects of locoweed should be reversible if tissue damage has not become extensive and permanent. This would suggest that intermittent grazing of locoweed should be an effective means of reducing locoweed poisoning. There is also an apparent threshold dosage where severity of cell damage is more time dependent than dosage dependent. Once the threshold dosage is reached, which appears to be relatively low (0.35 mg/kg in the rat), eating more locoweed does not accelerate the toxicosis (Stegelmeier *et al.*, 1995). Therefore, increasing animal numbers on loco pastures and reducing time of grazing is a logical method to reduce adverse effects.

Many locoweeds are biennials or perennials that flourish periodically under optimum environmental conditions. Historically, losses are regional and sporadic, with large regional economic impact. Individual cases of significant losses are frequent and reported in James and Nielsen (1988). Recently, 25% of over 500 mother cows of a University Foundation herd either aborted or apparently resorbed their fetuses after grazing pastures with *Oxytropis sericea*. Necropsy of aborted fetuses showed pathological lesions characteristic of locoweed poisoning and 50 calves born alive showed outward signs of toxicity (personal communication, 1994). Followup communication indicated that locoed calves were immunologically compromised and 3–4% of these calves died after weaning. The remainder of the loco calves remained about 200 lb behind calves not exposed to loco even though after the first 28 days in the feedlot the rate of gain was about the same.

### Conditions of grazing

The early literature suggested locoweeds were distasteful and animals were forced to start eating them because of hunger (Kingsbury, 1964). However, once started, animals seemed to become addicted to locoweeds. Recent research showed that locoweeds are not addicting, but are relatively more palatable than associated forages during various seasons of the year (Ralphs *et al.*, 1991b).

Preference for locoweed is relative to what other forage is available. Many locoweeds are cool-season species that green-up and start growth early in the spring, flower, set seed, and go dormant in summer, then resume growth in fall. Livestock generally prefer the green, growing locoweeds to dormant grass. Sheep preferred the regrowth foliage of Green River milkvetch to dormant grasses during late fall and early winter on desert range in eastern Utah (James *et al.*, 1968). Horses selected green-spotted locoweed to dormant grasses in spring in Arizona (Pfister *et al.*, 2003). Cattle readily grazed Wahweap milkvetch in proportion to its availability on desert winter range in southeastern Utah (Ralphs and Bagley, 1988). In a series of grazing studies in northeast New Mexico, cattle readily grazed white locoweed in March, April,

and May, but stopped grazing it in June as warm-season grasses became abundant and white locoweed matured and became coarse and rank (Ralphs *et al*., 1993, 2000). Stocker cattle grazed white locoweed on short-grass prairies in May and early June (Ralphs, 1987), but the weight loss continued throughout the summer, even though they were not eating locoweed (Ralphs *et al*., 2000; Torell *et al*., 2000). On mixed-grass prairies on the eastern foothills of the Rocky Mountains in northern Colorado, cattle ceased grazing white locoweed when it matured following flowering in mid June, and became rank and unpalatable in 1998. However, they continued to graze it throughout the summer in 1999 when abundant summer precipitation caused locoweed leaves to remain succulent (Ralphs *et al*., 2001).

### *Prevention of poisoning and management recommendations*

Prevention of poisoning remains a matter of management strategy adapted to individual grazing programs to minimize grazing of locoweed plants. Currently no broad management schemes or methods of treatment are known to generally prevent locoweed poisoning. Management strategies for individual operations have been developed once the grazing practices and options are identified, allowing utilization of the particular range and yet minimizing losses (Ralphs *et al*., 1984, 1993). It was determined that cattle generally rejected woolly loco even under extreme grazing conditions, but once they were forced to start eating it they continued to graze it and became intoxicated. Ranchers should watch for these "loco eaters" and remove them to clean pastures. Shortage of feed with high grazing pressure, social facilitation "loco eaters" teaching non-loco eaters to eat loco, or supplementing with alfalfa hay or cubes may compel cattle to start grazing woolly locoweed (Ralphs *et al*., 1993). White locoweed is more palatable than woolly locoweed and is green before spring grasses begin to grow in northeastern New Mexico. Therefore, cattle readily graze white loco in early spring while grasses are dormant and once green grass starts to grow cattle switch-off of loco. Recommendations include creating loco-free pastures through spraying, fencing, or selection of low loco-infested pastures for early spring grazing and also to provide a place to move the identified loco-eaters too. This practice appears to reduce the impact of locoweed on these ranges (Ralphs *et al*., 1993).

Many minerals, feed additives, and clay minerals have been investigated to prevent poisoning, but none have been effective. The poisonous plant literature is filled with statements that native livestock are less likely to be poisoned than new, inexperienced livestock. Locoweed poisoning does not follow this general trend. Cattle that are familiar with locoweed will likely select it first (Ralphs *et al*., 1987). Early observations by Marsh (1909) suggested that black cattle and black-faced sheep were more inclined to be poisoned by locoweed than white-faced cattle and sheep. In a recent grazing study comparing breeds, Brangus steers consumed more locoweed than Hereford and Charolais steers (Duff *et al*., 2001). The gregarious nature of Brangus cattle may have facilitated the social acceptance of locoweed among the steers.

Livestock should be denied access to locoweeds during critical periods when they are relatively more palatable than associated forages. On short-grass prairies of northeastern New Mexico, stocker cattle should not be turned onto locoweed-infested rangelands until warm-season grasses start growth in late May or early June. Cattle on rangeland year-round should be removed from locoweed-infested areas in the spring when it is green and growing, and warm-season grasses remain dormant. They can be returned to locoweed-infested pastures in summer when warm-season grasses are abundant.

Most locoweed species are endemic, growing only in certain habitats or on specific soils. Fences could be constructed on soil or vegetation boundaries to provide seasonal control of grazing. Reserving locoweed-free pastures for grazing during critical periods in spring and fall can prevent locoweed poisoning.

Locoweed-free areas can be created by strategic herbicide use (Ralphs and Ueckert, 1988; McDaniel, 1999). White locoweed is most susceptible to Clopyralid, but Picloram and Escort are also effective. However, natural population cycles should be considered to determine the practicality of spraying large areas and the potential lifetime of control. With the abundant seed bank in the soil, locoweeds are sure to germinate and reestablish when environmental conditions are favorable.

Animals that start eating locoweed may influence others to start. Social facilitation or peer pressure is a very strong influence inducing others to start eating locoweed (Ralphs *et al*., 1994b). Graham developed the "eat and pull" management strategy, whereby livestock should be watched closely and removed if they start eating locoweed to prevent poisoning and prevent them from influencing others to start (Torell *et al*., 2000).

Grazing pressure can also force cattle to begin grazing locoweed when they run short of desirable forage (Ralphs, 1987; Ralphs *et al*., 1994a). Ranchers should not overstock locoweed-infested ranges, but rather should ensure adequate forage is always available. Improper use of some grazing systems can cause livestock to graze locoweed. Rest-rotation grazing systems are designed to force livestock to uniformly graze all forage in a pasture. This caused cattle and horses to start grazing spotted locoweed in western Utah (James *et al*., 1969). Changing to a three-herd, four-pasture deferred rotation grazing system stopped locoweed poisoning by reducing the grazing pressure and allowing the cattle to select alternative forages in preference to white locoweed (Ralphs *et al*., 1984a). The

heavy grazing pressure associated with short-duration grazing systems may also induce poisoning problems.

Conditioned food aversion can be used as a management tool to train animals to avoid grazing locoweed (Ralphs *et al.*, 1997a). In the conditioning protocol, animals are brought into a pen and fed fresh-picked locoweed, and then lithium chloride (an emetic that causes gastrointestinal (GI) distress) is administered by stomach tube. The animals associate the induced illness with the taste of the plant and subsequently avoid eating it. Naive animals that are unfamiliar with the target plant form strong and lasting (>3 years) aversions following a single dose. Averted animals must be kept separate from non-averted animals on locoweed areas to prevent social facilitation from extinguishing the aversions. Aversion conditioning may be feasible where losses are heavy and persist year after year.

#### Summary

Locoweed is the most widespread poisonous plant problem in the western United States. Knowledge of sites where locoweeds grow and environmental conditions when they cause problems is necessary to manage livestock and prevent poisoning. Locoweeds are relatively palatable. Many locoweeds are the first plants to start growing in the spring and they may also regrow in the fall. Livestock generally prefer the green-growing locoweeds to other forage that is dormant in the fall, winter, and spring. The most effective management strategy is to deny livestock access to locoweeds during critical periods when they are more palatable than associated forage. Reserving locoweed-free pastures or controlling existing locoweed populations with herbicides can provide "safe" pastures for critical periods. Watching animals closely and removing those that begin eating locoweed can prevent further intoxication and prevent them from influencing others to start. Finally, conditioned food aversion is an effective practice to train animals to avoid eating locoweeds, and may be economical where losses are large and persistent. Good range management and wise grazing strategies can provide adequate forage for livestock and avoid critical periods of the year when locoweed is relatively more palatable than associated forages.

## Nitro-containing *Astragalus* (milkvetches)

There are over 260 species and varieties (356 taxa) of nitro-containing *Astragalus* in North America (Barneby, 1964; Williams and Barneby, 1977; Welsh *et al.*, 2007). They are frequently referred to as milkvetches as are some of the other *Astragalus* species. Nitrotoxins are, therefore, the most common toxin in the *Astragalus* followed by swainsonine (loco) and selenium (as discussed later). Major livestock losses occur in many regions of the western United States. These plants are very diverse and concentrated on the deserts, foothills, and mountains of the west.

#### Description

The description of these plants is the same as the locoweeds as discussed earlier. The milkvetches emerge from late April to June depending on elevation and snow cover. After seed dispersal in July or August the stems and leaves become dry and less dangerous as toxicity and palatability is reduced.

#### Distribution

The aliphatic nitro-containing *Astragalus* are distributed throughout North America with substantial livestock losses occurring in the 17 western states where cattle or sheep are concentrated in areas of milkvetch infestation (Table 66.1). There are examples of a number of *Astragalus* spp. such as *A. cicer* that are good forages and apparently contain low levels of the toxins discussed. Because of the extent of this group of *Astragalus* only a few of those implicated in poisonings will be mentioned here. For a more comprehensive review of the nitro-containing *Astragalus* the reader is referred to Williams and Barneby (1977). Examples of these include *A. emoryanus* (emory milkvetch) in New Mexico, Texas, and Arizona (*A. emoryanus* also contains swainsonine; Molyneux and James, 1991); *A. tetrapterus* (four-winged milkvetch) in Oregon, Utah, Nevada, and Arizona; *A. pterocarpus* (winged milkvetch) in Nevada; and *A. miser* var. *serotinus* var. *oblongifolius* and var. *hylophyilus* (collectively referred to as timber milkvetch) in western Canada, Montana, Idaho, Utah, Colorado, and Wyoming (James *et al.*, 1981).

#### Toxicology

The nitro-containing *Astragalus* cause an acute and chronic type of poisoning in sheep and cattle. The acute form results in weakness, increased heart rate, respiratory distress, coma, and death. Even though blood methemoglobin is high (induced from nitrotoxin metabolism to nitrites) and is a contributing factor to the respiratory difficulties, administration of methylene blue in cattle does not prevent death. Therefore, the methemoglobinemia is apparently not the primary cause of death (James *et al.*, 1981). The chronic form is the most frequent form of poisoning observed and follows a course of general weakness, incoordination, central nervous system involvement resulting in knuckling of the fetlocks, goose-stepping, clicking of the hooves, "cracker heels" progressing to paralysis, and death. A respiratory syndrome is also present in the chronic and acute signs with emphysema-like signs causing the animals to force respiration: "roaring disease." Sheep manifest the respiratory syndrome more than the central nervous syndrome and are more resistant to poisoning compared to cattle.

The toxic principles are β-D-glycosides of 3-nitro-1-propanol (3-NPOH) or 3-nitropropionic acid (3-NPA). The glycoside conversion occurs more readily in the ruminant because of the microflora and is apparently the reason for increased toxicity in ruminants. The glycoside (miserotoxin) is metabolized to the highly toxic 3-NPOH in the GI tract of ruminants (Williams *et al.*, 1970). Thus NPOH is absorbed in the gut and apparently converted to NPA by the liver (Majak *et al.*, 1985). Further metabolism yields inorganic nitrite and an unidentified metabolite which may be involved in toxicity (Majak *et al.*, 1985). It appears that NPOH is more rapidly absorbed from the gut than is NPA therefore forage containing the alcohol form is the most toxic (Williams and James, 1975).

### Prevention and treatment

There is no preferred treatment for milkvetch poisoning, although treatment with methylene blue appears to reverse the methemoglobinemia but does not prevent death in cattle (James *et al.*, 1981). Oxidation of NPOH to NPA was prevented if alcohol dehydrogenase was saturated with ethanol or inhibited with 4-methylpyrazole before NPOH was given (Majak *et al.*, 1985). This suggests that NPOH is a good substrate for the enzyme alcohol dehydrogenase. This information could be useful in acute cases, however, its value in treatment of poisoning in the field is unknown.

Livestock losses can be reduced by decreasing the density of the *Astragalus* species with herbicides or avoid grazing livestock on infested areas when the plant is most poisonous. Wasatch milkvetch contains the highest concentration of miserotoxin from bloom to immature pod stage of growth (Williams and Norris, 1969). Nitro compounds are found in all parts of the plant, but the leaves contain the highest concentration. Once the leaves begin to dry and lose their green color, the nitro levels drop very rapidly and the plant is relatively non-toxic (Williams and Binns, 1967). However, the toxins in plants pressed green and preserved in herbaria appear to remain stable for years (Williams and Barneby, 1977). Herbicide treatment decreases the density of plants and also decreases the toxicity of the plants once they start to dry, therefore spraying milkvetch appears to be the best method to reduce losses and still utilize infested ranges.

## Seleniferous *Astragalus*

About 22–24 species of *Astragalus* known to accumulate selenium have been identified (Rosenfeld and Beath, 1964; Welsh *et al.*, 2007). These are less numerous and more geographically restricted than the nitro-containing species. Many of these species are referred to as indicator plants because they only grow on soils high in selenium, therefore they are helpful in locating and identifying areas or soils high in selenium. The *Astragalus* are generally deep-rooted plants and may bring selenium from deeper soil profiles in a form unavailable to other plants and convert it to an available form subsequently taken up by grasses and other forbs (Olson, 1978). It is these secondary absorbers that create most of the subacute or chronic toxicity problems for livestock.

### Description

These *Astragalus* appear similar to those of locoweed and nitro-containing species as discussed earlier. One identifying feature of the selenium *Astragalus* species is an unpleasant garlic-like odor especially if picked and allowed to sit in a warm car or window. They are generally distasteful to livestock. Many selenium *Astragalus* may contain other toxins; e.g. *A. praelongus* and *A. bisulcatus* which contain selenium and swainsonine (the loco toxin) (Molyneux *et al.*, 1991) and *A. toanus* which contains all three: the nitrotoxins, selenium, and swainsonine (Williams and Parker, 1974).

### Distribution

The selenium *Astragalus* are limited in their distribution to geographical locations and soil sites of high selenium (Table 66.1). Major seleniferous areas of the west are in North and South Dakota, Montana, Wyoming, Colorado, and Utah with minor isolated pockets of seleniferous soils in most of the other western states.

The *Astragalus* species mostly associated with selenium poisoning include *A. bisulcatus* (two-grooved milkvetch), *A. praelongus* (stinking milkvetch), *A. pattersonii* (Patterson milkvetch), *A. pectinatus* (tiny-leaved milkvetch), and *A. racemosus* (alkali milkvetch).

### Toxicity

The syndromes of selenium poisoning are acute and chronic. Acute cases of selenium poisoning are rare and usually involve animals forced to graze indicator plants which are very disagreeable or animals accidentally eating chemical selenium preparations. Acute poisoning by overdosing with O-se or Bo-se in the treatment of white muscle disease may occur if label directions are not carefully followed. The signs of acute selenium poisoning include diarrhea, unusual postures, increased temperature and heart rate, dyspnea, tachypnea, respiratory distress, prostration, and death (James *et al.*, 1981; Tiwary *et al.*, 2005, 2006). Gross pathological findings are usually limited to pulmonary congestion and hemorrhage and pulmonary edema (James *et al.*, 1981). Histologically, multi-focal myocardial necrosis and pulmonary alveolar vasculitis is common (Tiwary *et al.*, 2006).

Chronic selenium poisoning is common and referred to as alkali disease because in most areas where selenium

is found there are alkali flats. This form of intoxication occurs from prolonged ingestion of forage containing 5–40 ppm selenium. Clinical signs include rough coat, hair or wool loss, poor growth, emaciation, abnormal hoof growth and lameness, dermatitis, and depressed reproduction (James *et al*., 1981). In swine a condition of paralysis (poliomyelomalacia or polioencephalomalacia) often occurs with cervical or lumbar involvement (Panter *et al*., 1996b). The description of a second chronic syndrome in cattle called "blind staggers" has been redefined and is now believed to be polioencephalomalacia induced by high-sulfate water or high-sulfate forage sources (James *et al*., 1989a).

Selenium is found in the inorganic and organic forms. The organic forms are found in plants and are more bioavailable than the inorganic forms resulting in higher tissues levels when administered at equivalent doses (Tiwary *et al*., 2006). While a dramatic difference in tissue selenium uptake between organic (seleno-methionine) and plant (*A. bisulcatus*) forms and inorganic (sodium selenate) forms occurs, the clinical and pathological syndromes are similar, i.e., poliomyelomalacia in pigs (Panter *et al*., 1996b), and pulmonary edema and hemorrhage in sheep (Tiwary *et al*., 2006).

Recent research demonstrated that a significant amount of selenium is eliminated in respired air especially when high doses are administered (Tiwary *et al*., 2006). Respiratory elimination kinetics were much different between sheep administered Se-methionine compared to sodium selenite. Significantly higher levels of Se were eliminated and for significantly longer periods of time after Se-methionine administration compared to sodium selenite. Grasses, forbs, and certain vegetables assimilate selenium from the soils at levels high enough to induce chronic selenium poisoning in animals and occasionally in man and this is of the organic form.

#### *Prevention of poisoning*

There is no treatment for selenium poisoning except the removal of the source allowing spontaneous recovery in the chronic cases. Monitoring soils in a particular area and understanding the plant communities can provide the management information to avoid poisoning. In areas where selenium is a problem many ranchers have switched to grazing steers because of the reproductive problems in cows. Sheep seem to be more resistant to selenium compared to cattle and are better adapted for some of these ranges; however, acute selenium poisoning in sheep after brief grazing on mine reclamation sites where high soil selenium occurred was recently reported (personal communications, 2004). Monitoring of soil selenium, vegetation, and animal selenium levels will help avoid poisoning. Likewise, deficiency problems can be rapidly resolved with frequent monitoring.

## LARKSPURS (*DELPHINIUM* SPP.)

There are over 80 wild species of larkspurs in North America (Kingsbury, 1964) and there are a larger number of domestic horticultural varieties. Wild larkspurs are divided into three general categories based primarily on mature plant height and distribution: low, tall, and plains larkspurs (Figure 66.2). The dominant larkspur species in the western United States are shown in Table 66.2. The larkspurs are a major cause of cattle losses on western ranges. In 1913, Marsh *et al*. (1916) stated that more cattle deaths on western ranges are caused by *Delphinium* spp. than any other poisonous plant except locoweed.

### Description

Most of the wild larkspurs have flowers of blue or purple, bilaterally symmetrical, in erect terminal racemes or panicles: five sepals, the upper prolonged backwards in a prominent spur; petals smaller and variable among species, usually four and sometimes two, the upper pair projecting back inside the spur; carpels one to five, sometimes fused, ripening into many-seeded follicles (Kingsbury, 1964).

The tall larkspurs (Figure 66.2) are 1–2 m tall or more, flower in summer and set seed in late summer subsequently senescing in the fall. In the west the tall larkspurs typically inhabit higher mountain elevations in the more open moist areas of canyons, draws, and meadows. *Delphiniums* are frequently confused with *Aconitum* but certain features distinguish them. As implied by the common names *Delphiniums* have a distinct spur (hence larkspur)

**FIGURE 66.2** Low larkspur (*Delphinium bicolor*) is on the left and tall larkspur (*D. barbeyi*) is on the right.

whereas *Aconitum* has no spur but a hood (hence monkshood). Tall larkspurs have hollow stems and their leaves are relatively long petioled whereas monkshood has stems that are solid and pithy and leaves are short petioled.

Low larkspurs (Figure 66.2) appear similar to tall except they grow in different habitats and locations, i.e. lower elevations in drier habitats, on the foothills and flats. They appear first as a rosette-like clump in early spring, soon producing an erect flowering stem, usually not more than 1 m in height, then die back in early summer.

Plains larkspur falls between the low and tall larkspur classifications. Plants range from 0.3 to 2 m tall. It grows on the short-grass plains of Wyoming into Nebraska and in the sagebrush and juniper woodlands of the Colorado Plateau.

## Distribution and habitat (Table 66.2; Kingsbury, 1964; Nielsen and Ralphs, 1988)

### *Low larkspur*

*D. nelsonii*: Idaho, South Dakota, Wyoming, Colorado, and Utah

*D. bicolor*: North Dakota, Montana, Wyoming, Oregon, and Washington

*D. andersonii*: Oregon, California, Nevada, Utah, and Idaho

*D. tricorne*: Nebraska and Oklahoma eastward

*D. virescens*: Great plains, east of the Rockies

### *Intermediate larkspur*

*D. geyeri*: Colorado, Wyoming, Nebraska, and Utah

### *Tall larkspur*

*D. barbeyi*: Utah, Wyoming, Colorado, and New Mexico

*D. occidentale*: Washington, Idaho, Wyoming, Nevada, Utah, and Colorado

*D. glaucescens*: Idaho and Montana

*D. glaucum*: Washington, Oregon, California, Nevada, Idaho, and Montana

*D. trolliifolium*: Washington, Oregon, and California

*D. robustum*: Colorado and New Mexico

## Toxicology

Larkspurs (*Delphinium* spp.) are a serious toxicity problem for cattle on foothill and mountain rangelands in western North America. Larkspurs contain numerous norditerpenoid alkaloids that vary greatly in toxicity. Three alkaloids are of primary concern (Manners *et al.*, 1995): methyllycaconitine (MLA **(2)**), 14-deacetylnudicauline (DAN), and nudicauline (NUD). MLA and DAN occur to some extent in all classes of larkspurs, whereas NUD occurs only in low and plains larkspurs (Majak and Engelsjord, 1988). The $LD_{50}$ for NUD, MLA, and DAN in mice is 2.7, 4.8, and 4.0 mg/kg i.v., respectively (Table 66.3; Panter

**TABLE 66.2 Characteristics of the dominant larkspur species in western North America**

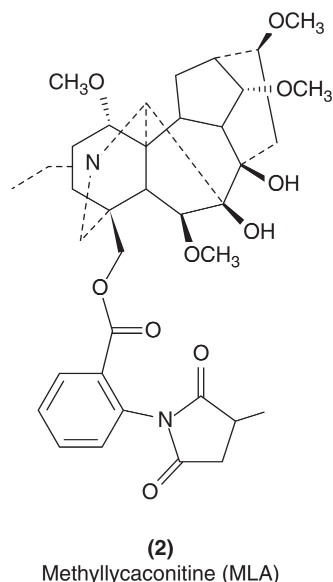
| Class/species | Height at maturity | Elevation | Associated plant communities | Toxicity ranking[a] | Typical risk of losses[b] |
|---|---|---|---|---|---|
| **Tall larkspurs** | | | | | |
| *D. glaucum*[c] | 90–200 | >2000 | Aspen, conifers, alpine meadows | 1 | Low |
| *D. barbeyi* | 90–180 | >2200 | Aspen, conifers, alpine meadow, mountain brush, alpine tundra | 2 | Moderate to severe |
| *D. glaucescens* | 76–90 | >2000 | Mountain meadows, sagebrush | 3 | Low to moderate |
| *D. occidentale* | 90–180 | >2000 | Mountain brush, sagebrush, conifer, aspen | 4 | Low to severe |
| **Low larkspurs** | | | | | |
| *D. nuttallianum* | 20–60 | >1200 | Mountain brush, sagebrush, aspen, conifer, mountain and foothill meadows | 2 | – |
| *D. bicolor* | 20–40 | >800 | Mountain brush, sagebrush | – | – |
| *D. andersonii* | 10–60 | >1200 | Desert shrub, mountain brush, sagebrush, pinyon-juniper | 1 | – |
| **Plains larkspur** | | | | | |
| *D. geyeri* | 40–80 | >1500 | Desert shrub, mountain brush, sagebrush, short-grass prairie | – | – |

[a] Tall larkspur and low larkspur species each ranked according to concentration of toxic alkaloids, from greatest (1) to least (4).

[b] The risk of losing cattle to tall larkspur species is a subjective evaluation based on plant toxicity, numbers of grazing cattle threatened during the growing season, and the geographical distribution of the larkspur species. *D. glaucescens* is relatively more toxic late in the growing season compared to mature plants of the other tall larkspur species. Spatial and temporal variability in plant density and toxicity preclude making such a risk assessment ranking for low larkspurs and plains larkspur.

[c] *D. glaucum* = *D. brownii* in Canada; livestock losses to *D. brownii* in Canada may greatly exceed those of *D. glaucum* in the United States (Majak and McDiarmid, 2000).

*et al.*, 2002). The toxic alkaloid concentration of the tall larkspur (*D. barbeyi*) dosed to cattle in the lethality study of Olsen (1978) was not determined. Assuming that the plant material (vegetative and early flowering tall larkspur) contained ~12 mg/g of toxic alkaloid, the $LD_{50}$ of MLA + DAN in cattle is approximately 30 mg/kg. The lethal dose is undoubtedly lower when tall larkspur is ingested repeatedly over 2–4 days, as often happens in field cases of intoxication (Pfister *et al.*, 1997).




**(2)**
Methyllycaconitine (MLA)

There are two primary structural features necessary for toxicity: (1) an *N*-ethyl bicyclo tertiary alkaloid nitrogen atom; and (2) a C-18 anthranilic acid ester (Kukel and Jennings, 1994). Other studies have also shown that MLA's **(2)** aromatic ester function is a significant haptophore (Coates *et al.*, 1994), and that the succinimide group imparts significant toxicity to alkaloids (Blagbrough *et al.*, 1994; Hardick *et al.*, 1996). Two other structural features also enhance toxicity: (1) functionality at the anthranilic acid amine nitrogen and (2) functionality at C-14 (Manners *et al.*, 1995).

Tall larkspur species vary substantially in toxicity, with a relative ranking (most to least toxic) of *D. glaucum* (*D. brownii* in Canada), *D. barbeyi*, *D. glaucescens*, and *D. occidentale* (Ralphs *et al.*, 1997b). Generally in tall larkspurs, the concentration of MLA and DAN is highest in immature plant tissues. MLA concentrations in immature tall larkspurs may exceed 20 mg/g. Before shattering, tall larkspur pods are relatively high in toxicity (MLA + DAN = 7–12 mg/g). Toxicity declines rapidly in tall larkspurs once pods begin to shatter. Measuring plant toxicity early in the growing season may allow prediction of season-long toxicity and risk (Ralphs *et al.*, 2002).

**TABLE 66.3 Relative toxicity of individual larkspur alkaloids: in the order of toxicity according to alkaloid class and determined by mouse bioassay (Manners *et al.*, 1993)**

| **MSAL[a] class alkaloid; $LD_{50}$ mg/kg** | **Lycoctonine class alkaloid; $LD_{50}$ mg/kg** | **MDL[b] class alkaloid; $LD_{50}$ mg/kg** |
|---|---|---|
| Nudicauline; 2.5 i.v.[c] | Anthronyllycoctonine; <365 sc[d] | Deltaline; 720 sc, 133 i.v. |
| 14-deacetylnudicauline; 4.5 i.v. | Lycoctonine; <450 sc | 14-*o*-acetyldictyocarpine; >765 sc |
| Methyllycaconitine; 7.5 i.v. | 14-dehydrobrownine; >254 sc | Dictyocarpine; >2000 sc |
| Barbinine; >115.5 i.v. | Brownine; >720 sc | |

[a]MSAL = methylsuccinimido anthranoyllycoctonine (most toxic).
[b]7,8-methylene dioxyllycoctonine (least toxic).
[c]i.v. = intravenous.
[d]*>sc = subcutaneous.

MLA and NUD are the dominant toxic alkaloids in low larkspurs with concentration ranges from 0.8 to 4.5 mg/g and 1 to 4 mg/g, respectively, for MLA and NUD in low larkspur populations (*D. nuttallianum* and *D. andersonii*) in Utah, Colorado, and Arizona (Gardner, unpublished data). Majak (1993) reported very high concentrations of MLA (up to 8.7 mg/g) in vegetative low larkspur (*D. nuttallianum*) from Canada, contrasted to 2 mg/g in flowering plants (Bai *et al.*, 1994). Unlike tall larkspurs, concentrations of toxic alkaloids in low larkspurs do not decline precipitously after senescence (Majak, 1993; Gardner, unpublished data). MLA is the major toxic alkaloid in plains larkspur (*D. geyeri*), with concentrations ranging from 1 to 4 mg/g in Wyoming and Colorado (Pfister *et al.*, 2002a). We emphasize that NUD is more toxic than MLA, and both alkaloids are frequently found together in low larkspurs (Gardner, unpublished data). Thus, the potential lethality of low larkspurs can be highly variable depending on alkaloid concentrations and plant density.

The primary result of larkspur toxicosis is neuromuscular paralysis from blockage at the post-synaptic neuromuscular junction (Aiyar *et al.*, 1979; Benn and Jacyno, 1983). MLA also elicits central effects in mice and rats (Alkondon *et al.*, 1992; Stegelmeier *et al.*, 1998). Larkspur alkaloids compete as post-synaptic inhibitors of acetylcholine particularly at $\alpha$-1-nicotinic sites (Dobelis *et al.*, 1999). MLA strongly competes with $\alpha$-bungarotoxin at nicotinic ACh (nACh) receptors (Kukel and Jennings, 1994). Larkspur alkaloid binding to nAch receptors appears to be correlated to toxicity in various tissues (Kukel and Jennings, 1994), and may explain sheep tolerance to larkspur if larkspur toxins bind less avidly to nAch receptors in sheep (Ward *et al.*, 1990; Stegelmeier *et al.*, 1998).

Clinical signs of intoxication include muscular weakness and trembling, straddled stance, periodic collapse into sternal recumbency, respiratory difficulty, and finally death while in lateral recumbency. An effective dose of larkspur causes labored breathing, rapid and irregular heartbeat and collapse, but not death. The effective i.v. dose for purified MLA is 2 mg/kg b.w. in calves versus 10 mg/kg in sheep given a single injection (Panter, unpublished data). Nation *et al.* (1982) reported that an effective dose of MLA in cattle receiving two i.v. injections was 1.1 mg/kg. Conversely, the effective i.v. dose for deltaline in both calves and sheep is 50 mg/kg (Panter, unpublished data). Cattle typically show clinical signs (i.e. tremors and periodic collapse) when given an MLA + DAN dose (i.e. via ground plant) of 20 + 3 mg/kg b.w. (Pfister *et al.*, 1994a, b). Stress and/or exertion will reduce the effective dose. Assuming an MLA + DAN concentration of 5 mg/g (dry weight (d.w.)), a 450 kg cow may show clinical signs after rapidly eating 1.8 kg (d.w.) of tall larkspur ($\approx$7.2 kg wet weight (w.w.)).

Key factors in larkspur intoxication are the amount eaten and the rate of ingestion. Studies utilizing direct observations of grazing animals have provided a number of insights into the amount and timing of tall larkspur ingestion (Pfister *et al.*, 1988). Over 10 such studies have been conducted since 1986 and have led to two major conclusions: (1) cattle eat little or no tall larkspur before the plant has elongated flowering racemes (Pfister *et al.*, 1997) and (2) weather patterns are very important determinants of larkspur consumption (Ralphs *et al.*, 1994c; Pfister *et al.*, 1999). Cattle often eat more tall larkspur during summer storms, and reduce larkspur consumption during drought for reasons that are not clear.

Cattle generally begin consuming tall larkspur after flowering racemes are elongated, and consumption increases as larkspur matures. Consumption usually peaks during the pod stage of growth in late summer, when cattle may eat large quantities (25–30% of diet as herd average; >60% on some days by individual animals).

Because larkspur toxicity generally declines throughout the growing season and cattle tend to eat more larkspur after flowering, we have termed the period of greatest danger a "toxic window" (Pfister *et al.*, 2002b). This "toxic window" extends from the flower stage into the pod stage, or about 5 weeks depending on temperature and elevation (Figure 66.3). Many ranchers typically defer grazing on tall larkspur-infested ranges until the flower stage to avoid death losses. This approach wastes much valuable forage, and often places cattle into larkspur-infested pastures when risk of losses is high. An additional 4–6 weeks of grazing may be obtained by grazing these ranges early, before larkspur elongates flowering racemes (Pfister *et al.*,

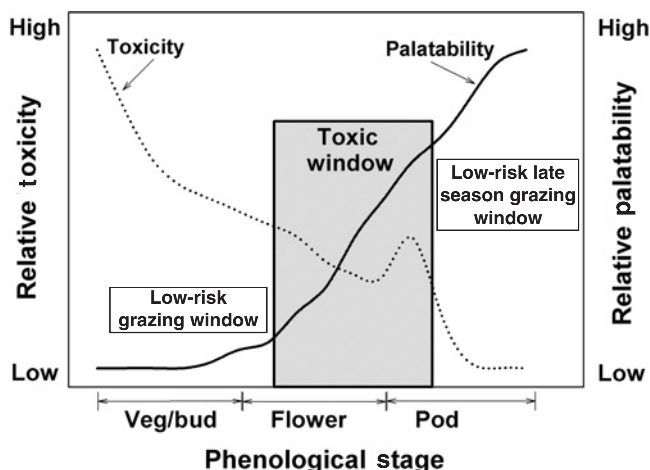



**FIGURE 66.3** Relationship between toxicity and palatability in tall larkspurs. Most cattle deaths are predicted to occur during the toxic window when concentration of toxic alkaloids is relatively high, and consumption by cattle begins to increase after flowering racemes elongate. There is a low-risk grazing window early in the season before flowering when larkspur is generally very toxic but risk is low because cattle typically eat little tall larkspur during this phenological stage. There is also a low-risk grazing window in late summer after pods shatter.

1997). The risk of losing cattle is low when grazing before flowering even though larkspur is very toxic, because larkspur consumption is typically very low. Once pods are mature and begin to shatter, larkspur ranges can usually be grazed with impunity because pod toxicity declines rapidly, and leaf toxicity is low. Based on limited study, cattle increase consumption of low larkspur after flowering, and increases in grazing pressure increase amounts of low larkspur eaten by cattle (Pfister and Gardner, 1999). No consistent consumption patterns of plains larkspur (*D. geyeri*) by cattle were found, thus management recommendations need to be tailored to each specific year and location (Pfister *et al.*, 2002a, b).

## Prevention and management of poisoning

### *Grazing management*

A simple and low-risk grazing management scheme can often be used based simply on tall larkspur growth and phenology: (1) graze during early summer when sufficient forage is available until larkspur elongates flowering racemes (4–6 weeks depending on elevation and weather); (2) remove livestock, or contend with potentially high risk from flowering to early pod stages of growth (4–5 weeks); and (3) graze with low risk during the late season when larkspur pods begin to shatter (4–6 weeks). This scheme can be refined substantially if livestock producers obtain an estimate periodically of the toxicity of tall larkspur, and if ranchers spend time to periodically observe and document larkspur consumption by grazing cattle (Pfister *et al.*, 1999).

Management to reduce losses to low larkspur begins with recognition of the plant during spring. Vegetative low larkspur plants will typically begin growth before the major forage grasses. Low larkspur populations fluctuate with environmental conditions (Pfister, unpublished data). Risk of losing cattle is much higher during years with dense populations. During those years, recognizing the plant, and finding alternative pasture or waiting to graze infested pastures for 4–6 weeks, until the low larkspur has dried up, will reduce losses.

### *Graze sheep before cattle*

Sheep can be herded into or bedded on the patches to reduce larkspur availability or acceptability to cattle on tall larkspur-infested ranges where larkspur grows as discrete patches (Ralphs *et al.*, 1991a; Ralphs and Olsen, 1992). In those areas where larkspur is uniformly spaced over a pasture, sheep must eat immature larkspur and leave sufficient feed for subsequent grazing by cattle. This can be problematic, since early growth tall larkspur may not be palatable to sheep. Marsh *et al*. (1916) also noted that sheep grazing may reduce cattle losses to low larkspur. Our observations (Pfister, unpublished data) indicate that sheep eat little low larkspur (*D. nuttallianum*) unless a high stock density is used.

### *Drug intervention*

A variety of remedies have been applied in the field when ranchers find intoxicated animals (e.g. bleeding by cutting the tail), but most are without a solid scientific rationale. Any imagined success with these treatments was probably related to the dose. If less than a lethal dose were ingested, the animal would likely recover in spite of any treatment, unless bloat or aspiration pneumonia occurred during recumbency. Drugs that increase ACh effectiveness at the neuromuscular junction have potential for reversing larkspur toxicity or reducing susceptibility. The cholinergic drug, physostigmine (0.08 mg/kg i.v.), has been successfully used under field and pen conditions to reverse clinical larkspur intoxication (Nation *et al.*, 1982; Pfister *et al.*, 1994b). This reversal lasts about 2 h, and repeated injections of physostigmine are sometimes required. Under field conditions, physostigmine temporarily abates clinical signs and animals quickly (~15 min) become ambulatory. Depending on the larkspur dose, the intoxication may recur. The use of physostigmine-based treatments may aggravate losses in the absence of further treatment if suddenly ambulatory animals later develop increased muscular fatigue, dyspnea, and death.

### *Herbicidal control*

Larkspur losses can be reduced greatly if dense larkspur populations are reduced by herbicides. Picloram, metsulfuron, and glyphosate have proven to be effective in killing tall larkspurs when applied at specific growth stages (Mickelsen *et al.*, 1990; Ralphs *et al.*, 1992). These herbicides do not reduce toxic alkaloid concentrations in treated larkspur plants, and metsulfuron may increase toxicity. Therefore, sprayed areas should not be grazed until the following growing season.

# LUPINES (*LUPINUS* SPP.)

The *Lupinus* genus contains more than 150 species of annual, perennial, or soft woody shrub lupines (Cronquist *et al.*, 1989). Over 95 species occur in California alone (Riggins and Sholars, 1993). The lupines are rich in alkaloids, responsible for most of the toxic and teratogenic properties. There are domestic lupines that through plant breeding are low in alkaloid content and have been selected for ornamental purposes or for animal and human food. Only those range lupines known to cause poisoning or birth defects will be discussed here.

Stockman have long recognized the toxicity of lupines when livestock, particularly sheep, were poisoned in the fall by the pods and seeds of lupine (Chesnut and Wilcox, 1901). Sampson (1952) and Stoddart and Smith (1955) reported that lupine was a major cause of sheep losses in the west and indeed single flock losses of hundreds and even thousands were reported (Chesnut and Wilcox, 1901). Lupines are poisonous to other livestock also and field cases of poisoning in cattle, horses, and goats have been reported. However, the most recognized condition of lupine ingestion is the "crooked calf syndrome," a congenital condition in calves resulting in skeletal contracture-type malformations and cleft palate after their mothers have grazed lupines during sensitive periods of pregnancy (Panter *et al*., 1999). The condition was first reported by Palotay (1959) and Wagnon (1960) and experimentally confirmed after large outbreaks in Oregon and Montana (Shupe *et al*., 1967a, b).

## Description

Lupines belong to the Leguminosae family, with alternate palmately compound leaves with 5–17 oblong to lanceolate leaflets (Figure 66.4). Flowers are terminal legume-like and can be blue, purple, white, yellow, or reddish. The seeds are flattened in legume-like pods. Range lupines are generally low, perennial, or annual forbs and species and varieties are taxonomically difficult because extensive hybridization occurs and literature may be somewhat confusing in this regard. Chemical profiles (chemo-taxonomy) can support or contradict taxonomic identification and this is a valuable resource if included in a voucher specimen, especially if the identified species is implicated in toxicoses or teratogenicity. The plant grows early in spring, flowering in early to mid-summer and forms pods in late summer or early fall. Seedlings may germinate in fall if temperature and moisture are conducive for seed germination. Some lupines are annuals and others are woody and shrub-like, however, these have not been reported in livestock poisoning. The reader is referred to Cronquist *et al*. (1989) or Riggins and Sholars (1993) for detailed descriptions of species and varieties.

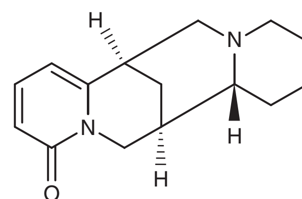
## Distribution

Most species of lupines grow in states and provinces from the rocky mountains, westward. They are classified as increaser species, i.e., they increase in abundance following disturbance from overgrazing or fire, to the point they may dominate the plant community at times. Their populations also cycle, increasing in wet years and dying back during drought. Table 66.4 lists species involved in toxicity, their common names, habitats, and geographical distribution.

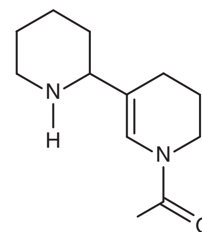
**FIGURE 66.4** *Lupinus leucophyllus*

## Toxicology

Most lupine species contain quinolizidine alkaloids, a few contain piperidine alkaloids and some contain both. The specific alkaloids responsible for "crooked calf disease"




**(3)**
Anagyrine




**(4)**
Ammodendrine

are anagyrine **(3)**, ammodendrine **(4)**, and *N*-methyl ammodendrine. Hence, risk is based on chemical profile and presence and concentration of these teratogenic alkaloids. We know that chemical profile and concentration differs, resulting in changing levels of toxicity within and between species and populations (Davis and Stout, 1986). The chemical phenology has been studied in *L. caudatus* (Keeler, 1976)

**TABLE 66.4 Lupine species known to be toxic or teratogenic**

| Scientific name | Common name | Habitat | Distribution |
|---|---|---|---|
| *L. alpestris* | Mountain silvery lupine | Dry rocky soils | CA to MT, CO and AZ |
| *L. arcticus* | | | AK, BC |
| *L. andersonii* | Anderson's lupine | Mountain meadows and clearings | Sierra Nevada, CA to 2900 m |
| *L. arbustus* | Spur lupine | Dry open hillsides | NV, CA to CO; western MT |
| *L. argenteus* | Silvery lupine | 1200–1500 m elevation; dry flats, slopes, woods, open hillsides | CA to MT, NM, OR, ID, UT, NV |
| *L. caudatus* | Tail cup lupine | | CA, OR, ID to UT, NV |
| *L. cyaneus* | | | MT |
| *L. formosus* | Lunara lupine | Dry flats | CA |
| *L. greenei* | | Plains and hills | WY, NV, CO, AZ |
| *L. latifolius* | Broad-leaved lupine | Mountain meadows and stream banks | Pacific states |
| *L. laxiflorus* | Spurred lupine | Hillsides, dry soils | WA, OR, ID |
| *L. leucophyllus* | White-leaved lupine, poison lupine | Dry soils | WA, MT, UT, CA |
| *L. nootkatensis* | Nootka lupine | | AK |
| *L. onustus* | Plumes lupine | Open pine wood | CA |
| *L. polyphyllus* (five varieties) | Meadow lupine | Mountain meadows, banks | WA, BC |
| *L. pusillus* | Rusty lupine, small lupine | Dry plains | BC to KS, NM, AZ |
| *L. sericeus* | Silky lupine | Dry hills, valleys | UT, WY to MT, BC |
| *L. sulphureus* | Yellow lupine | Open hillsides | OR |

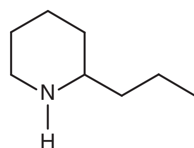
and *L. leucophyllus* (Lee *et al*., 2006). Total alkaloid concentration is high in the new early growth but diluted as the plant biomass increases. Pools of total alkaloids increase during the phenological growth stages and peak at the pod stage. The teratogenic alkaloid anagyrine appears to be an end product in the biosynthetic pathway and accumulates in the floral parts and is stored in the seed. Following seed shatter both concentration and pools of all alkaloids decline precipitously, leaving the senescent plant relatively non-toxic.

Stockmen recognized the toxicity of lupines in the fall when the pods and seeds are ripe. Historically, lupines were responsible for more sheep deaths than any other single plant in Montana, Idaho, and Utah (Kingsbury, 1964). Most losses occurred from hungry sheep grazing seedpods. Poisoning occurred following trucking or trailing bands in late summer or fall, or getting caught in early snowstorms that covered herbaceous vegetation. Hungry sheep non-selectively grazed lupine pods, which are highest in alkaloids, and were poisoned. Large losses have also occurred when lupine hay harvested in the seedpod stage was fed in winter. Seeds alone can be toxic to sheep at 0.25–1.5% of their body weight depending on alkaloid composition. A few cases of poisoning occurred on young plants. Recent losses of 80–100 sheep in multiple bands have been reported the last 5 years in Idaho and Wyoming (Panter, personal communication, 2005).

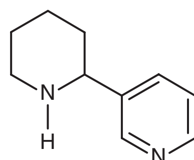
Poisoning by lupine plants should not be confused with lupinosis reported in Australia. This condition is entirely different and is a mycotoxicosis of livestock caused by toxins produced by the fungus *Phomopsis leptostromiformis* which colonizes domestic lupine stubble. It affects livestock which graze lupin stubble and limits the use of this animal feed in Australia (Allen and Cowling, 1992).

The lupine-induced "crooked calf disease" was first reported in 1959 (Palotay) and 1960 (Wagnon) and experimentally confirmed in 1967 (Shupe *et al*., 1967a, b). Crooked calf disease includes various skeletal contracture-type birth defects and occasionally cleft palate (Shupe *et al*., 1967a, b; 1968). The skeletal defects are similar to an inherited genetic condition reported in Charolais cattle (Nawrot *et al*., 1980). Through epidemiological evidence and chemical comparison of teratogenic and non-teratogenic lupines the quinolizidine alkaloid anagyrine was determined to be the teratogen (Keeler, 1973). A second teratogen, a piperidine alkaloid called ammodendrine found in *Lupinus formosus*, was also demonstrated to cause the condition (Keeler and Panter, 1989). Further research determined that the anagyrine-containing lupines only caused birth defects in cattle and did not affect sheep or goats, however, the piperidine-containing lupine *L. formosus* induced similar birth defects in cattle and goats (Keeler and Panter, 1989; Panter *et al*., 1998). This led to interesting speculation about possible metabolism or absorption differences between cattle and small ruminants. Keeler and Panter (1989) hypothesized that perhaps the cow was metabolizing the anagyrine to a complex piperidine, meeting the structural characteristics determined for a teratogenic piperidine (Keeler and Balls, 1978). This was supported by feeding trials with other piperidine-containing plants, extracts, and pure compounds. Coniine **(5)**, a simple piperidine from poison-hemlock, and anabasine **(6)**, a simple piperidine from tree tobacco (*N. glauca*), induced the same defects in cattle, sheep, and goats. Even though comparative studies support the hypothesis that the cow may metabolize the quinolizidine anagyrine to a complex piperidine, recent evidence reporting the absorption and elimination patterns

of many of the quinolizidine alkaloids, including anagyrine, in cattle, sheep, and goats, did not support the metabolism theory (Gardner and Panter, 1993). This research is currently ongoing at the Poisonous Plant Research Laboratory in Logan, Utah.




**(5)**
Coniine




**(6)**
Anabasine

Different lupines produce varying toxic syndromes in a given species of livestock, apparently because the alkaloid profile varies remarkably among species. Season and environment influenced alkaloid concentration in a given species of lupine. Generally alkaloid content is highest in young plants and in mature seeds and alkaloids are not lost upon drying, so wild hay may be highly toxic if young lupine plants or especially seedpods are present. For many lupines, the time and degree of seeding varies from year to year. Most losses occur under conditions in which animals consume large amounts of pods in a brief period, such as when they are being driven through an area of heavy lupine growth, unloaded into such an area, trailed through an area where the grass is covered by snow, but the lupine is not, or when feeding podded lupine hay, which is apparently palatable. Most serious poisonings may occur in the late summer or early fall because lupine remains green after other forage has dried and seedpods are present. Once the poisonings were understood the practice of harvesting lupine hay for winter sheep feed was discontinued.

### *Cattle grazing*

Lupine is not very palatable to cattle, although it has been considered fair feed on some ranges that are heavily utilized. Its palatability or acceptability depends on availability and maturity of other forage. In a grazing study of velvet lupine (*L. leucophyllus*) on annual cheatgrass ranges in eastern Washington (Ralphs *et al.*, 2006), cows selected lupine only in July and August after cheatgrass dried and other forbs were depleted or matured and became rank. The deep-rooted lupine remained green and succulent

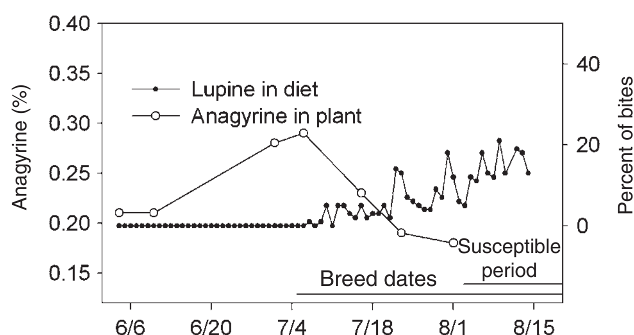



**FIGURE 66.5** The relationship between the concentration of the teratogen anagyrine in the plant and the amount of lupine in the diets of cattle during breeding and susceptible gestational stages.

longer into the summer than the other forage. Lupine was higher in crude protein (CP) and lower in fiber (neutral detergent fiber (NDF)) than the other forages throughout the season (CP level in foliage was 15% and in seeds 36%). However, the thick velvety pubescence was believed to be partially responsible for its lack of palatability. The high alkaloid concentration in the floral parts and seedpods may also have contributed to its lack of palatability. Once the seeds shattered in early July, the alkaloid concentration declined, and the cows began selecting lupine. Figure 66.5 shows the overlap of lupine consumption with the susceptible period of gestation, in relation to the anagyrine concentration in the lupine plants. Lopez-Ortiz *et al.* (2006) also confirmed that cattle selected velvet lupine in July after cheatgrass and other forbs matured.

The abundance of lupine is another factor influencing the amount of lupine consumed. Lupine population cycles are influenced by weather patterns. Catastrophic losses from lupine-induced crooked calves occurred in the Channel Scabland region of eastern Washington in 1997 (Warnock, 1997). Annual precipitation from 1995 to 1997 was 33% above average, initiating an outbreak of lupine throughout the region. Density of velvet lupine plants has declined since then (Ralphs, unpublished data) and incidence of crooked calves has been minimal.

Clinical signs of poisoning are those of muscular weakness (neuromuscular blockade) beginning with nervousness, frequent urination and defecation, depression, frothing at the mouth, relaxation of the nictitating membrane, ataxia, muscular fasciculations, weakness, lethargy, collapse, sternal recumbency followed by lateral recumbency, respiratory failure, and death. Signs may appear within 15 min to an hour after ingestion or as late as 24 h depending on amount and rate of ingestion. Death usually results from respiratory paralysis.

Incidence of crooked calves is variable geographically and from year to year within a given herd. Up to 100% of a given calf crop may be affected and individuals may be more severely affected than others. Affected calves are generally born alive at full term. Dystocia may occur when

calves are severely deformed and assistance is required, often by cross-section.

Arthrogryposis is the most common malformation observed often accompanied by one or more of the following: scoliosis, torticollis, kyphosis, or cleft palate (Shupe *et al.*, 1967a). Elbow joints are often immobile because of malalignment of the ulna with the articular surfaces of the distal extremity of the humerus. The part of the limb distal to the elbow joint is often rotated laterally. In crooked calf disease the osseous changes observed are permanent and generally become progressively worse as the calf grows and its limbs are subjected to greater load-bearing stress. Frequently, minor contractions such as "buck knees" often attributed to lupine will resolve on their own and the calf will appear relatively normal.

No breed predilection or genetic susceptibility to the lupine-induced condition has been determined. However, body condition was shown to affect the absorption and elimination half-life of anagyrine in sheep (Lopez-Ortiz *et al.*, 2004). Research to compare alkaloid disposition in cattle based on body condition is ongoing at the Poisonous Plant Research Laboratory. Preliminary data would suggest that body condition may have a significant impact on absorption, distribution, and elimination of toxins and therefore could impact teratogenic outcome of lupines. The fetal disposition of teratogenic alkaloids is unknown at this time.

The dangerous gestational period in the pregnant cow for exposure is 40–70 days with suspicious periods extending to day 100 (Panter *et al.*, 1997). The condition has been experimentally induced with dried ground lupine at 1 g/kg b.w. and with semi-purified preparations of anagyrine (the apparent teratogen) at 30 mg anagyrine/kg b.w. fed daily from 30 to 70 days gestation (Keeler, 1976; Keeler *et al.*, 1976). The dose range of anagyrine to cause crooked calves is 6.5–11.9 mg/kg b.w./day for 3–4 weeks during gestation day 40–70 (Keeler, 1976). Crooked calf disease has also been induced by feeding the piperidine alkaloid-containing lupine, *L. formosus* (Keeler and Panter, 1989). The teratogenic piperidines, ammodendrine, *N*-acetylhystrine, and *N*-methyl ammodendrine are absorbed quickly after ingestion and can be detected in blood plasma by 0.5 h and peak levels maintained for over 24 h (Gardner and Panter, 1993, 1994; Panter and Gardner, 1994). The mechanism of action has been determined to be an alkaloid-induced reduction in fetal movement by a neuromuscular blocking effect during the critical stages of gestation (Panter *et al.*, 1990a). This inhibited fetal activity is responsible for the skeletal malformations and cleft palates (Panter and Keeler, 1992).

## Prevention, management, and treatment

Keeler *et al.* (1977) proposed a simple management solution to prevent crooked calves: stagger grazing of lupine infested pastures so that the susceptible period of gestation (40–70 days) does not overlap the flower and pod stage of growth when anagyrine is highest. Ralphs *et al.* (2006) refined Keeler's recommendations to restrict access during the susceptible period of gestation, when anagyrine concentration is still high in the flower and pod stage, only when cattle are likely to eat lupine, and in years when it is abundant. Panter (unpublished data) has suggested that intermittent grazing between lupine pastures and clean pastures would allow the fetus to regain normal movement for a few days during the sensitive stage of gestation. It has been hypothesized that inhibited fetal movement over a prolonged period of time is required for severe malformations to occur (Panter *et al.*, 1999).

Lupines are easily controlled with 2,4-D-type broad-leaf herbicides (Ralphs *et al.*, 1991c), however, herbicide treatment alone rarely provides long-term solutions to poisonous plant problems. Seed reserves in the soil will rapidly reestablish the stands if grazing management practices are not implemented.

Death losses in sheep can be reduced by recognizing the variability in lupine toxicity with stage of growth and the conditions under which animals graze the plant. Providing a choice of other quality forages usually prevents excess lupine grazing. The dangerous period of plant growth for sheep exists mainly with podded plants. The hazard increases if sheep are hungry as often exists with crowding, hauling, driving, or overgrazed conditions. The hazard is reduced or eliminated when lupine is in post-seed stage.

Treatment for overt poisoning is usually symptomatic and recovery is often spontaneous if animals are not stressed further by driving. Once the animal is observed showing muscular tremors it should be allowed to drop back and proceed at its own pace. Never force poisoned animals to continue moving as this will exacerbate the clinical effects and can result in death. The elimination of the toxic alkaloids in the urine is quite rapid ($t_{1/2}$ = 6.32 – 6.88 h) and begins within hours of ingestion (Lopez-Ortiz *et al.*, 2004). Therefore, allowing the animal to rest and move slowly will often result in full recovery within 24 h. There is no treatment for the malformations and euthanasia is recommended for the serious skeletal defects and cleft palate. However, less severe contracture defects, particularly of the front legs (buck knees), will often resolve if the knee joint can be locked within a week after birth. If not, the defect generally becomes worse with growth and size and while the animal will continue to grow, the front legs will break down and the animal will not be adequate for the feedlot.

## Summary

A reduction in incidence can be expected and has been achieved by using one or more of the following: (1) coordinating grazing periods according to plant growth stage;

(2) changing time of breeding by either advancing or delaying or changing from spring to fall calving; and (3) reducing lupine populations through herbicide treatment; and (4) intermittent grazing between clean pastures and lupine pastures to break the cycle of lupine ingestion.

## POISON-HEMLOCK (*CONIUM MACULATUM*)

Poison-hemlock was introduced into the United States as an ornamental herb and grows throughout the United States. Generally, poison-hemlock grows in waste areas where adequate moisture will sustain the biennial stands. Four species are recognized worldwide but only one (*Conium maculatum*) is found in the United States. Historically, poison-hemlock has been associated with human poisoning more than livestock and is believed to be the tea used to execute Socrates (Daugherty, 1995; Reynolds, 2005).

### Description

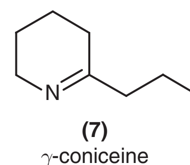
Unlike the lupines, locoweeds, etc., multiple *Conium* species are few worldwide and only one species is described in the United States, that being *C. maculatum*. *Conium maculatum* is a biennial plant 1–2.5 m tall (Figure 66.6). The stems are stout, rigid, smooth, and hollow except at the nodes. A distinguishing characteristic of the plant is the purple spots found up and down the main stem and a single carrot-like taproot. The leaves are large, triangular, fern like, and alternate on the erect stem. The stem grows the 2nd year from a rosette and taproot established from seed. Frequently, seed will germinate in the fall before winter then complete its reproductive cycle the following year. The flowers are small, white or cream–colored, and in umbellate clusters. The fruits are grayish-brown with conspicuous wavy, knotted ridges. The plant has a fleshy, usually unbranched, white taproot which looks like parsnips. Roots have been mistaken for parsnip, seeds for anise and leaves for parsley (Kingsbury, 1964).

**FIGURE 66.6** The hemlocks, poison-hemlock (*Conium maculatum*) on the left and water hemlock (*Cicuta douglasii*) on the right. Note the tubers and partitioned stalk on water hemlock versus the single taproot on poison-hemlock. Leaf structure is different and poison-hemlock has purple spots on the stems.

The geographical distribution of poison-hemlock is throughout the United States, restricted only by cultivation and adequate moisture to sustain stands of the plant from year to year. The plant usually grows in waste places where moisture may accumulate and protected from cultivation.

### Toxicology

Eight piperidine alkaloids are known in poison-hemlock, five of which are commonly discussed in the literature (Roberts and Brown, 1981). Two alkaloids (coniine **(5)** and $\gamma$-coniceine **(7)**) are prevalent and likely responsible for toxicity and teratogenicity of the plant. $\gamma$-coniceine **(7)** is the predominant alkaloid in the early vegetative stage of plant growth and is a biochemical precursor to the other *Conium* alkaloids (Panter *et al.*, 1988a; Panter and Keeler, 1989). Coniine **(5)** predominates in late growth and is found mainly in the seeds. $\gamma$-coniceine **(7)** is 7–8 times more toxic than coniine in mice (Bowman and Sanghvi, 1963). This makes the early growth plant most dangerous in the early spring and the seedlings and regrowth again in the fall. This is also the time when green feed is limited to livestock and may impact their propensity to graze this plant. Seeds, which are very toxic, can contaminate poultry and swine cereal grains (Panter and Keeler, 1989). Plants often lose their toxicity upon drying but seeds remain toxic as long as the seed coat is intact.




A recent analysis of a single plant of *Conium*, second year rosette, revealed $\gamma$-coniceine levels of 387, 326, 198, 176, and 850 mg/g fresh plant for whole plant, root crown, stem, leaf, and green seed, respectively. Coniine was only

detected in the leaf at 12 mg/g fresh plant (Panter and Gardner, unpublished data, 1994).

The clinical signs of toxicity are the same in all species and include initial stimulation (nervousness) resulting in frequent urination and defecation (no diarrhea), rapid pulse, temporarily impaired vision from the nictitating membrane covering the eyes, muscular weakness, muscle fasciculations, ataxia, incoordination followed by depression, recumbency, collapse, and death from respiratory failure (Panter *et al.*, 1988b).

*Conium* plant and seed are teratogenic, causing contracture-type skeletal defects and cleft palate like those of lupine. Field cases of teratogenesis have been reported in cattle and swine and experimentally induced in cattle, swine, sheep, and goats (Panter *et al.*, 1999). The birth defects include arthrogryposis (twisting of front legs), scoliosis (deviation of spine), torticollis (twisted neck), and cleft palate. Field cases of skeletal defects and cleft palate in swine and cattle have been confirmed experimentally (Keeler, 1978).

In cattle, the susceptible period for *Conium* induced terata is the same as that described for lupine and is between the 40th and 70th day of gestation. The defects, susceptible period of pregnancy and probable mechanism of action is the same as "crooked calf disease" induced by lupines (Panter *et al.*, 1999). In swine, sheep, and goats the susceptible period of gestation is 30–60 days. Cleft palate only has been induced in goats when plant or toxins were fed from 35 to 41 days gestation (Panter and Keeler, 1992).

Field cases of poisoning have been reported in cattle, swine, horses, goats, elk, turkeys, quail, and chickens (Copithorne, 1937; MacDonald, 1937; Widmer, 1984; Jessup *et al.*, 1986; Frank and Reed, 1987, 1990; Galey *et al.*, 1992; Frank *et al.*, 1995). Poisoning in wild geese eating small seedlings in early spring was recently reported (Panter, personal communication). Human cases of poisoning are frequently reported in the literature and recently a case of a child and his father mistakenly ingesting the plant was reported (Frank *et al.*, 1995). Field cases of teratogenesis have been reported in cattle and swine and experimentally induced in cattle, sheep, goats, and swine (Keeler and Balls, 1978; Panter *et al.*, 1990a). Pigs become habituated to poison-hemlock and if access to the plant is not limited they will eat lethal amounts within a short time (Panter, 1983).

There are no diagnostic lesions in poisoned animals and diagnosis is based on clinical history of exposure and/or alkaloid detection in liver, urine, or blood. At necropsy, presence of plant in stomach and a characteristic pungent odor in contents with chemical confirmation of the alkaloids may be diagnostic.

## Prevention and treatment

Prevention of poisoning is based on recognizing the plant and its toxicity and avoidance of livestock exposure when hungry. If a lethal dose has not been ingested, the clinical signs will pass spontaneously and a full recovery can be expected. Avoidance of stressing animals poisoned on *Conium* is recommended. However, if lethal doses have been ingested, supporting respiration, gastric lavage, and activated charcoal are recommended. Control of plants is easily accomplished using broad-leaf herbicides, however persistent control measures are recommended as seed reserves in the soil will quickly reestablish a population.

The mechanism of action of the *Conium* alkaloids is twofold. The most serious effect occurs at the neuromuscular junction where they act as non-depolarizing blockers like curare (Bowman and Sanghvi, 1963). Systemically, the toxins cause biphasic nicotinic effects, including salivation, mydriasis, and tachycardia followed by bradycardia as a result of their action at the autonomic ganglia. The teratogenic effects are undoubtedly related to the neuromuscular effects on the fetus and have been shown to be related to reduction in fetal movement (Panter *et al.*, 1990a). Likewise, cleft palate is caused by the tongue interfering in palate closure during the reduced fetal movement and occurs during 30–40 days of gestation in swine and 32–41 days in goats and 40–50 days in cattle (Panter and Keeler, 1992; Panter *et al.*, 1998).

# WATER HEMLOCK (*CICUTA* SPP.)

Water hemlock (*Cicuta* spp.) is among the most violently poisonous plants known to man. It is often confused with poison-hemlock because of its name, growth patterns, and appearance. There are distinct differences in appearance as shown in Figure 66.6.

## Distribution

There are about 20 species of *Cicuta* throughout the world and all are poisonous (Shishkin, 1973). Most of these species are found in North America; 9 are common in the United States, and 7 are found in the western United States (Table 66.5).

## Description

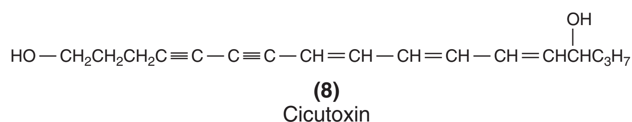
Water hemlock is often confused with poison-hemlock and, in fact, there are similar plant characteristics and both belong to the Umbelliferae family (Figure 66.6). However, their toxic effects are dramatically different and when toxicoses occur differentiation between the two genera is important.

Water hemlock (*Cicuta*) is a biennial, 1–2 m tall, with thickened tubers possessing long slender parsnip-like

**TABLE 66.5 Water hemlock species and geographical distribution**

| Species | Geographical distribution |
|---|---|
| *C. bulbifera* | Northern US, southern Canada |
| *C. bolanderi* | Central to western CA |
| *C. californica* | Central to western CA |
| *C. douglasii* | Throughout US |
| *C. machenziana* | Southeastern AK north to the Brooks Range |
| *C. occidentalis* | Rocky Mountains, Black Hills, and WA; south into NV and NM |
| *C. vagans* | Pacific northwest US and Canada |

roots radiating out from the main tuber. Water hemlock frequently grows directly in streambeds or water sources and is limited to wet habitats. The tubers are bulbous and exhibit several chambers or cavities separated by cross-partitions as seen in a vertical cut through the tuber at the base of the stem. The cut surface of the stem or tuber exudes a yellowish, thick, oily liquid believed to be predominantly cicutoxin **(8)**. This oily substance has a parsnip-like odor. The chambered tuber is a distinguishing feature of this plant (Figure 66.6). The roots extending from the tuber may be solid, white, and fleshy, closely resembling a parsnip. The stems are hollow except at the nodes, hairless, and occasionally have purple stripes up the stem. Leaf stalks alternate up the stem and leaves 2–3 pinnately divided and may be 30–60 cm long. Flowers are small, white, or cream colored and in terminal umbrella-shaped clusters. The fruits are small, with prominent ribs, and encased in a hard brownish shell very similar to poison-hemlock. The primary distinguishing feature separating water and poison-hemlock is the tuberous roots with the very distinct partitions in water hemlock (Figure 66.6). Poison-hemlock lacks the large tubers, although occasionally there are very small partitions observed in the area where the root and stem connect if the plant is cut vertically. The parsnip-like root is a single taproot in poison-hemlock whereas there are often multiple white parsnip-like roots radiating off the main tubers in water hemlock (Figure 66.6).




**(8)**
Cicutoxin

## Toxicology

The toxic principle in water hemlock is a long chain, highly unsaturated alcohol called cicutoxin **(8)**. Water hemlock acts on the central nervous system as a stimulant, inducing violent grand mal seizures and death from respiratory failure.

Tubers are the most toxic part of the plant especially in early spring. The parsnip-like roots extending from the tuber are 2–4 times less toxic and as the vegetative parts of the plant grow and mature they become less toxic. Preliminary studies suggest that mature leaves and stems are much less toxic and after drying are non-toxic (Panter *et al.*, 1988b). Historically, water hemlock was believed to be most dangerous in early spring, and poisoning usually occurred when animals milled around in streambeds or sloughs and exposed tubers which were then ingested. While this is true, a recent case of poisoning and death in cattle after ingesting flower and green-seed heads implicates this phenological stage as dangerous also (Panter *et al.*, 2007a, b). Chemical comparison of green seed and tubers and mouse bioassay studies showed that green seed was equally as toxic as tubers. Like tubers, the more mature vegetation including leaves, flowers, and green-seed heads were very palatable. Free choice exposure of hamsters to the white parsnip-like roots suggest they are quite palatable and are less toxic than is the tuber itself. Observations of cattle grazing early in spring suggest the young shoots of water hemlock are very palatable as young plants growing in streambeds were frequently and extensively grazed (Panter, personal observation).

Clinical signs of poisoning appear within 10–15 min after ingestion and progress from nervousness, frothing, ataxia, dyspnea, muscular tremors and weakness to involuntary, spastic head and neck movements accompanied by rapid eye blinking and partial occlusion of the eyes from the nictitating membranes. This is quickly followed by collapse and intermittent grand mal seizures lasting 1–2 min each followed by relaxation periods of 8–10 min. Depending on the dosage, recovery may occur or seizures continue until death from exhaustion or respiratory failure. There appears to be a threshold response in which very small increases in dosage will induce an apparently normal animal into grand mal seizures (Panter *et al.*, 1996a).

Upon necropsy, gross lesions are confined to pale areas in heart muscle and skeletal muscles, particularly the long digital extensor muscle groups (Panter *et al.*, 1996a). Microscopic lesions include multifocal, subacute to chronic myocardial degeneration characterized by granular degeneration of myofiber cytoplasm necrosis and replacement fibrosis in the heart. These areas correspond to the pale areas observed grossly. There is bilateral symmetrical, subacute to chronic, myofiber degeneration and necrosis of the long digital extensor muscle groups. Clinical serum chemistry changes of elevated lactic dehydrogenase (LDH), aspartate aminotransferase (AST), and creatine kinase (CK) occur in relation to severity of seizures. The extent of gross and microscopic lesions and clinical chemistries are a result of the severity of the seizures. Experimentally, barbiturates prevented seizures, death and lesions in sheep and a 3 × lethal dose of water hemlock could be reversed with pentobarbital and no death occurred (Panter *et al.*, 1996a). In animals in which seizures were prevented by barbiturates there were no lesions observed

even though doses of 2 and 3 times a lethal dose were administered.

## Prevention and treatment

Prevention of poisoning is accomplished by recognizing the plant and avoiding exposing animals to them early in the spring or when in flower/seed stage. Water hemlock is easily controlled with herbicides (2,4-D per manufacturers specification) however herbicide use is often restricted near natural water sources. If few plants are present hand pulling may be accomplished using caution to discard tubers away from possible exposure to animals or humans.

Successful treatment with barbiturates or perhaps tranquilizers prevents death and the lesions and serum chemistry changes, however treatment must be prompt (Panter *et al.*, 1996a). This treatment has been successful in humans, but in animals has never been demonstrated in the field and would require a veterinarian to be on sight quickly after the ingestion of this plant.

# PONDEROSA PINE NEEDLES (*PINUS SPP.*)

The needles of ponderosa pine have been known for years to induce abortion in pregnant cows when grazed, particularly during the last trimester of pregnancy (James *et al.*, 1989b). Occasional toxicity in pregnant cows occurs (Panter *et al.*, 1990b), however cases of toxicity in non-pregnant cows, steers, or bulls are not reported.

## Description

Ponderosa pine (*Pinus ponderosa*) is one of the most prevalent species of *Pinus* in the western United States. Under ideal conditions it grows to heights of over 60 m and 1.5–2.5 m in diameter. It is extensively harvested for lumber. During early growth the bark is dark brown to black, hence the name "black jack" pine. Older trees have a bark of cinnamon brown to yellow hence the name "yellow pine." Ponderosa pine is a three-needled pine although groups of two and three can be found on the same tree. The needles are about 8–20 cm long growing in clusters. They are soft and quite palatable especially in the winter when green feed is limited. The cones are brown, 7–15 cm long, and frequently grow in clusters. Two varieties of ponderosa pine have been identified including var. *ponderosa*, var. *scopulorum*, and a separate five-needled species *Pinus arizonica* (Conkle and Critchfield, 1987).

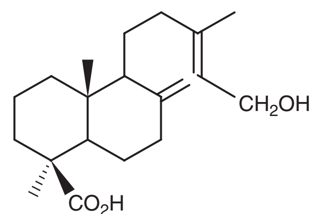
## Distribution

Ponderosa pine grows in every state west of the Great Plains and western Canada. It has a total stand greater than any native tree in the western United States except Douglas fir. It grows at elevations between 1500 and 2500 m although populations in California do extend almost to sea level. Ponderosa pine is drought resistant and is extensively harvested for lumber. Gardner and James (1999) surveyed numerous other *Pinus, Juniperus, Abies* spp., etc. for isocupressic acid (ICA) content and Table 66.6 lists common names, location, and ICA concentration of many species of trees or shrubs.

*Pinus ponderosa* var. *ponderosa* (western variety) extends from the mountains of southern California, northward on the western and eastern sides of the Sierra Nevada–Cascade crest to Canada. The eastern variety *P. ponderosa* var. *scopulorum* meets the western variety near the Continental Divide in west-central Montana and extends southward throughout mountains, plains, and basins to scattered stands in the Sierra Madre Occidental and Sierra Madre Oriental of Northern Mexico. *Pinus arizonica* (once considered a third variety) has scattered populations in southern Arizona and New Mexico (Conkle and Critchfield, 1987).

## Toxicology

The toxin in ponderosa pine that induces abortion in cattle is the labdane resin acid ICA **(9)** (Gardner *et al.*, 1994). Two related derivatives (succinyl ICA and acetyl ICA) also contribute to the induction of abortion after hydrolytic conversion to ICA in the rumen (Gardner *et al.*, 1996). Other related labdane acids (imbricataloic acid and dihydroagathic acid) that are found in ponderosa pine needles (PN) at low levels may also contain abortifacient properties based on their similar chemical structure to ICA. Other genera and species have also been implicated in abortions such as Monterey cypress (Parton *et al.*, 1996), Korean pine (Kim *et al.*, 2003), California juniper, and lodgepole pine (Panter, personal communications).




**(9)**
Isocupressic acid

The primary toxicological effects of ponderosa PN in cattle are abortion and complications associated with the abortion, such as retained fetal membranes (RFM), metritis and

**TABLE 66.6 ICA content of selected species and location (Gardner and James, 1999; Gardner, unpublished data)**

| Species | Common name | Location | ICA content (% d.w.) |
|---|---|---|---|
| *Pinus ponderosa* | Ponderosa pine | Oregon | 0.74–1.30 |
| | | Arizona | 0.49 |
| | | California | 0.42–1.35 |
| | | Utah | 0.51 |
| | | Colorado | 0.49 |
| | | Wyoming | 0.58–1.11 |
| *Pinus contorta* | Lodgepole pine | Oregon | 0.28 |
| | | Idaho | 0.11 |
| | | Colorado | 0.29–0.47 |
| | | Utah | 0.66 |
| | | Canada (BC) | 0.45 |
| | | Arkansas | n.d. |
| *Pinus jeffreyi* | Jeffrey pine | California | 0.04–0.54 |
| *Pinus arizonica* | Arizona pine | California | n.d. |
| | | Arizona | 0.49 |
| *Pinus edulis* | Pinyon pine | Arizona | n.d. |
| | | Colorado | 0.12 |
| | | New Mexico | 0.10 |
| | | Utah | 0.45 |
| *Pinus monophylla* | White pine | Nevada | 0.32 |
| *Pinus echinata* | | Arkansas | n.d. |
| *Pinus taeda* | Loblolly pine | Arizona | <0.01 |
| | | Arkansas | n.d. |
| *Pinus elliottii* | Limber pine | Arkansas | n.d. |
| | | Colorado | n.d. to 0.06 |
| | | Utah | n.d. |
| *Pinus palustris* | Long-leaf pine | Arkansas | n.d. |
| *Pinus montezume* | Montezuma pine | California | n.d. |
| *Pinus koraiensis* | Korean pine | Utah (Red Butte Gardens) | Positive |
| | | Korea | 0.02 |
| *Abies concolor* | White fir | California | <0.01 |
| | | Colorado | 0.042 |
| | | Utah | n.d. |
| *Abies lasiocarpa* | Subalpine fir | Oregon | <0.01 |
| | | Idaho | 0.043 |
| *Pseudotsuga menziesii* | Douglas fir | Utah | 0.035 |
| | | California | n.d. |
| | | Idaho | n.d. |
| | | Arizona | n.d. |
| | | Oregon | n.d. |
| *Juniperus communis* | Mountain common juniper | Colorado | 2.05–2.88 |
| *Juniperus scopulorum* | Rocky Mountain juniper | Utah | 0.84 |
| | | New Mexico | 0.25 |
| | | Arizona | 0.42 |
| *Juniperus osteosperma* | Utah juniper | Utah | n.d. |
| | | Nevada | 0.065 |
| | | Utah | Agathic acid |
| *Juniperus californicum* | California juniper | California | 0.93 needles; 1.4 berries; 0.05 bark |
| *Juniperus monosperma* | One seed juniper | Arizona | 0.14 |
| | | New Mexico | n.d. |
| *Thuja plicata* | Western red cedar | Arizona | 0.42 |
| | | New Mexico | 0.33 |
| | | Utah | 0.84 |
| *Picea engelmannii* | Engelmann spruce | California | 0.27 |

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**TABLE 66.6 (Continued)**

| Species | Common name | Location | ICA content (% d.w.) |
|---|---|---|---|
| | | Colorado | n.d. |
| | | Idaho | 0.04 |
| | | Montana | 0.31 |
| | | Oregon | n.d. |
| | | Utah | n.d. |
| *Picea pungens* | Colorado blue spruce | Utah | 0.17 |
| | | Colorado | n.d. |
| *Cupressus macrocarpa* | Monterey cypress | California | n.d. |
| | | New Zealand | 0.89–1.24 |
| *Pinus echinata* | Short-leaf pine | Arkansas | <0.01 |
| *Pinus elliottii* | Slash pine | Arkansas | <0.01 |
| *Pinus aristata* | Bristle cone pine | Colorado | 0.01–0.05 |
| *Abies grandis* | Grand fir | Oregon | <0.01 |
| *Abies magnifica* | Red fir | California | 0.052 |
| *Tsuga mertensiana* | Mountain hemlock | Oregon | <0.01 |
| *Larix occidentalis* | Western larch | Oregon | n.d. |
| *Libocedrus decurrencs* | Incense cedar | Oregon | 0.065 |

occasional overt toxicity, and death (James *et al*., 1989b; Panter *et al*., 1990b, 1992b; Gardner *et al*., 1999). The abortions generally occur in the last trimester of pregnancy in the late fall, winter, or early spring. Abortions have been induced as early as 3 months gestation and have been reported by ranchers to occur anytime; however, the closer to the time of normal parturition that ingestion of PN occurs the higher the risk of abortion. Abortions may occur following a single exposure to the needles but results from controlled experiments indicate the highest incidence of abortion is in cows eating the needles over a period of days. Abortions have been associated with grazing of green needles from trees, slash from the lumber industry, and dead, dry needles from the ground.

Abortions are generally characterized by weak uterine contractions, uterine bleeding, incomplete cervical dilation, dystocia, birth of weak but viable calves, agalactia and RFM. Two syndromes seem to occur depending on the amount of PN eaten. Either abortion occurs relatively quickly (3–6 days) in which no signs of pending abortion such as udder filling, vulvar swelling, or pelvic relaxation occur but a small weak calf is quickly delivered or the second syndrome in which abortion occurs after 6–14 days and all the before-mentioned signs occur before the calf is born. Unless complications occur, most calves are born alive and the closer to normal parturition the higher the survival rate. Calves born after 255 days gestation will often survive with extra care but need to be supplemented with colostrum and milk from other sources until the dam begins to lactate. Cows with RFM may need antibiotic therapy to avoid uterine infections.

The PN will induce abortion in buffalo (Short *et al*., 1992) but sheep and goats do not abort. The PN, pine bark, and new growth tips of branches are all abortifacient and new growth tips were toxic also (Panter *et al*., 1990b).

A separate toxic syndrome has been described in addition to the abortion in which the abietane-type diterpene resin acids cause depression, feed refusal, weakness, neurological problems, and eventually death. Specific compounds include abietic acid, dehydro abietic acid, and other related compounds (Stegelmeier *et al*., 1996). At 15–30% of the diet PN have been shown to alter rumen microflora and affect the rumen fermentation (Pfister *et al*., 1992; Pfister and Adams, 1993). Rumen stasis is part of the toxic syndrome (Stegelmeier *et al*., 1996).

PN-induced abortion appears to mimic normal parturition except premature. The mechanism of action appears to be a reduction in blood flow to the caruncular vascular bed stimulating the fetal parturition mechanism (Christensen *et al*., 1992a, b; Ford *et al*., 1992). ICA has not been found to be directly vasoactive (Short *et al*., 1996) and it is unclear if ICA metabolites are vasoactive or if the vasoconstriction is a secondary response to ICA or ICA metabolites. Further research is needed to determine the cellular or biochemical mechanism and if metabolic alteration of the ICA occurs.

Extensive vasoconstriction of the caruncular vascular bed with accompanying necrosis and hemorrhage have been the only reported pathological changes in maternal tissues (Stuart *et al*., 1989). This was supported by Christensen *et al*. (1992 a, b), when serum from pregnant cows fed PN showed vaso (vasoconstriction) activity *in vitro*.

## Prevention and treatment

Currently the only recommendation to prevent PN abortion is to avoid grazing pregnant cows in pine trees especially in the third trimester. There is no known treatment for cattle once ingestion of PN has occurred. Open cows,

steers, or bulls are apparently unaffected by PN and likewise sheep, goats (pregnant or not), and horses can graze PN with impunity and experience no adverse effects. Supportive therapy (antibiotic treatment or uterine infusion for RFM) is recommended for cows that have aborted and intensive care of the calf may save the life of the calf. Grazing of PN intensifies during cold inclement weather (Pfister and Adams, 1993) and if other forage is in short supply. In spring before green grass is available cows will leave feeding grounds in search of new green grass and frequently graze old dry needles from surrounding trees where the snow has melted. These cows are at risk and should be kept away from the pines. Anecdotal information suggests that pregnant llamas may be at risk from PN but no experimental support has been done (Panter, personal communications).

## BROOM SNAKEWEED (*GUTIERREZIA* SPP.)

Broom snakeweed causes significant losses to cattle, sheep, and goat producers in Texas and New Mexico from abortions and toxicoses. There are some similarities with that of ponderosa PN except PN apparently only affect cattle.

### Description and distribution

There are two major species of broom snakeweed, G*utierrezia sarothrae* (perennial snakeweed or turpentine weed) and *G. microcephala* (threadleaf broomweed). Snakeweeds are short-lived perennial half shrubs ranging from 15 to 60 cm tall (Parker, 1982). Many unbranched erect stems originate from a woody base and die back when the plant enters dormancy. They have a suffrutescent growth form with new stems originating from the crown each year without becoming woody. The stems originate from the crown and are unbranched, giving rise to the common name of broom snakeweed, with the stems resembling the straight straws of a broom. Leaves are narrow and linear and alternately arranged on stems. These species are of the Composite family, thus they have two types of flowers. Disk flowers are tightly clustered in the center of the head, while long yellow petal ray flowers radiate out from the head (Lane, 1985; McDaniel and Loomis, 1985).

#### *Broom snakeweed (Gutierrezia sarothrae (Pursh) Britt. & Rusby)*

It has more than three florets (usually seven) per involucre or flowering cluster. Broom snakeweed is one of the most ubiquitous range plants and widely distributed throughout North America, ranging from the cold temperate climate of Canada to subtropical areas of Mexico; from the subhumid Great Plains, up to the montane Rocky Mountains and Sierras, to the arid Great Basin. It ranges in elevation from 50 to 2900 m, rainfall from 20 to 50 cm, and temperatures from 4°C to 21°C (McDaniel and Sosebee, 1987). It is a principal component of the following plant communities: desert creosote, desert grassland, short-grass prairie, salt-desert shrub, sagebrush, pinyon-juniper, and mountain brush. Its wide tolerance limits allow it to inhabit a broad range of environments, and can dominate and cause significant problems as both a noxious and poisonous weed in the short-grass prairies and sagebrush plant communities.

#### *Threadleaf snakeweed (G. microcephala (DC) Gray)*

It has one floret per flowering cluster. Threadleaf snakeweed is restricted to the Mojave, Sonoran, and Chihuahuan deserts of the southwestern United States and northern Mexico. It grows in dry, desert habitat and favors sandy soils and has a greater tolerance for arid climates than broom snakeweed.

### Ecology

Broom snakeweed is a native plant and was a minor component of pristine plant communities (5–10%; Campbell and Bamberger, 1934). It was restricted to rocky ridges, gravely slopes and immature or infertile soils (Parker, 1982). However, it aggressively invades disturbed areas. The early literature associated it with overgrazing, which reduced desirable vegetation and allowed broom snakeweed to increase where it already existed, and invade deeper soils and more productive sites (reviewed by McDaniel, and Torell, 1987). Other disturbances such as fire, drought, and chaining can also cause it to increase (USFS, 1937; Parker, 1939; Arnold *et al.*, 1964). It is very competitive with desirable grasses and greatly suppresses forage production (Ueckert, 1979; McDaniel *et al.*, 1982).

A two-tier root system allows the deep taproot to extract deep-stored water, and the extensive adventitious roots near the surface take advantage of the light sporadic rain showers. It has little leaf stomatal control (Depuit and Caldwell, 1975), thus is a luxuriant water user for maximum growth. It grows early in the spring and depletes moisture from the entire soil profile before warm-season grasses break dormancy. Platt (1959) ranked it one of the most undesirable plants in the various regions of the West. On many localized rangelands of the southwest, it is the most significant problem limiting forage and livestock production.

Broom snakeweed is short-lived and experiences dramatic population cycles, which appear to be related to climatic patterns. Although it is very competitive for soil

moisture, it is not particularly drought tolerant. Its populations die-off in drought and from insect depredation, but is one of the first plants to germinate and establish when rains resume, forming widespread monospecific stands (McDaniel and Ross, 2002; Ralphs and Sanders, 2002).

## Toxicology

The snakeweeds are toxic and abortifacient to cattle, sheep, and goats. Abortions and RFM in cattle are among the most serious problems in livestock. In 1985, McGinty estimated losses in excess of $15 million annually to the cattle industry in Texas alone and over $30 million when losses in New Mexico and Arizona were included. This does not account for indirect losses such as loss of useable forage, management changes, increased calving intervals, or added veterinary care.

Snakeweed contains toxic and abortifacient compounds. Dollahite *et al*. (1962) extracted what they called a saponin fraction from threadleaf snakeweed which caused abortions in rabbits, goats and cattle at low doses, and caused death at high doses. Molyneux *et al*. (1980) identified some major monoterpenes and sesquiterpenes in the essential oil fraction of snakeweed and these included α-pinene, myrcene, linalool, *cis*-verbenol, *trans*-verbenol, verbenone, geraniol, caryophyllene, and γ-humulene. Roitman *et al*. (1994) extracted several furano-diterpene acids and flavones from the resinous exudate in trichomes on leaves of broom snakeweed. These diterpene acids were structurally similar to ICA, the abortifacient compound in ponderosa pine (*Pinus ponderosa* Laws) needles (Gardner *et al*., 1994). Gardner *et al*. (1999) speculated that some of the furano-diterpene acids may be abortifacient, while others may be toxic, and the relative concentrations determine whether animals are poisoned or abort. The crude resin content of broom snakeweed, which includes the diterpene acids along with other monoterpenes, increased from 5% in early growth up to 13% at flowering in August (Ralphs and Wiedmeier, 2004, 2007).

Snakeweeds are both abortifacient and toxic. In West Texas in the 1930s, 10–60% of cattle ranches experienced abortions, and cows retained placentas which led to infection and death of the cows. Many calves were born small and weak. Mathews (1936) fed threadleaf snakeweed to cattle, sheep, and goats and reported it damaged the liver and kidneys, but no abortions were produced. Dollahite and Anthony (1957) and others found threadleaf snakeweed did cause abortions, retained placenta and weak calves, and was more toxic on sandy soils (Shaver *et al*., 1964). Low levels of snakeweed caused abortions and high levels are toxic. Clinical signs of poisoning include anorexia, mucopurulent nasal discharge, loss of appetite and listlessness, diarrhea, then constipation and rumen stasis, which may lead to death.

There appears to be a relationship between nutrition and fertility problems caused by broom snakeweed (Flores-Rodriguez *et al*., 1989; Strickland *et al*., 1998). Smith *et al*. (1991) summarized research in rats at New Mexico State University and concluded that increasing amounts of snakeweed in rat diets reduced intake, which led to malnutrition and contributed to diminished fertility and increased fetal mortality. Edrington *et al*. (1993a, b) confirmed that increasing amounts of snakeweed in rat diets reduced intake and contributed to problems of malnutrition and toxicity. However, they determined the overriding factor in reducing fertility and reproduction was the impaired hormone balance and disruption of blood flow to the uterus and developing embryos. Ewes on a high-quality alfalfa diet (18% CP) consumed snakeweed for up to 25% of the ration with no adverse effects on estrus; while ewes fed blue grama hay (11% CP) would not consume rations containing more than 10% snakeweed, and 43% of these ewes did not show estrus and did not breed (Oetting *et al*., 1990). Heifers fed snakeweed as 15% of a balanced diet before breeding and during early gestation had no effect on progesterone levels or conception rates (Williams *et al*., 1993). During the last trimester of gestation, snakeweed added up to 30% of this same diet did not cause abortion or lower calf birth weight (Martinez *et al*., 1993). In a grazing trial on snakeweed-infested crested wheatgrass, cows in the last trimester of gestation were forced to graze snakeweed as a biological control. Snakeweed consumption averaged 10% of bites over the day and peaked at 20% of bites in the evening grazing periods. There were no signs of toxicity or abortions, even though their feed intake was severely restricted (Ralphs and Wiedmeier, 2007).

## Management and treatment

Broom snakeweed is not palatable to most large ungulates (Pieper, 1989). Cattle will not graze snakeweed unless all other vegetation is depleted (Ralphs and Wiedmeier, 2007).Thus the management strategy is simple – ensure adequate feed is available. This can be accomplished by maintaining range in good condition and moving animals when proper utilization is reached.

Broom snakeweed can be controlled by the common rangeland herbicides: Tordon (0.25–0.5 lb/ac) is most consistent; Escort (3–6 oz/ac) is a new and promising herbicide; and 2,4-D (1–2 lb/ac) can be applied for two successive years when soil moisture is not limiting growth (McDaniel *et al*., 1982; McDaniel and Duncan, 1987; Whitson and Freeburn, 1990). Better control is obtained in fall after flowering, when the carbohydrate stream is going down and carries the herbicide to the roots (Sosebee *et al*., 1982).

Snakeweed is readily killed by fire. McDaniel and Ross (2002) suggested prescribed burning is an effective control

in the early stages of the population cycle while there was sufficient grass to carry a fire and respond to the released resources. In the latter part of the population cycle, grasses will be crowded out and spraying with herbicides may be the only alternative. In this case, spraying must be followed by seeding, to ensure a weed-resistant plant community is established that will resist reinvasion of snakeweed.

Insects may play a role in snakeweed population die-off. There are many insects that are associated with snakeweed: defoliators (leaf tyers, grasshoppers, weevils), sap suckers (scale insects and mealy bugs), root boring weevils, and gall formers (Wangberg, 1982). Individually and collectively, they contribute to the demise of mature snakeweed populations. However, it is unlikely that they will contribute to preventing a population buildup because of the time lag of their populations in relation to that of snakeweed.

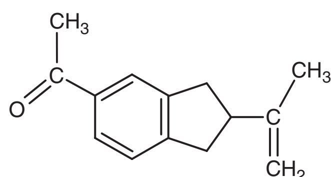
Treatment of sick animals is only symptomatic providing supplementation to weak calves and antibiotic therapy to cows with RFM to avoid infection.

# RAYLESS GOLDENROD (*HAPLOPAPPUS HETEROPHYLLUS*)

Rayless goldenrod (*Haplopappus heterophyllus*) is a toxic range plant of the southwestern United States. The disease associated with toxicity has been referred to as "alkali disease" because originally it was associated with drinking of alkali water (Kingsbury, 1964). Currently, it is referred to as "milk sickness" or "trembles" (the same as white snakeroot in the Midwest) because the toxin tremetone **(10)** is excreted in the milk and subsequently results in poisoning of humans and nursing offspring. *Haplopappus acradenius* was most recently implicated in poisoning in cattle in southern California (Galey *et al.*, 1991).

## Description

Rayless goldenrod is an erect, bushy, unbranched perennial shrub growing 0.5–1.5 m tall. The base is woody and leaves alternate linearly up the stem. Heads are numerous, small, and clustered at the top of the stem with 7–15 yellow flowers on each head.




**(10)**
Tremetone

## Distribution

Rayless goldenrod grows in the desert rangelands of the southwestern United States from southern Colorado into Texas, New Mexico, Arizona, and California into Mexico. The plant grows abundantly on alkaline and gypsic soils in western texas and the Pecos River Valley (Ueckert, 1984).

## Toxicology

The toxic constituent of rayless goldenrod is the same as that of white snakeroot and was originally termed tremetol (a mixture of ketones and alcohols) in white snakeroot (Panter *et al.*, 1992a). Tremetone **(10)** (5-acetyl-2,3-dihydro-2-isopropenyl-benzofuran) was determined to be the principal toxic factor (Zalkow *et al.*, 1962; Beier *et al.*, 1993).

The toxicity of rayless goldenrod mimics that of white snakeroot. Clinical signs of poisoning may occur after ingestion of 1–15% body weight over a 1–3 week period (Schuster and James, 1988). Signs begin with depression or inactivity, followed by noticeable trembling of the fine muscles of the nose and legs. Most cases of poisoning reported constipation, nausea, vomition, rapid labored respiration, progressive muscular weakness, stiff gait, standing in a humped-up position, dribbling urine, inability to stand, coma, and death. Signs are similar in cattle, sheep, and goats (Beier and Norman, 1990). The disease is often more acute and severe in horses than cattle and horses may die of heart failure after subacute ingestion of white snakeroot and presumably rayless goldenrod (Smetzer *et al.*, 1983). Cattle have also been poisoned on a related plant (*Haplopappus acradenius*) in southern California (Galey *et al.*, 1991). In this case 21 of 60 cattle died and 15 of 60 were affected but recovered. Creatine phosphokinase and ketones were elevated and severe myonecrosis was described in the dead animals.

## Prevention and treatment

Rayless goldenrod is not readily palatable and toxicity results from animals being forced to graze the plant due to lack of good quality forage. Avoiding overgrazing will usually minimize poisoning in livestock.

Control of rayless goldenrod can be accomplished with herbicide applications (Ueckert, 1984). Late summer and early fall application of picloram or 2, 4-D ester are successful in reducing plant populations.

Treatment is generally symptomatic and supportive providing dry bedding, good shelter, and fresh feed and water (Beasley *et al.*, 1997). Activated charcoal and saline cathartic may be beneficial. Treatment may include fluids, B-vitamins, ketosis therapy, and tube feeding. Hay and water should be placed within reach if the animal is recumbent. In lactating cows, frequent milking may facilitate a more rapid

elimination of the toxins. In horses, monitoring of cardiac arrhythmias and EKG will provide information concerning heart damage and associated circulatory dysfunction. Treatment is the same for rayless goldenrod and white snakeroot as the toxins are the same.

## HALOGETON (HALOGETON GLOMERATUS)

Halogeton is an alien, invasive, noxious, and poisonous weed introduced from central Asia in the early part of the 20th century. It was first collected along a railroad spur near Wells, Nevada in 1934, and rapidly invaded 11.2 million acres of the cold deserts of the western United States (Young, 1999). There has been no appreciable spread since the 1980s, because halogeton has filled all the suitable niches within its tolerance limits. It currently infests disturbed areas within the salt-desert shrub and sagebrush plant communities in the Great Basin, Colorado Plateau, and Wyoming's Red Desert physiographic provinces, which range from 3 to 15 in. annual precipitation (Pemberton, 1986).

Halogeton's infamy began in the 1940s and 1950s by causing large, catastrophic sheep losses. There were many instances of large dramatic losses; sometimes entire bands of sheep died over night from halogeton poisoning. *Life Magazine* ran a cover story "Stock Killing Weed" which focused national attention on halogeton. Congress passed the Halogeton Act in 1952 with the intent to:

1 Detect the presence of halogeton.
2 Determine its effects on livestock.
3 Control, suppress, and eradicate this stock killing weed.

Federal research was reallocated from the Forest Service Experiment Stations to the Bureau of Plant Industries, creating the Range Research unit devoted specifically to "solving" the halogeton problem. It was realized that halogeton was not the problem but a symptom of a larger problem; that of degradation of desert rangelands (Young, 1999). It invaded disturbed sites where sheep congregated: around railroad loading sites, trailheads, stock drive ways, and around water holes. When hungry sheep were turned loose to graze, halogeton was the only feed available, and they consumed too much, too rapidly, and were poisoned.

### Description and ecology

Halogeton is an annual plant germinating from seed each year. Its stems are branched from the base and tinged with red and purple. Its leaves are fleshy and "hot-dog" shaped, with the distinguishing feature of a single spine on its tip. It can be distinguished from Russian thistle and pigweed (with which it grows) by the tubular, spine tipped leaf. It does not have flowers, but bracteoles formed in the axils of leaves from which seed clusters develop. These seed clusters occur throughout the length of its stems. A robust plant may have 1500 linear inches of stem, with 75 seeds/in., producing 200–400 lb seed/ac (Cronin and Williams, 1966). The seeds are winged, and are spread by wind and rodents.

Its seeds are dimorphic (Williams, 1960), which is key to its successful survival strategy. The majority of its seeds are black, and readily germinate anytime temperature and soil moisture are favorable. However, the black seeds are viable for less than a year. A small percentage of the seeds are hard and brown with low germination rates but they survive for long periods in the soil, and germinate when favorable conditions returned. The dimorphic seed provided abundant seed for germination each year, but also a reserve if drought killed its populations over successive years.

Although halogeton will germinate whenever conditions are favorable, it remains as a seedling until May, at which time lateral branches develop and ascend. It reaches its reproductive stage by mid summer, or when soil moisture becomes limiting. When mature, the winged fruits are blown by wind or carried and deposited by rodents (Cronin, 1965).

Halogeton is not competitive with perennial shrubs and grasses. It will not invade healthy desert plant communities. Only when a site is disturbed will halogeton invade and establish. It is also allelopathic (Eckert and Kinsinger, 1960). It takes up sodium and potassium from saline soils forming the respective oxalates. These oxalates provide an important metabolic function to maintain high cell sap osmotic potential to allow the plant to take up saline water (Kinsinger and Eckert, 1961). Oxalates accumulate over the growing season, reaching peak concentration in the fall (20–36% of plant dry weight). Soluble oxalates leach out of the senescent foliage over the winter and accumulate on the soil surface, thus increasing its salinity. Thus halogeton modifies its environment, making it more saline to meet its requirements, while exceeding the tolerance limits of associated species.

### Toxicology

The toxins are sodium and potassium oxalates (Cook and Stoddart, 1953). The plants are high in these oxalates in the fall and early winter when sheep enter the desert winter ranges. Poisoning occurs when sheep consume more oxalates than the body can detoxify (James, 1972 1999a). Rumen microbes can detoxify the oxalates, and their populations can be induced to accommodate increasing levels of oxalates (James *et al.*, 1967). Furthermore, calcium in the native plants or Ca supplements will rapidly combine with the oxalates in the rumen to form Ca oxalates that cannot

be absorbed and are excreted in feces. If the Na oxalates are absorbed, they can be flushed out in urine (James and Johnson, 1970). If they reach the bloodstream, they precipitate the Ca from the blood, creating Ca oxalate crystals, causing hypocalcemia and shock (James, 1968). The Ca oxalate crystals physically damage the tubules of the kidney (Van Kampen and James, 1969). The Na oxalates interfere with two key enzymes (succinic dehydrogenase and lactic dehydrogenase) in the Krebs cycle, disrupting energy metabolism (James, 1968). Combined, they cause rapid and acute death.

Clinical signs of poisoning include depression, anorexia, weakness, incoordination, recumbency, blood-tinged nasal discharge, coma, and rapid death (Cook and Stoddart, 1953). Gross pathological changes include hemorrhage and edema of the rumen wall, hyperemia of the abomasal wall and intestinal mucosa and ascites (Shupe and James, 1968). Morphological changes include hemorrhage and calcium oxalate crystal formation in the rumen wall and oxalate crystals with accompanying cellular damage in the renal tubules of the kidney (Van Kampen and James, 1969).

## Treatment of poisoned animals

Animals can be drenched with water to flush oxalates out in the urine (James and Johnson, 1970). They can be drenched with dicalcium phosphate (Cook and Stoddart, 1953); the Ca will combine with oxalates in the rumen and can be excreted. An i.v. injection of calcium gluconate can maintain blood Ca levels, but the forming Ca oxalate crystals will continue to damage kidneys. However, it is generally impractical to treat a severely poisoned animal. Prevention is the key to avoid poisoning. Only hungry sheep are poisoned. Research demonstrated that as little as 1 oz of soluble oxalates can be lethal to fasted, hungry sheep. Well-fed sheep grazing nutritious forage throughout the day can tolerate more than 4 oz of soluble oxalate (Cook and Stoddart, 1953). Sheep grazed in a desert plant community infested with halogeton consumed it from 5% to 25% of their diets without ill effect. If other forage is available, they will likely not get a lethal dose.

## Management to prevent poisoning

Never turn hungry sheep onto dense halogeton-infested sites. Provide good feed following trucking or trailing. Ensure there is good feed available following watering. Introduce sheep gradually to halogeton to allow rumen microbes to adjust. Some sheep producers graze their sheep on shadscale ranges (which contains low oxalate levels) before going into halogeton areas. Do not overgraze; maintain desert range in good condition. This prevents halogeton invasion, as well as provides an alternative food source (James and Cronin, 1974).

Herbicide control is not recommended (Cronin, 1965) as the waxy surface of its leaves hinders absorption of most herbicides. But more importantly, desirable desert shrubs are killed, leaving the site open for further invasion and degradation by halogeton and other invasive weeds.

# OAK POISONING

Toxicoses in cattle from ingestion of oak buds, leaves, and acorns occur in many parts of the United States and Europe (Cheeke and Shull, 1985). Poisoning is usually seasonal with ingestion of buds and leaves in spring and acorns in fall.

All oaks should be considered potentially toxic (Table 66.7). Oaks are perennial trees or woody shrubs. They grow in all parts of the United States especially in the southwestern states of Texas, Arizona, and Utah and in Europe and New Zealand.

## Conditions of poisoning

The most dangerous period for oak poisoning is during March and April when new foliage is sprouting. Poisoning of cattle and less frequently sheep and goats occurs on ranges of the southwest and especially during drought years when the forage is limited. Supplemental feeding so that oak ingestion is below 50% of their diet will usually prevent poisoning. Acorn poisoning occurs in the fall, particularly when an acorn crop is heavy. Cattle, sheep, horses, and swine have been involved and craving for acorns has been observed in which animals appear to seek them out (Kingsbury, 1964).

Swine seem to be somewhat resistant and goats apparently utilize oak browse and are used to control oak brush. Feeding high levels of immature gambel oak to goats did not induce any toxicosis (Nastis and Malechek, 1981).

The toxins in oak are tannins, particularly tannic acid and the phenolic acid (gallic acid), and these are highest

**TABLE 66.7 Oak species believed to contribute to poisoning**

| Species | Common name |
|---|---|
| *Quercus gambelii* | Gambel's oak |
| *Q. harvardii* | Shin or shinnery oak |
| *Q. marilandica* | Jack oak |
| *Q. stellata* | Post oak |
| *Q. breviloba* or *durandii* | |
| *Q. coccinea* | Scarlet oak |
| *Q. pedunculata* or *robur* | European oak |
| *Q. prinus* | Chestnut oak |
| *Q. xubra* | Northern red oak |
| *Q. velutina* | Yellow-barked oak |

in new spring growth. Clinical signs of poisoning begin with depression, nasal discharge, abdominal pain, constipation, thirst, frequent urination, rapid pulse, progressing over 1–3 days to rumen atony, anorexia, emaciation, weakness, prostration, rough coat, dry muzzle, subnormal temperature, bloody diarrhea, and death. Diagnosis is based on history of ingestion, clinical signs, elevated SGOT (serum glutamic oxaloacetic transaminase) and BUN (blood urea nitrogen), creatinine, serum potassium, and lower urine specific gravity. Death may occur within 24 h of the onset of clinical signs.

Gross and microscopic lesions include gastritis, nephritis, increased peritoneal and pleural fluids, perirenal edema and hemorrhages with pale swollen kidneys. The proximal convoluted tubules of the kidneys may be damaged and abundant hyline or granular casts may be evident. Mucous and blood are common in voided feces.

## Prevention and treatment

Prevention is the best policy by providing adequate feed to reduce oak ingestion to less than 50% of the diet. Supplemental high-energy feed at 0.5–1 kg/head/day of a 54% cottonseed or soybean meal with 30% alfalfa meal, 6% vegetable oil, and 10% calcium hydroxide may be beneficial.

Treatment includes blood transfusions if anemic, fluid therapy, rumenotomy if warranted, activated charcoal, plenty of fresh water, and avoid stressing animals as much as possible.

# PYRROLIZIDINE ALKALOID-CONTAINING PLANTS

Pyrrolizidine alkaloid (PA) containing plants are numerous and worldwide in distribution and in toxic significance (Cheeke, 1998). Three plant families predominate in PA producing genera and species: Compositae (*Senecio* spp.), Leguminosae (*Crotalaria* spp.) and Boraginaceae (*Heliotropium, Cynoglossum, Amsinckia, Echium*, and *Symphytum* spp.). All of these do not occur in the western United States. For plant descriptions readers are referred to those species causing losses in the western United States and are listed in Table 66.8 with common names and distribution.

## Toxicology

Over 150 PAs have been identified and structural characteristics elucidated (Mattocks, 1986). The PAs contain the pyrrolizidine nucleus and can be represented by the basic structures of senecionine and heliotrine. The toxic effects of all PAs are somewhat similar although their potency varies and are due to their bioactivation in the liver to toxic metabolites called pyrroles (Figure 66.7). These pyrroles are powerful alkylating agents that react with cellular proteins and cross-link DNA resulting in cellular dysfunction, abnormal mitosis and tissue necrosis. The primary effect is hepatic damage, however, many alkaloid and species-specific extra hepatic lesions have been described

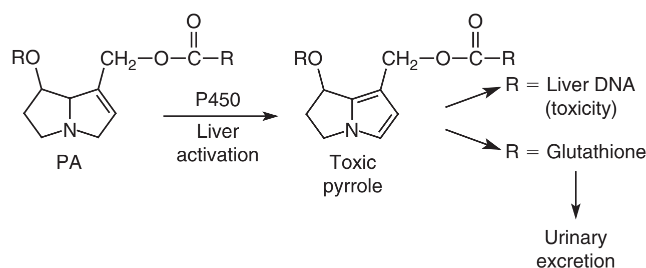



**FIGURE 66.7** Metabolic pathway of PAs showing the toxic pyrrole pathway and glutathione conjugate pathway for excretion.

**TABLE 66.8 PA-containing plants in the western United States, common names, habitat, and distribution**

| Scientific name | Common name | Habitat | Distribution |
|---|---|---|---|
| *Amsinckia intermedia* | Tarweed, fiddleneck | Dry, open cultivated fields | Pacific Coast; CA, WA, OR, ID |
| *Cynoglossum officinale* | Houndstongue | Dry, open areas; cultivated fields | Throughout West |
| *Echium vulgare* | Vipers bugloss | | CA |
| *Symphytum officinale* | Comfrey | | |
| *Senecio brasiliensis* | | | |
| *Senecio cineraria* | Dusty miller | | Ornamental |
| *Senecio glabellus* | Bitterweed | Wet soils | TX and East |
| *Senecio integerrimus* | | | MT; Rocky Mountains, CA |
| *Senecio jacobaea* | Stinking willy, Tansy ragwort | Open fields and woods | Pacific Northwest |
| *Senecio longilobus* | Woody or groundleaf groundsel | Deserts | WY to NE, south to AZ and TX |
| *Senecio spartioides* | Broom groundsel | | CO, UT south to TX, AZ, NM |
| *Senecio riddellii* | Riddell groundsel | Deserts | CO, UT south to TX, AZ, NM |
| *Senecio vulgaris* | Common groundsel | | CA, OR |
| *Crotalaria sagittalis* | | | Pacific Northwest |

(Hooper, 1978). Small amounts of pyrrole may enter the blood and be transported to other tissues, but there is some debate on this issue as most pyrroles are super reactive and not likely to even make it into the circulation (Stegelmeier *et al.*, 1999). When PA metabolites circulate, they are probably protein adducts that may be recycled. Some alkaloids (monocrotaline) may come off their carrier blood proteins and damage other tissues such as lung (Lame *et al.*, 2004). Pigs seem more prone to develop extra hepatic lesions.

Toxicity of *Senecio, Heliotropium*, and *Echium* is largely confined to the liver while *Crotalaria* will cause significant lung damage also (Hooper, 1978; Lame *et al.*, 2004). Typical histological lesions are swelling of hepatocytes, hepatocyte necrosis, periportal necrosis, megalocytosis (enlarged parenchymal cells), karyomegaly (enlarged nuclei) fibrosis, bile duct proliferation, and vascular fibrosis and occlusion. Hepatic cells may be 10–30 times normal size and DNA content may be 200 times normal.

In most species affected by PA poisoning, the liver becomes hard, fibrotic, and smaller. Because of decreased bile secretion, bilirubin levels in the blood rise, causing jaundice. Common clinical signs include ill thrift, depression, diarrhea, prolapsed rectum, ascites edema in the GI tract, photosensitization, and aberrant behavior. In horses, "head pressing" or walking in straight lines regardless of obstacles in the path may occur. These neurological signs in horses are due to elevated blood ammonia from reduced liver function (Knight *et al.*, 1984). PA poisoning may cause elevated blood ammonia resulting in spongy degeneration of the central nervous system.

Elevated levels of serum enzymes such as alanine aminotransferase, AST, $\gamma$-glutamyl transferase, and alkaline phosphatase are reported (Craig *et al.*, 1991; Stegelmeier *et al.*, 1999). Use of these tests for diagnosis is supportive but should not be relied upon exclusively as they vary with animal species and other conditions. They may also be in the normal range even though liver damage had occurred and they tend to be transient. Liver function tests such as bilirubin, bile acids, or sulfobromophthalein (BSP) clearance may be useful estimates of the extent of liver damage.

There are marked differences in susceptibility of livestock and laboratory animals to PA toxicosis. Cows are most sensitive followed by the horse, goat, and sheep, respectively. In small laboratory animals, rats are most sensitive followed by rabbits, hamster, guinea pig, and gerbil, respectively. Among avian species chickens and turkeys are highly susceptible while Japanese quail are resistant (Shull *et al.*, 1976).

Detoxification mechanisms of PAs generally involve the liver and GI tract. Evidence of ruminal detoxification in sheep suggests this contributes to the reduced toxicity in that species. There are also substantial species-specific differences in rate of PA metabolism. Both probably contribute to species susceptibility. For example, *Echium* and *Heliotropium* PAs are easily degraded by certain rumen microflora, but there is little evidence of ruminal degradation of *Senecio* PAs. The PAs in *Senecio* are macrocyclic-closed esters of retronecine as opposed to the open esters found in heliotridine (Kim *et al.*, 1993). Therefore, it is unlikely to be the rumen detoxification but more likely differences in species-specific enzymatic activation of *Senecio* PAs that is the reason for the difference in *Senecio* toxicity between sheep and cattle (Cheeke, 1998). For example, *in vitro* studies of retrorsine metabolism have been shown to be high in those species that are most susceptible and lowest in animals of least susceptibility. Additionally, using *in vivo* studies, Shull *et al.* (1976) demonstrated a higher pyrrole production rate in cattle than sheep. Simple induction of liver microsomal enzymes by phenobarbitone increases pyrrole production and increased PA toxicity ($LD_{50}$ in guinea pigs from >800 to 216 mg/kg). PA toxicity may disrupt other hepatic functions. Abnormal copper metabolism coagulation, $NH_3$ metabolism, protein metabolism, etc. may be affected in PA poisoning.

## Prevention and treatment

As there are no proven effective methods of prevention or treatment, avoidance of the plant and controlling plant populations with herbicides or biological control are essential. Resistance to PA toxicosis in some species suggests that possibilities may exist to increase resistance to PAs. Dietary factors, such as increased protein particularly those high in sulfur amino acids, had small protective effects in some species. Antioxidants such as butylated hydroxytoluene (BHT) and ethoxyquin-induced increased detoxifying enzymes such as glutathione *S*-transferase and epoxide hydrolase. Zinc salts have been shown to provide some protection against hepatotoxicosis from sporidesmin or lupinosis in New Zealand and Australia and zinc supplementation reduced toxicity in rats from *Senecio* alkaloids (Cheeke, 1998; Burrows and Tyrl, 2001; Knight and Walter, 2001).

Many of these plants were introduced either inadvertently or intentionally. Without natural predators to keep populations in check they experienced explosive growth and distribution followed by epidemic proportions of toxicity. Introduction of biological controls and natural population controls have reduced many of the plant populations and thus toxicoses have declined. Sheep, a resistant species, have been used to graze plants particularly *S. jacobaea* (Cheeke, 1998).

# PHOTOSENSITIZING PLANTS

There are numerous plants that cause photosensitization resulting in losses to the livestock industry. Photosensitization is the development of abnormally high reactivity

to ultraviolet radiation or natural sunlight in the skin or mucous membranes. Primarily induced in livestock by various poisonous plants, the syndrome in livestock has been defined as primary and secondary photosensitization.

## Description and distribution

Photosensitizing plants are too numerous to describe individually and readers are referred to taxonomic texts for plant description. Photosensitizing plants occur throughout the world and are common in the diets of livestock and people. Their distribution in the western United States, common names, and toxins are listed in Table 66.9.

## Toxicology

### *Primary*

In primary photosensitization, the photoreactive agent is absorbed directly from the plant and reaches the peripheral circulation and skin where it reacts with the ultraviolet rays of the sun and results in sunburn, particularly of the unprotected areas of the body (Rowe and Norman, 1989). Hypericin and fagopyrin are polyphenolic derivatives from St. John's wort and buckwheat, respectively, and are primary photodynamic agents (Cheeke and Shull, 1985). By definition, primary photosensitization does not induce hepatic damage. Most agents are ingested, but some may induce lesions through skin contact. Several of these plants are weedy in nature and can contaminate pastures and feed. Exposure to some plants is increasing as they are becoming widely used as herbal remedies and holistic medicines. In most cases the photodynamic agent is absorbed from the digestive tract unchanged and reaches the skin in its "native" form (Stegelmeier, 2002)

There are drugs and other toxins known to cause primary photosensitization and should be considered in the differential diagnosis. Phenothiazine-induced photosensitization is most common in ruminants because the photodynamic agent is phenothiazine sulfoxide, a rumen metabolite. Clinical signs in addition to photosensitivity include corneal edema and kerato conjunctivitis from the phenothiazine sulfoxide excreted in tears and the aqueous humor. Other toxins associated with primary photosensitivity include thiazides, acriflavins, sulfonamides, tertracyclines, methylene blue, coal-tar derivatives, furosemide, promazine, chlorpromazine, quinindine, and some antimicrobial soaps (Stegelmeier, 2002).

### *Secondary*

In secondary or hepatogenous photosensitization, the photoreactive agent is phylloerythrin, a degradation product of chlorophyll. Phylloerythrin is produced in the stomach of the animals especially in ruminants and absorbed into the bloodstream. In normal animals, the hepatocytes conjugate phylloerythrin and excrete it in the bile. However, if the liver is damaged or bile secretion is impaired, phylloerythrin accumulates in the liver, the blood, and subsequently the skin, causing photosensitivity. This is the most common

**TABLE 66.9 Photosensitizing plants of the western United States, listed as primary and secondary photosensitizers (Kingsbury, 1964; Johnson, 1982; Cheeke and Shull, 1985)**

| Scientific name | Common name | Distribution | Toxin |
|---|---|---|---|
| **Primary photosensitizers** | | | |
| *Hypericum perforatum* | St. John's wort, Klamath weed | Pacific Coast states | Hypericin |
| *Fagopyrum sagittatum* | Buckwheat | Northwestern US | Fagopyrin |
| *Cymopterus watsoni* | Spring parsley | Southwestern US | Furocoumarins |
| *Ammi majus* | Bishop's weed | Southwestern US | Furocoumarins |
| **Secondary photosensitizers** | | | |
| *Artemesia* | Sagebrush | Western US | |
| *Tetradymia glabrata* | Spineless horsebrush | Western US | |
| *Tetradymia canescens* | Gray horsebrush | Western US | |
| *Agave lecheguilla* | Lechuguilla | Southwest | Saponins |
| *Nolina texana* | Sacahuiste | Southwest | Saponins |
| *Tribulus terrestris* | Puncture vine | Southwest | Saponins |
| *Trifolium hybridum* | Alsike clover | North, Midwest | |
| *Lantana* spp. | Lantana | Southwest | Saponins |
| *Panicum* spp. | Panic grass, Kleingrass | Western US | |
| *Brassica napus* | Rape | Western US | |
| *Senecio* spp. | Senecios | Western US | PAs |
| *Cynoglossum officinale* | Houndstongue | Western US | PAs |
| *Cooperia pedunculata* | Amaryllis family | Southwest US | |
| *Thamnosma texana* | Dutchmans breeches | Southwest US | Psoralens |
| *Kohia scoparia* | Kochia, Burning bush | | Saponins |
| *Descurania pinnata* | Tansymustard | | Unknown |

cause of photosensitization in livestock and horses (Knight and Walter, 2001). Since chlorophyll is almost always present in the diet of livestock the etiological agent of secondary photosensitization is the hepatotoxic agent.

The dermatological signs of photosensitization in livestock are similar regardless of the plant or toxicant involved. Degree or severity varies, depending on the amount of toxin or reactive phylloerythrin in the skin, degree of exposure to sunlight, and amount of normal physical photo protection (hair and pigmentation). First signs in most animals are restlessness or discomfort from irritated skin followed by photophobia, squinting, tearing, erythema, itching, and sloughing of skin in exposed areas, i.e. lips, ears, eyelids, udder, external genitalia, or white pigmented areas (Burrows and Tyrl, 2001). Swelling in the head and ears (edema) of sheep after ingestion of *Tetradymia* has been referred to as bighead. It was determined that sheep grazing black sagebrush (*Artemesia nova*) before *Tetradymia* were 3 times more likely to develop this photosensitization (Johnson, 1974). Tissue sloughing and serum leakage may occur where tissue damage is extensive. Primary photosensitization rarely results in death. However, in secondary or hepatogenic photosensitization the severity of liver damage and secondary metabolic and neurological changes of hepatic failure may ultimately result in death. Recovery may leave sunburned animals debilitated from scar tissue formation and wool or hair loss.

#### *Prevention and treatment*

Prevention of poisoning lies in controlling plants with photosensitizing potential and providing adequate quality forage to animals. Treatment after poisoning involves removing animals from sun exposure, treating areas of necrosis and sunburn, antibiotic therapy and supplementing young animals when access to sunburned udders is prevented because of nursing discomfort to dams. Identifying chronic hepatic disease is complicated because many of the serum markers for hepatic disease have returned to normal. As normal hepatocytes become replaced with fibrous connective tissue there are fewer damaged cells to elevate serum enzymes. Percutaneous liver biopsies are invaluable in identifying and diagnosing these cases (Stegelmeier *et al*., 1999).

Plant-induced hepatopathy generally results in characteristic histological lesions. For example, PAs generally cause bridging portal fibrosis with hepatocellular necrosis, biliary proliferation, and megalocytosis. *Panicum* and *Tribulus* species generally produce a crystalline cholangiohepatitis. Liver biopsy also provides prognostic information. The degree of damage is correlated directly with the animal's ability to compensate, recover, and provide useful production. Keep in mind that the liver reacts to insult in a limited number of ways, and most histological changes are not pathognomonic. Hepatic cirrhosis (necrosis, fibrosis, and biliary proliferation) involves non-specific changes that can be initiated by a variety of toxic and infectious agents (Stegelmeier *et al*., 1999).

## DEATH CAMAS

All death camas species are assumed to be toxic; however, variation in toxicity exists between species and even within species depending on season, climate, soils, and geographical locations. Poisoning in sheep, cattle, horses, pigs, fowl, and humans has been reported (Kingsbury, 1964; Wagstaff and Case, 1987). The largest losses generally occur in sheep. Sheep are primarily affected because of their tendency to select forbs, particularly in early spring when they are turned onto range before grasses have emerged.

Death camas is generally not palatable to livestock but is one of the earliest species to emerge in the spring. Poisoning most frequently occurs in spring when other more palatable forage is not available, or on over-grazed ranges where there is a lack of more desirable forage. Poisonings have resulted due to management errors in which hungry animals were placed in death camas-infested areas (Panter *et al*., 1987).

### Description, habitat, and geographical distribution

Foothill death camas is typical of the 15–20 species of *Zigadenus* in North America and Asia (Figure 66.8). A list of death camas species, habitat, distribution, and growth period is given in Table 66.10. It is difficult to distinguish between species because they are taxonomically similar. A member of the lily family, death camas is a perennial, glabrous herb with basal V-shaped grass-like leaves growing from an onion-like bulb with a dark-colored outer coat. Stems produced at flowering are single, unbranched, sparingly leafed, and terminated by a terminal raceme of greenish-white, cream-colored, or pink inflorescence. The perianth is six-membered, consisting of three lanceolate or ovate sepals and three petals separate or united below, with one or two glands just above the base; stamens six; styles three; floral parts are persistent but winter as the fruits develop. The seed is a three-cavitied capsule, separating into three members and opening inwardly at maturity (Kingsbury, 1964; Muenscher, 1975). Death camas is easily confused with wild onion, mariposa lily, or common camas particularly before flowering. Wild onions are distinguished by tubular leaves and their onion-like odor. Mariposa lilies have leaves more U-shaped in cross-section and common camas has a blue flower.

**FIGURE 66.8** Foothill death camas with leaf structure, flower head, and bulb.

Death camas is prevalent in the western part of North America and is native to the open plains and foothills of the United States. Table 66.10 lists species of death camas with available information about common names, geographical distribution, growth period, and habitat.

## Toxicity of death camas to livestock

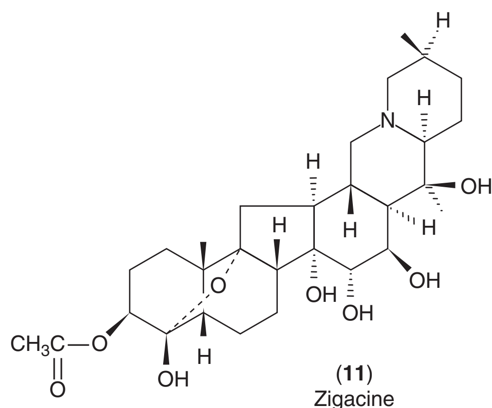
The toxins in death camas are of the cevanine steroidal alkaloid type, i.e., zigacine **(11)**. Clinical signs of toxicity are similar in all livestock poisoned by *Zigadenus*, irrespective of the species of plant involved (Kingsbury, 1964). Excessive salivation is noted first, with foamy froth around the nose and muzzle which persists, followed by nausea and occasionally vomiting in ruminants (Kingsbury, 1964; Panter *et al*., 1987). Intestinal peristalsis is dramatically increased, accompanied by frequent defecation and urination. Muscular weakness with accompanying ataxia, muscular fasciculations, prostration, and eventual death may follow. The pulse becomes rapid and weak, and the respiration rate increases but the amplitude is reduced. Some animals become cyanotic and the spasmodic struggling for

**TABLE 66.10** ***Zigadenus* spp.: common names, distribution, habitat, and growth periods**

| Species | Common name | Distribution | Habitat | Growth period |
|---|---|---|---|---|
| *Z. paniculatus* S. Watson | Foothill death camas; sand-corn; panicled death camas | Eastern WA, OR, ID, UT, WY, NV, eastern CA, northern AZ and NM | Foothills and benches | May to July |
| *Z. venenosus* S. Watson = *Z. gramineus* Rydb. = *Z. intermedium* Rydb. = *Z. salinus* Rydb. | Meadow death camas; grassy death camas | WA, OR, ID, MT, ND; south to NE, UT, CO, NV, CA; north to western Canada | Plains, prairies, meadows and open coniferous woods | May to July |
| *Z. nuttallii* A. Gray | Nuttall's death camas; poison camas | TN, AR, OK, KS, and northern TX | Prairies and rocky sites | April to June |
| *Z. gramineus* Rydb. | Grassy death camas | Southwestern Canada, MT, ID, WA; south through WY, CO, UT, NV, AZ, and NM | Open hills and plains | April to July |
| *Z. elegans* Pursh = *Z. glaucus* Nutt. | White camas, elegant death camas, mountain death camas | AK, western Canada, MT, south into AZ and east into MN and IA | Prairies and meadows | June to August |
| *Z. leimanthoides* A. Grey | – | Southwestern US and coastal plains of DE, NJ, and RI | Sandy pine lands and bogs of the coastal plains | June to August |
| *Z. fremontii* Torr. | – | Southwestern OR and western CA | Dry grassy or brushy slopes | May to July |
| *Z. glaberrimus* Michx. | – | Southeastern US | Savannas and wet pine lands | July to September |
| *Z. densus* Desr. | Black snakeroot, crow poison, St. Agnes' feather, black death camas | Southeastern US | Damp soils, pine woods and bogs | May to June |
| *Z. exaltatus* Eastw. | – | Central CA | | May to June |
| *Z. micranthus* Eastw. | – | Northwestern CA, southwestern OR | | |
| *Z. vaginatus* Rydb. | Alcove death camas | Northwestern CA, southwestern OR | | |
| *Z. virescens* Kunth. | | AZ, NM, and northern Mexico | | |

breath may be confused with convulsions. The heart fails before respiration, and at necropsy the heart is usually found in diastole. A comatose period may range from a few hours to a few days before death.

Pathological lesions are those of pulmonary congestion. Gross lesions of sheep include severe pulmonary congestion, hemorrhage, edema, and subcutaneous hemorrhage in the thoracic regions. Microscopic lesions include severe pulmonary congestion with infiltration of red blood cells in the alveolar spaces and edema. Diagnosis of poisoning may be established by clinical signs of toxicity, evidence of death camas being grazed, histopathological analysis of tissues from necropsied animals, and identification of death camas in the rumen or stomach contents (Panter *et al*., 1987).



(**11**)
Zigacine

Similarity in clinical signs of toxicity between certain species of these plants suggests the same alkaloids are present; however, differences in concentrations can explain the differences in relative toxicity of different species (Table 66.11).

## Management and prevention

Conditions conducive to poisoning by death camas include driving animals through death camas-infested ranges; not allowing animals to graze selectively; unloading hungry animals in infested areas; lambing, bedding, watering or salting livestock in death camas-infested areas; or placing animals on range where little forage is available. Poisoning generally occurs in the early spring when death camas is the first green forage available and the young immature foliage is the most toxic. Single losses of 300–500 sheep have been reported (Kingsbury, 1964; Panter *et al*., 1987). In the 1987 case 80% of the dead sheep were 80–90 lb lambs. Three key factors contributed to the losses: (1) ewes with lambs were driven through a heavily infested area of death camas when the sheep were hungry; (2) the sheep were bedded down for the night near the death camas area providing immediate access to death camas the following morning; and (3) the herder panicked and rapidly forced the sheep out of the area contributing to the stress and probably exacerbating the toxic effects and increasing the losses.

**TABLE 66.11 Relative toxicity in sheep of five *Zigadenus* spp.**

| *Zigadenus* species | Average minimum toxic dose* | Average minimum lethal dose* |
|---|---|---|
| *Z. gramineus* | 4 | 6 |
| *Z. paniculatus* | 10 | 25 |
| *Z. venenosus* | 4 | 20 |
| *Z. elegans* | 20 | 60 |
| *Z. nuttallii* | 2 | 5 |

Adapted from Kingsbury (1964).
*Grams of green plant per kg body weight.

# *VERATRUM* SPP.

*Veratrum* belongs to the Liliaceae (Lily) family and is comprised of at least five species in North America. During the mid- 20th century, up to 25% of pregnant ewes that grazed on pastures infested with *Veratrum californicum* in the mountains of central Idaho gave birth to lambs with serious craniofacial malformations (James, 1999b). These malformations ranged from the gross anomaly of cyclops to less severe deformities of the upper and lower jaws. The Basque shepherds called the cyclopic defect "chatto" which translated as "monkey-faced" lamb disease. While losses from *Veratrum* have long been reduced or eliminated on these ranges because of the research and recommended management strategies, biomedical research using the alkaloids, isolated and identified at the Poisonous Plant Research Laboratory, as molecular probes have opened a new frontier for human medical research (Gaffield and Keeler, 1996; James *et al*., 2004).

## Distribution

*Veratrum californicum* grows primarily in the high mountain ranges of the western United States (Kingsbury, 1964; Knight and Walter, 2001). *Veratrum viride* is the most widespread species and grows in the northwestern United States north through western Canada into Alaska and is also widespread in the northeastern United States; *V. insolitum* grows in a relatively small region of northwestern California and southwestern Oregon; *V. parviflorum* grows in the central southeastern states; and *V. woodii* grows from Ohio to Missouri, Oklahoma, and Arkansas. Two other species have been reported to cause poisoning in other countries, *V. japonicum* in Korea and *V. album* in Europe.

Common names include western false hellebore, hellebore, skunk cabbage, corn lily, Indian poke, wolfsbane, etc. Caution should be used with common names as they may be used interchangeably within this genus but also in unrelated genera. For example, the name hellebore is also used for the genus *Helleborus* in the buttercup family.

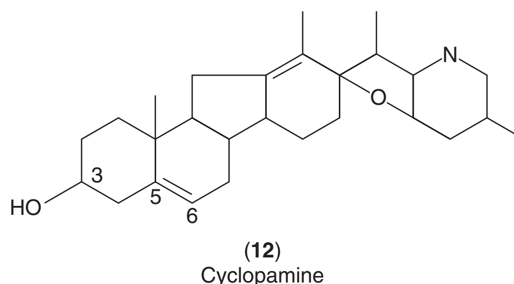
## Habitat and description

Most *Veratrum* spp. are found in similar habitats of moist, open alpine meadows or open woodlands, marshes, along waterways, in swamps or bogs, and along lake edges in high mountain ranges (Kingsbury, 1964). Most species grow at higher elevations. All species are similar with coarse, erect plants about 1–2.5 m tall with short perennial rootstalks. The leaves are smooth, alternate, parallel veined, broadly oval to lanceolate, up to 30 cm long, 15 cm wide, in three ranks and sheathed at the base. The inflorescence is panicle flowers, the lower ones often staminate and the upper ones perfect. The flowers of *V. viride* are distinctly green and the fruit is three-chambered with several seeds.

## Toxicology

Over 50 complex steroidal alkaloids have been identified from the *Veratrum* spp. Five classes of steroidal alkaloids have been characterized: veratrines, cevanines, jervanines, solanidines, and cholestanes. The veratrines and cevanines are of considerable interest in toxicology as they are neurological toxins and hypotensive agents that bind to sodium channels delaying closure and causing cardiotoxic and respiratory effects. The cevanine alkaloids, like zigacine **(11)** are also found in *Zigadenus* spp., also members of the Lily family. The jervanines are most significant for their teratogenic effects; the most notable alkaloids were named cyclopamine **(12)** and jervine, both potent inducers of the congenital cyclopia "monkey-faced" lamb syndrome reported in many flocks of sheep in the late 1950s in central Idaho (Binns *et al.*, 1965). This cyclopic defect is induced in the sheep embryo during the blastocyst stage of development when the pregnant mother ingests the plant during the 14th day of gestation. Early embryonic death up to the 19th day of gestation and other defects such as limb defects and tracheal stenosis occur when maternal ingestion includes days 28–33 of gestation (Keeler *et al.*, 1985; Keeler and Stuart, 1987; Keeler, 1990). The solanidine alkaloids are also found in many *Solanum* spp. and are toxic and teratogenic. The cholestanes have been used as hypotensive drugs but are much less likely to induce the birth defects (Gaffield and Keeler, 1994). Structure activity relationship is very important in potency to produce birth defects. It is now known that this structure activity relationship is key in the mechanism of action which is the inhibition of the sonic hedgehog signaling pathway (Gaffield and Keeler, 1996). This sonic hedgehog gene pathway and the subsequent down stream regulation of other gene's expression have now been implicated in numerous cancers, birth defects and other anomalies. The toxin, cyclopamine, has become a significant tool in the study of this very complex sonic hedgehog pathway. Clinical trials have been proposed and studies are ongoing to further identify the hedgehog complex of genes and to understand its mechanism and function in formation and growth of numerous cancers, childhood birth defects, and manipulation of regulatory pathways.

Clinical signs of poisoning are most likely caused by the neurotoxic cevanine alkaloids present in most species of *Veratrum*. Typical signs begin with excess salivation with froth around the mouth, slobbering and vomiting progressing to ataxia, collapse, and death.




(**12**)
Cyclopamine

## Prevention and treatment

Control of *Veratrum* is relatively easy with herbicides such as broad-leaf herbicides and long-term control has been demonstrated (Williams, 1991). The teratogenic effects of *Veratrum* can be avoided by keeping sheep and other livestock species off pastures containing the plants during the first trimester of pregnancy. Observation of toxicoses in the field is rare unless herders move the animals shortly after exposure. The neurological signs, which are likely produced by the cevanine alkaloids (both *Veratrum* and *Zigadenus* spp.) can be treated with atropine to improve the cardiovascular output. Activated charcoal to adsorb toxins and administration of picrotoxin to improve respiration have been recommended (Burrows and Tyrl, 2001).

# BRACKEN FERN (*PTERIDIUM AQUILINUM*)

## Distribution and habitat

The bracken fern family is worldwide in distribution and include about 20 genera and over 400 species. While most species described are found in the tropics, there are 4 genera

and 6 species described in North America (Burrows and Tyrl, 2001). The bracken fern most associated with toxicoses in the United States is *Pteridium aquilinum* (Kuhn) and is distributed throughout North America. It is reported to be one of the most widespread species of vascular plants only exceeded in geographical range by a few annual weeds. Four varieties of *P. aquilinum* have been described including var. *pubescens* (western bracken fern) found throughout the western United States; var. *caudatum* (lacy bracken) restricted to southern Florida; var. *latiusculum* (eastern bracken) distributed throughout the eastern United States; and var. *pseudocaudatum* (tailed bracken) mostly located in the southeastern quarter of the United States.

Western bracken grows best in rocky, gravelly, well-drained soils in dry open woodlands to semi-shaded habitats. Extensive stands frequently grow along pasture edges, in logged areas or where fires have opened up the canopy. Moisture needs are modest in comparison with many of the ferns and good soil drainage is important.

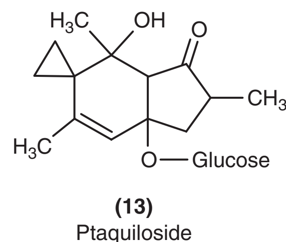
## Description

Bracken plants are deciduous and grow from brown to black woody rhizomes, forming large often dense patches. The leaves emerge from erect fronds and are pinnately compound, scattered, erect, coarse, narrowly or broadly triangular, to 2 m in height. Fronds (leaves) are pinnules (ultimate segments) oblong, entire in the apices of the pinnae, lobed toward the stalk. Reproduction is by spores produced in sporangia lining the under surface margins of the photosynthetic fronds when reproductive, covered by the narrow recurved edge of the leaf (Kingsbury, 1964; Burrows and Tyrl, 2001).

## Toxicology

Toxicity of bracken fern was first recognized in the 1800s and described in horses as a neurological condition. Contaminated hay was believed to be the cause and the condition was described in the United Kingdom and Pacific Coast states of the United States (Taylor, 1990; Burrows and Tyrl, 2001). Early research determined that 20–25% bracken for 3 or 4 weeks would induce a neurological disease followed soon after by death. While the neurological condition is mostly described in horses, low hematocrits and reddish-brown urine has also been described in suspected cases of bracken poisoning (Kelleway and Geovjian, 1978).

Bracken causes a wide range of syndromes that have been described in livestock including thiamine deficiency in monogastrics, acute hemorrhagic disease associated with bone marrow aplasia and ulceration of the upper GI tract, "bright blindness" progressive retinal degeneration, and neoplasia of the urinary bladder and upper digestive tract (Smith, 2004). The major toxin is the sesquiterpene glucoside, ptaquiloside **(13)**. Other toxins, carcinogens and mutagens may be implicated in the disease conditions also.




**(13)**
Ptaquiloside

People have consumed the rhizomes or croziers of bracken fern as a traditional food or out of necessity, and the toxin is transferred through milk of cows grazing the plant. Epidemiological evidence suggests that some cancers in man probably result from primary or secondary consumption of the carcinogens. Ptaquilosides form adducts with DNA, binding to certain base sequences, especially those associated with adenine. Ptaquiloside mutates codons associated with known oncogenes and this hypothesis has been proposed in the pathogenesis of the disease (Smith, 2004).

Syndromes of bracken poisoning are well recognized in livestock. Many factors must be considered in the genesis of the disease such as quantity consumed, phenological stage of the plant, time of year, consumption rate and length of time consumption occurs, animal species, age, sex of the animal and other factors. The disease conditions described include thiamine deficiency in monogastrics, acute hemorrhagic disease, bright blindness, enzootic hematuria, and small intestine carcinoma (Smith, 2004). These conditions have been experimentally produced by feeding bracken fern and ptaquiloside to livestock species and rodent models. Bracken fern feeding studies in rodents have produced neoplasms in the ileum, urinary bladder, mammary glands, and lungs of rodents.

The toxin ptaquiloside **(13)** was isolated, characterized and the structure published in the early 1980s (Niwa *et al.*, 1983; Hirono *et al.*, 1984). Different species and varieties of *Pteridium* have been compared for ptaquiloside concentration and all examined to date contained ptaquiloside. Large variations in concentration were demonstrated between locations, altitude, season, etc., suggesting a genetic component or another factor such as endophyte-produced compounds. These differences were maintained when bracken fern rhizomes were transferred to a greenhouse and soils were changed, thus the ptaquiloside differences were still evident for the next 3 years. Ptaquiloside concentrations are highest in young growing parts, i.e. the tips of the croziers and immature fronds while the concentration diminishes as the plant matures and as samples are taken from more mature pinnae and towards the base of the lamina. All

samples of rhizome, the apices of the fronds or primordia taken from below the soil surface had no ptaquiloside; however, very immature croziers only a few centimeters above the surface showed the presence of the toxin (Smith, 2004). The emergence of the crozier apices and exposure to light apparently influence the biosynthesis of the toxin. The spores have not been shown to contain ptaquiloside but they have caused cancer and formed DNA adducts suggesting carcinogenic or mutagenic derivatives are present. Ptaquiloside and other ptaquiloside-like compounds with carcinogenic activity have been isolated from other ferns from the genera *Histiopteris, Cheilanthes, Cibotium, Dennstadtia, Hypolepis, Pteris*, and *Pityrogramma* (Saito *et al*., 1990).

While bracken fern poisoning occurs in the United States the most serious risk is in countries such as Australia, New Zealand, Japan, and the United Kingdom where bracken is more prevalent and utilized for human food or animal feed. Epidemiology studies have associated increased esophageal and gastric cancers with direct ingestion, secondary ingestion (i.e. milk from cows grazing bracken) or just living in bracken fern-infested areas. Bracken fronds steeped in water or treated with wood ash or sodium bicarbonate have reduced toxicity but this process only reduces the concentration and does not eliminate the toxins (Hirono *et al*., 1972).

Lesions in horses poisoned by bracken fern are indicative of thiamine deficiency and include congestion of the brain, a swollen and edematous cerebrum grossly and necrosis of some neurons microscopically. Acute hemorrhagic disease in cattle is characterized by extensive hemorrhage of the mucous membranes and subcutaneous hemorrhage and edema.

Pathology of the bracken-induced enzootic hematuria include desquamated and proliferative bladder epithelium and areas of vascularized epithelial proliferation appear as polyploid, papillary, or fungoid reddened foci. Microscopically, columns of transitional epithelium infiltrate into the lamina propria along with mononuclear cells. Neoplasia, when it occurs, is most commonly the non-invasive papillomatous type, but other papillary types of transitional cell carcinoma, squamous cell carcinoma, adenocarcinoma, or hemangiomas may also develop (Burrows and Tyrl, 2001).

## Treatment

Bracken-induced thiamine deficiency in horses is treatable with administration of thiamine parenterally at 0.5–1 g followed by decreasing doses over the next few days. Symptomatic care with good feed and fresh water accompanied by administration of a laxative but *not* mineral oil is helpful. In ruminants, the bone marrow suppression and deficiency of blood platelets and neutrophils is best treated with antimicrobials to counteract any bacterial infection that might occur because of diminished immune function. Good veterinary care, symptomatic treatment, clean water and quality feed in a quiet clean environment is recommended.

# MILKWEEDS: *ASCLEPIAS* SPP.

## Description

Milkweeds are divided into two broad groups: narrow-leaved with narrow, linear, lanceolate leaves; and broad-leaved with leaves about 4 cm wide throughout much of their length (Figure 66.9). There are over 150 species of *Asclepias* of which 108 occur in North America (Woodson, 1954). Milkweeds are perennial, summer or early autumn flowering-herbs with a milky latex-like cream in the stems. Of the many species found in North America several are important toxicologically and represented in Table 66.12. The stems of the plants are erect and range from 4 to 150 cm tall, leaves opposite or whorled. Flowers are of various colors, umbellate clusters or solitary, and terminal or extra-axillary. Numerous seeds are contained in an inflated milkweed-type pod and are flat, large and each bearing a tuft of long, silk-like hairs for wind distribution.

**FIGURE 66.9** Narrow-leaf (left) and broad-leaf (right) milkweeds (*Asclepias* spp.) represented.

**TABLE 66.12 Milkweeds: *Asclepias* spp., common names, distribution, habitat and toxin**

| Species | Common name | Distribution | Habitat | Toxin; content* |
|---|---|---|---|---|
| **Narrow-leaved milkweeds** | | | | |
| *A. labriformis* Jones | Labriform milkweed | Southeastern UT | Along old stream beds in sandy soils | Cardiac glycosides, digitoxin; very high |
| *A. verticillata* L. | Eastern whorled milkweed, spider milkweed | From TX to MI, east to FL and MA | Dry open areas | Cardenolides; very low |
| *A. subverticillata* Vail | Western whorled milkweed, horsetail milkweed | Western KS and OK, UT, AZ, TX and into Mexico | Dry plains and foothills, spreads rapidly along waterways and canals | Cardenolides; very low |
| *A. pumila* Vail | Low whorled milkweed, plains whorled milkweed | East of the Rockies from TX and NM north to southeast MT and southwest ND | Small patches in draws and ravines | Cardenolides; very low |
| *A. mexicana* Cav. | Mexican whorled milkweed | Southern TX to central Mexico | Open areas and dry soils | Cardenolides; unknown |
| *A. asperula* Woodson | Antelope horn milkweed | KS, AR to NV and AZ | Open areas and dry soils | Cardenolides; very high |
| *A. fascicularis* Decne. | Mexican milkweed | CA, western OR through eastern WA, ID and NV | | Cardenolides; very low |
| *A. brachystephana* Engelm. | Short-crown milkweed | West central TX, southern AZ into NM and Mexico | | Cardenolides; very high |
| *A. subulata* Decne. | Desert milkweed, yamate, ajamete | Southern CA, AZ, west coastal region of Mexico | | Cardenolides; very high |
| **Broad-leaved milkweeds** | | | | |
| *A. eriocarpa* Benth. | Woolly-pod milkweed | CA | Dry soils | Cardenolides; very high |
| *A. latifolia* Brit. | Broad-leaf milkweed | KS, CO to TX and AZ | Dry plains | Cardenolides; high |
| *A. speciosa* Torr. | Showy milkweed | MN south to MI and TX; west to CA | Prairies and open spaces | Cardenolides; intermediate |
| *A. syriaca* L. | Common milkweed | Widely distributed in central and eastern US | Open areas and along roadsides | Cardenolides; intermediate |
| *A. incarnata* L. | Swamp milkweed | IN | Cool damp soils | Cardenolides; very low |
| *A. vestita* Hook and Am. | Woolly milkweed | Central and southwestern CA | | Cardenolides; very high |
| *A. virdis* Walter | Green milkweed, spider milkweed | Southcentral states, FL | | Cardenolides; high |

Adapted from Kingsbury (1964), Burrows and Tyrl (2001), and Knight and Walter (2001).
*Cardenolide content mg/g plant from <0.25 (very low) to >4 (very high).

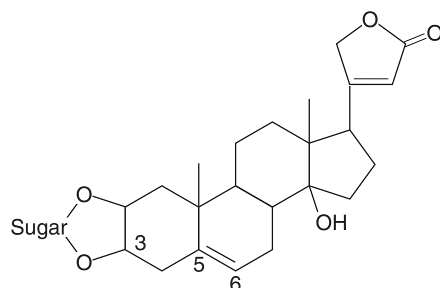
## Distribution and habitat

Milkweeds are adapted to a broad range of habitats and environmental conditions and are found in most plant communities. Occupied habitats include prairies, woodlands, open areas, rocky plains, sand dunes, swamps, marshes, seeps, canyon bottoms, dry washes, desert lands, and wastelands. Most species are weedy and form small to large patches often along roadsides, in disturbed areas or in overgrazed pastures.

## Toxicology

Some species of milkweeds have been used as medicines, food or a source of poison. Most species contain a mixture of steroidal glycosidic cardenolides **(14)** that are toxic and induce two syndromes: GI/cardiotoxic effects and the other neurological (Radeleff, 1970). Earlier literature suggested that the broad-leaved group was cardiotoxic while the narrow leave-type was neurotoxic. However, most narrow-leaved species have now been shown to contain cardiotoxins also (Ogden *et al.*, 1992).

Historically, naturalists observed as early as the mid 1800s that birds avoided eating certain butterflies, such as the monarchs whose larvae fed on milkweeds. It was later learned that the feeding larvae were able to concentrate some of the cardenolides that were potent emetics and thus created an effective defense against herbivory. Interestingly, other butterfly species mimicked the color configuration of the monarch as an effective defense mechanism against herbivory by birds. As of the late 1980s, 27 species of milkweed




(**14**)
Cardenolide

were recognized as common feed for butterfly larvae, and the most toxic species were often preferred (Malcolm *et al.*, 1989). The milkweeds are host to many other insect species also.

The basic structure of the series of identified cardenolides **(14)** is a 23-C steroidal backbone with a five-membered, singly unsaturated lactone ring at the C-17 position, a hydroxyl group at C-14, and methyl groups at C-10 and C-13. Glycosidic linkage usually occurs at the C-3 or C-2 positions and may include glucose, rhamnose or thevetose as the sugars. Additional functionalities attached to the backbone further influence solubility, and binding thus increasing or decreasing toxicity. The cardenolides inhibit $Na^+$, $K^+$-ATPase, and the structure activity relationship is believed to reside in the unsaturated lactone ring at C-17 and the hydroxyl group at C-14 (Joubert, 1989).

Sheep, goats, cattle, horses, and domestic fowl have been poisoned with milkweeds (Kingsbury, 1964). Clinical signs usually begin with depression, weakness and labored breathing accompanied by ataxia and loss of balance, progressing to recumbency, seizures and death. The order of clinical signs is dependent on the type of animal affected, toxicity of the plant, time of ingestion and amount of the toxin consumed. Clinical effects appear within a few hours of ingestion and death may follow within one to a few days later in fatal cases.

## Treatment and control

There are two syndromes of milkweed poisoning, a neurological one and a cardiac one. Treatment for the cardiotoxic signs is approached similar to that of digitalis glycoside toxicity, i.e. activated charcoal, atropine for atrioventricular (AV) block, and/or antiarrhythmic drugs. There is no specific treatment for the neurological syndrome except symptomatic treatment to control seizures and supportive therapy. A field test with a detection threshold of 0.057% is available for detecting cardenolides in the latex of milkweeds (Sady and Seiber, 1991). Knowledge of the milkweed species in the pasture, stage of plant growth when consumed and history of grazing and clinical effects are all important in developing a diagnosis and eliminating other causes.

In most cases, knowledge that the plant exists in your pasture and prevention of ingestion is the best way to avoid poisoning. Purchase of weed-free hay to avoid poisoning via contamination is also important. Know where the hay comes from and always be alert as to the content of the hay. Control of milkweeds can be accomplished with herbicides such as 2,4-D, picloram, amitrole or glyphosate depending on the other forage it has infested and the circumstances. Always follow the manufacturer's recommendation.

# NIGHTSHADES

The nightshade family comprises over 80 or 90 genera and more than 2300 species worldwide. Some of the more common poisonous genera include *Brugmansia, Brunfelsia, Capsicum, Cestrum, Datura, Hyoscyamus, Lycium, Lycopersicon* (tomato), *Nicotiana* (tobacco), *Solandra, Solanum, Nicandra*, and *Physalis*. Some common nightshade species are listed in Table 66.13. In the United States and Canada the nightshades can be annual, perennial or shrubs with alternate, simple or compound leaves, axillary inflorescences, radially symmetrical flowers composed of five free sepals, five free petals, usually five stamens with anthers grouped conically about the stigma and berry-like fruits of variable colors.

## Toxins

The nightshades have a variety of toxins known to affect people and animals and include the tropane alkaloids, affecting the autonomic nervous system by blocking the action of cholinesterase (Knight and Walter, 2001). This results in accumulation of acetylcholine and subsequent inhibition of the parasympathetic nervous system, causing dry mouth, decreased intestinal motility, dilated pupils, and tachycardia. The tropane alkaloids also affect the GI tract causing colic in horses, constipation and/or hemorrhagic diarrhea. The *Nicotiana* spp. contain neurological toxins including the pyridine and piperidine alkaloids responsible for toxicoses and teratogenesis. Some species of *Solanum* cause a neurological disease in cattle, sheep, and goats characterized by loss of equilibrium, tremors, ataxia, collapse, opisthotonus, seizures, and death. *Solanum malacoxylon* and *Cestrum* spp. contain vitamin D-like compounds that cause abnormal calcium absorption and metabolism resulting in calcified tissues, lameness, and weight loss. Green potatoes, sprouts, and vines contain steroidal glycoalkaloids that are both toxic and teratogenic. Table 66.13 provides a limited

**TABLE 66.13 Nightshade species with known links to toxicosis in humans or animals: common names, distribution, and toxins**

| Species | Common name | Distribution | Toxins |
|---|---|---|---|
| *Solanum elaeagnifolium* | Silverleaf nightshade | Southern US | Solanine and solanidine |
| *S. nigrum* | Black nightshade | Throughout North America | Tropane and glycoalkaloids |
| *S. dulcamara* | Bittersweet; climbing nightshade | Eastern half of US; parts of OR, WA, NV, CA, UT and WY | Glycoalkaloids |
| *S. rostratum* | Buffalo bur | Central US to north and south borders | Glycoalkaloids |
| *S. triflorum* | Cutleaf nightshade | Central US to north border | Glycoalkaloids |
| *S. americanum* | Huckleberry; wonderberry | Southern US | Glycoalkaloids |
| *S. physalifolium* | Tropical soda apple | Throughout US | Glycoalkaloids |
| *S. sarrachoides* | Hairy nightshade | | Glycoalkaloids |
| *S. carolinense* | Horse or bull nettle | Eastern half of US; west coast | Tropane alkaloid solanine |
| *S. tuberosum* | White or Irish potato | Worldwide | Glycoalkaloids |
| *Datura wrightii* | Sacred datura | Southwestern US | Tropane alkaloids |
| *D. stramonium* | Jimson weed; thornapple | Southeastern half of US; west coast | Tropane alkaloids |
| *Nicotiana tabacum* | Cultivated or burley tobacco | Southern US | Pyridine alkaloids (nicotine) |
| *N. glauca* | Tree tobacco | Southern US | Piperidine alkaloids |
| *Capsicum annum* | Green or chile pepper | Cultivated in US | Capsaicinoids |
| *Cestrum parqui* | Willow-leaved jessamine | Southern Gulf states | Glycoside of vitamin D |
| *Atropa belladonna* | Deadly nightshade; belladonna | Cultivated as an ornamental | Tropane alkaloids |
| *Hyoscyamus niger* | Black henbane | Northern US | Tropane alkaloids, calystegins |
| *Physalis virginiana* | Ground cherry | Plains states and eastern US | Glycoalkaloids, calystegins |
| *P. lobota* | Chinese lantern | Southern regions; cultivated throughout US | Glycoalkaloids |
| *Lycopersicon esculentum* | Tomato | Worldwide | Glycoalkaloids |

Adapted from Kingsbury (1964), Burrows and Tyrl (2001), and Knight and Walter (2001).

overview of selected species, their distribution, and toxin (Burrows and Tyrl, 2001; Knight and Walter, 2001).

## Clinical signs

The toxins in the Solanaceae family contribute to various clinical effects depending on the amount of plant/toxin ingested, the plant species eaten and the animal species consuming the plant. Signs range from mild digestive upset to severe colic in horses when contaminated hay is fed, to neurological dysfunction, seizures and death in sheep and cattle, to "big head" and calcification of the blood vessels to teratogenesis.

## Treatment

Animals showing severe neurological signs such as tremors, ataxia, dilated pupils, etc. may be treated with physostigmine. Oral activated charcoal as an adsorbent may be effective if administered in a timely fashion. However, most animals will recover if treated symptomatically and if the animals are not overly stressed.

# KNAPWEEDS: *CENTAUREA* SPP.

The knapweeds are a large group with primarily noxious, invasive characteristics. While this genus is not a great risk for livestock producers, a serious disease of horses called nigropallidal encephalomalacia warrants its inclusion in this chapter. There are 450–500 species of *Centaurea* and 29 have been described in North America (Burrows and Tyrl, 2001). Most of these have been introduced and have made a huge negative impact on rangelands in the western United States. While most species are opportunists and will aggressively invade rangelands, especially those that have been overgrazed, burned or disturbed, only two are of any toxicological significance, *Centaurea repens* (Russian knapweed) and *C. solstitialis* (yellow star thistle).

## Habitat and distribution

Yellow star thistle is most abundant in the western United States from central California north through Oregon, Washington, and Idaho. Smaller invasions are reported in many states east of the Intermountain Region and while these have not received the attention of the larger invasions in the west, they have the potential to rapidly spread under the right environmental conditions (Panter, 1991; Burrows and Tyrl, 2001; Knight and Walter, 2001). Russian knapweed has invaded very large areas of the Intermountain Region of the western United States and the Great Plains. Again, smaller populations have invaded areas of the Midwest and northeastern United States and have the potential to expand rapidly. While the aggressive nature of these species threatens rangelands and prohibits optimum utilization, a greater threat is the risk to sensitive or threatened native plant species and the balance of plant biodiversity.

## Toxicology

The compounds isolated from knapweeds include a large class called sesquiterpene lactones. While the putative toxin causing the neurological disease in horses has not been specifically identified, six of these compounds have been screened for cytotoxicity in an *in vitro* neuronal cell bioassay (Riopelle *et al*., 1992). The rank order of activity is repen > subluteolidejanerin > cynaropicrin > acroptilin > solstitial in (Riopelle and Stevens, 1993). Toxicity of solstitialin A 13-acetate and cynaropicrin has been demonstrated to primary cultures of fetal rat substantia nigra cells (Cheng *et al*., 1992). These sesquiterpene lactones are quite unstable and it has been hypothesized that they are precursors to the ultimate neurotoxin. Also, there are aspartic and glutamic acids present in these plants and they possess neuroexcitatory properties (Roy *et al*., 1995).

## Clinical signs

Thus far, only yellow star thistle and Russian knapweed have been implicated in toxicoses in the United States and only in horses. Ruminants are apparently not affected and the *Centaurea* spp. may be useful forage for sheep and goats. However, in other countries toxicoses in ruminants have been reported. For example, in South Africa, *C. repens* fed to sheep at 600 g dosages for 2 days caused an acute digestive upset and pulmonary edema and acsites (Steyn, 1933). In Azerbaijan, *C. repens* is reported to cause a neurological disease in buffalo similar to what has been described in horses (Dil'bazi, 1974). However, no neuropathology similar to what was seen in horses was observed in the buffalo.

Toxicity generally occurs in summer and fall when forage is depleted and horses are forced to graze less palatable species. Ingestion often occurs for several months or more before an abrupt onset of neurological dysfunction is observed. Impaired eating and drinking are often the first observable signs. Depression and hypertonicity of the lips and tongue follow and a constant chewing may be observed hence the name "chewing disease." Abnormal tongue and lip postures may be observed and other neurological signs including locomotor difficulties such as aimless walking, drowsy appearance, and inactivity with the head held low. The neurological disease is considered permanent and while some improvement may be seen, difficulty eating and drinking may preclude long-term recovery. Often the disease progresses to dehydration, starvation, bizarre behavior including submergence of the head in water to allow water to flow into the esophagus or lapping water like a dog. *C. repens* appears to be more toxic than *C. solstitialis* but prolonged ingestion is required by both before disease appears. Amounts of plant ingested to induce the clinical effects is reported to be 60% or more of body weight for *C. repens* and 100% or more of body weight of *C. solstitialis*. Intermittent grazing can prevent disease suggesting there is not a cumulative effect but rather a threshold must be exceeded before neurological signs are observed (Cordy, 1954, 1978). Once neurological signs are observed in horses, prognosis for recovery is poor and euthansia should be considered.

## Pathology

The lesions are very specific and limited to the globus pallidus and the substantia nigra (nigropallidal encephalomalacia), where distinct pale yellowish to buff-colored foci or softening and cavitation are seen (Cordy, 1954, 1978). The lesions are typically bilateral and symmetrical. This specificity of the lesions for the basal ganglia has prompted more investigations into unraveling the mysteries of human diseases associated with dopaminergic pathways, such as Parkinson's or Huntington's disease, and tardive dyskinesia. This disease in horses is often called equine Parkinsonism. This unusual disease is manifest by an almost immediate onset after prolonged ingestion suggesting an all or none type of acute neurological crisis. The lesions develop quickly and completely and progressive stages of degeneration rarely occur except for some changes in the adjoining neurons adjacent to the necrotic foci in the globus pallidus and the pars reticularis of the substantia nigra (Cordy, 1954). Microscopically there is extensive necrosis of neurons, glia and capillaries within sharply defined margins of the involved brain centers. Occasionally, lesions may be observed in the gray and white matter of the brain.

## Prevention and treatment

Good veterinary care and supportive therapy including good feed, easy access to water, supplemental vitamins, and good nursing care is essential for survival. Treatment of the disease once it is manifest is not generally successful. However, in Argentina affected horses have been treated with glutamine synthetase and a bovine brain ganglioside extract given daily i.m. for a month with some success (Selfero *et al*., 1989). When animals are first observed grazing *Centaurea* spp. they should be immediately removed to better pastures. Prevention of the disease is easily accomplished by knowing the plants that exist in your pastures, by providing good quality and adequate amounts of forages and feed and by frequent observation of your animal's grazing patterns and behavior.

Control of plant invasion by good range/pasture management to prevent overgrazing and loss of other competitive grasses and forbs is important. Herbicide control is quite easily accomplished with broad-leaf products including 2,4-D, dicamba, picloram, etc. applied according to

label. These plants are prolific seed producers and follow-up treatment will be required to eliminate the populations. Seeds are often distributed through contaminated hay or other feed sources and initial populations often start near feed bunks and spread from there. Because of their morphology, size and parachute-like structures, seeds are easily spread by wind and water. Understanding your weeds and close monitoring of populations will help in control of these highly invasive species.

# CONCLUSION

Even with our ever increasing knowledge about poisonous plants and their toxins, poisonings continue to occur, some catastrophic, on livestock operations. Poisoning in humans and companion animals from toxic plants also continues to be a significant risk, especially to pets and children. As the influx of small acreage farmers onto native rangelands increases and ranchettes become more common, the potential risk for poisonous plant problems increases. Lack of understanding and increased grazing pressure on these small acreages often contribute to the consumption of toxic plants by animals. In some cases, novel or unusual animal species are exposed to unfamiliar forages further contributing to potential poisonings. Plant poisonings will undoubtedly increase in wildlife populations also, as humans continue to encroach on their native ranges and interrupt their migratory pathways.

There are a few basic concepts that can help reduce risk of poisoning:

1. Understand and recognize the plants on your range or pastures and know the potential hazards of grazing where poisonous plants grow. Know the conditions under which poisoning may occur.
2. Do not introduce unfamiliar animals onto ranges where poisonous plants may present a hazard.
3. Avoid introducing animals to poisonous plant-infested ranges when adequate, good quality forage is not available.
4. Do not throw grass, shrub, or tree clippings into paddocks where animals reside (yew clippings are a common cause of poisoning in many animals).
5. Provide free access to fresh water and minerals/salt.
6. Do not overstock the range or pastures.
7. Avoid bedding, lambing/calving, watering, salting, or unloading hungry animals near poisonous plant populations.
8. Avoid excess stress to those animals showing clinical signs of poisoning and contact your veterinarian.
9. Control poisonous plants if economically feasible, either through hand grubbing, mechanical clipping or herbicide treatment.

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# REFERENCES

Acamovic T, Stewart CS, Pennycott TW (eds) (2004) *Poisonous Plants and Related Toxins (ISOPP6)*. CAB International, New York.

Aiyar VN, Benn MH, Hanna T, Jacyno J, Roth SH, Wilkens J (1979) The principal toxin of *Delphinium brownii* Rydb., and its mode of action. *Experientia* **35**: 1367–8.

Alkondon M, Pereira EFR, Wonnacott S, Albuquerque EX (1992) Blockade of nicotinic currents in hippocampal neurons defines methyllycaconitine as a potent and specific receptor antagonist. *Mol Pharmacol* **41**: 802–8.

Allen JG, Cowling WA (1992) A preliminary report on the use of new phomopsis-resistant cultivars of *Lupinus angustifolius* to prevent lupinosis. In *Poisonous Plants*, James LF, Keeler RF, Bailey Jr EM, Cheeke PR, Hegarty MP (eds). Iowa State University Press, Ames, IA, pp. 539–41.

Arnold JF, Jameson DA, Reid EH (1964) The pinyon-juniper type of Arizona: effects of grazing fire and tree control. USDA Forest Service Production Research Report 84.

Bai Y, Sun F, Benn M, Majak W (1994) Diterpenoid and norditerpenoid alkaloids from *Delphinium nuttallianum. Phytochemistry* **37**: pp. 1717–24.

Barneby RC (1952) A Revision of the North America species of Oxytropis DC. *Proceedings of the California Academy of Sciences,* vol. 27(7), 177–312.

Barneby RC (1964) *Atlas of North American Astragalus*, Parts I and II, vol. 13. Memoirs New York Botanical Garden, New York..

Beasley VR, Dorman DC, Fikes JD, Diana SG, Woshner V (1997) *A Systems Affected Approach to Veterinary Toxicology*. University of Illinois, Urbana, Illinois.

Beier RC, Norman JO (1990) The toxic factor in white snakeroot: identity, analysis and prevention. *Vet Hum Toxicol* **32**(Suppl.): 81–8.

Beier RC, Norman JO, Reagor JC, Rees MS, Mundy BP (1993) Isolation of the major component in white snakeroot that is toxic after microsomal activation: possible explanation of sporadic toxicity of white snakeroot plants and extracts. *Nat Toxin* **1**: 1–8.

Benn MH, Jacyno JM (1983) The toxicology and pharmacology of the diterpenoid alkaloids In *Alkaloids: Chemical and Biological Perspectives*, vol. 1, Pelletier SW (ed.). John Wiley, New York, pp. 153–210.

Binns W, Shupe JL, Keeler RF, James LF (1965) Chronologic evaluation of teratogenicity in sheep fed *Veratrum californicum. J Am Vet Med Assoc* **147**: 839–42.

Blagbrough IS, Coates PA, Hardick DJ, Lewis T, Rowan MG, Wonnacott S, Potter BVL (1994) Acylation of lycoctonine: semi-synthesis of inuline, delsemine analogues and methyllycaconitine. *Tetrahedron Lett* **35**(46): 8705–8.

Bowman WC, Sanghvi IS (1963) Pharmacological actions of hemlock (*Conium maculatum*) alkaloids. *J Pharm Pharmacol* **15**: 1–25.

Burrows GE, Tyrl RJ (2001) *Toxic Plants of North America*. Iowa State University Press, Ames, IA.

Campbell RS, Bamberger EH (1934) The occurrence of *Gutierrezia sarothrae* on *Bouteloua eropoda* ranges in southern New Mexico. *Ecology* **15**: 49–61.

Cheeke PR (1998) *Natural Toxicants in Feeds, Forages, and Poisonous Plants*. Interstate Publishers, Inc., Danville, IL.

Cheeke PR, Shull LR (1985) *Natural Toxicants in Feeds and Poisonous Plants*. AVI Publishing Company, Inc., Westport, CT.

Cheng CH, Costall B, Hamburger M, Hostettmann K, Naylor RJ, Wang Y, Jenner P (1992). Toxic effects of solstitialin A 13-acetate and cynaropicrin from *Centaurea solstitialis* L. (Asteraceae) in cell cultures of foetal rat brain. *Neuropharmacology* **31**: 271–7.

Chesnut VK, Wilcox EV (1901) The stock-poisoning plants of Montana. *Washington Printing Office Bulletin #26*, pp. 151.

Christensen LK, Short RE, Ford SP (1992a) Effects of ingestion of ponderosa pine needles by late-pregnant cows on uterine blood flow and steroid secretion. *J Anim Sci* **70**: 531–7.

Christensen LK, Short RE, Rosazza JP, Ford SP (1992b) Specific effects of blood plasma from beef cows fed pine needles during late pregnancy on increasing tone of caruncular arteries *in vitro. J Anim Sci* **70**: 525–30.

Coates PA, Bragbrough IS, Hardick DJ, Rowan MG, Wonnacott S, Potter BVL (1994) Rapid and efficient isolation of the nicotinic receptor antagonist methyllycaconitine from *Delphinium*: assignment of the methylsuccinimide absolute stereochemistry as S. *Tetrahedron Lett* **35**(46): 8701–4.

Colegate SM, Dorling PR (eds) (1994) *Plant-Associated Toxins, Agricultural, Phytochemical and Ecological Aspects (ISOPP4)*. CAB International, Wallingford, UK.

Colegate SM, Dorling PR, Huxtable CR (1979) A spectroscopic investigation of swainsonine: an α-mannosidase inhibitor isolated from *Swainsona canescens. Aust J Chem* **32**: 2257–64.

Conkle MT, Critchfield WB (1987) Genetic variation and hybridization of ponderosa pine. In *Ponderosa Pine the Species and Its Management*, Baumgartner DM, Lotan JE (eds). Washington State University Office of Conferences and Institutes, Pullman, pp. 27–43.

Cook CW, Stoddart LA (1953) The Halogeton problem in Utah. *Utah Agricultural state Experimental Station Bulletin 364*.

Copithorne B (1937) Suspected poisoning of goats by hemlock (*Conium maculatum*). *Vet Rec* **49**(33): 1018–19.

Cordy DR (1954) Nigropallidal encephalomalacia in horses associated with ingestion of yellow star thistle. *J Neuropathol Exp Neurol* **13**: 330–42.

Cordy DR (1978) Centaurea species and equine nigropallidal encephalomalacia. In *Effects of Poisonous Plants on Livestock*, Keeler RF, Van Kampen KR, James LF (eds). Academic Press, New York, pp. 327–36.

Craig AM, Pearson EG, Meyer C, Schmitz JA (1991) Serum liver enzyme and histopathologic changes in calves with chronic and chronic-delayed *Senecio jacobaea* toxicosis. *Am J Vet Res* **52**: 1969–78.

Cronin EH (1965) Ecological and physiological factors influencing chemical control of *Halogeton glomeratus. USDA Technical Bulletin 1325*.

Cronin EH, Williams MC (1966) Principles for managing ranges infested with Halogeton. *J Range Manage* **19**: 226–7.

Cronquist A, Holmgren AH, Holmgren NH, Reveal JL, Holmgren PK (1989) *Intermountain Flora; Vascular Plants of the Intermountain West, USA*, vol. 3(B). The New York Botanical Garden, Bronx, NY, pp. 236–68.

Daugherty CG (1995) The death of Socrates and the toxicology of hemlock. *J Med Biograph* **3**: 178–82.

Davis AM, Stout DM (1986) Anagyrine in western American lupines. *J Range Manage* **39**(1): 29–30.

DePuit EJ, Caldwell MM (1975) Gas exchange of three cool semi-desert species in relation to temperature and water stress. *J Ecol* **63**: 835–58.

Dil'bazi GI (1974) Poisoning of buffalos eating hay containing choking mustard. *Veterinariya* (*Moscow*) **2**: 106–7.

Dobelis P, Madl JE, Pfister JA, Manners GD, Walrond JP (1999) Effects of *Delphinium* alkaloids on neuromuscular transmission. *J Pharmacol Exp Ther* **291**: 538–46.

Dollahite JW, Anthony WV (1957) Poisoning of cattle with *Gutierrezia microcephala*, a perennial broomweed. *J Am Vet Med Assoc* **130**: 525–30.

Dollahite JW, Shaver T, Camp BJ (1962) Injected saponins as abortifacients. *J Am Vet Res* **23**: 1261–3.

Dorling PR, Colegate SM, Huxtable CR (1989) Toxic species of the plant genus *Swainsona*. In *Swainsonine and Related Grycosidase Inhibitors*, James LF, Elbein AD, Molyneux RJ, Warren CD (eds). Iowa State University Press, Ames, IA, pp. 14–22.

Duff GC, Ralphs MH, Walker D, Rivera JD, James LF (2001) Influence of beef breeds (Hereford, Charolais, Brangus) on locoweed consumption. *Prof Anim Sci* **18**: 33–7.

Edrington TS, Flores-Rodriguez GI, Smith GS, Hallford DM (1993a) Effect of ingested snakeweed (*Gutierrezia microcephala*) foliage on reproduction, semen quality, and serum clinical profiles of male rats. *J Anim Sci* **71**: 1520–5.

Edrington TS, Smith GS, Ross TT, Hallford DM, Samford MD, Tilsted JP (1993b) Embryonic mortality in Sprangue–Dawley rats induced by snakeweed. *J Anim Sci* **71**: 2193–8.

Eckert Jr RE, Kinsinger FE (1960) Effects of *Halogeton glomeratus* leachate on chemical and physical characteristics of soils. *Ecology* **41**: 785–90.

Flores-Rodriguez GI, Smith GS, McDaniel KC (1989) Effects of ingested snakeweed (*Gutierrezia microcephala*) herbage on reproduction, serum progesterone, and blood constituents of female albino rats. *Proc West Sect Am Soc Anim Sci* **40**: 217–21.

Ford SP, Christenson LK, Rosazza JP, Short RE (1992) Effects of ponderosa pine needle ingestion on uterine vascular function in lategestation beef cows. *J Anim Sci* **70**: 1609–14.

Fox WE, Allred KW, Roalson EH (1998) *A Guide to the Common Locoweeds and Milkvetches of New Mexico*. Circular 557, New Mexico State University, College of Agriculture and Home Economics, Las Cruses.

Frank AA, Reed WM (1987) *Conium maculatum* (poison hemlock) toxicosis in a flock of range turkeys. *Avian Dis* **31**: 386–8.

Frank AA, Reed WM (1990) Comparative toxicity of coniine, an alkaloid of *Conium maculatum* (poison hemlock), in chickens, quails, and turkeys. *Avian Dis* **34**: 433–7.

Frank BS, Michelson WB, Panter KE, Gardner DR (1995) Ingestion of poison-hemlock (*Conium maculatum*). *West J Med* **163**: 573–4.

Gaffield W, Keeler RF (1994) Structure–activity relations of teratogenic natural products. *Pure Appl Chem* **66**: 2407–10.

Gaffield W, Keeler RF (1996) Steroidal alkaloid teratogens: molecular probes for investigation of craniofacial malformations. *J Toxicol Toxin Rev* **15**: 303–26.

Galey FD, Hoffman, R, Maas J, Barr B, Holstege D, Giacomazzi R (1991) Suspected *Haplopappus acradenius* toxicosis in beef heifers. *American Association of Veterinary Laboratory Diagnosticians, 34th Annual Meeting*, October, San Diego, CA.

Galey FD, Holstege DM, Fisher EG (1992) Toxicosis in dairy cattle exposed to poison hemlock (*Conium maculatum*) in hay: isolation of *Conium* alkaloids in plants, hay and urine. *J Vet Diagn Invest* **4**: 60–4.

Gardner DR, James LF (1999) Pine needle abortion in cattle: analysis of isocupressic acid in North American gymnosperms. *Phytochem Anal* **10**: 1–5.

Gardner DR, Panter KE (1993) Comparison of blood plasma alkaloid levels in cattle, sheep and goats fed *Lupinus caudatus. J Nat Toxin* **2**: 1–11.

Gardner DR, Panter KE (1994) Ammodendrine and related piperidine alkaloid levels in the blood plasma of cattle, sheep and goats fed *Lupinus formosus. J Nat Toxin* **3**: 107–16.

Gardner DR, James LF, Molyneux RJ, Panter KE, Stegelmeier BL (1994) Ponderosa pine needle-induced abortion in beef cattle: identification of isocupressic acid as the principal active compound. *J Agric Food Chem* **42**: 756–61.

Gardner DR, Panter KE, Molyneux RJ, James LF, Stegelmeier BL (1996) Abortifacient activity in beef cattle of acetyl- and succinylisocupressic acid from ponderosa pine. *J Agric Food Chem* **44**: 3257–61.

Gardner DR, James LF, Panter KE, Pfister JA, Ralphs MH, Stegelmeier BL (1999) Ponderosa pine and broom snakeweed: poisonous plants that affect livestock. *J Nat Toxin* **8**: 27–34.

Garland T, Barr CA (eds) (1998) *Toxic Plants and Other Natural Toxicants (ISOPP5)*. CAB International, New York.

Hardick DJ, Blagbrough IS, Cooper G, Potter BVL, Critchley T, Wonnacott S (1996) Nudicauline and elatine as potent norditerpenoid ligands at rat neuronal alpha bungarotoxin binding sites: importance of the 2 (methylsuccinimido) benzoyl moiety for neuronal nicotinic acetylcholine receptor binding. *J Med Chem* **39**: 4860–6.

Hirono I, Shiguya C, Shizmu M, Fushmi K (1972) Carcinogenic activity of processed bracken as human food. *J Natl Cancer Inst* **48**: 1245.

Hirono I, Kono Y, Takahashi K, Yamada K, Niwa H, Ojika M, Kigoshi K, Niiyama H, Uosaki Y (1984) Reproduction of acute bracken poisoning in a calf with ptaquiloside, a bracken constituent. *Vet Rec* **115**: 375–8.

Hooper PT (1978) Pyrrolizidine alkaloid poisoning – pathology with particular reference to differences in animal and plant species. In *Effects of Poisonous Plants on Livestock (ISOPP1)*, Keeler RF, Van Kampen KR, James LF (eds). Academic Press, New York, pp. 161–76.

James LF (1968) Serum electrolyte, acid–base balance and enzyme changes in acute *Halogeton glomeratus* poisoning in sheep. *Can J Comp Med* **32**: 539–43.

James LF (1972) Oxalate toxicosis. *Clin Toxicol* **5**: 231–43.

James LF (1999a) Halogeton poisoning in livestock. *J Nat Toxin* **8**: 395–403.

James LF (1999b) Teratological research at the USDA-ARS Poisonous Plant Research Laboratory. *J Nat Toxin* **8**: 63–80.

James LF, Cronin EH (1974) Management practices to minimize death losses of sheep grazing Halogeton-infested range. *J Range Manage* **27**: 424–6.

James LF, Johnson AE (1970) Prevention of fatal *Halogeton glomeratus* poisoning in sheep. *J Am Vet Med Assoc* **157**: 437–42.

James LF, Nielsen DB (1988) Locoweeds: assessment of the problem on Western US rangelands. In *The Ecology and Economic Impact of Poisonous Plants on Livestock Production*, James LF, Ralphs MH, Nielsen DB (eds). Westview Press, Boulder, CO, pp. 119–29.

James LF, Street JC, Butcher JE (1967) *In vitro* degradation of oxalate and of cellulose by rumen ingesta from sheep fed *Halogeton glomeratus. J Anim Sci* **26**: 1438.

James LF, Bennett KL, Parker KG, Keeler RF, Binns W, Lindsay B (1968) Loco plant poisoning in sheep. *J Range Manage* **21**: 360–5.

James LF, Van Kampen KR, Stacker GR (1969) Locoweed (*Astragalus lentiginosus*) poisoning in cattle and horses. *J Am Vet Med Assoc* **155**: 525–30.

James LF, Hartley WJ, Van Kampen KR (1981) Syndromes of *Astragalus* poisoning in livestock. *J Am Vet Med Assoc* **178**: 146–50.

James LF, Panter KE, Mayland HF, Miller MR, Baker DC (1989a) *Selenium Poisoning in Livestock: A Review and Progress*. Soil Science Society of America, Madison, WI, pp. 123–31.

James LF, Short RE, Panter KE, Molyneux RJ, Stuart LD, Bellows RA (1989b) A review and report of 1973–1984 research. *Cornell Vet* **79**: 39–52.

James LF, Keeler RF, Bailey Jr EM, Cheeke PR, Hegarty MP (1992) *Poisonous Plants, Proceedings of the Third International Symposium (ISOPP3)*. Iowa State University Press, Ames, IA.

James LF, Panter KE, Gaffield W, Molyneux RJ (2004) Biomedical applications of poisonous plant research. *J Agric Food Chem* **52**: 3211–30.

Jessup DA, Boermans HJ, Kock ND (1986) Toxicosis in tule elk caused by ingestion of poison hemlock. *J Am Vet Med Assoc* **189**: 1173–5.

Johnson AE (1974) Experimental photosensitization and toxicity in sheep produced by *Tetradymia glabrata. Can J Comp Med* **38**: 405–10.

Johnson AE (1982) Toxicologic aspects of photosensitization in livestock. *J Natl Cancer Inst* **69**: 253–8.

Joubert JPJ (1989) Cardiac glycosides. In *Toxicants of Plant Origin, Vol. 2. Glycosides*, Cheeke PR (ed.). CRC Press, Boca Raton, FL, pp. 61–96.

Keeler RF (1973) Lupin alkaloids from teratogenic and nonteratogenic lupines. 2. Identification of the major alkaloids by tandem gas chromatography-mass spectrometry in plants producing crooked calf disease. *Teratology* **7**: 31–6.

Keeler RF (1976) Lupin alkaloids from teratogenic and nonteratogenic lupines. 3. Identification of anagyrine as the probable teratogen by feeding trials. *J Toxicol Environ Health* **1**: 887–98.

Keeler RF (1978) Alkaloid teratogens from *Lupinus, Conium, Veratrum* and related genera. In *Effects of Poisonous Plants on Livestock (ISOPP1)*, Keeler RF, Van Kampen KR, James LF (eds). Academic Press, New York, pp. 397–408.

Keeler RF (1990) Early embryonic death in lambs induced by *Veratrum californicum. Cornell Vet* **80**: 203–7.

Keeler RF, Balls LD (1978) Teratogenic effects in cattle of *Conium maculatum* and *Conium* alkaloids and analogs. *Clin Toxicol* **12**: 49–64.

Keeler RF, Panter KE (1989) Piperidine alkaloid composition and relation to crooked calf disease-inducing potential of *Lupinus formosus. Teratology* **40**: 423–32.

Keeler RF, Stuart LD (1987) The nature of congenital limb defects induced in lambs by maternal ingestion of *Veratrum californicum. Clin Toxicol* **25**: 273–86.

Keeler RF, Cronin EH, Shupe JL (1976) Lupin alkaloids from teratogenic and nonteratogenic lupins. 4. Concentration of total alkaloids, individual major alkaloids, and the teratogen anagyrine as a function of plant part and stage of growth and their relationship to crooked calf disease. *J Toxicol Environ Health* **1**: 899–908.

Keeler RF, James LF, Shupe JL, Van Kampen KR (1977) Lupine-induced crooked calf disease and a management method to reduce incidence. *J Range Manage* **30**: 97–102.

Keeler RF, Van Kampen KR, James LF (eds) (1978). *Effects of Poisonous Plants on Livestock (ISOPP1)*. Academic Press, New York.

Keeler RF, Young S, Smart R (1985) Congenital tracheal stenosis in lambs induced by maternal ingestion of *Veratrum californicum. Teratology* **31**: 83–8.

Kelleway RA, Geovjian L (1978) Acute bracken fern poisoning in a 14-month-old horse. *Vet Med Small Anim Clin* **73**: 295–6.

Kim H-Y, Stermitz FR, Molyneux RJ, Wilson CW, Taylor D, Coulombe Jr RA (1993). Structural influences on pyrrolizidine alkaloid-induced cytopathology. *Toxicol Appl Pharm* **122**: 61–9.

Kim I-H, Choi K-C, An B-S, Choi I-G, Kim B-K, Oh Y-K, Jeung E-B (2003) Effect on abortion of feeding Korean pine needles to pregnant Korean native cows. *Can J Vet Res* **67**: 194–7.

Kingsbury JM (1964) *Poisonous Plants of the United States and Canada*. Prentice-Hall, Inc., Englewood Cliffs, NJ.

Kinsinger FE, Eckert Jr RE (1961) Emergence and growth of annual and perennial grasses and forbs in soils altered by Halogeton leachate. *J Range Manage* **14**: 1194–7.

Knight AP, Walter RG (2001) *A Guide to Plant Poisoning of Animals in North America*. Teton New Media, Jackson, WY.

Knight AP, Kimberling CV, Stermitz FR, Roby MR (1984) *Cynoglossum officinale* (hounds' tongue) – a cause of pyrrolizidine alkaloid poisoning in horses. *J Am Vet Med Assoc* **185**: 647–50.

Kukel CF, Jennings KR (1994) Delphinium alkaloids as inhibitors of $\alpha$-bungarotoxin binding to rat and insect neural membranes. *Can J Physiol Pharmacol* **72**: 104–7.

Lame MW, Jones AD, Wilson DW, Dunston SK, Segall HJ (2004) Monocrotaline pyrrole protein targets in pulmonary artery endothelial cells. In *Poisonous Plants and Related Toxins (ISOPP6)*, Acamovic T, Stewart CS, Pennycott TW (eds). CAB International, New York, pp. 394–401.

Lane M (1985) Taxonomy of *Gutierrezia* Lag (Compositeae: Astereae) in North America. *Syst Bot* **10**: 7–28.

Lee ST, Ralphs MH, Panter KE, Cook DC, Gardner DR (2006) Alkaloid profiles, concentration and pools in velvet lupine (*Lupinus leucophyllus*) over the growing season. *J Chem Ecol* (accepted Sept. 2001, 2006).

Lopez-Ortiz SL, Panter KE, Pfister JA, Launchbaugh KL (2004) The effect of body condition on disposition of alkaloids from silvery lupine (*Lupinus argenteus* Pursh) in sheep. *J Anim Sci* **82**: 2798–895.

Lopez-Ortiz SL, Pfister JA, Launchbaugh KL, Gay CC (2007) Forage variability and body condition affect intake of lupine (*Lupinus leucophyllus*) by grazing cattle. *The Professional Animal Scientist* (in review).

MacDonald H (1937) Hemlock poisoning in horses. *Vet Rec* **49**: 1211–12.

Majak W (1993) Alkaloid levels in a species of low larkspur and their stability in rumen fluid. *J Range Manage* **46**: 100–3.

Majak W, Engelsjord M (1988) Levels of a neurotoxic alkaloid in a species of low larkspur. *J Range Manage* **41**: 224–6.

Majak W, McDiarmid RE (2000) Alkaloid levels of a tall larkspur species in southwestern Alberta. *J Range Manage* **53**: 207–10.

Majak W, Cheng KJ, Muir AD (1985) Analysis and metabolism of nitrotoxins in cattle and sheep. In *Plant Toxicology*, Seawright AA, Hegarty MP, James LF, Keeler RF (eds). Dominion Press-Hedges and Bell, Melbourne, pp. 446–52.

Malcolm SB, Cockrell BJ, Brower LP (1989) Cardenolide fingerprint of monarch butterflies reared on common milkweed, *Asclepias syriaca* L. *J Chem Ecol* **15**: 819–53.

Manners GD, Panter KE, Ralphs MH, Pfister JA, Olsen JD (1993) The occurrence and toxic evaluation of norditerpenoid alkaloids in the tall larkspurs (*Delphinium* spp.). *J Agric Food Chem* **41**: 96–100.

Manners GD, Panter KE, Pelletier SW (1995) Structure – activity relationships of norditerpenoid alkaloids occurring in toxic larkspur (*Delphinium*) species. *J Nat Prod* **58**: 863–9.

Marsh CD (1909) The locoweed disease of the plains. *USDA Bureau Animal Industry, Bulletin #112*, p. 130.

Marsh CD (1924) Stock-poisoning plants of the range. *USDA Bulletin #1245*, pp. 24–44.

Marsh CD, Clawson AB, Marsh H (1916) Larkspur poisoning of live stock. *USDA Bureau of Animal Industry Bulletin 365*.

Martin CJ (1897) Report of an investigation into the effects of darling pea (*Swainsona galegifolia*) upon sheep. *Agric Gazet NSW* **8**(Part 6): 363–9.

Martinez JH, Ross TT, Becker DA, Smith GS (1993) Ingested dry snakeweed foliage did not impair reproduction in ewes and heifers during late gestation. *West Sect Am Soc Anim Sci* **44**: 32–5.

Mathews FP (1936) The toxicity of broomweed (*Gutierrezia microcephala*) for sheep, cattle and goats. *J Am Vet Med Assoc* **88**: 55–61.

Mattocks AR (1986) *Chemistry and Toxicology of Pyrrolizidine Alkaloids*. Academic Press, San Diego, CA.

McDaniel KC (1999) Controlling locoweed with herbicides. In *Locoweed Research Updates and Highlights*, Sterling TM, Thompson DC (eds). New Mexico Agricultural Experimental Station Research Report 730, pp. 52–3.

McDaniel KC, Duncan KW (1987) Broom snakeweed (*Gutierrezia sarothrae*) control with picloram and metsulfuron. *Weed Sci* **35**: 837–41.

McDaniel KC, Loomis LE (1985) Livestock poisoning by perennial snakeweeds. *Weed Today* **16**: 9–11.

McDaniel KC, Ross TT (2002) Snakeweed: poisonous properties, livestock loss and management considerations. *J Range Manage* **55**: 277–84.

McDaniel KC, Sosebee RE (1987) Ecology, management and poisonous properties associated with perennial snakeweeds. In *The Ecology and Economic Impact of Poisonous Plants on Livestock Production*, James LF, Ralphs MH, Nielsen DB (eds). Westview Press, Boulder, CO, pp. 43–56.

McDaniel KC, Torell LA (1987) Ecology and management of broom snakeweed. In *Integrated Pest Management on Rangeland, A Shortgrass Prairie Perspective*, Capinera JL (ed.). Westview Press, Boulder, CO, pp. 101–15.

McDaniel KC, Pieper RD, Donart GB (1982) Grass response following thinning of broom snakeweed. *J Range Manage* **35**: 219–22.

McGinty A (1985) Survey suggests broomweed costs far more than previously thought. *Livestock Weekly*, September 5–6.

Mickelsen LV, Ralphs MH, Turner DL, Evans JO, Dewey SA (1990) Herbicidal control of duncecap larkspur (*Delphinium occidentale*). *Weed Sci* **38**: 153–7.

Molyneux RJ, James LF (1982) Loco intoxication: indolizidine alkaloids of spotted locoweed (*Astragalus lentiginosus*). *Science* **216**: 190–1.

Molyneux RJ, James LF (1991) Swainsonine, the locoweed toxin: analysis and distribution. In *Toxicology of Plant and Fungal Compounds; Handbook of Natural Toxins*, vol. 6, Keeler RF, Tu AT (eds). Marcel Dekker, New York, pp. 41–60.

Molyneux RJ, Stevens KL, James LF (1980) Chemistry of toxic range plants: volatile constituents of broomweed (*Gutierrezia sarothrae*). *J Agric Food Chem* **28**: 1332–3.

Molyneux RJ, James LF, Panter KE, Ralphs MH (1991) Analysis and distribution of swainsonine and related polyhydroxyindolizidine alkaloids by thin-layer chromatography. *Phytochem Anal* **2**: 125–9.

Muenscher WC (1975) *Poisonous Plants of the United States*. Macmillan Publishing Co. Inc., New York.

Nastis AS, Malechek JC (1981) Digestion and utilization of nutrients in oak browse by goats. *J Anim Sci* **53**: 283–90.

Nation PN, Benn MH, Roth SH, Wilkens JL (1982) Clinical signs and studies of the site of action of purified larkspur alkaloid, methyllycaconitine, administered parenterally to calves. *Can Vet J* **23**: 264–6.

Nawrot PS, Howell WE, Leipold HW (1980) Arthrogryposis: an inherited defect in newborn calves. *Aust Vet J* **56**: 359–64.

Nielsen DB, James LF (1992) The economic impacts of livestock poisonings by plants. In *The Ecology and Economic Impact of Poisonous Plants on Livestock Production*, James LF, Keeler RF, Bailey Jr EM, Cheeke PR, Hegarty MP (eds). Iowa State University Press, Ames, IA, pp. 3–10.

Nielsen DB, Ralphs MH (1988) Larkspur, economic considerations. In *The Ecology and Economic Impact of Poisonous Plants on Livestock Production*, James LF, Keeler RF, Bailey Jr EM, Cheeke PR, Hegarty MP (eds). Westview Press, Boulder, CO, pp. 119–29.

Nielsen DB, Rimbey NR, James LF (1988) Economic considerations of poisonous plants on livestock. In *The Ecology and Economic Impact of Poisonous Plants on Livestock Production*, James LF, Keeler RF, Bailey Jr EM, Cheeke PR, Hegarty MP (eds). Westview Press, Boulder, CO, pp. 5–15.

Niwa H, Ojika M, Wakamatsu K, Yamada K, Ohba S, Saito Y, Hirono I, Matsushita K (1983) Stereochemistry of ptaquiloside, a novel norsequiterpene glucoside from bracken. *Pteridium aquilinum* var. *latiusculum. Tetrahedron Lett* **24**: 5371–2.

Oetting BC, Ross TT Walraven K, Kloppenburg P, Smith GS Hallford DM (1990) Effects of ingested snakeweed herbage on estrous activity, blood progesterone and serum clinical profiles of fine-wool ewes. *West Sect Am Soc Anim Sci* **41**: 23–6.

Ogden L, Burrows GE, Tyrl RJ, Ely RW (1992) Experimental intoxication in sheep by *Asclepias*. In *Poisonous Plants: Proceedings of the Third International Symposium on Poisonous Plants (ISOPP3)*, James LF, Keeler RF, Bailey Jr EM, Cheeke PR, Hegarty MP (eds). Iowa State University Press, Ames, IA, pp. 495–9.

Olsen JD (1978) Tall larkspur poisoning in cattle and sheep. *J Am Vet Med Assoc* **173**: 762–5.

Olson OE (1978) Selenium in plants as a cause of livestock poisoning. In *Effects of Poisonous Plants on Livestock*, Keeler RF, Van Kampen KR, James LF (eds). Academic Press, New York, pp. 121–33.

Palotay JL (1959) Crooked calves. *Western Vet* **6**: 16–20.

Panter KE (1983) *Toxicity and Teratogenicity of Conium maculatum in Swine and Hamsters*. PhD Dissertation, University of Illinois, Urbana, IL.

Panter KE (1991) Neurotoxicity of the knapweeds (*Centaurea* spp.) in horses. In *Noxious Range Weeds*, James LF, Evans JO, Ralphs MH, Child RD (eds). Westview Press, Boulder, CO, pp. 316–24.

Panter KE, Gardner DR (1994) Comparison of toxic and teratogenic effects of *Lupinus formosus*, *L. arbustus* and *L. caudatus* in goats. *J Nat Toxin* **3**: 83–93.

Panter KE, Keeler RF (1989) Piperidine alkaloids of poison hemlock (*Conium maculatum*). In *Toxicants of Plant Origin, Vol. I. Alkaloids*, Cheeke PR (ed.). CRC Press, Boca Raton, FL, pp. 109–32.

Panter KE, Keeler RF (1992) Induction of cleft palate in goats by *Nicotiana glauca* during a narrow gestational period and the relation to reduction in fetal movement. *J Nat Toxin* **1**: 25–32.

Panter KE, Ralphs MH, Smart RA, Duelke B (1987) Death camas poisoning in sheep: a case report. *Vet Hum Toxicol* **29**: 45–8.

Panter KE, Bunch TD, Keeler RF, Sisson DV (1988a) Maternal and fetal toxicity of poison hemlock (*Conium maculatum*) in sheep. *Am J Vet Res* **49**: 281–3.

Panter KE, Keeler RF, Baker DC (1988b). Toxicoses in livestock from the hemlocks (*Conium* and *Cicuta* spp.). *J Anim Sci* **66**: 2407–13.

Panter KE, Bunch TD, Keeler RF, Sisson DV, Callan RJ (1990a) Multiple congenital contractures (MCC) and cleft palate induced in goats by ingestion of piperidine alkaloid-containing plants: reduction in fetal movement as the probably cause. *Clin Toxicol* **28**: 69–83.

Panter KE, James LF, Short RE, Molyneux RJ, Sisson DV (1990b) Premature bovine parturition induced by ponderosa pine: effects of pine needles, bark, and branch tips. *Cornell Vet* **80**: 329–33.

Panter KE, Gardner DR, James LF (1992a) Rayless goldenrod toxicity in livestock. *Rangelands* **14**: 284–5.

Panter KE, James LF, Molyneux RJ (1992b) Ponderosa pine needle-induced parturition in cattle. *J Anim Sci* **70**: 1604–8.

Panter KE, Baker DC, Kechele PO (1996a) Water hemlock (*Cicuta douglasii*) toxicoses in sheep: pathologic description and prevention of lesions and death. *J Vet Diagn Invest* **8**: 474–80.

Panter KE, Hartley WJ, James LF, Mayland HF, Stegelmeier BL, Kechele PO (1996b) Comparative toxicity of selenium from seleno-DL-methionine, sodium selenate, and *Astragalus bisulcatus* in pigs. *Fundam Appl Toxicol* **32**: 217–23.

Panter KE, Gardner DR, Gay CC, James LF, Mills R, Gay JM, Baldwin TJ (1997) Observations of *Lupinus sulphureus*-induced crooked calf disease. *J Range Manage* **50**: 587–92.

Panter KE, Gardner DR, Molyneux RJ (1998) Teratogenic and fetotoxic effects of two piperidine alkaloid-containing lupines (*L. formosus* and *L. arbustus*) in cows. *J Nat Toxins* **7**: 131–40.

Panter KE, James LF, Gardner DR (1999) Lupines, poison-hemlock and *Nicotiana* spp.: toxicity and teratogenicity in livestock. *J Nat Toxins* **8**(1): 117–34.

Panter KE, Manners GD, Stegelmeier BL, Lee ST, Gardner DR, Ralphs MH, Pfister JA, James LF (2002) Larkspur poisoning: toxicology and alkaloid structure–activity relationships. *Biochem Syst Ecol* **30**: 113–28.

Panter KE, Wierenga TL, Pfister JA (eds) (2007a). *Poisonous Plants: Global Research and Solutions*. CAB International, Wallingsford, UK (in press).

Panter, KE, Gardner DR, Holstege D, James LF, Stegelmeier BL (2007b) A case of acute water hemlock (*Cicuta maculata*) poisoning and death in cattle after ingestion of green seed heads. In *Poisonous Plants: Global Research and Solutions*, Panter KE, Wierenga TL, Pfister JA (eds.) CAB International, Wallingsford, UK (in press).

Parker KE (1939) The control of snakeweed in the southwest. Southwest Forages Range Experiment Station Research Note 76.

Parker MA (1982) Association with mature plants protects seedlings from predation in an arid grassland shrub (*Gutierrezia microcephala*). *Oecologia* **53**: 276–80.

Parton K, Gardner D, William NB (1996) Isocupressic acid, an abortifacient component of *Cupressus macrocarpa. New Zeal Vet J* **44**: 109–11.

Pemberton RW (1986) The distribution of Halogeton in North America. *J Range Manage* **39**: 281–3.

Pfister JA, Adams DC (1993) Factors influencing pine needle consumption by grazing cattle during winter. *J Range Manage* **46**: 394–8.

Pfister JA, Gardner DR (1999) Consumption of low larkspur (*Delphinium nuttallianum*) by cattle. *J Range Manage* **52**: 378–83.

Pfister JA, Manners GD, Ralphs MH, Hong ZX, Lane MA (1988) Effects of phenology, site, and rumen fill on tall larkspur consumption by cattle. *J Range Manage* **41**: 509–14.

Pfister JA, Adams DC, Wiedmeier RD, Cates RG (1992) Adverse effects of pine needles on aspects of digestive performance in cattle. *J Range Manage* **45**: 528–33.

Pfister JA, Panter KE, Manners GD (1994a) Effective dose in cattle of toxic alkaloids from tall larkspur (*Delphinium barbeyi*). *Vet Hum Toxicol* **36**: 10–11.

Pfister JA, Panter KE, Manners GD, Cheney CD (1994b) Reversal of tall larkspur (*Delphinium barbeyi*) toxicity with physostigmine. *Vet Hum Toxicol* **36**: 511–14.

Pfister JA, Ralphs MH, Manners GD, Gardner DR, Price KW, James LF (1997) Early season grazing by cattle of tall larkspur (*Delphinium* spp.)-infested rangeland. *J Range Manage* **50**: 391–8.

Pfister JA, Gardner DR, Panter KE, Ralphs MH, Manners GD, Stegelmeier BL, Schoch TK (1999) Larkspur (*Delphinium* spp.) toxicity to livestock. *J Nat Toxin* **8**: 81–94.

Pfister JA, Gardner DR, Stegelmeier BL, Knight AP, Waggoner Jr JW, Hall JO (2002a) Plains larkspur (*Delphinium geyeri*) grazing by cattle in Wyoming. *J Range Manage* **55**: 350–9.

Pfister JA, Ralphs MH, Gardner DR, Stegelmeier BL, Manners GD, Panter KE, Lee ST (2002b) Management of three toxic *Delphinium* species based on alkaloid concentration. *Biochem Syst Ecol* **30**: 129–38.

Pfister JA, Stegelmeier BL, Gardner DR, James LF (2003) Grazing of spotted locoweed (*Astragalus lentiginosus*) by cattle and horses in Arizona. *J Anim Sci* **81**: 2285–93.

Pieper RD (1989) Broom snakeweed content in herbivore diets. In *Snakeweed: Problems and Perspectives*, Huddleston EW, Pieper RD (eds). *New Mexico Agricultural Experimental Station Bulletin* **751**, pp. 203–10.

Platt KB (1959) Plant control – some possibilities and limitations. II. Vital statistics for range management. *J Range Manage* **12**: 194–200.

Radeleff RD (1970) *Veterinary Toxicology,* 2nd edn. Lea and Febiger, Philadelphia, PA, pp. 118–21.

Ralphs MH (1987) Cattle grazing white locoweed: influence of grazing pressure and palatability associated with phenological growth stage. *J Range Manage* **40**: 330–2.

Ralphs MH, Bagley VL (1988) Population cycles of Wahweap milkvetch on the Henry mountains and seed reserve in the soil. *Great Basin Nat* **48**: 541–7.

Ralphs MH, Cronin EH (1987) Locoweed seed in soil: density, longevity, germination, and viability. *Weed Sci* **35**: 792–5.

Ralphs MH, Olsen JD (1992) Prior grazing by sheep reduces waxy larkspur consumption by cattle. *J Range Manage* **45**: 136–9.

Ralphs MH, Sanders KD (2002) Population cycles of broom snakeweed in the Colorado Plateau and Snake River Plains. *J Range Manage* **55**: 406–11.

Ralphs MH, Ueckert DN (1988) Herbicide control of locoweeds: a review. *Weed Tech* **2**: 460–5.

Ralphs MH, Wiedmeier RD (2004) Conditioning cattle to graze broom snakeweed (*Gutierrezia sarothrae*). *J Anim Sci* **82**: 3100–6.

Ralphs MH, Wiedmeier RD (2007) Increasing grazing pressure can force cattle to graze broom snakeweed as a biological control. *J Rangeland Ecol and Manage* (in review).

Ralphs MH, James LF, Nielsen DB, Panter KE (1984) Management practices reduce cattle loss to locoweed on high mountain range. *Rangelands* **6**: 175–7.

Ralphs MH, Mickelsen LV, Turner DL (1987) Cattle grazing white locoweed: diet selection patterns of native and introduced cattle. *J Range Manage* **40**: 333–5.

Ralphs MH, Bowns JE, Manners GD (1991a) Utilization of larkspur by sheep. *J Range Manage* **44**: 619–22.

Ralphs MH, Panter KE, James LF (1991b) Grazing behavior and forage preference of sheep with chronic locoweed toxicosis suggest no addiction. *J Range Manage* **44**: 208–9.

Ralphs MH, Whitson TD, Ueckert DN (1991c) Herbicide control of poisonous plants. *Rangelands* **13**(2): 73–7.

Ralphs MH, Evans JO, Dewey SA. (1992) Timing of herbicide applications for control of larkspurs. *Weed Sci* **40**: 264–9.

Ralphs MH, Graham D, Molyneux RJ, James LF (1993) Seasonal grazing of locoweeds by cattle in northeastern New Mexico. *J Range Manage* **46**: 416–20.

Ralphs MH, Graham D, James LF (1994a) Cattle grazing white locoweed in New Mexico: influence of grazing pressure and phenological growth stage. *J Range Manage* **47**: 270–4.

Ralphs MH, Graham D, James LF (1994b) Social facilitation influences cattle to graze locoweed. *J Range Manage* **47**: 123–6.

Ralphs MH, Jensen DT, Pfister JA, Nielsen DB, James LF (1994c) Storms influence cattle to graze larkspur: an observation. *J Range Manage* **47**: 275–8.

Ralphs MH, Graham D, Galyean ML, James LF (1997a) Creating aversions to locoweed in naive and familiar cattle. *J Range Manage* **50**: 361–6.

Ralphs MH, Manners GD, Pfister JA, Gardner DR, James LF (1997b) Toxic alkaloid concentration in tall larkspur species in the western United States. *J Range Manage* **50**: 497–502.

Ralphs MH, Graham D, Duff G, Stegelmeier BL, James LF (2000) Impact of locoweed poisoning on grazing steer weight gains. *J Range Manage* **53**: 86–90.

Ralphs MH, Greathouse G, Knight AP, James LF (2001) Cattle preference for Lambert locoweed over white locoweed throughout their phenological stages. *J Range Manage* **54**: 265–8.

Ralphs MH, Gardner DR, Turner DL, Pfister JA, Thacker E (2002) Predicting toxicity of tall larkspur (*Delphinium barbeyi*): measurement of the variation in alkaloid concentration among plants and among years. *J Chem Ecol* **28**: 2327–41.

Ralphs MH, Pfister JA, Welsh SL, Graham D, Purvines J, Jensen DT, James LF (2003) Locoweed population cycles. *Rangelands* **25**: 14–18.

Ralphs MH, Panter KE, Gay CC, Motteram E, Lee ST (2006) Cattle consumption of velvet lupine (*Lupinus leucophyllus*) in the Channel Scablands of eastern Washington. *J Range Ecol Manage* **59**: 204–207.

Reynolds T (2005) Hemlock alkaloids from Socrates to poison aloes. *Phytochemistry* **66**: 1399–406.

Riggins R, Sholars T (1993) *Lupinus* Lupine. In *The Jepson Manual, Higher Plants of California*, Hickman JC (ed.). University of California Press, Berkeley, CA, pp. 622–36.

Riopelle RJ, Stevens KL (1993) *In vitro* neurotoxicity bioassay: neurotoxicity of sesquiterpene lactones. In *Bioactive Natural Products – Detection, Isolation, and Structural Determination*, Colegate SM, Molyneux RJ (eds). CRC Press, Boca Raton, FL, pp. 457–63.

Riopelle RJ, Boegman RJ, Little PB, Stevens KL (1992) Neurotoxicity of sesquiterpene lactones. In *Poisonous Plants (ISOPP3)*, Keeler RF, Bailey Jr EM, Cheeke PR, Hegarty MP (eds). Iowa State University Press, Ames, IA, pp. 298–303.

Roberts MF, Brown RT (1981) A new alkaloid from South African *Conium* species. *Phytochemistry* **20**: 447–9.

Roitman JN, James LF, Panter KE (1994) Constituents of broom snakeweed (*Gutierrezia sarothrae*), an abortifacient rangeland plant. In *Plant Associated Toxins: Agricultural, Phytochemical and Ecological Aspects*, Colegate SM, Dorling PR (eds). CAB International, Wallingford, Oxon, UK, pp. 345–50.

Rosenfeld I, Beath OA (1964) *Selenium. Geobotany, Biochemistry, Toxicity and Nutrition*. Academic Press, New York.

Rowe LD, Norman JO (1989) Detection of phototoxic activity in plant specimens associated with primary photosensitization in livestock using a simple microbiological test. *J Vet Diagn Invest* **1**: 269–70.

Roy DN, Peyton DH, Spencer PS (1995) Isolation and identification of two potent neurotoxins, aspartic acid and glutamic acid from yellow star thistle (*Centaurea solstitialis*). *Nat Toxin* **3**: 174–80.

Sady MB, Seiber JN (1991) Field test for screening milkweed latex for cardenolides. *J Nat Prod* **54**: 1105–7.

Saito K, Nagao T, Takasuki S, Koyama K, Natori S (1990) The sesquiterpenoid carcinogen of bracken fern, and some analogs, from the Pteridaceae. *Phytochemistry* **29**: 1475.

Sampson AW (1952) *Range Management, Principles and Practices*. John Wiley and Sons, Inc., Indianapolis, IN.

Schuster JL, James LF (1988) Some other major poisonous plants of the Western United States. In *The Ecology and Economic Impact of Poisonous Plants on Livestock Production*, James LF, Ralphs MH, Nielsen DB (eds). Westview Press, Boulder, CO, pp. 295–307.

Seawright AA, Hegarty MP, James LF, Keeler RF (1985) *Plant Toxicology, Proceedings of the Australia–USA Poisonous Plants Symposium (ISOPP2)*. Queensland Poisonous Plant Committee, Yeerongpilly, Australia.

Selfero NA, Merlassina JL, Audisio S (1989) Intoxication by *Centaurea solstitialis*, therapeutic evaluation. *Therios* **13**: 42–4.

Shaver TN, Camp BJ, Dollahite JW (1964) The chemistry of a toxic constituent of *Xanthocephalum* species. *Ann NY Acad Sci* **111**: 737–43.

Shishkin BK (1973) *Flora of the USSR*. Botanical Institute of the Academy of Sciences of the USSR, Moscow, Russia, pp. 271–2.

Short RE, James LF, Panter KE, Staigmiller RB, Bellows RA, Malcolm J, Ford SP (1992) Effects of feeding pine needles during pregnancy: comparative studies with buffalo, cattle, goats, and sheep. *J Anim Sci* **70**: 3498–504.

Short RE, Ford SP, Rosazza JPN, Farley DB, Klavons JA, Hall JB (1996) Effects of feeding pine needles and pine needle components to late pregnant cattle. *Proc Western Sect Am Soc Anim Sci* **47**: 193–6.

Shull LR, Buckmaster GW, Cheeke PR (1976) Factors influencing pyrrolizidine (*Senecio*) alkaloids metabolism: species, liver sulfhydryls, and rumen fermentation. *J Anim Sci* **43**: 1247–53.

Shupe JL, James LF (1968) Additional physiopathologic changes in *Halogeton glomeratus* (oxalate) poisoning in sheep. *Cornell Vet Coll Ser* 41–55.

Shupe JL, James LF, Binns W (1967a) Observations on crooked calf disease. *J Am Vet Med Assoc* **151**: 191–7.

Shupe JL, Binns W, James LF, Keeler RF (1967b) Lupine, a cause of crooked calf disease. *J Am Vet Med Assoc* **151**: 198–203.

Shupe JL, Binns W, James LF, Keeler RF (1968) A congenital deformity in calves induced by the maternal consumption of lupine. *Aust J Agric Res* **19**: 335–40.

Smetzer DL, Coppock RW, Ely RW, Duckett WM, Buck WB (1983) Cardiac effects of white snakeroot intoxication in horses. *Equine Pract* **5**: 26.

Smith BL (2004) Bracken fern (genus *Pteridium*) toxicity: a global problem. In *Poisonous Plants and Related Toxins*, Acamovic T, Stewart CS, Pennycott TW (eds). CAB International, New York, pp. 227–40.

Smith GS, Ross TT, Flores-Rodriguez GI, Oetting BC, Edrington TS (1991) Toxicology of snakeweeds *Gutierrezia microcephala* and *G. sarothrae*. In *Noxious Range Weeds*, James LF, Evans JO, Ralphs MH, Child RD (eds). Westview Press, Boulder, CO, pp. 236–46.

Smith GS, Allred KW, Kiehl DE (1992) Swainsonine content of New Mexican locoweeds. *J Anim Sci* **70** (Suppl. 1): 124.

Sosebee RE, Seipp WE, Alliney J (1982) Effect of timing of herbicide application on broom snakeweed control. In *Noxious Brush and*

*Weed Control Research Highlight*, vol. 13. Range and Wildlife Department, Texas Tech University, Lubbock, pp. 19–21.

Stegelmeier BL (2002) Equine photosensitization. *Clin Tech Equine Pract* **1**(2): 81–8.

Stegelmeier BL, James LF, Panter KE, Molyneux RJ (1995) Tissue and serum swainsonine concentrations in sheep ingesting *Astragalus lentiginosus* (locoweed). *Vet Hum Toxicol* **37**: 336–9.

Stegelmeier BL, Gardner DR, James LF, Panter KE, Molyneux RJ (1996) The toxic and abortifacient effects of ponderosa pine. *Vet Pathol* **33**: 22–8.

Stegelmeier BL, Panter KE, Pfister JA, James LF, Manners GD, Gardner DR, Ralphs MH, Olsen JD (1998) Experimental modification of larkspur (*Delphinium* spp.) toxicity. In *Toxic Plants and Other Natural Toxicants*, Garland T, Barr C (eds). CAB International, New York, pp. 205–10.

Stegelmeier BL, Edgar JA, Colegate SM, Gardner DR, Schoch TK, Coulombe RA, Molyneux RJ (1999) Pyrrolizidine alkaloid plants, metabolism and toxicity. *J Nat Toxin* **8**: 95–116.

Steyn DG (1933) Plant poisoning in stock and the development of tolerance. *Onderstepoort J Vet Sci Anim Ind* **1**: 141–3.

Stoddart LA, Smith AD (1955) *Range Management*, 2nd edn. McGraw-Hill, New York.

Strickland JR, Gulino-Klein LF, Ross TT, Slate S, Peterson MK, May T, Taylor JB (1998) Effects of nutrient supplementation in beef cows of poor body condition fed snakeweed (*Gutierrezia* spp.). *Vet Hum Toxicol* **40**: 278–84.

Stuart LD, James LF, Panter KE, Call JW, Short RE (1989) Pine needle abortion in cattle: pathological observations. *Cornell Vet* **79**: 61–9.

Taylor JA (1990) The bracken problem: a global perspective. In *Bracken Biology and Management*, Thomson JA, Smith BL (eds). Australian Institute of Agricultural Science Occasional Publication 40, Sydney, Australia, pp. 3–19.

Tiwary AK, Panter KE, Stegelmeier BL, James LF, Hall JO (2005) Evaluation of the respiratory elimination kinetics of selenium after oral administration in sheep. *Am J Vet Res* **66**: 2142–8.

Tiwary AK, Stegelmeier BL, Panter KE, James LF, Hall JO (2006) Comparative toxicosis of sodium selenite and selenomethionine in lambs. *J Vet Diagn Invest* **18**: 61–70.

Torell LA, Owen LP, McDaniel KC, Graham D (2000) Perceptions and economic losses from locoweed in northeastern New Mexico. *J Range Manage* **53**: 376–83.

Ueckert DN (1979) Broom snakeweed: effect on shortgrass forage production and soil water depletion. *J Range Manage* **32**: 216–20.

Ueckert DN (1984) Management of selected poisonous plants on semiarid rangelands in west Texas with herbicides. In *Plant Toxicology; Proceedings of the Australia, USA Poisonous Plants Symposium*, Seawright AA, Hegarty MP, James LF, Keeler RF (eds). Queensland Poisonous Plant Committee, Yeerongpilly, Australia, pp. 32–41.

USDA FS (1937) *Range Plant Handbook*. US Government Printing Office, Washington, DC.

Van Kampen KR, James LF (1969) Acute Halogeton poisoning of sheep: pathogenesis of lesions. *Am J Vet Res* **30**: 1779–83.

Wagnon KA (1960) Lupine poisoning as a possible factor in congenital deformities in cattle. *J Range Manage* **13**: 89–91.

Wagstaff DJ, Case AA (1987) Human poisoning by *Zigadenus. Clin Toxicol* **25**: 361–7.

Walkley SU, Siegel DA (1989) Comparative studies of the CNS in swainsonine-induced and inherited feline α-mannosidosis. In *Swainsonine and Related Glycosidase Inhibitors*, James LF, Elbein AD, Molyneux RJ, Warren CD (eds). Iowa State University Press, Ames, IA, pp. 57–75.

Wangberg JK (1982) Destructive and potentially destructive insects of snakeweed in western Texas and eastern New Mexico. *J Range Manage* **35**: 235–8.

Ward JM, Cockcroft VB, Lunt GG, Smillie FS, Wonnacott S (1990) Methyllycaconitine: a selective probe for neuronal α-bungarotoxin binding sites. *FEBS Lett* **270**: 45–8.

Warnock D (1997) Lupine toxicity. *Western Beef Producer*, May 20–22.

Welsh SL, Ralphs MH, Panter KE, Pfister JA, James LF (2007) Locoweeds of North America. In *Poisonous Plants: Global Research and Solutions*, Panter KE, Wierenga TL, Pfister JA (eds.) CAB International, Wallingsford, UK (in press).

Whitson TD, Freeburn JW (1990) Broom snakeweed control two years following herbicide treatments. *Western Society Weed Science Progress Report*, pp. 54–5.

Widmer WR (1984) Poison hemlock toxicosis in swine. *Vet Med* **79**: 405–8.

Williams JL, Campos D, Ross TT, Smith GS, Martinez JM, Becker KA (1993) Heifer reproduction is not impaired by snakeweed consumption. In *Snakeweed Research Updates and Highlights*, Sterling TM, Thompson DC (eds). New Mexico State University Experimental Station Research Report 647, pp. 46–7.

Williams MC (1960) Biochemical analysis, germination and production of black and brown seed of *Halogeton glomeratus. Weeds* **8**: 452–61.

Williams MC (1991) Twenty year control of California false hellebore. *Weed Technol* **5**: 40–2.

Williams MC, Barneby RC (1977) The occurrence of nitro-toxins in North American *Astragalus* (Fabaceae). *Brittonia* **29**: 310–26.

Williams MC, Binns W (1967) Toxicity of *Astragalus miser* Dougl., var. *oblongifolius* (Rydb.) Crong. *Weeds* **15**: 359–62.

Williams MC, James LF (1975) Toxicity of nitro-containing *Astragalus* to sheep and chicks. *J Range Manage* **28**: 260–3.

Williams MC, Norris FA (1969) Distribution of miserotoxin in varieties of *Astragalus miser* Dougl. ex Hook. *Weed Sci* **17**: 236–8.

Williams MC, Parker R (1974) Distribution of organic nitrites in *Astragalus. Weed Sci* **22**: 259–62.

Williams MC, Norris FA, Van Kampen KR (1970) Metabolism of miserotoxin to 3-nitro-1-propanol in bovine and ovine ruminal fluids. *Am J Vet Res* **31**: 259–62.

Woodson RE (1954) The North American species of *Asclepias* L. *Ann MO Bot Gard* **41**: 1–211.

Young JA (1999) *Halogeton: A History of Mid-20th Century Range Conservation in the Intermountain Area.* USDA ARS Miscellaneous Publication.

Zalkow LH, Burke N, Cabat G, Grula EA (1962) Toxic constituents of Rayless goldenrod. *J Med Pharm Chem* **5**: 1342–3.

CHAPTER

# 67

# Cyanogenic plants

*Steven S. Nicholson*

## INTRODUCTION

Cyanide, hydrocyanic acid, hydrogen cyanide (HCN) and prussic acid are all terms relating to the same toxic principle. Cyanide is used as a fumigant and in chemical synthesis; 50–60 ppm in air, as in fumigants, may cause poisoning. Cyanide salts are used in metal cleaning, hardening, refining and in the recovery of gold from ores. Burning nitrogen-based polymers used in plastics, fabrics and seat covers releases HCN. Cyanide blocks molecular oxygen transfer in cytochrome oxidase systems in mitochondria causing tissue anoxia. The process is reversible. Various cyanogenic glycosides, which can hydrolyze to form HCN, are present in a number of plant species. These compounds probably developed as a defense against excessive grazing by herbivores. Only a few of these plants are a significant risk to livestock. Some are grasses cultivated as forage for livestock and horses and others are ornamentals, commercial fruit trees, shrubs, weeds and range plants. All animal species are susceptible to cyanide poisoning. The ability of rumen microbial flora to rapidly hydrolyze cyanogenic glycosides makes ruminants particularly at risk of cyanide intoxication from plant sources. A recent review of the risks and effects of cyanogenic plants in animals was published by Burrows and Tyrl (2001).

## BACKGROUND

In general, the location of cyanogenic glycoside in plants is the epidermis, with highest levels in seeds, leaves, bark and twigs, to lowest in fruit. Seeds of grain sorghum and other grasses do not contain these glycosides. Seeds of members of the Rosaceae family including apple, cherry, peach and apricot do contain cyanogenic glycosides. Laboratory test results of plant tissues for presence of cyanide are reported as cyanide potential since free cyanide is not present in plants but is generated from glycoside during testing.

HCN potential of cyanogenic plants ranges from a few parts per million to 8000 ppm dry weight from the glycoside dhurrin in foliage of a grain sorghum (Burrows and Tyrl, 2001). Similar levels may occur in *Sorghum halapense* (Johnsongrass), considered a weed but also utilized for grazing and hay. Related sorghum hybrids and Sudangrass forages were developed which have less cyanide potential but most could be a hazard under certain conditions. Nitrogen fertilization increases glycoside content. Cyanide potential in these forages is greatest during early growth. Concentrations of the glycoside prunasin in leaves of *Prunus virginiana* may be as high as 6% dry weight (Majak *et al.*, 1981). Within a growing season glycoside levels in plants are generally highest in early growth and decline as maturity approaches. Fluctuations in glycoside levels during a growing season occur associated with climatic changes such as periods of drought.

Physical damage to plant tissue (freezing, crushing, macerating, cutting and drying) allows plant enzyme $\beta$-glycosidase and hydroxynitrile lyase to come in contact with and hydrolyze the glycoside to hydroxynitrile and free, volatile HCN. The first step in this process yields a sugar and an aliphatic or aromatic $\alpha$-hydroxynitrile aglycone (cyanohydrin), then formation of an aldehyde or a ketone, e.g. benzaldehyde, and HCN (Conn, 1979). Generally, hay loses most of the HCN prior to feeding. In some situations toxic levels may remain in large bales of *Sorghum* spp. where the cut forage dries rapidly and is immediately

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baled. Properly ensiled silage loses cyanide potential. Green chopping immature sorghum forage and feeding it the same day to ruminants is a serious potential hazard.

## PHARMACOKINETICS/ TOXICOKINETICS AND MECHANISM OF ACTION

Rumen microflora rapidly hydrolyze cyanogenic glycosides releasing HCN which is quickly absorbed and distributed to the tissues. Lower ruminal pH in cattle fed high-grain diets reduces the action of microbial enzymatic activity and release of HCN (Majak *et al*., 1990). Acid stomach contents in monogastrics limit hydrolysis and release of HCN. HCN is rapidly absorbed from the gastrointestinal tract, lungs and slowly through the skin.

Cyanide ion combines with ferric (trivalent) iron in the cytochrome oxidase system, blocking electron transport and molecular oxygen transfer from oxyhemoglobin to tissues. The effect is cellular hypoxia or histotoxic anoxia. This is a reversible action.

Arterial blood is normally bright red because of the presence of oxyhemoglobin. In cyanide toxicosis oxygen is not released from oxyhemoglobin to the tissues and the bright red color remains in venous blood. This process, from ingestion of a toxic dose of plant material, release of cyanide in the rumen and onset of clinical signs, can occur within a few minutes.

Some cyanide is detoxified by an endogenous thiosulfate limiting process. Thiosulfate combines with cyanide to form thiocyanate which is excreted in the urine. The reaction is catalyzed by the enzyme rhodanese. The ability to detoxify cyanide allows animals to safely metabolize small amounts of cyanide.

## TOXICITY

Ruminants are more likely to be poisoned by plant origin cyanide than other animals because rumen microorganisms readily release cyanide from the glycoside. An active microbial flora in the gut allows considerable but somewhat delayed and slower hydrolysis of cyanogenic glycosides in humans and hamsters, and lesser amounts in mice, rats, guinea pigs and monkeys (Stavric and Klassen, 1980; Adewusi and Oke, 1985; Frakes *et al*., 1986). Although uncommon there are reports of cyanide toxicosis in horses, pigs and dogs. Eating raw plant material containing $\beta$-glycosidase along with crushed apricot or apple seeds has proved fatal in humans. Generally speaking, monogastric animals including horses are poisoned by 1–3 mg/kg b.w. of preformed HCN or cyanide salts. The lethal dose of sodium cyanide, 3–4 mg/kg b.w. to ruminants (Burrows and Way, 1977), is quite similar to the lethal dose of cyanide from the glycoside prunasin in plant material, 5 mg/kg b.w. in cattle (Majak *et al*., 1990). Plant material containing more than 20 mg/100 g (200 ppm) cyanide potential is considered hazardous. A level of 500 ppm has been used more specifically for the sorghums (Burrows and Tyrl, 2001).

The effects are not cumulative. One-half the lethal dose can be given repeatedly during the course of a day such that a total dose of 4–5 times the single lethal dose can be tolerated (Burrows and Tyrl, 2001). Tolerance does not develop.

Death may occur within minutes after ingestion of a toxic amount of a plant containing high cyanide potential. An exception may occur if ruminants grazing arid rangelands ingest a toxic amount of chokecherry or arrow grass while rumen contents are quite dry, with release of HCN by rumen flora delayed until the animal drinks water later in the day. Affected animals may be found dead in or near the water source.

Ruminants may exhibit signs within minutes to less an hour after commencing ingestion of toxic plant material. Cattle may become apprehensive and excitable at the sight of herdmates that are suddenly affected and collapsing. Onset of clinical signs is peracute and includes apprehension, pronounced polypnea then dyspnea because initially there is stimulation of chemoreceptors in the carotid body and respiratory centers. The pupils dilate and mucous membranes may be pink and venous blood a bright cherry red. Weakness, voiding of urine, collapse, paddling and death follow within a few minutes. Sublethal cases may recover within the hour.

Lesions are few. Mucous membranes may be pink, venous blood bright red and clot slowly. Subendocardial and subepicardial petecchial and ecchymotic hemorrhages typical of an agonal death may be present. A bitter almond or "cherry coke" odor from stomach contents is detectable in some cases. Venous blood may not be bright red in animals dead several hours. At necropsy, blood of animals that died of some other cause may develop bright red color when exposed to the air for several minutes.

Differential diagnosis of cyanide toxicosis in ruminants may include acute toxicoses caused by nitrate–nitrites, urea–ammonia, ipomeanol, perilla ketone, 3-methylindole, bluegreen algae and electrical shock or lightning strike.

When cyanide toxicosis is suspected submit to the laboratory, along with the usual specimens, refrigerated heart or skeletal muscle and rumen contents for cyanide ion detection. In blood, concentrations of 1 ppm or more are consistent with severe intoxication in mammals and birds (Burrows and Tyr, 2001). Blood should be kept in air-tight containers at 4°C (Egekeze and Oehme, 1979). Continued hydrolysis of glycoside and loss of HCN may make necropsy samples less useful for confirming the diagnosis.

Specimens can be immersed in mercuric chloride to prevent hydrolysis from continuing. Rumen or stomach contents can be examined for presence of material from cyanogenic plants. A sensitive field test using alkaline picrate treated filter paper strips can be prepared (Burrows and Tyrl, 2001) for testing plant materials and fresh rumen contents.

Chronic cyanide poisoning (or a nitrile compound) may be involved in equine ataxia–urinary incontinence seen in horses, grazing sorghum hybrid pastures (Van Kampen, 1970; Wheeler and Mulcahy, 1989). There are reports of a similar condition in cattle and sheep (Bourke, 1995). After grazing forage sorghum and grain sorghum regrowth, 54 of 330 breeding cows became ataxic and developed urinary incontinence. Wallerian degeneration of the white matter of the spinal cord, cerebellar peduncles and cerebellum was seen histologically (McKenzie and McMicking, 1977). In humans, long-term consumption of the cyanide containing plants tropical lima beans and cassava root are associated with well-documented conditions involving the spinal cord, optic nerves and other lesions. A study of children in Mosambique evaluated the possible association of high cyanide and low sulfur intake in cassava-induced spastic paraparesis. The study results supported the hypothesis that the epidemic was due to the combined effects of high dietary cyanide exposure and sulfur deficiency (Cliff *et al.*, 1985). Teratogenic effects of cyanide were demonstrated in hamsters (Frakes *et al.*, 1986).

## TREATMENT

Rapid response to intravenous antidote solution can be striking although the opportunity to treat is rare because of the peracute nature of the poisoning. The antidote of choice in humans, dogs and probably most other animals is 10–20 mg/kg b.w. of sodium nitrate in combination with 250–500 mg/kg b.w. of sodium thiosulfate (Baskin *et al.*, 1992; Burrows and Tyrl, 2001). Ruminants can be treated with thiosulfate alone using a 30–40% solution intravenous at a dose of 25–50 g/100 kg b.w.

## CONCLUDING REMARKS AND FUTURE DIRECTION

Cyanide poisoning is an uncommon event in livestock production but the potential for sudden economic loss to individual farmers and ranchers is significant. Veterinarians, agricultural educators and consultants should continue to remind clientele of the risks to ruminant species associated with forages, shrubs and trees of known cyanide potential.

## REFERENCES


Adewusi Sr A, Oke OL (1985) On the metabolism of amygdaline. 2. The distribution of $\beta$-glucosidase activity and orally administered amygdalin in rats. *Can J Physiol* **63**: 1084–7.

Baskin SJ, Horowitz AM, Nealley EW (1992) The antidotal action of sodium nitrate and sodium thiosulfate against cyanide poisoning. *J Clin Pharmacol* **32**: 368–75.

Bourke C (1995) *Sorghum* spp. Neurotoxicity in sheep. *Aust Vet J* **72**: 467.

Burrows GE, Tyrl RL (2001) *Toxic Plants of North America.* Iowa State University Press, Ames, IA, pp. 1043–1074.

Burrows GE, Way JL (1977) Cyanide intoxication in sheep: therapeutic value of oxygen or cobalt. *Am J Vet Res* **38**: 223–7.

Cliff I, Lundquist P, Martensson I, Rosling H, Sorbo B (1985) Association of high cyanide and low sulfur intake in cassava-induced spastic paraparesis. *Lancet* **II**: 1211–13.

Conn EE (1979) Cyanogenic glycosides. *Rev Biochem, Biochem Nutr 1A* **27**: 21–43.

Frakes RA, Sharma RP, Willhite CC (1986) Comparative metabolism of linamarin and amygdalin in hamsters. *Food Chem Toxicol* **24**: 417–20.

Egekeze JO, Oehme FW (1979) Blood and liver cyanide concentrations in rats poisoned with oral doses of potassium cyanide. *Toxicol Lett* **3**: 243–7.

Majak W, McDiarmid RE, Hall JW (1981) The cyanide potential of saskatoon serviceberry (*Amelanchier alnifolia*) and chokecherry (*Prunus virginiana*). *Can J Anim Sci* **61**: 681–6.

Majak W, McDiarmid RE, Hall JW, Cheng K-J (1990) Factors that determine rates of cyanogenesis in bovine ruminal fluid *in vitro*. *J Anim Sci* **68**: 1648–55.

McKenzie RA, McMicking LI (1977) Ataxia and urinary incontinence in cattle grazing sorghum. *Aust Vet J* **53**: 496–7.

Stavric B, Klassen R (1980) Enzymatic hydrolysis of amygdalin by fecal samples and some foods. In *Natural Toxins*: *6th International Symposium on Animal, Plant and Microbial Toxins*, Eaker D, Wadstrom T (eds). Pergamon Press, New York, pp. 655–660.

Wheeler JL, Mulcahy C (1989) Consequences for animal production of cyanogenesis in sorghum forage and hay – a review. *Trop Grassl* **23**: 193.

Van Kampen KR (1970) Sudan grass and sorghum poisoning of horses: a possible lathyrogenic disease. *J Am Vet Med Assoc* **156**: 629–30.

C H A P T E R

# 68

# Nitrate and nitrite accumulating plants

*Steven S. Nicholson*

## INTRODUCTION

Ruminants are particularly at risk of acute, fatal nitrate–nitrite poisoning. Microorganisms in the rumen reduce nitrates to nitrites then ammonia for microbial growth. Excess intake of nitrates may cause toxic levels of nitrite to accumulate and be absorbed into the blood. Cattle graze a variety of grasses and weeds which, under certain conditions, especially excessive fertilization, can accumulate levels of nitrate in the stems that may prove toxic to animals that eat a sufficient dose. Excess levels of nitrate may be present in grazed forages and weeds, in hay or fresh cut forage brought to the animals as greenchop. Ensiling may reduce nitrate levels by 30% or more. Sheep and rabbits can convert nitrate to toxic levels of nitrite. Goats browse leafy portions of plants and may not ingest toxic levels of nitrates in stalks and stems of forages. In horses some bacterial reduction of nitrate to nitrite does occur in the large bowel (Bruning-Fann and Kaneene, 1993). Nitrate toxicosis in horses is rarely reported as a clinical entity (Hintz and Thompson, 1998).

Monogastric species are susceptible to the toxic effects of nitrites ingested as nitrites from non plant sources. Nitrates and nitrites are water soluble and may contaminate water sources. Forages and weeds growing in soil rich in manure waste or in holding pens are a potential source of poisoning. Nitrate fertilizers are commonly found on farms and ranches and accidental ingestion or feed contamination does occasionally occur.

## BACKGROUND

Plants take up nitrogen from the soil primarily in the form of nitrate. Nitrate accumulation in the stems and leaves of plants may be associated with high levels of nitrates, or ammonia, in the soil. Plants growing in soil where livestock manure and urine were applied as fertilizer or where accumulations occur in holding pens may accumulate nitrates. Hungry cattle and sheep introduced to stockyards containing a dominant or pure growth of button grass (*Dactyloctenium radulans*) suffered acute nitrate–nitrite toxicity in four incidents in inland Queensland between 1993 and 2001 (McKenzie *et al.*, 2004). The nitrate content of the button grass from within the stockyards ranged from 4.0% to 12.9% as $KNO_3$ in dry matter and from outside the stockyards ranged from <0.2% to 0.4%. After harvesting corn in Nebraska cornstalks remaining in fields had an average decrease in potassium nitrate content of only 30% in 90 days (Johnson *et al.*, 1992). Young plants are more likely to have high nitrate levels than are more mature plants. Nitrate concentrations decline considerably in all parts of Sudangrasses following heading (Mizukami *et al.*, 1997).

Plant growth may be slowed, and nitrate accumulation increased, when growing in soil that contains nutrient deficiencies or excesses. For example, molybdenum is a component in enzymatic reactions of nitrate reductase in plants. Nitrate accumulation in stalks and stems may follow herbicide damage to plants or loss of leaves due to hail.

A major reason plants accumulate nitrates is drought. During periods of drought the growth of forages and weeds is reduced but the roots may continue to collect and store nitrate in the stems. This is particularly true of well-fertilized sorghum hybrid (*Sorghum* spp.) and millet (*Pennisetum* spp.) forages grown for temporary summer grazing and for hay production (Clay *et al.*, 1976). Plants may accumulate nitrates during periods of reduced sunlight. Sunlight is needed to drive photosynthesis and the energy-dependent nitrate reductase system in the plant. Forage or weeds growing in the shade of trees in an orchard may be subject to nitrate accumulation. Nitrate poisoning is occasionally a problem in areas of the United States where

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winter grazing for cattle consists of fertilized pastures of ryegrass (*Lolium multiflorum*), oats (*Avena* spp.), turnips (*Brassica rapa*) or wheat (*Triticum* spp.). During extended periods, perhaps several days, of overcast weather the nitrate content of the forage may increase to potentially toxic levels. Accumulation is more likely when temperatures are mild (>55°F) and the root systems are actively taking up nitrates. Growth slows or stops but the roots continue uptake of nitrate which is stored in the stems until there is adequate sunlight and growth resumes. Generally, a day or two of sunlight with temperatures above 55°F allows plant growth to continue, converting excess stored nitrate to plant protein.

To reduce trampling of forage farm management may employ limited grazing periods allowing hungry cattle to consume a large amount of green forage for 2 h or so each day. This increases the risk because of the time–dose relationship that exists when excess nitrate is present and conversion of nitrate to nitrite exceeds the ability of the rumen flora to convert nitrite to ammonia. Total dietary intake of nitrate should be considered. Supplemental feeding of hay that has increased nitrate levels to cattle grazing forages with elevated nitrate levels increases the risk of toxicosis in this situation. Nitrate in drinking water adds to dietary intake. Nitrate concentrations in water in excess of 1000 ppm may cause nitrate poisoning in livestock.

Nitrate levels are not reduced by drying and baling as hay. High nitrate hay (>1.5% $KNO_3$; 1.0% nitrate) fed to cattle months after baling can cause multiple deaths and possibly abortions. Mortality can be striking as in a case in Nebraska where *Amaranthus*/*Kochia* hay with 4.9% $KNO_3$ and Sudangrass with 8% $KNO_3$ were fed to 390 cattle resulting in death of 226 and 42 abortions (Hibbs *et al.*, 1978). High nitrate summer hay fed during the winter of 1977–1978 killed cattle in Oklahoma (Haliburton and Edwards, 1978).

Determining the nitrate status of bales of stored hay can be a challenge because only the forage growing in a portion of a hay field may have been affected. Bales must be labeled, sampled and tested for nitrate content. Obtaining a representative sample requires using a hollow hand-held commercial tool which cuts through to the center of the bale and recovers a core sample of an ounce or so of hay. At least two samples from each bale should be collected. Investigation might reveal that one-third of the bales have nitrate levels, say <0.5%, a third perhaps 0.5–1.0%, and the remaining portion >1%.

Rumen microorganisms can adapt to and utilize increasing levels of nitrate in the diet. The period of maximum acclimation occurs within 6 days (Allison and Reddy, 1984). Adaptation can be lost within a few days. The ability of rumen microorganisms to safely reduce nitrate and nitrite can be increased by feeding corn-based supplements to cattle (Burrows *et al.*, 1987). Nitrate content in properly ensiled forage may be reduced by 30% or more during the ensilage process. The silage should be tested before feeding. Silage juices draining from the silo may be high in nitrates. Nitrogen dioxide ($NO_2$) and nitrogen tetroxide ($NO_4$) gases may be formed from oxides of nitrogen generated during anaerobic fermentation of high nitrate forages. These pulmonary toxicants are heavy yellow–brown gases.

## PHARMACOKINETICS/ TOXICOKINETICS

Action of the rumen flora reduces nitrate to the much more toxic nitrite which normally is converted to ammonia and further utilized by the microorganisms. Nitrite is absorbed into the blood when the intake of nitrates and the production of nitrite exceed the capacity of the rumen flora to further reduce nitrite. In some cases preformed nitrite in hay may shorten the period from ingestion to onset of signs. Nitrates are absorbed into the blood as well but are much less toxic than nitrite. In adult cattle the half-life of nitrate is estimated to be 9 h and in the bovine fetus more than 24 h (Johnson *et al.*, 1992). The half-lives of nitrate and nitrite in the blood of sheep are 4.2 and 0.5 h (Schneider and Yeary, 1975).

## MECHANISM OF ACTION

The nitrite anion causes vasodilation and oxidizes ferrous iron in hemoglobin to the ferric (trivalent) state forming methemoglobin which cannot accept molecular oxygen. As the percentage of methemoglobinemia rises oxygen starvation to tissues increases and blood becomes chocolate brown in color. In sheep the half-life of methemoglobinemia is about 1.5 h (Schneider and Yeary, 1975). Clinical signs such as exercise intolerance appear at 30–40% methemoglobinemia with death from hypoxia likely when concentrations exceed 80% (Burrows, 1980). In non-fatal cases a red blood cell intrinsic NADH-dependent diaphorase or reductase system gradually reduces methemoglobin to hemoglobin.

## TOXICITY

Nitrate level in edible stalks and stems of plants generally accepted as safe for all classes of cattle is <0.5%. It is recommended that pregnant animals not be fed forage or hay with nitrate content between 0.2% and 1.0% nitrate levels. Forage nitrate above 1% (1.5% $KNO_3$) is considered dangerous. The rate of conversion of nitrate to nitrite then

to ammonia is a limiting factor in safe utilization of nitrates by ruminants. Hungry cattle are at greater risk and intake of dry matter from hay may be faster than from grazing. The additive effect of nitrates in water and other feed sources must be considered when evaluating total dietary nitrate.

The rumen flora can safely utilize higher amounts of nitrate if sufficient dietary energy is present to promote reductive activity. Feeding corn-based supplements to cattle reduced nitrite accumulation (Burrows *et al.*, 1987). In this study feeding of 3.2 kg of corn protected against nitrate poisoning by reducing intraruminal nitrite and blood methemoglobin ($p > 0.05$).

Clinical signs of nitrate–nitrite toxicosis in cattle include weakness, cyanosis of mucous membranes, ataxia, collapse and death. Increased respiratory rate may be noted in some animals. Affected animals may remain standing then collapse and die within minutes. Dead animals may be found in sternal recumbancy or lying on their side. Blood is dark and may have an obvious brown color when drawn into a syringe or spread on a white cloth. At necropsy of animals dead several hours this color may not be as apparent.

In cattle abortions may occur in the herd 3–7 days after the acute toxicosis episode. Less oxygen is available to the fetus because of methemoglobinemia in the cow and nitrite induces methemoglobinemia in fetal blood (Bruning-Fann and Kaneene, 1993). Bovine abortion has been reported to occur with forages containing 0.61–1% nitrate (van't Klooster *et al.*, 1990). Differential diagnoses to consider include acute toxicoses caused by insecticides, carbohydrate overload, hypomagnesemia in lactating cattle, cyanide, bluegreen algae, urea (ammonia) and potent oxidizing agents such as sodium chlorate herbicide and aniline dyes. Lesions are not diagnostic. Blood and tissues may appear brown at time of death but this becomes less obvious as autolysis proceeds. Dark blood may suggest septicemia. Agonal hemorrhages in the epicardium may be present.

Ocular fluid is an excellent body fluid for nitrate analysis. Plasma and serum are acceptable. Ocular fluid nitrate levels are 35% lower than serum levels (Boermans, 1990). The diphenylamine blue test is widely used for testing fluids and plant tissues (Burrows, 1980; Bhikane and Singh, 1990). Another test that has been used is the diazotization test (Bhikane and Singh, 1991). Nitrate concentrations in ocular fluid of 10 ppm are indicative of excessive nitrate exposure and $> 20$ ppm are considered positive diagnosis of poisoning (Burrows and Tyrl, 2001). In abortion and stillbirth situations interpreting bovine fetal ocular fluid nitrate levels is more problematic because normal levels may approach 20 ppm in weak or stillborn calves (Johnson *et al.*, 1994). Level of 30 ppm or more and additional diagnostic information such as elevated forage nitrates may be needed to confirm nitrate-induced abortion (Casteel and Evans, 2004). The clinical history may suggest nitrates as a possible cause. All sources of forages, weeds, water, feed supplements and fertilizers to which the animals had access should be determined and sampled for analysis.

## TREATMENT

Treatment is with intravenous methylene blue in a 1% or 2% aqueous solution at a rate of 1–2 mg/kg b.w (Bhikane *et al.*, 1990). Up to 10 mg/kg b.w. can be administered in severe cases. The response to intravenous treatment of a 2% solution of methylene blue at a dosage of 20 ml/100 kg b.w. is rapid with reversal of the clinical signs within several minutes (Burrows and Tyrl, 2001). In severe cases treatment at a lower dose can be repeated. Methylene blue serves as an electron carrier for an NADPH-dependent system to reduce methemoglobin to hemoglobin. Methylene blue is most effective in humans and ruminants (Burrows and Tyrl, 2001). Tissues in the treated animals are stained and the urine becomes dark green. Treated animals should not be sold for slaughter for 180 days. Other dyes such as tolonium chloride (tolonium blue) are effective in reducing methemoglobin to hemoglobin but have a narrow therapeutic index (Gupta *et al.*, 1992; Cudd *et al.*, 1996).

## CONCLUDING REMARKS

Safe use and storage of nitrate fertilizers is essential if accidental poisoning of livestock, especially cattle, is to be avoided. Use of liquid fertilizer tanks to deliver water to livestock is a documented hazard for nitrate or urea toxicosis. The potential for nitrate accumulation in weeds and forages intended for feeding or grazing and the risks this poses to ruminants, especially cattle, should be pointed out to farmers and ranchers by university extension and farm industry personnel. Cutter blades can be raised to reduce the amount of edible stalk in harvested forages if conditions warrant. Pre-feeding testing of potentially high nitrate forages is especially important when environmental conditions affect growth. Feeding cattle an energy source like corn or providing oral product containing *Proprionibacterium* can increase the rate of nitrite reduction by rumen flora.

## REFERENCES

Allison MJ, Reddy CA (1984) Adaptations of gastrointestinal bacteria in response to changes in dietary oxalate and nitrate. In *Current Perspectives in Microbial Ecology*, Klug MJ, Reddy CA (eds). American Society for Microbiology, Washington, DC, pp. 248–56.

Bhikane AU, Singh B (1990) Diphenylamine blue test for diagnosis of experimental nitrite poisoning in crossbred calves. *Indian Vet J* **67**: 808–12.
Bhikane AU, Singh B (1991) Diazotization test for diagnosis of experimental nitrite poisoning in cross bred cattle. *Indian J Anim Sci* **61**: 61–2.
Bhikane AU, Singh B, Salabat Ali M (1990) Therapeutic efficacy of methylene blue against experimental nitrite poisoning in crossbred calves. *Indian Vet J* **67**: 459–62.
Boermans HJ (1990) Diagnosis of nitrate toxicosis in cattle, using biological fluids and a rapid ion chromatographic method. *Am J Vet Res* **51**: 491–5.
Bruning-Fann CS, Kaneene JF (1993) The effects of nitrate, nitrite, and *N*-nitroso compounds on animal health. *Vet Hum Toxicol* **35**: 237.
Burrows GE (1980) Nitrate intoxication. *J Am Vet Med Assoc* **177**: 82.
Burrows GE, Tyrl RL (2001) *Toxic Plants of North America*. Iowa State University Press, Ames, IA, pp. 934–41.
Burrows GE, Horn GW, McNew RW, Croy LI, Keeton RD, Kyle J (1987) The prophylactic effect of corn supplementation on experimental nitrate intoxication in cattle. *J Anim Sci* **64**: 1682–9.
Casteel SW, Evans TJ (2004) Feed associated toxicants: nitrate. In *Clinical Veterinary Toxicology*, Plumlee KH (ed.). Mosby Inc, St. Louis, MO, pp. 127–30.
Clay BR, Edwards WC, Peterson DR (1976) Toxic nitrate accumulation in sorghums. *Bovine Pract* **11**: 28–32.
Cudd LA, Burrows GE, Clarke CR (1996) Pharmacokinetics of and toxicity of tolonium chloride in sheep. *Vet Hum Toxicol* **38**: 329–32.
Gupta D, Singh B, Bhikane AU, Rajgura DN (1992) Treatment of experimental nitrate poisoning with tolonium chloride in buffalo calves. *Indian J Anim Sci* **62**: 1180–2.
Haliburton JC, Edwards WC (1978) Nitrate poisoning in Oklahoma cattle during the winter of 1977–1978. *Vet Hum Toxicol* **20**: 401–3.
Hibbs CM, Stencel EL, Hill RM (1978) Nitrate toxicosis in cattle. *Vet Hum Toxicol* **20**: 1–2.
Hintz HF, Thompson LJ (1998) Nitrate toxicosis in horses. *Equine Prac* **20**: 5.
Johnson JL, Hergert GW, Schneider NR, Grabouski PH (1992) Post harvest change in cornstalk nitrate and its relationship to bovine fetal nitrate/nitrite exposure. In *Poisonous Plants: Proceedings of the Third International Symposium*, James LF, Keeler RF, Bailey Jr EM, Cheeke PR, Hegarty MP (eds). Iowa State University Press, Ames, IA, pp. 423–30.
Johnson JL, Grotelueschen DM, Knott M (1994) Evaluation of bovine perinatal nitrate accumulation in western Nebraska. *Vet Hum Toxicol* **36**: 467–71.
McKenzie RA, Rayner AC, Thompson GK, Pidgeon GF, Burren BR (2004) Nitrate–nitrite toxicity in cattle and sheep grazing *Dactyloctenium radulans* (button grass) in stockyards. *Aust Vet J* **82**: 630–40.
Mizukami Y, Kanbe M, Inami S, Fukaya K (1997) Changes in the nitrate content of millets and Sudangrass. *Res Bull Aichi-ken Agri Res Cent* **29**: 71–6.
Schneider NR, Yeary RA (1975) Nitrate and nitrite pharmacokinetics in the dog, sheep, and pony. *Am J Vet Res* **36**: 941–7.
van't Klooster AT, Taverne MA, Malestein A, Akkersdijk EM (1990) On the pathogenesis of abortion in acute nitrite toxicosis of pregnant dairy cows. *Theriogenology* **33**: 1075–89.

C H A P T E R

# 69

# Oxalates-containing plants

*Theuns W. Naudé and V. Naidoo*

## TREATMENT

Oxalates are of importance in veterinary toxicology for two reasons.

The first and most important reason is that absorbed *soluble oxalates* cause serious primary nephropathy and kidney shut down by severely damaging the renal tubular epithelium. They form insoluble complexes with calcium and magnesium and cause hypocalcaemia and hypomagnesaemia and form crystalline deposits in especially the kidneys. They also contribute to the formation of urinary calculi.

The second reason is that highly irritating *insoluble calcium oxalate raphides* form in certain plants as protection against herbivory and may cause alarming signs if the plants are eaten, or in the case of those with a "nettle action", touched.

These two syndromes are discussed in this chapter separately.

## PART I: SOLUBLE OXALATE POISONING

## BACKGROUND

Oxalates (Figure 69.1) occur in many plants and formation is via the glyoxylate cycle or from ascorbic acid (*vide infra*). The toxic levels of soluble oxalates in plants are given as 0.5% for *Osteodystrophia fibrosa* in the horse and 10% for nephropathy (Cheeke, 1998). The latter level is supported by other authors (Seawright, 1989 cited by Kellerman *et al.*, 2005).

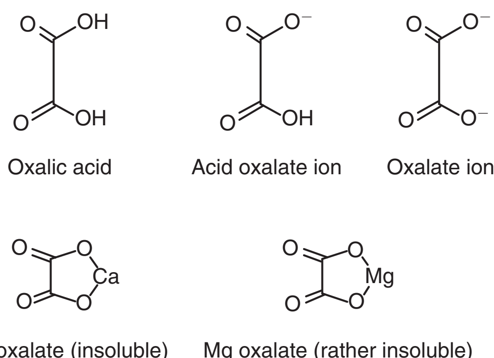


**FIGURE 69.1** Oxalic acid and its salt.

The families and genera which are of toxicological significance differ from country to country but any plant high in oxalates eaten by animals in quantity could potentially be toxic. What appears to be the generally important families and genera involved are listed in Table 69.1.

In addition to plants, a variety of saprophytic fungi may produce oxalates and render hay toxic even without any obvious moldy appearance (Wilson and Wilson, 1961; Cheeke, 1995).

All species, including man, are susceptible to intoxication but it is generally sheep and cattle that succumb from nephropathy and horses that develop *osteodystrophia fibrosa.*

In all cases of soluble oxalate toxicity unadapted animals (*vide infra*) have to be rather abruptly exposed to large quantities of oxalate-containing plants these often being the only, or largely, the plants eaten. It is often a problem in low rainfall areas that some of these are the dominant plants that animals have to browse. During feed shortage the cut-up leaves of agave and cathodes of prickly pear (*Opuntia ficus indica*), particularly the thornless variety,

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**TABLE 69.1 Significant plants involved in oxalate poisoning**

| Family | Genus | Common name |
|---|---|---|
| Agavaceae | *Agave* | Agave |
| Amaranthaceae | *Amaranthus* | Pigweed |
| Araceae* | For example *Dieffenbachia** | Dumb cane* |
| Cactaceae | *Opuntia* | Prickly pear |
| Chenopodiaceae | *Beta* | Beetroot, mangold, sugar beet |
| | *Bassia* | Bassia |
| | *Chenopodium* | White goosefoot |
| | *Spinacia* | Spinach |
| | *Halogeton* | Halogeton |
| | *Sarcobatus* | Greasewood |
| Euphorbiaceae* | *Tragia** | Noseburn* |
| Mesebryanthemaceae | *Mesembryanthemum* | Mesems |
| Oxalidaceaea | *Oxalis* | Sorrel |
| Poaceae | *Cenchrus* | Blue buffelgrass |
| | *Setaria* | Bristlegrass |
| | *Digitaria* | Pangolagrass |
| | *Pennisetum* | Kikuyu |
| Polygonaceae | *Rumex* | Sorrel, dock |
| | *Rheum* | Rhubarb |
| Fungi | Especially *Aspergillus* and *Pennicillium* (also *Botrytis, Rhizopus, Pithomyces, and Mucor*) | Moldy hay and silage |

*Insoluble raphides.

two species specially cultivated for this purpose, are fed to stock and in excessive quantities, may result in oxalate toxicosis.

## STRUCTURAL FORMULAE

Those of the acid and its two salts and of insoluble calcium and magnesium oxalate are given in Figure 69.1. Note that in the plants having oxalic acid and the acid oxalate ion occur if the plant sap has a pH of *ca.* 2 (e.g. *Oxalis* and *Rumex*), whereas if the plant sap's pH is *ca.* 6 the oxalate ion is found (e.g. *Halogeton* and *Mesembryanthemum*). With acid oxalate both acute and chronic toxicity occur but with oxalate only acute toxicity (Cheeke, 1998).

## BIOSYNTHESIS OF OXALATES IN PLANTS

This complex synthesis is summarized by Franceschi and Nakata (2005). Oxalic acid can be formed through the oxidation of glycolate and glyoxylate by the activity of glycolate oxidase or by the activity of isocitrate lyase on isocitrate. By C2/C3 cleavage, ascorbate is also a substrate for synthesis (Libert and Franceschi, 1987).

## TOXICOKINETICS

At present the toxicokinetics of oxalates have only been studied in any detail in people. Since the oral bioavailability of chemicals in people may be used as a model for monogastric physiology, the toxicokinetics of oxalates may be applicable to the veterinary situation for all non-ruminants. The bioavailability of oxalates in people is dependent on the person, oxalate content in the plant and most likely the method of cooking. Depending on the plant, studies have shown the bioavailability from grilled spinach is $0.75 \pm 0.48\%$ and for *Oxalis tuberosa*, $1.44 \pm 1.31\%$ after the first 6 h of ingestion (Albihn and Savage, 2001; Brogren and Savage, 2003). Furthermore they are absorbed from the gastric mucosa.

The reason for preferential gastric absorption is linked to the specific conditions required for the reaction of calcium with oxalic acid. For the insoluble calcium oxalate to form, an alkaline environment in required. Therefore the acidic environment favors the absorption of oxalic acid before the formation of the insoluble salts as in ruminants (Zhiqiang *et al.*, 2003).

In the normal healthy rumen, a portion of the oxalic acid and soluble oxalates can combine with calcium and become insoluble thereby reducing the chance of toxicity, the pH of *ca.* 5–7 of the rumen appears to favor the formation of Ca oxalate. This is in contrast to non-ruminants with a gastric pH of 1–2 (Phillis, 1976).

Ruminants have, however, developed an additional protective mechanism via a variety their rumen microorganisms. If the rumen is adapted by gradual exposure to oxalic acid and oxalates, these microorganisms (such as *Oxalobacter formigenes*) can use oxalic acid as an energy source and produce the by-products carbon dioxide and formate. It is only when the microbes are unable to break down oxalic acid that toxicity results (Rekhis, 1994).

In studies using guinea pigs previously adapted to a diet high in oxalates (2%), as a model for hindgut fermenters, it has been shown that the caecal bacteria played an important role in adaptation. This adaptation could be negated by treating animals with gut active antibiotics and certain secondary bile salts associated with ileal diseases (Argenzio *et al.*, 1988).

With ruminants being able to adapt to a higher concentration of oxalates in their diets, it has been questioned why a similar degree of protection fails to occur in horses. By combining the predominant gastric absorption of the soluble oxalates and the level of adaptation in hindgut fermenters, it may be concluded that if tolerance did develop

it would occur too low down the gastrointestinal tract (GIT) to allow for sufficient protection from absorption.

In humans oxalates are eliminated mainly by urinary excretion. Excretion is biphasic and peaked at 40 min and 3 h after the consumption of a warmed, commercially bought frozen spinach meal in normal healthy patient. The initial peak was however absent in patients that had undergone gastrotomy due to cancer. This once again indicated the importance of the stomach in initial absorption, i.e. the first major peak. The second peak would as such be related to delayed absorption from the intestines. When comparing oral bioavailability between the two groups, it was seen that area under the curve (AUC) was 50% greater in the patients with a functional stomach. At the 3.5 h post-feeding period these patients still had a greater extent of absorption with a difference of 20%. The excretion of oxalates was determined to be 0.0732 ± 0.0294 mg/min (Zhiqiang *et al.*, 2003).

# MECHANISM OF TOXICITY

There are four ways in which soluble oxalate-containing plants may cause toxicity or have adverse effects.

## Cellular toxicity

The organ systems most commonly affected are the kidneys, GIT and neural tissue and generally it was assumed that it resulted from the precipitation of calcium oxalate crystals in these specific organs (Andrews, 1971). Van Kampen and James (1969), however, in an experiment with sheep poisoned with a lethal dose of *Halogeton* (containing mainly the oxalate ion) and slaughtered sequentially every 2 h up to 8 h, that Ca oxalate crystal deposition is secondary to vascular and renal cellular damage. Their postulation is that a deficiency in intracellular Ca and Mg (due to its removal as insoluble oxalates, *vide infra*) results in inactivation of vitally essential Ca- and Mg-dependent enzymes and consequent cell damage. In a review in 1972, James summarizes literature that oxalate competitively inhibits oxidation of lactate and non-competitively interferes with pyruvate reduction. This is further supported by Absan (1997) who states that *ca.* half the Mg in the body is intracellular and that it is an essential co-factor to catalyze some 300 enzymatic reactions, particularly those involving ATP production.

The exact cellular pathophysiology for cellular toxicity, hereafter, has not been further elucidated despite the importance of ethylene glycol toxicity in people and dogs. At present the pathophysiology of nephrotoxicity is believed to be related to the following mechanism.

Once the absorbed, soluble oxalate is freely available to filter out through the glomerulus, where in the tubuli it binds to calcium to precipitate out as crystals on the damaged cells. Although the physical injury to the tubules may account for the nephrotoxicity, it at this stage believed unlikely to be the only mechanism, as the rapid transit time of 3–4 min in the tubules hardly seems long enough for the crystals to grow, in sufficient size to block tubules (Corley *et al.*, 2005; Jonassen *et al.*, 2005). James *et al.* (1971), however, pointed out that the original site where the crystals actually form is in the filtered fluid in the lumens of the tubules and not necessarily those associated with the oxalate-damaged cells. These crystals may, however, grow to such sizes that secondary mechanical damage is caused.

Apart from the kidney, high oxalate levels also occur in the blood vessels of the rumen and here extensive deposition of crystals in the walls of small arteries of the submucosa with thrombosis results in severe diphtheritic inflammation and invasion with secondary microorganisms. The vasculature damage, again, precedes the crystal deposition (Van Kampen and James, 1969).

In tissue culture, it has been shown that certain chemical changes occur in the renal tubular cells once exposed to the oxalates. One of these is to produce phosphatidylserine which would normally recruit macrophages to remove damaged cells. However, in cases of toxicity, the latter appears to promote the attachment of oxalates to the cell membrane. Other changes induced in the cell, include the activation of phospholipase $A_2$ that eventually lead to the release of bioactive lipids that alter mitochondrial function, activate caspases and result in apoptosis (Kohjimoto *et al.*, 1999; Cao *et al.*, 2004; Jonassen *et al.*, 2005).

A third pathway may also be present as calcium oxalate induces lipid peroxidation in both renal cell cultures and rodents (Maroni *et al.*, 2005). The latter was deduced from an increase in malondialdehyde (Malini *et al.*, 2000; Thamilselvan *et al.*, 2003) and changes in the redox index (Farooq *et al.*, 2006) following the exposure of the renal tubules to oxalates. It has been deduced that the compromised cells, undergo apoptosis or necrosis as a result and subsequently slough off into the mucosa to form the nidus over which further crystals may precipitate (Jonassen *et al.*, 2005).

# INFLUENCE ON SERUM ELECTROLYTES

## Hypocalcaemia

This occurs in all species exposed to intake of plants containing high soluble oxalates. With oxalic acid and acid oxalate ion (Figure 69.1) poisoning, it is responsive to

treatment with calcium borogluconate but with oxalate ions not (James, 1972a; Cheeke, 1998). The soluble oxalates, including oxalic acid, are free to enter into the circulation and combine with calcium to result in hypocalcaemia similar to that seen with eclampsia in the bitch (Rekhis, 1994). In a review article, James (1972b), points out that it is unlikely that hypocalcaemia is the principal cause of death as there is proof that hypocalcaemia due to EDTA infusion or dialysis, does not result in mortality.

In foals the exposure to *Rumex* spp. resulted in signs of hypocalcaemia (Laan *et al.*, 2000). In people and animals the exposure to oxalates has been associated with muscle paralysis which may be related to the decrease in serum calcium (Benitez *et al.*, 1991).

In addition, hypocalcaemia may also result in a decreased blood-clotting time, as calcium is vitally important in the blood-clotting cascade (Steyn, 1934; Kingsbury, 1964).

## Hypomagnesaemia

Wilson and Wilson (1961) suggest that oxalates from a series of oxalate-producing fungi (Table 69.1) in spoiled hay, may produce frank hypomagnesaemia ("grass tetany") in cattle. An alarming fact is that such hay may not even appear obviously moldy. This is certainly an aspect to consider.

Hypomagnesaemia would imply low intracellular Mg too (*vide supra*).

## Nutrient deficiency

Nutrient deficiencies ascribed to oxalates have long been known in people (von Unruh *et al.*, 2004). This occurs from the ability of the oxalates to bind to various minerals such as calcium, iron and magnesium. By binding to these minerals in the plants, their bioavailability is decreased (Quinteros *et al.*, 2003). James *et al*. (1968) found that low levels of *Halogeton glomeratus* had a deleterious influence on nutrient balance in sheep. Additionally oxalates can also decrease the absorption of calcium from other dietary sources such as milk. In one study in ponies fed on a diet rich in oxalate it was shown that calcium absorption is decreased in animals as well. This increase in calcium excretion occurs only from dietary sources. For this trial the radio-tagged $Ca^{2+}$ circulating in the blood, following intravenous (IV) administration, did not end up in the faeces and was excreted by the kidneys. It was also noted that magnesium reten-tion by the animals was decreased while overall the plasma calcium and phosphorous levels were unchanged (Swartzman et al., 1978). With long-term exposure to plants with oxalates (such as various grasses, *vide* Table 69.1) horses will mobilize large amounts of calcium from the bony appetite and this will eventually precipitate O*steodystrophia fibrosa* (*vide infra*) (Walthall and McKenzie, 1976).

## Kidney and bladder stones

Together with silicates, calcium oxalate plays a major role in the formation of kidney and bladder stones of livestock, 75% of such stones consisting of calcium oxalate (Libert and Franceschi, 1987; Chai and Liebman, 2005).

# TOXICITY SYNDROMES

Two *acute syndromes* occur: death in 8–12 h due to *oxalate ion* poisoning (as in *Halogeton* intoxication) or death due to, arguably, *hypocalcaemia* in *acid oxalate* and *oxalic acid* poisoning (as in *Oxalis* and *Rumex* intoxication).

Subacute to chronic poisoning occurs only with the latter.

## Oxalate ion toxicity

Distinction between the two ion-dependent syndromes is not always specified in the literature as the specific ion involved and the pH of the plant sap are not specified, for example that of *Halogeton* in the Chenopodiaceae is specified but that of beet and white goosefoot (of the same family *vide* Table 69.1) is not. The position in, or example, Amaranthaceae, Agavaceae and Cactaceae (Table 69.1) is not known.

In the case of *Mesembryanthemum nodiflorum* of Western Australia the pH of the plant sap is also 6, and the active ion there would thus be oxalate. The same acute syndrome as with *Halogeton* is experienced and poisoning with *Mesembryanthemum crystallinum* in Australia (J.G. Allen, Perth, personal communication, 2006).

In South Africa mesems of the Aizoaceae are widely distributed in our western, semi-desert areas and although they are known to contain toxic quantities of oxalates (Steyn, 1934) they are so widely occurring that stock (especially sheep and goats) just consume it in small quantities as part of their daily diet and frank toxicity like in Western Australia is not seen (D.J. Schneider, S.A. Stellenbosch, personal communication, 2006).

*Setaria sphacelata*, with an acidic sap, on the contrary, is believed to contain ammonium oxalate (Douglas and Birch, 1967, cited by James *et al.*, 1971). In Australia it causes the typical acute syndrome in sheep due to the oxalate ion (James *et al.*, 1971).

According to Burrows and Tyrl (2001) the Chenopodiaceae contain both the oxalate and the acid oxalate ions as toxic principles and they point out that there are

subtle differences in the syndromes caused by *Halogeton glomeratus* and other soluble oxalate-containing plants.

### Sheep

Under circumstances where sheep have grazed *Halogeton* extensively, up to 1200 have been poisoned at a time and under many conditions 100–800 sheep have died (James, 1972a). *Halogeton*, as summarized by Burrows and Tyrl (2001), affects mainly sheep and to a lesser extent cattle. They summarize the literature with the following: ". . . sheep within a few hours (exhibit) dullness, head held low, anorexia, white froth from mouth; shortly, weakness, stiffness (and) rapid respiration sets in (as well as) ataxia (animals becoming) comatose with extensive jerky extensor rigidity".

### Cattle

Burrows and Tyrl (2001) describe that the clinical signs in cattle are similar to those of sheep but more subacute: incoordination, apprehension, belligerence, excess salivation, recumbency, coma, bloat, cyanosis and death. Subacute locomotor disturbances are mainly evident when animals are forced to move and start with the forelimbs. In calves, however, more severe signs of hypersensitivity to stimulation and seizures are possible.

These signs are typically associated with hypocalcaemia (as low as 1.4 mg/dl) during which time blood magnesium and phosphorus may double.

In one incident in South Africa cited in Kellerman *et al.* (2005) 4 out of 40 nursing beef cows developed acute flaccid paralysis 24 h after introduction to a harvested wheat land heavily infested with the chenopodiaeceous *Chenopodium album* (white goosefoot). The paralysis was so marked that the investigating veterinarian (E. Albertyn, S.A. Winburg, personal communication, 2006) suspected botulism and sent the owner to Onderstepoort for antitoxin. Fortunately, he also sent the heavily grazed weed with. After identification and advice to use calcium borogluconate IV, he used MFC ("Merial", calcium borogluconate: magnesium hypophosphite (20%:4%) (Swan, 2005/2006) to which he had added 15 g $MgSO_4$/500 ml IV (a practice he had been using for years with recalcitrant "milk fever" with total recovery). Despite the warning that the four cows should preferably be slaughtered as eventual kidney lesions were still a probability, he specifically kept track of the four animals and informs that they had experienced total recover. The plant contained 16% oxalate (total) on dry matter basis.

According to Shupe and James (1967) *Halogeton* is a highly toxic to sheep and produces an acute syndrome where animals die within 8–12 h of exposure, rarely any longer and no chronic syndrome is seen. The clinical signs are dullness, lowered heads, anorexia and ruminal stasis, blood-tinged frothing from the mouth, weakness, stiffness, polypnea, ataxia with jerky extensor rigidity and coma and death with sheep lying dead where they had grazed.

At necropsy marked edema and hemorrhages are encountered in the rumen which is caused by severe rumenitis with vascular damage. Kidneys are pale edematous and enlarged and histopathology reveals hyalinization of the glomeruli and marked tubular dilation. Calcium oxalate crystals are encountered in both the ruminal wall around the damaged vasculature as well as in the kidneys.

Van Kampen and James (1969) determined the pathogenesis by dosing a group of 12 sheep with a known lethal dose of *Halogeton*, sacrificing two animals every 2 h and studying the sequential development of the lesion in relation to the deposition of calcium (and magnesium oxalate?) crystals. It is clear that the deposition of crystals is secondary to cellular damage and not *vice versa*, although secondary mechanical damage also occurs. Some sheep died peracutely with insignificant morphologic kidney lesions and even in sheep with severe kidney damage, death occurred too rapidly to be attributed to renal dysfunction. They state that sheep with a bilateral nephrectomy live longer than those with acute oxalate intoxication.

They maintain that in *Halogeton* poisoning the serum calcium is within the range where tetany should occur but it does not. Signs preceding death are in fact extreme weakness and flaccidity of all skeletal muscles. Calcium borogluconate is the standard treatment for most oxalate plant intoxications but in *Halogeton* in sheep it may delay death but not necessarily result in survival. Van Kampen and James (1969) postulate that intracellular inactivation of Ca- and Mg-dependent enzyme systems may be significant in causing death (*vide supra*).

An interesting, aberrant syndrome is reported by James (1972a, b) with *Bassia actinophylla* of the Chenopodiaceae where on an oxalate basis the lethal dose for sheep is about one-half of that of *Halogeton*. The signs resembled those of *Halogeton* but there was a greater tendency to develop tetany and incoordination and less than half the lethal amount fed per day resulted in a cumulative effect and mortality. Compared to *Halogeton* this plant was higher in potassium and lower in sodium suggesting a difference due to cations.

The effect of acutely toxic doses of the different cations of oxalate fed daily by rumen fistula to unadapted sheep was reported by James (1972a, b). In a preliminary experiment prior to testing the adaptation of sheep to *Halogeton* he stated that sodium oxalate at *ca.* 25–45 g oxalic acid equivalent on the first day and 40–74 g on the last day, killed three sheeps in 3–6 days after they had been off feed all the time. Diarrhea and severe edema and hyperemia of the rumen wall were evident. Two sheep dosed with potassium oxalate at *ca.* 25 and 42 g on the first day and 56 and 67 g oxalic acid equivalent on the last day took

6 days to die and they were only slightly off feed for the last 3 days. There was less diarrhea and the effect on the rumen wall was less severe.

Magnesium oxalate, on the contrary, at 70.6 g oxalic acid equivalent dosed to one sheep for 1 day caused only diarrhea.

If the oxalate ion is in fact the toxic substance, these differences in cations are difficult to explain.

## Oxalic acid and acid oxalates

The nephrotoxic syndrome occurring here is basically the same as with predominantly the oxalate ion (*vide supra*) but seems to be delayed: it develops slower, is subacute with mortalities and signs more typically a day or more after exposure. Chronic toxicity also occurs.

### Species-specific toxicity

#### *Sheep*

According to Panciera *et al.* (1990), acute exposure to *Rumex crispus* may result through calcium deficiency in sudden death or in animals showing severe clinical signs such as depression, salivation, coarse head tremors and stilted, ataxic gait and recumbency. When excited some animals become severely ataxic, fall and struggled to arise. Generally, clinical signs observed in sheep are polypnea, dyspnea, anorexia, dullness and depression and sometimes muscle fasciculation, tremor, loss of coordination, teeth grinding, pulmonary edema, tetany, seizures, recumbency, prostration and death (Panciera *et al.*, 1990). In animals affected with the more chronic form of the disease may show signs of azotemia and hypocalcaemia on clinical pathology.

Littledike *et al.* (1976) describe virtually the same changes in acute *Halogeton* poisoning of sheep.

On necropsy animals show acute renal tubular necrosis. The kidneys are pale swollen and moist. On histopathology the predominant lesion is nephrosis and is characterized by the widespread dilation of the convoluted and collecting tubules in the cortex. Although necrosis of the tubular epithelium is rarely seen, the tubular epithelium is generally flat and appears degenerate, particularly where the crystals impact the tubular walls. Birefringent crystals are also observed in the mucosa of the abomasums (Panciera *et al.*, 1990).

Chronic toxicity has also been reported to occur in sheep. Animals demonstrated clinical signs over 2–12 months, the principal clinical signs being anemia and loss of condition and appetite. On postmortem the kidneys were half the normal size and weight and were pale and mottled. In animals that were anemic, the hearts were enlarged (McIntosh, 1972).

#### *Cattle*

In cattle clinical sings reported included catarrhal abomasitis, enteritis, pale edematous kidneys and congested lungs (Dickie *et al.*, 1978). According to Walthall and McKenzie (1976), affected cattle on oxalate-containing pasture grasses (probably ammonium oxalate), much like sheep, tend to show signs of depression, anorexia and diarrhea. On pathology the kidneys are pale and firm. On cut surface the renal medulla is thin and the calyces dilated (confirmed histopathologically). Cortical and medullary tubules distended with crystalline casts. The crystals were typical in appearance of birefringence under polarized light. The crystals were specifically stained with Pizzolato's technique (peroxide–silver staining) with which they appeared brown to black.

Although the origin of the oxalates was not known, Gopal *et al.* (1978) were of the opinion that it may also play a role in abortions and possibly teratogenicity. In one random survey on pre- and perinatal mortalities of cattle, 56 of 142 dead calves had oxalate crystals on kidney sections. Although the presence of the crystals might have been incidental, the occurrence was higher in the calves showing a variety of congenital disorders. The fetal presence of crystals was believed to originate from the cow and therefore suggested that oxalate can cross the placenta. It was also believed that exposure to higher concentrations may have caused the teratogenicity seen in some of the calves.

In their sequential sacrificing experiments (*vide supra*) Shupe and James (1967), however, found no crystalline deposits in the tissues of fetuses of ewes that had been pregnant during exposure to *Halogeton*.

#### *Congenital primary hyperoxaluria in Beefmasters*

It needs to be mentioned that the presence of oxalate crystals in cattle may not be due to oxalates from plants only. Rhyan *et al.* (1992) describes this condition in the above breed in the United States in the absence of oxalates of plant origin. The general term is used for an inherited metabolic disorder that results in early death by renal failure due to a recessive inherited metabolic disorder.

#### *Horses*

Both acute and chronic toxicity can occur in horses. For experimental acute toxicity to occur, animals need to be exposed to a high dose of 454 g of either Na-, K- or ammonium oxalate. This toxicity is characterized by hypocalcaemia and will result in muscle rigidity and a stiff gait (Laan *et al.*, 2000). Non-fatal toxicity occurs when animals are exposed to 200 g of oxalic acid/day for 8 days (Andrews, 1971).

Chronic poisoning, following 2–8 months of exposure, to oxalate-containing grasses resulted in nutritional secondary hyperparathyroidism (NSHP) or *osteodystrophia fibrosa.* Clinical signs observed in these animals were lameness, ill thrift (harsh coats, loss of condition) and in some animals, swelling of the osseous structure of the head. Mildly affected horses showed a decreased ability to work while the more severely affected animals became cachectic and even died. The swelling to the head was bilateral and involved the nasal bones or the maxillae. At necropsy there was swelling of the maxillae and mandibular rami. Fibrous proliferation occurred and extended from the original cortices of the bone. Histopathologically a decrease in osseous tissue could be confirmed. Fibrous tissue surrounded small fragments of old bone in which sites of osteoclastic activity were detected (Walthall and McKenzie, 1976).

Nephrotoxicity has also been reported. The clinical signs are anorexia and gradual weight loss. Although the kidney appeared normal, on histopathology the renal cortex contained dilated tubules lined with flattened or degenerated epithelial cells. Tubular structures were displaced by fibrous tissue while the glomeruli had undergone various degrees of degeneration. Crystals were present in the tubules, particularly the proximal convoluted tubule (PCT) and were yellowish brown and aggregated into rosettes having radial symmetry. The crystals were anisotropic under polarized light (Andrews, 1971).

### *Pigs*

This species has a strongly acidic gastric pH (like the horse) but only one incident of oxalate poisoning in 1932 is on record where acute poisoning was reported in pigs following the consumption of beet. There is, however, uncertainty of the correct diagnosis as beet also contains nitrates and a "brown discoloration of the blood" is also mentioned (Rupprecht, 1932). Baxter (1956) describes fodder beet poisoning in pigs but the signs were not those of oxalosis but he cites that Gregor (1953) found oxalic acid in toxic amounts in sugar-beet tops.

Although Beasley (1999) states that convulsions may also occur with this in pigs, one would have expected more reports of intoxication in this exceptionally widely farmed commodity and species (especially where they are farmed free range) if oxalosis was indeed a problem in swine.

### *Dogs*

The predominant form of oxalate poisoning in dogs is due to consumption of ethylene glycol (antifreeze). Although no confirmed instance of poisoning due to plant oxalates were found, it must be considered as a possibility (Stowe and Fangmann, 1975).

### *Fowls*

It is hardly likely that chickens, as other farm stock, will be as exposed to oxalate poisoning as other farm stock. However, Williams and Olsen (1992), during an investigation into the contribution of each of a mixture of miserotoxin, nitrates and oxalate involved in a particular syndrome, established the $LD_{50}$ of sodium oxalate for 1-week-old chicks to be 984 mg/kg.

### *Humans*

This fatal nephrotoxic syndrome also occurs in people. Cooked rhubarb stems owe their pleasant, acrid taste to its oxalic acid content. During World War I, shortage of greens in Britain lead to rhubarb leaves (where the concentration of soluble oxalates is much higher than the stems) being widely advocated as a substitute for spinach and other greens until several deaths due to renal damage were attributed to it (Ellenhorn and Barceloux, 1988).

Acute fatalities due to soup containing 500 g of *Rumex crispus* in an adult man within 4 h and that of a 4-year-old child who had eaten an unspecified quantity of rhubarb leaves within 1½ h, are recorded (Tallqvist and Vaananen, 1960 and Farre *et al.*, 1989 cited by Ellenhorn *et al.*, 1997).

## TREATMENT

In Animals suffering from a decrease in calcium and showing signs of eclampsia or of muscle stiffness may be treated with IV calcium (Laan *et al.*, 2000; Kellerman *et al.*, 2005). Following the development of acute clinical signs, oral dosing with lime water/milk or calcium lactate followed later by an emetic (in appropriate species) may be helpful. The rationale is to bind the unabsorbed oxalates to calcium in the GIT and remove it from the system. Activated charcoal may also be used. In animals already convulsing, the use of emetics (where applicable) is not recommended. In these animals, the plasma calcium levels should be monitored and treatment with calcium borogluconate instituted.

Although this is not practicable in the veterinary situation it must be noted that heat treatment diminishes the oxalate content of green vegetables considerably. In one study using beans, chickpeas and lentils it was shown that standard cooking on a hot plate could reduce oxalate content by 0–40%, while microwave cooking reduced oxalate content from 60% to 85% and industrial cooking reduced oxalate content from 80% to 92% (Quinteros *et al.*, 2003). The fatalities in humans after consuming rhubarb

leaves (*vide supra*) was most probably due to insufficient heat treatment.

## ADAPTATION

It has been shown that the constant exposure of rumen microorganism, i.e. *Oxalobacter formigenes*, can handle the large concentration of oxalates. In one study goats were adapted to a concentration of 0.6 mmol oxalic acid by exposing the animals to equal increases over a 5-day period, and maintenance over 2 days prior to receiving a ration high in oxalate concentrations. Exposure was obtained by dosing the animals with gelatin capsules filled with oxalic acid. This study indicated that artificial exposure of ruminants to oxalates can create adequate microorganism adaptation to allow an animal to cope on high oxalate-containing plants. Alternatively, the same may be achieved by gradually exposing animals artificially to the plants that contain oxalates (Frutos *et al.*, 1998). With *Halogeton* James (1972a, b) experimentally determined that sheep were 5–10 times more resistant after 8–25 days' feeding and they advise an adaptation period of 10 days prior to known field exposure which would increase the lethal dose by some 30%.

## PROPHYLAXIS

Dicalcium phosphate has been recommended to reduce the likelihood of *Halogeton* toxicity when mixed into the ration at a ratio of 1:3 with salt. Alternatively, 5% dicalcium phosphate-containing alfalfa pellets should be fed at a rate of 100 g to 2 kg (3–5 lb) per sheep per day (Beasley, 1999). The purpose of this is to have all the soluble oxalates precipitated in the rumen.

## CONCLUSION

Fatal soluble oxalate poisoning is caused by exposure of unadapted ruminants to a large intake of plants high in oxalates. Due to occasional shortage of feed or other unforeseen circumstances it may be difficult to avoid such incidents. The fact that apparently good quality hay may be contaminated with oxalate-producing fungi may also be discovered too late.

When horses graze pastures containing grass species high in oxalates precautionary measures should be taken to prevent *osteodystrophia fibrosa*.

## POSSIBLE FUTURE RESEARCH DIRECTIONS

### The role of Ca and Mg at cellular level on enzyme activity and the pathogenesis of the oxalate syndrome

Practically all authors on oxalates, *vide supra*, refer to tetany as a result of hypocalcaemia in ruminants. In addition the serum Mg levels reported are also exceptionally variable between authors.

Radostits *et al.* (1999) refer to the production of tetany in cows infused with Ca-EDTA and regard this as a suitable model for experimental production of hypocalcaemia. It must, however, be pointed out that EDTA complexes both Ca and Mg (although the latter less strongly) and, therefore, the tetany may have been produced by a combination of both hypocalcaemia and hypomagnesaemia.

In contrast, Findlay (1998) describes uncomplicated hypocalcaemia ("milk fever") in the cow and ewe as a flaccid paralytic condition and that tetany (signs of "grass staggers") may be seen briefly only if concurrent hypomagnesaemia is present. This is in stark contrast to non-ruminants where eclampsia in the bitch and the queen results in excitement and tetanic muscular activity. In would indeed be interesting to know how often the magnesium status has been assessed in small animal "eclampsia".

In contrast to calcium, magnesium homeostasis is not regulated by a hormonal feedback system and is simply dependent on inflow and outflow across membranes (Martens and Schweigel, 2000). The analytical techniques of determining serum magnesium levels are often reported to be unreliable with only the referenced atomic absorption method atomic absorption and the enzymatic methods giving consistently reliable results. However, this is cumbersome and difficult to set up and calibrate. Photometric or colorimetric techniques are unreliable as they are influenced by a number of factors, including the calcium level and bilirubin (Saur *et al.*, 1994; Prof (Emeritus) Reyers, Specialist Clinical Pathologist, Pretoria: personal communication, 2006).

### Triterpenes

In one study it has been shown that rodents treated with triterpenes had reduced formation of urinary calculi. With the plants' diuretic effect, it causes a decrease in the accumulation of calcium in the renal tubular environment decrease the super-saturation typical in cases of stone formation. Since the triterpenes are also known to be anti-oxidant in nature, they have the ability to decrease

the peroxidative injury seen with calcium oxalate stone formation (Malini *et al.*, 2000). This must, however, be weighed up against their potential toxicity.

### Thiazide diuretics

In dogs suffering from calcium oxalate urolithiasis, surgical and medical treatment has been used to treat animals. Although surgery is in most cases required, the thiazide diuretics have been beneficial as they decrease the overall calcium excretion by the kidneys thereby reduces the calcium available to bind the oxalic acid. Added benefits arise from treating the animal with potassium citrate. In addition to alkalinizing the urine and rendering oxalic acid more soluble, calcium shows an increased tendency to bind to citrate and thereby forms a more soluble complex than with oxalic acid. As such in very valuable animals showing early signs of renal pathology, it may be possible to initiate clinical therapy to modulate the degree of nephrotoxicity (Booth, 2001).

### Speciation of oxalates

The exact speciation of soluble oxalate ions in the various plants involved in toxicosis seems to be directly related to the pH of the plant sap. This could be of assistance in explaining or even predicting the outcome of intoxication.

### Inclusion of Mg with Ca borogluconate as standard treatment of oxalosis

In view of the fact that magnesium (*vide supra*) is also complexed with oxalate and is unavailable at cellular level as a co-factor for essential enzyme activity, surely indicates experimental investigation of supplementation in intoxicated individuals.

### Role of Mg in Ca oxalate crystals

There does not appear to be any analyses of the contribution of magnesium (although slightly more soluble) to the well-known calcium oxalate crystals characteristic of this syndrome. These crystals can be readily collected as described by James *et al.* (1971).

### Pathogenesis of oxalic acid and acid oxalate poisoning

As the mechanism of intoxication with the oxalate ion, e.g. *Halogeton,* has been determined and it was proved by Van Kampen and James (1969) that cellular damage precedes calcium oxalate deposition, it should be determined if this is indeed the case with oxalic acid and the acid oxalate ion too.

### Greater toxicity of the oxalate ion

The reason why the oxalate ion appears to be more toxic than the acid oxalate ion and oxalic acid (Figure 69.1) to at least ruminants is intriguing. The intracellular body pH is around 7 and for oxalic acid the $pK_1 = 1.27$ and the $pK_2 = 4.28$ (Budavari, 1996) exists that the double negative ion (of which a 0.1 M solution in water has a neutral pH) will bind with $Mg^{2+}$ and $Ca^{2+}$ more readily and forthwith than the acid oxalate ion (0.1 M solution in water pH 2.7) and oxalic acid (0.1 M solution in water, pH 1.3 (Budavari, 1996)). In the rumen (pH 6.5) both these should, however, be rapidly be changed to the oxalate ion. The fact that the cations ($Na^+$, $K^+$ and $NH_4^+$) of the soluble oxalates may differ, should not be a factor either as they will be fully ionized in the rumen.

### The role of cations of oxalates

At present there are scientific apparent inexplicable differences in toxicity whether oxalate is bound to $Na^+$, $K^+$ or $NH_4^+$ (James, 1972a, b). This should be followed up. In addition as far as could be ascertained, potassium acid oxalate has not yet been used to simulate *Rumex* and *Oxalis* poisoning. It is interesting that the Merck Index lists the potassium salt (potassium binoxalate or "sal acetosella" [salt of *Rumex acetosella*]) but not the sodium acid oxalate (Budavari, 1996).

## CONCLUSION

In conclusion, we would like to echo the 30-year-old words of James (1972a,b), a scientist who has published most extensively in this field: "Oxalate poisoning remains a complex and poorly understood phenomenon". There is apparently as much dissimilarity as similarity among the effects of the different types of oxalates on the different species of animals.

# PART II: POISONING BY INSOLUBLE CALCIUM OXALATE RAPHIDES

## BACKGROUND

Calcium oxalate is a common substance found in some 250 species of plants. Since the calcium oxalate crystals

accounts for the majority of calcium in the plant it is possible that it is found in all plants (Franceschi and Nakata, 2005). Its exact function within the plant is at present unknown. It has been speculated that these substances may play a role in protection from chemicals produced by other plants, protection from herbivores/insects and perhaps it is the plant's natural calcium sink (Jauregui-Zuniga *et al.*, 2005; Korth *et al.*, 2006; Weir *et al.*, 2006).

Insoluble calcium oxalate is formed in plants by the simple combination of calcium and oxalic acid in the endogenous environment. The crystals can form in a number of different shapes which range from prismatic crystals (rhomboid), large elongate rectangular styloids, bundles of needle (acicular) raphide crystals, druse crystals or small angular crystals known as crystal sand (Webb, 1999; Nakata, 2002; Franceschi and Nakata, 2005). Of these only the raphide crystals (presumably formed as an antiherbivor maneuver) are of toxicological significance. At present the exact formation of the crystals in raphides is unknown (Volk *et al.*, 2004; Franceschi and Nakata, 2005; Xu *et al.*, 2006).

Members of the family Araceae are widely grown for either their flowers or for their particularly attractive foliage, e.g. *Diefenbachia* spp. (dumb cane), *Colocasia* spp. (elephant's ear), *Phillodendron* spp. (sweetheart vine) and *Monstera* spp. (delicious monster). They are also widely cultivated for culinary purposes of the starch-rich tuberous rhizomes or as green vegetables, e.g. *Colocasia esculenta* (cocojam, taro) and *Xanthosoma* (new cocojam, okumo). The whole family is known to have irritant properties on the buccal mucous membrane when the fresh plants are eaten or even just chewed and the syndrome is a common occurrence in humans and occasionally in animals (Frohne and Pfänder, 1983). Plants intended for eating, must, therefore, be cooked or baked beforehand to render them non-irritant (Purseglove, 1972).

The irritant properties are due to insoluble calcium oxalate raphides (needle-sharp crystals, 250 μm). They are formed and located in special vacuoles in microscopic lemon-shaped ideoblasts, (explosive ejector cells with an operculum) in the epidermis of these plants. The exact complex mechanism by which they are formed is not known (Franceschi and Nakata, 2005). The raphides are packed together lengthwise in a gelatinous mass and if the plant is damaged the operculum of the ideoblast is dislodged, sap of the plant or saliva causes the gelatinous mass to swell and the needles are expelled under the pressure (Frohne and Pfänder, 1983). Cheeke (1998) describes it: "They ... (the crystals) ... emerge like bullets one at a time, with sufficient force to cause the cell to recoil like a gun and (this) goes on for many minutes".

If the plant is chewed, prior to being swallowed, masses of these raphides penetrate the mucous membrane of the mouth causing alarming, severe (but usually transient) local irritation and clinical signs such as excessive salivation in people (Gardner, 1994; Chiou *et al.*, 1997; Salinas *et al.*, 2001) and even laryngospasm. Salivation, gagging, colic, bloody diarrhoea, depression, prostration and sometimes death are known to occur in animals (Knight and Walter, 2003) but the latter is rare.

This phenomenon can be experienced by taking a single experimental bite of the stem or leaf of one of these plants: after a latent period of *ca.* 30 s it results in this transient, somewhat uncomfortable sensation which may last up to 30 min.

Occasional intoxication in sheep kept in paddocks with no grazing and force-fed only, or mainly, these plants pruned in gardens, is seen (Kellerman *et al.*, 2005).

An extraordinary incident in a black rhino is reported by Wood *et al.* (1997). In a game reserve in Zimbabwe, an orphan black rhino (*Diceros bicornis*) calf of 12 months started chewing half a leaf of an ornamental araceous *Xanthosoma mafaffa* (elephant's ear, new cocoyam) in the conservation officer's garden but promptly spat it out. Irritation was manifested by severe salivation, flicking her tongue and rubbing her mouth for an extended period in mud. She tried to browse after a while but spat out the food. These signs lasted for 3–4 h and then she made an uneventful recovery.

The black rhino eats various *Euphorbia* spp. with impunity (Loutit *et al.*, 1987). The latter has severely caustic, ingenol- and phorbol-containing latex which results in serious blistering of the mouth and even skin in humans (Watt and Breyer-Brandwijk, 1962) and domestic stock (Kellerman *et al.*, 2005). It is amazing that the relatively mild calcium oxalate raphide irritation described above, caused such marked clinical signs in a rhino.

Burrows and Tyrl (2001) described that in *Tragia* spp. (noseburns) of the Euphorbiaceae, the stinging hair of this unobtrusive creeper contains a calcium oxalate raphide up to 4 mm long which on contact, penetrates the skin allowing the highly irritant proteinaceous contents to enter the small wound causing a transient pruritis very similar to but less severe than that caused by the well-known stinging nettles of the Urticaceae.

In summary, in the veterinary situation, Ca-oxalate raphide intoxication is rare but it is prudent to be aware of it in coming to a diagnosis of sudden acute mouth irritation in humans and diverse species of animals.

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## REFERENCES

Absan SK (1997) Metabolism of magnesium in health and disease. *J Indian Med Assoc* **95**: 507–10.

Albihn PB, Savage GP (2001) The bioavailability of oxalate from Oca (*Oxalis tuber ose*). *J Urol* **166**: 420–2.

Andrews EJ (1971) Oxalate nephropathy in a horse. *J Am Vet Med Assoc* **159**: 49–52.

Argenzio RA, Liacos JA, Allison ML (1988) Intestinal oxalate-degrading bacteria reduce oxalate absorption and toxicity in guinea pigs. *J Nutr* **118**: 787–92.

Baxter JT (1956) Suspected fodder-beet poisoning in pigs. *Vet Rec* **68**: 236–7.

Beasley V (1999) Nephrotoxic plants. In *Veterinary Toxicology*, 1st edn, Beasly V (ed.). IVIS, Ithaca, NY.

Benitez MA, Navarro E, Feria M, Trujillo J, Boada J (1991) Pharmacological study of the muscle paralyzing activity if the juice of the banana trunk. *Toxicon* **29**: 511–55.

Booth DM (2001) Drugs affecting the kidney and urination. In *Small Animal Clinical Pharmacology and Toxicology*, Booth DM (ed.). W.B. Saunders Co., Philadelphia, PA, pp. 515–27.

Brogren M, Savage GO (2003) Bioavailability of soluble oxalates from spinach eaten with and without milk products. *Asia Pacific J Clin Nutr* **12**: 219–24.

Budavari S (1996) *The Merck Index*, 12th edn. Merck & Co, Whitehouse Station, NJ.

Burrows GE, Tyrl RJ (2001) *Toxic Plants of North America*. Iowa State University Press, Ames, IA.

Cao L, Honeyman TW, Cooney R, Kennington L, Scheid CR, Jonassen JA (2004) Mitochondrial dysfunction is a primary event in renal cell oxalate toxicity. *Kidney Int* **66**: 1890–900.

Chai W, Liebman M (2005) Effect of different cooking methods on vegetable oxalate content. *J Agric Food Chem* **53**: 3027–30.

Cheeke PR (1995) Endogenous toxins and mycotoxins in forage grasses and their effects on livestock. *J Anim Sci* **73**: 909–18.

Cheeke PR (1998) *Natural Toxicants in Feeds, Forages and Poisonous Plants*, 2nd edn. Interstate Publishers, Danville, IL.

Chiou AG, Cadez R, Bohnke M (1997) Diagnosis of *Dieffenbachia* induced corneal injury by confocal microscopy. *Br J Ophthalmol* **81**: 168.

Corley RA, Meek ME, Carney EW (2005) Mode of action: oxalate crystal-induced renal tubule degeneration and glycolic acid-induced dysmorphogenesis – renal and developmental effects of ethylene glycol. *Crit Rev Toxicol* **35**: 691–702.

Dickie CW, Hamann MH, Carrol WD, Chow F (1978) Oxalate (*Rumex venosus*) poisoning in Cattle. *J Am Vet Med Assoc* **1**: 73–4.

Ellenhorn MJ, Barceloux DG (1988) *Medical Toxicology*: *Diagnosis and Treatment of Human Poisoning*. Elsevier, New York.

Ellenhorn MJ, Schonwald S, Ordog G, Wassenberg J (1997) *Ellenhorn's Medical Toxicology*: *Diagnosis and Treatment of Human Poisoning*, 2nd edn. Williams & Wilkens, Baltimore, MD.

Farooq SM, Ebrahim AS, Subramhanya KH, Sakthivel R, Rajesh NG, Varalakshmi P (2006) Oxalate mediated nephronal impairment and its inhibition by C-phycocyanin: a study in urolithic rats. *Mol Cell Biochem* **284**: 95–101.

Findlay ARL (1998) Veterinary Physiology, University of Cambridge, Available online: http://www.chu.cam.ac.uk/~ ALRF/calcium.htm

Franceschi VR, Nakata PA (2005) Calcium oxalate in plants: formation and function. *Annu Rev Plant Biol* **56**: 41–71.

Frohne D, Pfänder H (1983) *A Colour Atlas of Poisonous Plants.* Wolfe Publishing Co, London.

Frutos P, Duncan AJ, Kyriazakis I, Gordon IJ (1998) Learned aversion towards oxalic acid containing food by goats: does rumen adaptation to oxalic acid influence diet choice. *J Chem Ecol* **24**: 383–97.

Gardner DG (1994) Injury to the oral mucous membranes caused by the common houseplant, dieffenbachia. A review. *Ora Surg Oral Med Oral Pathol* **78**: 631–3.

Gopal T, Leipold HW, Cook JE (1978) Renal oxalosis in neonatal calves. *Vet Pathol* **15**: 519–24.

Gregor A (1953) Rye-grass staggers. Rape poisoning. Feeding of beet tops. Dietetic haematuria. Selenium poisoning. *Proc Conf Metab Disord Brit Vet Ass Publ* **23**: 132–6.

James LF (1972a) Oxalate toxicosis. *Clin Toxicol* **52**: 231–43.

James LF (1972b) Oxalate poisoning of livestock. In *Effects of Poisonous Plants on Livestock*, Keeler RF, Van Kampen KR (eds). Academic Press, New York.

James MP, Seawright AA, Steele DP (1971) Experimental acute ammonium oxalate poisoning of sheep. *Aust Vet J* **47**: 9–17.

Jauregui-Zuniga D, Ferrer MA, Calderon AA, Munoz R, Moreno A (2005) Heavy metal stress reduces the deposition of calcium oxalate crystals in leaves of Phaseolus vulgaris. *J Plant Physiol* **162**: 1183–7.

Jonassen JA, Kohjimoto Y, Scheid CR, Schmidt M (2005) Oxalate toxicity in renal cells. *Urol Res* **33**: 329–39.

Kellerman TS, Coetzer JAW, Naudè TW, Botha CJ (2005) *Plant Poisonings and Mycotoxicoses of Livestock in Southern Africa*, 2nd edn. Oxford University Press, Cape Town.

Kingsbury JM (1964) *Poisonous Plants of the United States and Canada.* Prentice-Hall Inc, Englewood Cliffs, NJ.

Knight AP, Walter RG (2003) Plants affecting the digestive system. In *A Guide to Poisonings of Animals in North America*, 1st edn, Beasley V (ed.). IVIS, Ithaca, NY.

Kohjimoto Y, Kennington L, Scheid CS, Honeyman TW (1999) Role of phospholipase A2 in the cytotoxic effects of oxalate in cultured renal epithelial cells. *Kidney Int* **56**: 1432–41.

Korth KL, Doege SG, Park S, Goggin FL, Wang Q, Gomez SK, Liu G, Jia L, Nakata PA (2006) *Medicago truncatula* mutants demonstrate the role of plant calcium oxalate crystals as an effective defense against chewing insects. *Plant Physiol* **1104**: 706–737.

Laan TTJM, Spoorenberg JFM, van der Kolk JH (2000) Hypocalcaemie bij een vier weken oud veulen. *Tijdschr Diergeneeskd* **125**: 185–7.

Libert B, Franceschi R (1987) Oxalate in crop plants. *J Agric Food Chem* **35**: 926–38.

Littledike ET, James L, Cook H (1976) Oxalate (Halogeton) poisoning of sheep: certain physiopathologic changes. *Am J Vet Res* **37**: 661–6.

Loutit BD, Louw GN, Seely MK (1987) First approximation of food preferences and the chemical composition of the desert-dwelling black rhinoceros. *Madoqua* **15**: 35–54.

Malini MM, Lenin M, Varalakshmi P (2000) Protective effect of triterpenes on calcium oxalate crystal induced perioxidative changes in experimental urolithiasis. *Pharmacol Res* **41**: 413–18.

Maroni PD, Koul S, Chandhoke PS, Meacham RB, Koul HK (2005) Oxalate toxicity in cultured mouse inner medullary collecting duct cells. *J Urol* **174**: 757–60.

Martens H, Schweigel M (2000) Pathophysiology of grass tetany and other hypomagnesaemia. Implication for clinical management. *Vet Clin North Am Food Anim Pract* **16**: 339–68.

McIntosh GH (1972) Chronic oxalate poisoning in sheep. *Aust Vet J* **48**: 535.

Nakata PA (2002) Calcium oxalate crystal morphology. *Trend Plant Sci* **7**: 324.

Panciera RJ, Martin T, Burrows GE, Taylor DS, Rice LE (1990) Acute oxalate poisoning attributable to ingestion of curly dock (*Rumex crispus*) in sheep. *J Am Vet Med Assoc* **12**: 1981–4.

Phillis JW (1976) Motility and secretions of the various regions of the alimentary tract. In *Veterinary Physiology*, Phillis JW (ed.). Wright-Scientechnica, Bristol.

Purseglove JW (1972) *Tropical Crops: Monocotyledons*. Longmans Group, London.

Quinteros A, Farre R, Ladarda MJ (2003) Effect of cooking on oxalate content of pulses using an enzymatic procedure. *Int J Food Sci Nutr* **54**: 373–7.

Radostits OM, Gay CG, Blood DC, Hinchcliff KW (eds) (1999) Metabolic diseases chapter 28. Hypocalcaemia. *Veterinary Medicine. A Textbook of the Diseases of Cattle, Sheep, Pigs, Goats and Horses*, 9th edn. WB Saunders, New York, Chapter 28, pp. 1424–52.

Rekhis J (1994) The poisonous plant *Oxalis cernua*. *Vet Hum Toxicol* **36**: 23.

Rhyan JC, Sartin EA, Powers RD, Wolfe WD, Dowling PM, Spano JS (1992) Severe renal oxalosis in five young Beefmaster calves. *J Am Vet Med Assoc* **201**: 1907–10.

Rupprecht K (1932) Beet poisoning in Swine. *Wien Teirarztl Mschr* **19**: 557–8.

Salinas ML, Ogura T, Soffchi L (2001) Irritant contact dermatitis caused by needle-like calcium oxalate crystals, raphides, in *Agave tequilana* among workers in tequila distilleries and agave plantations. *Contact Dermatitis*, **44**(2): 94–96.

Saur PM, Zielmann S, Roth A, Frank L, Warneke G, Ensink FB, Radke A (1994) Comparison of the determination of magnesium by methylthymol blue spectrophotometry and atomic absorption spectrophotometry. *Eur J Clin Chem Clin Biochem* **32**(7): 539–42.

Seawright AA (1989) Distilleries and agave plantations. In *Animal Health in Australia. Chemical and Plant Poisons*, 2nd edn. *Contact Derm* **44**: 94–6.

Shupe JL, James LF (1967) Additional physiopathologic changes in *Halogeton glomeratus* (oxalate) poisoning in sheep. *Cornell Vet* **59**: 41–55.

Steyn DG (1934) *The Toxicology of Plants in South Africa*. Central News Agency, South Africa.

Stowe CM, Fangmann G (1975) Schefflera toxicosis in a dog. *J Am Vet Med Assoc* **167**: 74.

Swan G (ed.) (2005/2006) *IVS Desk Reference* **9**: 359.

Swartzman JA, Hintz HF, Schryver HF (1978) Inhibition of calcium absorption in ponies fed diets containing oxalic acid. *Am J Vet Res* **39**: 1621–3.

Thamilselvan S, Khan SR, Menon M (2003) Oxalate and calcium oxalate mediated free radical toxicity in renal epithelial cells: effect of antioxidants. *Urol Res* **31**: 3–9.

Van Kampen KR, James LF (1969) Acute halogeton poisoning of sheep: pathogenesis of lesions. *Am J Vet Res* **30**: 1779–83.

Volk GM, Goss LJ, Franceschi VR (2004) Calcium channels are involved in calcium oxalate crystal formation in specialized cells of *Pistia stratiotes*. *Ann Bot (London)* **93**: 741–53.

von Unruh GH, Voss S, Sauerbruch T, Hesse A (2004) Dependence of oxalate absorption of daily calcium intake. *J Am Soc Neph* **15**: 1567–73.

Walthall JC, McKenzie RA (1976) Osteodystrophia fibrosa in horses at pasture in Queensland: field and laboratory observations. *Aust Vet J* **52**: 11–16.

Watt JM, Breyer-Brandwijk MG (1962) *The Medicinal and Poisonous Plants of Southern and Eastern Africa.* E.S. Livingstone, Edinburgh.

Webb MA (1999) Cell-mediated crystallization of calcium oxalate in plants. *Plant Cell* **11**: 751–61.

Weir TL, Bais HP, Stull VJ, Callaway RM, Thelen GC, Ridenour WM, Bhamidi S, Stermitz FR, Vivanco JM (2006) Oxalate contributes to the resistance of *Gaillardia grandiflora* and *Lupinus sericeus* to a phytotoxin produced by *Centaurea maculosa*. *Planta* **233**: 785–795.

Williams MC, Olsen JD (1992) Toxicity to chicks of combinations of miserotoxin, nitrate, selenium and soluble oxalate. In *Poisonous Plants: Proceedings of the Third International Symposium*, James LF, Keeler RF, Bailey EM, Cheeke PR, Hegarty MP (eds). Iowa State University Press, Ames, IA.

Wilson BJ, Wilson CH (1961) Oxalate formation in moldy feedstuffs as a possible factor in livestock disease. *Am J Vet Res* **91**: 961–8.

Wood PA, Foggin DC, Naudé TW (1997) Suspected calcium oxalate raphide irritation in a black rhino (*Diceros bicornis*) due to ingestion of *Xanthosoma mafaffa*. *J S Afr Vet Assoc* **68**: 2.

Xu HW, Ji XM, He ZH, Shi WP, Zhu GH, Niu JK, Li BS, Peng XX (2006) Oxalate accumulation and regulation is independent of glycolate oxidase in rice leaves. *J Exp Bot* **57**(9): 1899–1908.

Zhiqiang C, Zhangqun YE, Lingqi Z, Weimin Y (2003) Clinical investigation on gastric oxalate absorption. *Chin Med J* **116**: 1749–51.

CHAPTER

# 70

# *Datura* spp. and other related plants

*Theuns W. Naudé*

## INTRODUCTION

*Datura* appears to be the main (species) genus involved in poisoning by tropane alkaloids (hyoscyamine, scopolamine and atropine). Although *Datura* poisoning is possible in most animal species, it is particularly a practical problem in equines. The reason for this is that equines are particularly sensitive to atropine and related tropane alkaloids. Being hindgut fermenters, the alkaloids are absorbed as the contaminated feed passes through the stomach. A paralytic ileus results with consequent death (from acute complications like torsion, strangulation or tympany) or recalcitrant impaction colic may develop. Swines appear to be also rather susceptible.

Being annuals, *Datura* weeds, particularly *D. stramonium* and *D. ferox*, invade and contaminate especially annual crops like corn (maize), sorghum, soybeans and linseed. The weeds ripen with the crop and the weed seed contaminates the finally harvested grain. With annual grass crops, such as tef, the equally toxic immature weeds are cut and dried with the hay. Other plants that contain tropane alkaloids usually only cause incidental poisoning in livestock.

## BACKGROUND

The family Solanaceae contains several genera that are toxic due to the tropane alkaloids atropine and scopolamine. The genera *Atropa* (belladonna), *Hyoscymus* (henbane), *Brugmansia* (angel's trumpet) and *Datura* (jimsonweed) are all of toxicological importance (Burrows and Tyrl, 2001). They can all cause incidental poisoning but only the genus *Datura* appears to be of veterinary significance.

**FIGURE 70.1** *Datura stramonium* L. (jimsonweed, common thorn apple) (courtesy South African National Biodiversity Research Institute (SANBRI), Pretoria, South Africa).

*Datura stramonium* L. (jimsonweed, common thorn apple) (Figure 70.1) and *D. ferox* L. (large thorn apple) (Figure 70.2) probably originate from the Tropical Americas (Henderson, 2001). Although the latter occurs in the United States

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**FIGURE 70.2** *Datura ferox* L. (large thorn apple) (courtesy SANBRI, Pretoria, South Africa).

**FIGURE 70.3** *Datura subspecies innoxia* C Mill. subspecies *innoxia* (*D. meteloides* Dunal) (downy thorn apple) (courtesy SANBRI, Pretoria, South Africa).

(Burrows and Tyrl, 2001) no distribution map for this species and the extent to which it occurs there is given. Both these spp. now have a cosmopolitan distribution and are annual pioneer weeds on wasteland and are particularly troublesome, serious invaders on fertilized soil with annual crops (Henderson, 2001) all over the world, thus their significance concerning livestock.

These plants have malodorous leaves (common name "stink leaf") and spiny fruit capsules by which the two species can be readily identified. *D. ferox* and *D. stramonium* both have erect, ovoid capsules with the longest spines in the former. They have erect, funnel shaped flowers, white in *D. ferox* and white, mauve or purplish ones in *D. stramonium*. The stems are green except that a purple-stemmed variety occurs with the latter (this, on drying e.g. in hay, turns brownish). The seeds of the two species are black, kidney shaped and flat with a characteristically pitted exterior (Henderson and Anderson, 1966) and the seed of the two species cannot be differentiated morphologically from each other. The seeds of *D. ferox* are, however, larger (average weight 14.1 mg ($n = 6 \times 1$ g)) than those of *D. stramonium* (average weight 6.6 mg (also $n = 6 \times 1$ g)) (SS de Kock, L Jonker, National Horse Racing Authority of South Africa) and TW Naudé (Veterinary Faculty at Onderstepoort, unpublished data, 2004).

A third species *D. innoxia* C Mill. subspecies *innoxia* [=*D. meteloides* Dunal] (downy thorn apple) (Figure 70.3), is primarily an invader of waste areas and sandy river banks and beds but may also occur in cultivated areas. It produces large, tubular, white flowers but in contrast to the glabrous previous two species this species is softly, grey-velvety on all parts. Furthermore it is differentiated by a pendulous, globose fruit capsule densely covered with small spines. Its seeds are of similar shape and size as those of *D. ferox* but are brown (Henderson, 2001). As it is quoted in literature on *Datura* spp., it is included here.

The problem with the seed of *D. ferox* and *D. stramonium* (no mention in the literature about the seed of *D. innoxia* was found) is that as the plants grow more rapidly than the crops they invade and, therefore, cannot be effectively controlled by mechanical means, the fruit ripen concurrently with those of the planted crop, e.g. corn (or maize) and are harvested at the same time. The seeds, therefore, occur admixed in the threshed corn seed. Their specific gravity is approximately the same as the corn and unless sifting is done in single layers it is not separated from the corn. It is, therefore, often a contaminant of

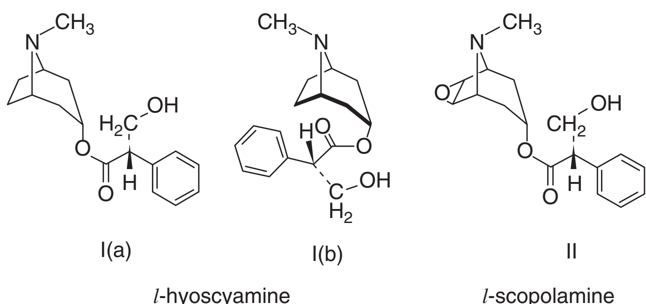


**FIGURE 70.4.1** (I) structures of hyoscyamine (conventional, (a) and stereo-specific (b) and (II) scopolamine (conventional).

grains such as corn, sorghum, soybeans and linseed. All counties have strict legislation about the number of *Datura* seeds allowable in grain for human consumption. Grain rejected for this reason usually finds its way into stock feed and further sifting to clear such seed for human consumption often results in the heavily contaminated siftings being used as stock feed (Kellerman *et al*., 2005).

## STRUCTURE OF TROPANE ALKALOID MUSCARINIC RECEPTOR ANTAGONISTS

These alkaloids basically consist of a bicyclic seven member tropane alkaloidal ring esterified through its hydroxyl group at C3 with the carboxyl group of L-tropic acid. (Figures 70.4.1 and 70.4.2).

The best known drug in this group in veterinary practice is atropine (a racemate of *d,l*-hyoscyamine) of which practically only the one isomer, l-hyoscyamine (Figures 70.4.1 and 70.4.2), is anti-muscarinic (Adams, 2001; Brown and Taylor, 2001).This is an ester of tropine and *l*-tropic acid (Figure 70.4.2). In the plant it occurs as this active isomer, *l*-hyoscyamine, but during commercial extraction it partially racemizes and is then further chemically converted to the stable racemate, atropine (Budavari, 1996). Most atropine is currently synthesized and, consequently, is the racemate (R. Smith, Phytex, Australia, personal communication, 2006). In this chapter the *l*-isomer will be referred to as hyoscine in contrast to atropine (which is *d,l*-hyoscyamine), which has only half the antimuslarinic potency of *l*-hyoscyamine. Its pharmacological potency in plant material is thus double that of atropine as used therapeutically.

Scopolamine (hyoscine) is similarly an ester of scopine and *l*-tropic acid (Figures 70.4.1 and 70.4.2). It is hardly, if ever, used in veterinary medicine but is well known in medical circles as a pre-anesthetic. Due to its potency as a hallucinogen, it has even been used at 1.5 mg/70 kg man as

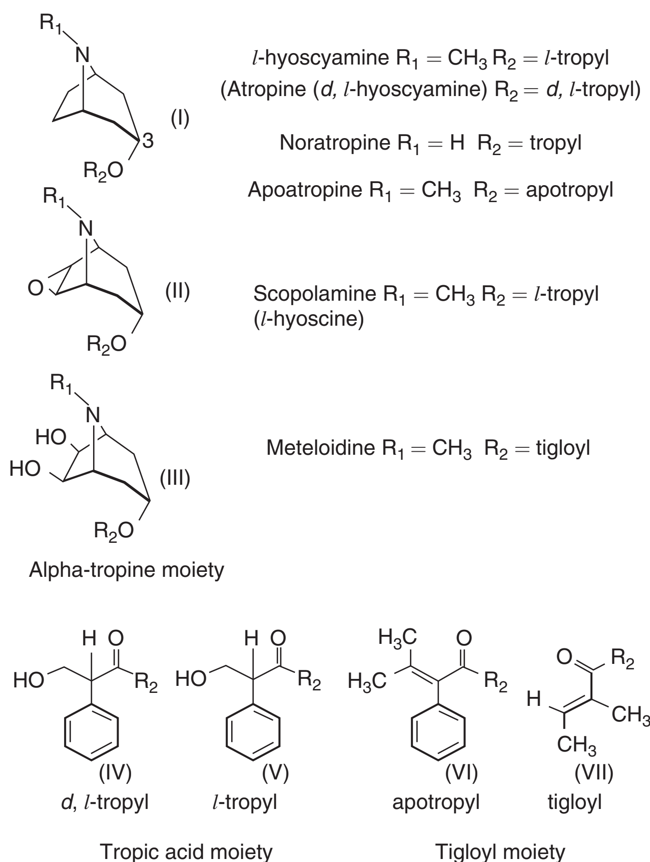


**FIGURE 70.4.2** Relationship between relevant tropane alkaloids.

an incapacitating agent in chemical warfare. It has now been replaced by an extremely potent, structurally related compound, 3-quinuclidinyl benzilate (BZ or QNB) which is 3-fold more potent than scopolamine (Ketchum and Sidell, 1997).

Hyoscyamine and scopolamine are the major toxic components of *D. stramonium* (Watt and Breyer-Brandwijk, 1962) and predominantly scopolamine that of *D. ferox* (Springhall and Seawright, 1972; cited by Everist, 1974). Also refer to Table 70.1.

## STRUCTURE–ACTIVITY RELATIONSHIP

The pharmacological anti-muscarinic action of atropine and scopolamine are both dependent on the presence and optical configuration of the levorotatory tropic acid moiety. The intact ester bond free hydroxyl group on the acyl portion is essential (Brown and Taylor, 2001). Furthermore the tropic acid moiety must be attached alpha to, and thus below, the plane of the tropane ring (Figure 70.4.1b).

**TABLE 70.1 Hyoscyamine and scopolamine determinations on South African weed – *Datura* spp.**

| *Datura* spp. and part | Alkaloid concentrations (μg/g) | | | | Ratio |
|---|---|---|---|---|---|
| | Hyoscyamine | | Scopolamine | | Scopolamine/ hyoscyamine. |
| | Average | Range | Average | Range | |
| *Datura stramonium* | | | | | |
| Young plants | | | | | |
| Purple stem | 1046 | 531–2291 | 1063 | 296–2844 | 1.01 |
| Green stem | 587 | 491–742 | 525 | 249–1155 | 0.89 |
| Seed | 557 | 273–908 | 587 | 254–800 | 1.1 |
| *Datura ferox* | | | | | |
| Young plants | 68 | 0–149 | 1048 | 82–2541 | 15.4 |
| Seed | 7.5 | 2–19 | 766 | 218–956 | 102 |
| *Datura innoxia* | | | | | |
| Young plants | 360 | 175–462 | 790 | 692–869 | 2.2 |
| Seed | 297 | 127–524 | 454 | 104–815 | 1.85 |

Apoatropine (Figure 70.4.2) differs from atropine only in having an apotropyl acid moiety at C3. Its acute toxicity to mice is, however, 180 times that of atropine by intraperitoneal injection and it is convulsive (Krantz *et al.*, 1954). Kobert (1905, cited by Krantz *et al.*, 1954) found it only mildly mydriatic and the latter confirmed mydriasis and vagal block at as little as 2 mg/kg i.v. in dogs. In addition, severe cardiac depression was observed. However, it has about the same potency as atropine in relaxing barium-induced spasm of rabbit intestine but was 40 times less effective in reducing intestinal motility in the rat (Krantz *et al.*, 1954).

In summary the replacement of the optically active tropyl group of atropine with an inactive apotropyl group does not abolish but definitely diminishes the anti-muscarinic potency of atropine.

No pharmacological or toxicological data could be obtained on meteloidine, found at significant concentration, and the other related tropane alkaloids (Figure 70.4.2) found at low concentrations in plant material (*vide infra*).

## BIOSYNTHESIS IN PLANTS AND METABOLISM IN MAMMALS

The biosynthesis of tropane alkaloids by plants is succinctly summarized by Dräger (2004). An ubiquitous 4-carbon diamine (putrescine) is methylated and enzymatically transformed into the bicyclic, seven-membered alkaloid, tropinone. The latter is enzymatically converted to either alpha tropine, the precursor of the tropane alkaloids under discussion (Figure 70.4.2) or into its stereo-isomer, beta pseudotropine, from which the polyhydroxylated glycosidase inhibitors, the calystegines, originate.

The intact esters of the tropane alkaloids (Figure 70.4.2) are essential for anti-muscarinic activity. Atropine esterases occur to varying extent in the livers of different animals and detoxify it by hydrolysis. It is interesting that the resistance to tropane alkaloid poisoning by guinea pigs and certain strains of the rabbit, where metabolic polymorphism occurs, is ascribed to high enzyme liver activity in these species. In addition, the enzyme detoxifies exclusively the *l*-isomers and has no activity in relation to scopolamine (Bernheim and Bernheim, 1938). In contrast, Wada *et al.* (1991), however, found in *in vivo* experiments that scopolamine is indeed hydrolyzed by this esterase in the guinea pig and certain strains of the rabbit but not in the rat and only to a minor extent in the mouse. According to Brown and Taylor (2001) hepatic metabolism accounts for the elimination of about half the dose of atropine in man and the remainder is excreted unchanged in the urine. No metabolism data are available for livestock (Adams, 2001).

## MECHANISM OF ACTION

Muscarinic receptor antagonists compete with acetylcholine (ACh) for a common binding site on the muscarinic receptors and thus block the action of ACh at muscarinic neuroeffector sites on smooth and cardiac muscle, gland cells, in peripheral ganglia and in the central nervous system (CNS). In general they cause little blockade at nicotinic receptor sites (Brown and Taylor, 2001).

## SIGNS OF INTOXICATION

In man, atropine at therapeutic doses has virtually no detectable effect on the CNS whereas scopolamine, which more readily crosses the blood–brain barrier, causes CNS

depression manifested as drowsiness, amnesia and fatigue. Euphoria is also encountered. The acute toxic effects commonly seen with atropine are mydriasis and cycloplegia, dry mucous membranes and tachycardia. At higher doses central nervous excitation becomes more prominent resulting in restlessness, irritability, disorientation, hallucination and delirium. At still larger doses, stimulation is followed by depression leading to circulatory and respiratory failure after a period of paralysis and coma (Brown and Taylor, 2001). Although humans are very susceptible to poisoning, atropine has an exceptionally wide therapeutic index in man, fatalities being rare even in instances of gross over dosage or poisoning. *Datura* poisoning, especially following ingestion of the seed, is often seen in man (Watt and Breyer-Brandwijk, 1962; Brown and Taylor, 2001).

In the horse, additionally, the motility of the large intestine is seriously affected and either an acute paralytic ileus results with consequent death from acute complications like torsion, strangulation or tympany, or in more chronic cases recalcitrant impaction colic develops (Naudé *et al.*, 2005).

## EXTRACTION, ISOLATION AND QUANTIFICATION OF TROPANE ALKALOIDS: SURVEY ON SOUTH AFRICAN WEED–*DATURA* SPECIES

As no such survey data seem to be available and as the procedure used may be universally applicable (or can be adapted), the basic technique employed by SS de Kock, L Jonker (National Horse Racing Authority) and TW Naudé (Faculty of Veterinary Science, Onderstepoort) (unpublished data, 2002), for this purpose, is given.

The standard procedure was used: after extracting the alkaloids in the dry, finely milled plant material into an aqueous acid medium, the latter was alkalinized and the alkaloids back-extracted in methylene chloride. Gas chromatography (GC) isolation was found to be less sensitive, less compound- specific and produced artifacts, thus high pressure liquid chromatography (HPLC) isolation was used in preference. This isolation entailed the use of a gradient of water and methanol both containing 10 mM ammonium formate and a C18 reverse phase column. LC/MS/MS was undertaken on a Thermo Finnigan LCQ ion trap instrument operated in positive ion mode with atmospheric pressure chemical ionization (APCI). Full scan LC/MS was undertaken for alkaloid presence and target MS/MS analyses were on the $[M + H]^+$ (protonated molecular ion) entities observed with this mild ionization technique. For hyoscyamine this corresponds to $m/z$ 290 and for scopolamine to $m/z$ 304.

The one standard used was analytically pure atropine sulphate (racemic *d,l*-hyoscyamine). These two diastereoisomers cannot be differentiated unless a chyral column is used. In the plant only the antimuscarinic *l*-hyoscyamine is produced. Atropine, if dosed or injected, thus has only half the potency of the plant's alkaloid.

The other standard was *l*-scopolamine.

## RESULTS OF *DATURA* SURVEY IN SOUTH AFRICA

The results of tropane alkaloid analyses on vegetative material and the seed of various species of *Datura* are summarized in Table 70.1.

Note the great variations in alkaloid concentrations between individual plants of the same species as reflected by the highest and lowest values found for that particular species. Note further that the vegetative parts of the purple-stemmed variety of *D. stramonium* had approximately twice the alkaloid content of the botanically identical green-stemmed variety. Selected plants were also investigated by LC/MS/MS for the presence of tropane alkaloids other than hyoscyamine and scopolamine. These included the compounds meteloidine, noratropine and apoatropine (Fig. 70.4.2).

Meteloidine was the most significant of these alkaloids, in many cases exceeding the levels of atropine and scopolamine. Noratropine and apoatropine levels were generally very similar at about 5% that observed for hyoscyamine.

## TOXICITY TO LIVESTOCK

### Equines

*Datura* poisoning, especially following ingestion of the seed, is often seen in man (Brown and Taylor, 2001). In animals it is rare and the only cases of significance have been reported in the horse although the pig is regarded as rather susceptible to tropane alkaloid poisoning. The serious consequences of gastrointestinal complications are, however, only mentioned in equines (Adams, 1995). Three field outbreaks occurred after ingestion of grain heavily contaminated with *Datura* seed (Barney and Wilson, 1963; Williams and Scott, 1984; Schulman and Bolton, 1998). The clinical signs and course of intoxication varied rather widely.

In the first case, 15 animals from a group of 34 were affected and 11 died, 2 as long as 6 days after withdrawal of the incriminated feed, cracked maize containing "an unusually large number" of jimsonweed seeds. The signs recorded were anorexia, hyper excitability, staggers, muscular spasms, frequent urination and mydriasis with

impaired vision progressing to convulsive seizures, rigor and coma preceding death. There is no mention of gastrointestinal complications. In the case reported by Williams and Scott (1984) two horses were fed a meal containing 0.5% *D. stramonium* seed. They exhibited depression, mydriasis, anorexia, tachycardia, polydipsia and polyuria, fever and brown, fetid diarrhea. Both recovered after supportive treatment. In the last case (Schulman and Bolton, 1998) one horse died from acute gastric dilation and rupture whereas the second had to be euthanized due to unresponsive paralytic ileus. As this had occurred in South Africa, it is assumed that the seed involved was probably a mixture of that of *D. stramonium* and *D. ferox*.

Only two cases of experimentally produced *Datura* intoxication were found. Barney and Wilson (1963), *vide supra*, also fed a Shetland pony two quarts (*ca.* 2.2 litres) of the cracked maize containing "an unusually large number of jimsonweed seeds" on a daily basis for an unspecified number of days. Inappetence, hyper excitability and mydriasis appeared on the 8th day. On the 10th day staggering and muscle spasms were exhibited and the animal died on the 10th day. Again the authors do not refer to any clinical signs associated with colic.

Following the discovery of scopolamine in the urine of horses post racing by Galey *et al*. (1996), it emerged that they had been on bedding containing *Datura*. Galey *et al*. (1996) dosed four adult mares with air-dried *D. meteloides* (*D. innoxia*) vegetative plant material containing *ca.* 550 mg scopolamine and 740 mg as atropine, thus (*vide supra*) 740 mg *l*-hyoscyamine/kg at 0.0125, 0.025, 0.1 and 0.5 g/kg. Clinical signs consisting of severe gastrointestinal atony, tachycardia, sweating and colic were seen at only the highest dose of 0.5 g/kg, equivalent to 0.275 mg scopolamine and 0.185 mg hyoscyamine/kg (in addition to the other unquantified related tropane alkaloids in the plant). Clinical signs were evident 2 h after dosing and had not resolved by 72 h after dosing. Urine alkaloid concentrations peaked within 1–2 h after dosing reaching the mg/ml range according to the graphs and at low dosages were mostly absent after 12–24 h. The $t_{1/2}$ of scopolamine and atropine (=hyoscyamine) were 1.7 and 2.3 h, respectively at the lowest dose.

Recently, two cases of *Datura* intoxication where the poisoning is attributed to dried vegetative plants in tef hay were described (Naudé *et al*., 2005; Gerber *et al*., 2006). The vegetative material was found to be as toxic as seed.

Atropine is widely used in veterinary practice especially for the treatment of poisoning by organophosphorus and carbamate cholinesterase inhibitors (Adams, 2001). However, even when used as a pre-anesthetic or mydriatic in the horse, the potential detrimental effects on intestinal activity should be considered (Roberts and Argenzio, 1986; Adams, 1995).

In the horse it is also used for the treatment of chronic obstructive pulmonary disease but with the proviso that it must be used with care as it may produce ileus and abdominal pain (Pearson and Rierbold, 1989). Similarly, Ducharme and Fubini (1983) found that the horse was especially sensitive to gastrointestinal atony on administration of atropine and concluded that it was contraindicated for the relief of intestinal spasm and may in fact lead to severe gastrointestinal complications. After experimental atropine i.v. injection of clinically normal ponies ($n = 5$) at 0.044 (=0.022 hyoscyamine) mg/kg and 2 weeks later again at 0.176 (=0.08 hyoscyamine) mg/kg, intestinal motility was decreased after 30 min and the horses had stopped eating, exhibited abdominal pain and were quiet and depressed at both dose levels. With the low dose they resumed eating after 1–2 h but after the higher dose, which also caused cecal distention, they only resumed eating 2–7.5 h later and intestinal motility had only gradually returned to normal by 12 h.

In an equine study on myoelectrical and mechanical intestinal activity in clinically normal ponies ($n = 4$), atropine at 0.044 (=0.022 hyoscyamine) mg/kg caused a marked decrease in jejunal and pelvic flexure motility for longer than 2 h and had increased pelvic flexure sphincter tone possibly indirectly by decreasing parasympathetic tone (Adams *et al*., 1984).

In man the gastrointestinal tract is, in contrast to the horse, rather insensitive to the effect of atropine. Only one report of paralytic ileus was traced and was due to protracted, excessively high, constant infusion of atropine (2600 mg over a 30-day period) to control excessive bronchial secretion after an organophosphorus suicide attempt (Beards *et al*., 1994). In contrast to man, scopolamine is hardly ever used in veterinary medicine (Adams, 2001) and in older textbooks is mentioned mainly as a soporific in man and the dog. It is stated that its use in horses appears to produce hallucination and excitability (Milks, 1949). Galey *et al*. (1996) administered scopolamine s.c. at 0.008 mg/kg to a horse. It caused transient sweating and tachycardia but not colic. No data are available on the dose of scopolamine which may result in colic. Its role and that of the other minor tropanes in *Datura* spp. can only be surmised to be additive and aggravating.

Apart from atropine, xylazine also decreases the myoelectrical and mechanical activity of the distal portion of the equine jejunum and the pelvic flexure of the colon (Adams *et al*., 1984) and fatal drug induced, refractory impaction colic in the equine can be related to the use of amitraz. These should be considered in the differential diagnoses of suspected tropane alkaloid poisoning.

### *A brief account of two recent outbreaks of* Datura *poisoning due to dry, young plants in hay is given below*

*Outbreak No. 1 (Naudé et al., 2005)*

Eighty-three riding horses were kept at an established, well-managed riding school. A spate of impaction colic, a

condition which had not been diagnosed there previously, suddenly occurred. Over a 6-week period 18 horses were affected of which 1 died. A new consignment of hay, received from the usual supplier had been fed to the horses. Impaction colic occurred in a total of 18 horses during the following 7 weeks several having up to three attacks. The attending veterinarian referred a number of affected animals to the Equine Clinic of the Faculty of Veterinary Science, Onderstepoort where a tentative diagnosis of atropine intoxication was made as some *Datura* plants were found in the hay submitted with the animals. Following an *in loco* inspection the suspect hay was withdrawn after it had been fed for 7 weeks. During the week following the withdrawal there were, however, still 11 cases of impaction colic. The withdrawal terminated the outbreak of colic. One horse, however, exhibited an abnormal *habitus* 6 days before withdrawal of the hay and was hospitalized for suspected colic but was discharged the following day. The same animal had to be readmitted 3 days after withdrawal of the suspect hay with severe, recalcitrant impaction colic and eventually had to be euthanized after 8 days, i.e. 11 days after the last possible exposure to toxic plant material. All other probable sources of *Datura,* except the hay were excluded.

**Physical examination of hay:** The two last consignments were examined for contaminating plants, six bales of each irrespective of whether it was *Eragrostis curvula* or tef were picked at random and the stable hands requested to collect all material other than grass for further inspection.

The second last batch was of poorer quality than the last. The following dried foreign plant material was identified: several maize stalks; stems of a large, hard grass and even tufts and roots of various veld grasses; numerous pieces of nut sedge and several broad-leafed weeds. Additionally *Datura* spp. were present and comprised some 30 small to large pieces of young, brownish stems (diameter 0.5–1 cm), which could initially not be identified as such. Fortunately, two of these stems with young fruit were present and were positively identified as *D. stramonium* by a botanist thus enabling probable identification of the remaining stems by comparison (Figure 70.5b).

The contamination was traced to the tef hay which was of inferior quality. On a second visit two bales were very carefully examined by gently taking it apart, separating layer by layer. This enabled recovery of three *Datura* plants. The young, extremely brittle, dried, pressed plants were intact and entire with leaves, of which one could, due to the presence of the typical young fruit, be positively identified as *D. ferox* (Figure 70.5a).

From the positive identification of *D. stramonium* from the previously inspected bales, the other two young botanically sterile plants appeared to be possibly *D. stramonium* (Figure 70.5c). Both the positively identified *D. stramonium* and *D. ferox* plants as well as the botanically

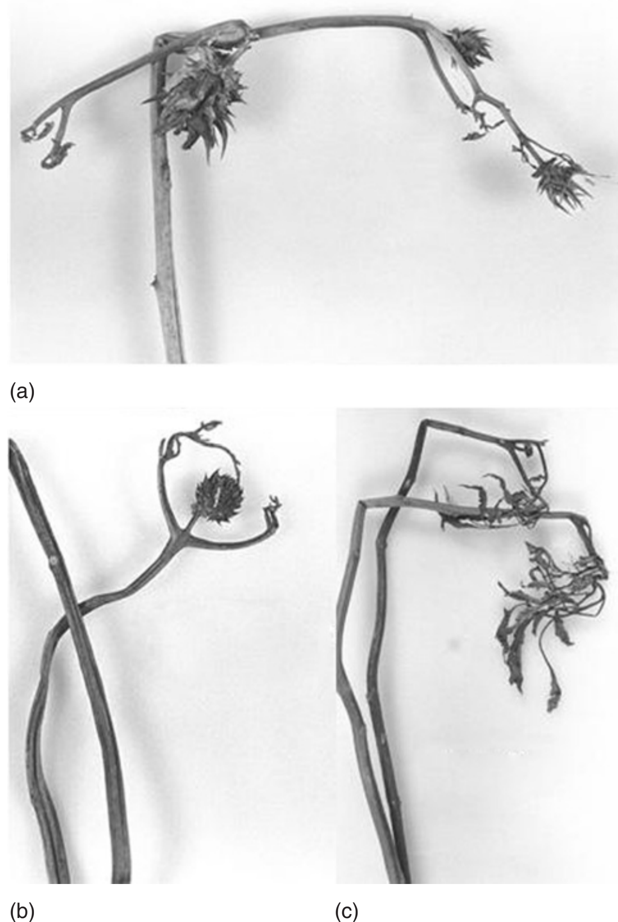


**FIGURE 70.5** Young plants from hay: botanically identifiable (a) *D. ferox*, (b) *D. stramonium* and (c) botanically sterile *Datura* spp.

sterile suspect entire plants taken from the hay were milled and chemically analyzed for tropane alkaloid content. In order to compare these analyses with that of the well-known toxic seed, material collected from waste areas as well as fertilized lands in the Onderstepoort vicinity were also subjected to chemical analysis for tropanes in Australia.

**Colic management and treatment:** Sixteen horses were referred to the Onderstepoort Equine Clinic over a period of 18 days. The standard tests for and treatment of impaction colic in horses (*vide infra)* were implemented. Apart from the impaction colic, and despite a tentative diagnosis of *Datura* poisoning, no other characteristic signs of parasympathetic blockade were observed. Treatment was symptomatic and administered according to the severity and extent of the signs of colic and rectal findings (*vide infra*). Neostigmine at 0.005 mg/kg s.c. was also given occasionally but with no dramatic effect. Treatment was continued until all signs of discomfort were absent and rectal examination revealed resolution of the impaction. Most of the animals needed repeated treatment over several days until all signs of discomfort were absent. Then tef and alfalfa

(lucerne) were reintroduced in small amounts several times a day and gradually increased. All responded well to treatment except for one which had undergone surgery for severe large colon impaction which was unresponsive to conservative treatment. Five days later it had to be euthanized due to recurrence of the same problem.

**Tropane alkaloid analysis:** This was done in Australia and the extraction and chromatographic procedure were more or less on the same lines as described (*vide supra*). The botanically identified vegetative *D. stramonium* from the hay contained respectively 1500 μg hyoscyamine and 1600 μg scopolamine/g. The identified *D. ferox* contained no hyoscyamine and 1100 μg scopolamine/g. The unknown vegetative young *Datura* plants contained 1400 μg hyoscyamine and 1200 μg scopolamine/g.

*Outbreak No. 2 (Gerber et al., 2006)*

A group of eight mares kept for student training at the Department of Theriogenology at the Veterinary Faculty of Onderstepoort had been kept on artificial pasture and were brought into a concrete paddock for a few days. Here they were fed cubed concentrate and tef hay and they had been in this paddock for 4 days when one developed acute, colic due to paralytic ileus. She exhibited severe, continuous pain and severe bilateral abdominal distention confirmed by rectal examination which revealed severe gas distention of the cecum and moderate distention of the large colon. She was unresponsive to treatment with xylazine and butorphanol and had to be euthanized for humane reasons. At necropsy the stomach was greatly distended with feed, the small intestine mildly fluid-distended and the cecum moderately and colon severely distended with gas and ingesta. A diagnosis of generalized gut atony was made. Eight days later (day 0) a mare from the same paddock and eating the same feed was admitted to the clinic after exhibiting moderate colic for a few hours. She only exhibited mild, bilateral abdominal distension, all other parameters being within normal limits. On rectal examination moderate gas distention of the cecum was evident. This mare, however, had severe bilateral mydriasis. She was treated symptomatically with flunixin and recovered from the colic without any further treatment. Mydriasis was, however, still very obvious the next day (day 1) and a moderate degree of mydriasis could be detected for the next 5 days. Urine was collected from her on days 1–3 and kept frozen until analyzed.

**Examination of the hay:** The hay being fed to the horses in the paddock was inspected on day 1. A piece of dried *Datura ferox,* identified by its characteristic, long-spined fruit capsule (Henderson, 2001), was found at the bottom of the metal trough in which the morning's tef hay had been eaten. The tef hay was immediately withdrawn and no further cases of colic developed in this group. Five bales of the tef hay from this particular consignment were subsequently carefully examined for any foreign material (*vide supra*) which revealed that this too was of poor quality with three containing *Datura* spp. One had one specimen of *D. ferox* with almost mature fruit, another contained a stem which could have been either *D. ferox* or *D. stramonium* and the last contained three specimens of *D. ferox* with fruit and numerous sterile specimens that could have been either species Specimens of the dried plant material identified as, or resembling *D. ferox*, were collected and analyzed for tropane alkaloids.

**Extraction, identification and quantification of tropane alkaloids:** Following overnight incubation of urine with β-glucuronidase to free the glucuronylated alkaloids, the acidified urine was once extracted with ethyl acetate and further like the plant extraction as described (*vide supra*). Extraction of plant material was done as described *(vide supra).* In the plant material the average concentrations of hyoscyamine and scopolamine were 18.5 and 1 553 μg/g, respectively. In the urine hyoscyamine was present at 0.4 ng/ml on Day 1 and barely detectable on day 2 whereas the scopolamine levels were 68, 5 and 0.4 ng/ml on respectively days 1–3.

## DISCUSSION ON BOTH CASES AND ON EQUINES IN GENERAL

Both incidents of intoxication were attributed to consignments of exceptionally poor quality *Datura* contaminated tef hay. Horses being fastidious feeders would normally avoid fresh *Datura* plants because of their offensive smell. However, when the plants are dry and contained in baled hay the smell largely dissipates. Additionally the dried young plants are brittle and disintegrate when the bales are opened and become so intermingled that the animals cannot avoid eating it. The young plants have, consequently, not been discovered in South Africa in contaminated hay in the past and the diagnosis not been confirmed despite strong evidence of suspected *Datura* poisoning.

The clinical signs encountered during the first outbreak were characterized by only gastrointestinal colic manifested as retarded motility. The usually expected signs of muscarinic receptor blockage, *inter alia* mydriasis, tachycardia, sweating and dry mucous membranes (Adams, 2001; Brown and Taylor, 2001) were not recorded at all. Furthermore, stimulatory CNS signs, e.g. what could be expected from hallucination, were absent and animals were, in fact, very docile. Repeated attacks on the farm were initially probably due to re-exposure to the contaminated hay. One horse, however, developed severe, recalcitrant

impaction colic 3 days after withdrawal of the hay and eventually had to be euthanized 11 days after the last possible exposure to *Datura.*

The spate of impaction colic of the first outbreak was, nevertheless, attributed to *Datura* intoxication as withdrawal of the contaminated hay, resulted in complete termination of its incidence. The case of serious colic that necessitated euthanasia 11 days after the last possible exposure to *Datura* contaminated hay was remarkable. A certain way to have confirmed the diagnosis in this outbreak would have been the determination of tropane alkaloid levels in the urine of affected horses. However, there was no access to this facility at that time.

In the second outbreak, apart from unresponsive gut atony, no other specific anti-muscarinic signs were noted in the first horse of this group that had to be euthanized due to unresponsive impaction colic. However, indications are that this mare had suffered from per acute *Datura* intoxication. Except for transient impaction colic, which responded well to conservative treatment, anti-muscarinic signs were indeed present in the second mare. The signs were, however, limited to a slight tachycardia, marked mydriasis for the first 2 days and mild mydriasis for 7 further days, a remarkably long period. In contrast to the first incident where colic kept on recurring even after the contaminated hay had been withdrawn, this mare's colic attack was very transient. This could possibly be linked to the fact that mainly scopolamine from predominantly *D. ferox*, as opposed to hyoscyamine and scopolamine from predominantly *D. stramonium* in the previous case could have been involved. The analytical method was so sensitive that hyoscyamine and scopolamine could be observed in urine specimens at even ng/ml concentrations. Using this methodology none of the other minor tropane alkaloids meteloidine, noratropine and apoatropine (Table 70.2) could be detected in the urine or in the *D. ferox* plant specimens. The tropane alkaloid analysis of this horse's urine as well as of the suspect plant material indicate that only (or mainly) *Datura ferox* might have been the cause of intoxication.

The fact that hyoscyamine was only detected in the urine of the horse at a very low concentration and ratio in comparison to scopolamine is in accordance with the ratio of these two alkaloids present in the leaves (arguably the main vegetative component ingested) of the plant. These levels and ratios are also in accordance with that data of the survey (Table 70.1) on *Datura ferox* occurring in South Africa.

The metabolism of anti-muscarinic tropanes varies greatly in different laboratory animals (*vide supra*) and comprehensive studies in various livestock species (*inter alia* the horse) do not appear to be available.

However, the administration to horses of *Datura innoxia* (*D. meteloides*) with a hyoscyamine:scopolamine ratio of *ca.* 1.5:1, resulted in urine concentration ratios of only *ca.* 1:10. Therefore, upon ingestion very little non-metabolized atropine (hyoscyamine?) is excreted in urine in comparison with comparable administration of scopolamine. Furthermore, Galey *et al*. (1996) found that both atropine (hyoscyamine?) ($t_{1/2} = 1.7\,\text{h}$) and scopolamine ($t_{1/2} = 2.3\,\text{h}$) were not present in the urine after 24 h in spite of the fact that one of the horses (administered the plant at 0.5 g/kg) had developed severe gastrointestinal atony, sweating and colic. This is in contrast to the present findings where, in this clinical case of *Datura* poisoning, scopolamine was present in the horse's urine at a level of 68 ng/ml 24 h after showing signs of intoxication and was still detectable (0.4 ng/ml) at 72 h. The fact that the mare still continued exhibiting mydriasis at a stage where the scopolamine was presumably depleted could indicate exceptional potency of these alkaloids due to very strong and long-acting receptor binding with the activity persisting even after the majority of the alkaloids had cleared the urine.

Galey and his associates (1996) are of opinion that tropanes behave erratically in the horse and any substantial exposure should be treated with extreme care. Despite the short half-life following low doses it seems that the residence time at higher levels may be prolonged and it is surmised that once atony sets in it seems to be intractable. This could explain why the one horse in the first incident had to be euthanized 11 days after the last possible exposure. It is, therefore, postulated that the horses during the first outbreak under discussion might have had just transient signs of mydriasis, etc., or that this was missed and that only the long-term effects on the gastrointestinal tract were noticed. In this incident both hyoscyamine

**TABLE 70.2 Tropane alkaloid profiles of selected *Datura* spp. plants and seed detected by LC/MS/MS**

| | Range of related tropane alkaloids (μg/g) detected in plants and seed | | | | |
|---|---|---|---|---|---|
| **Species** | **Hyoscyamine** | **Scopolamine** | **Meteloidine** | **Noratropine** | **Apoatropine** |
| *D. stramonium* | 336–800 | 226–1036 | 11–560 | ND–44 | 6–33 |
| *D. ferox* | 2–20 | 338–1800 | 1.4–321 | 0.8–31 | ND |
| *D. innoxia* | 175–393 | 384–845 | 72–590 | ND–22 | 3–24 |

ND: not detected.

and scopolamine were involved whereas in the second incident it was presumably mainly scopolamine from *D. ferox*.

During routine screening for drugs in thoroughbreds post racing (implying that they had been deemed fit for racing) by the laboratory of the South African National Horse Racing Authority (SS de Kock, personal observations, 2002) confirmed the observations by Galey *et al*. (1996). It was interesting that scopolamine had been detected in up to eight urine specimens annually for the past few years. In these specimens scopolamine was prominent at levels sometimes exceeding 100 ng/ml, most often with hyoscyamine also present, but at much lower concentrations. The presence of tropane alkaloids in apparently healthy thoroughbreds' urine suggests that feed or bedding (Galey *et al*., 1996) contamination with *Datura* may be more widespread than realized up to now. The etiology of many incidents of impaction colic are never ascertained and the possibly that it might be due to grain contamination with *Datura* seed or hay contamination with the plants, should be considered.

In the first outbreak (Naudé *et al*., 2005) affected animals were depressed and docile which is in contrast to the hyperexitability, muscular spasms, rigor and convulsive seizures described by Barney and Wilson (1963), but in accord with the signs described by Williams and Scott (1984) and Ducharme and Fubini (1983). Colic is the dominant sign described by other authors (Galey *et al*., 1996; Schulman and Bolton, 1998) and it is possible that the nervous signs mentioned by Barney and Wilson (1963) could have been due to per acute colic or even hallucination.

Galey *et al.* (1996) refer to atropine (*d,l*-hyoscyamine) and scopolamine found in *Datura innoxia*. As this alkaloid occurs as the levorotatory isomer in the plant, it may be assumed that they actually refer to *l*-hyoscyamine. The tropane alkaloid content (determined by GC/MS as the trimethylsilyl derivatives) of the plants dosed to horses in a paste was 370 mg atropine (probably as *l*-hyoscyamine) and 550 mg scopolamine/kg. They determined that only at 0.05% *Datura innoxia* plant material, colic was induced: "That horse developed gastrointestinal atonia, tachycardia, sweating and colic beginning within 2 h after dosing. Signs had not resolved by 72 h". This represents a dose of 0.185 mg atropine (probably *l*-hyoscyamine) and 0.225 mg scopolamine/kg. At 0.01% (equivalent to respectively 0.037 mg/kg L-hyoscyamine (atropine?) and 0.04 mg/kg scopolamine) there were no clinical signs of intoxication. However, scopolamine was detected in the urine of even the horse that had even received a dose as low as 0.00125% at more than 100 ng/ml after 1 h but it had disappeared all together at 10 h.

Ducharme and Fubini (1983) found atropine sulfate to cause colic at 0.044 mg/kg (equivalent to only 0.022 mg/kg of *l*-hyoscyamine) and marked atony lasting 12 h at a dose of 0.176 mg/kg. The latter would be equivalent to only 0.088 or *ca.* 0.1 mg *l*-hyoscyamine/kg. The toxic oral dose of hyoscyamine affecting gastrointestinal motility in the horse (not taking the possible additive effect of additional scopolamine into consideration), therefore, seems to be in the order of about 0.01–0.1 mg/kg (or 0.02–0.2 mg atropine/kg) if taken repeatedly.

In the first incident under discussion the dried *Datura* plants ingested by the horses contained an average of 1450 mg *l*-hyoscyamine and 1300 mg scopolamine/kg (*vide supra*). In terms of just *l*-hyoscyamine, the equivalent dose of plant material containing 0.1 mg of *l*-hyoscyamine/kg on a repeated basis could thus have resulted in intractable impaction colic would have been only 69 mg/kg (ca 70 mg/kg) or ca 0.007% body mass.

From a pharmacological viewpoint neostigmine appeared to be the drug of choice in hyoscyamine poisoning but it did not have any dramatic beneficial effect on the intestinal atony in the first outbreak. Mild cases receiving the drug recovered as well as those not receiving it. As the dose of neostigmine used in this case was slightly higher than that usually employed, the treated horses were walked after injection to obviate possible side effects. There were none and thus higher doses of neostigmine could possibly have been used (*vide infra*).

The *Datura* contamination was limited to the dry tef, hay in these two outbreaks. Tef is sown annually and *D. stramonium* and *D. ferox* are annual weeds and it must, consequently, be expected that tef (and any other annual grasses or first stands of perennial grasses or even alfalfa) would easily be contaminated with poisonous weeds. Tef should routinely be sprayed with broad-leaf herbicides a couple of weeks before the hay is cut in order to ensure freedom from noxious weeds (A. Lawrence, tef farmer, Greylingstad, South Africa – personal communication, 2002).

It is most difficult once *Datura* has been found in a few bales of hay of a particular consignment, to attempt to exactly quantify the extent of contamination. It is impractical to do a detailed, layer-by-layer examination of every bale. Problems may either be due to only one or two bales or most of the bales may be contaminated. Manual removal of all toxic plants in order to render contaminated bales safe is impractical. This procedure is both very labor intensive and, due to the brittle nature of the fragile dried young plants, impossible. Such hay should certainly not be used for equines. Provided the contamination is not gross there, however, appears to be only minor danger to ruminants fed such contaminated hay (Kellerman *et al*., 2005).

The danger of *Datura* seed to particularly equines (Kellerman *et al*., 2005) and man (Adams, 2001; Brown and Taylor, 2001) has always been recognized. References to the toxicity of vegetative parts of *Datura* to man is well-known (Steyn, 1934; Watt and Breyer-Brandwijk, 1962; Brown and Taylor, 2001) but have up to now, been largely ignored in relation to livestock as intoxication from it was not diagnosed.

# RUMINANTS

## Cattle

Fresh *Datura* is frequently grazed by ruminants in South Africa as part of their overall diet under grazing conditions (TW Naudé and TS Kellerman, Onderstepoort, personal observations past three decades). The vegetative part of the plant as such has, consequently, to date been regarded as of relatively minor toxicological importance (Kellerman *et al*., 2005). During the survey conducted in South Africa (Tale 70.1) single lush-growing specimens of both *D. ferox* and *D. stramonium* were collected in an overgrazed, well-fertilized area on a specific beef cattle farm. The plants contained, respectively, 149 and 260 μg/g hyoscyamine and 988 and 260 μg/g scopolamine. A few weeks later these 1–2 m high plants had been mowed down by the herd with no apparent deleterious effect (TW Naudé, personal observation, 2000).

An early report on jimsonweed toxicity in cattle is by Case (1956). A group of 7 hungry spring beef calves "mowed down" a patch of *D. stramonium* plants in a few hours: "They were dead within a week from . . . a textbook picture of poisoning by the Jimsonweed group . . . ". No data are, unfortunately, given on which to validate this statement. Burrows and Tyrl (2001) cites a Czechoslovakian incident reported by Ofukany *et al*. (1983) where mass intoxication of cows occurred on *D. stramonium* where out of 510 cows at risk, 44 died and 22 more had to be destroyed.

Nelson *et al*. (1982) exposed a control and 3 test groups ($n = 3$) of dairy type heifers to diets containing 0, 8.8, 880 and 4410 *D. stramonium* seeds/kg for 14 days. Only at the highest dose repeated cycles of marked anorexia, which abated after 2–3 days only to be repeated, occurred. During these spells bloat, dry muzzles, miosis (mydriasis?) and tenesmus were observed in two of the three heifers. It was deducted that the poisoning was self-limiting in that the anorexia allowed the heifers to metabolize the absorbed alkaloids to a level where they could start eating again. The seed contained 0.26% atropine (probably as *l*-hyoscyamine) and 0.55% scopolamine but the diets were not analyzed. Although the seed had not been milled and some seed might not have been digested, it was estimated that the toxic dose of alkaloids was 2.49 mg atropine (probably hyoscyamine thus actually 5 mg of atropine) plus 0.5 mg scopolamine/kg/day.

This is in accordance with the unpublished pilot experiment by TW Naudé and NPJ Kriek, Onderstepoort Veterinary Research Institute (1969) as cited by Kellerman *et al*. (2005), where a single dose 5 mg atropine sulfate/kg *per os* produced mild anti-muscarinic signs in a bovine. Cattle are probably susceptible to acute tropane alkaloid intoxication but it seems to be very dose specific and no cumulative effects are evident.

## Sheep and goats

In the pilot experiment by Naudé and Kriek (unpublished data, 1969) as cited by Kellerman *et al*. (2005), they could not produce acute toxicity in sheep with excessive doses of *Datura* seed with an alkaloid concentration of *ca*. 0.2% by rumen fistula at up to 50 g/kg. The susceptibility of a merino type sheep weighing approximately 30 kg to atropine was then tested by giving it an intravenous bolus injection of 1 g atropine sulfate (*ca*. 33 mg/kg, thus 16 mg hyoscyamine/kg). Its pupils dilated maximally, its heart rate shot up to a steady ca 100 per min and the next day it was clinically normal. An oral dose of atropine sulfate at 50 mg/kg evicted only mild, transient signs of muscarinic blockade.

El Dirdiri *et al*. (1981) dosed groups of male desert sheep and Nubian goats daily until death or disposal with fresh, green *D. stramonium* leaves or fruit. The sheep ($n = 2$) were dosed with both fruit or leaves at 10 g/kg/day and with leaves only at 1 g/kg/day. Signs set in after 2–6 days and continued up to death at 12–38 days at the higher dose whereas signs set in and at 35 and 45 days and the sheep were disposed of at 45 and 61 days at the lower dose. The goats ($n = 4$) were similarly dosed with both leaves or fruit at 10 g/kg/day and with fruit only at 2.5 g/kg/day. Signs set in after 2–101 days and continued up to death or disposal at 30–136 days at the higher dose, whereas at the lower dose signs set in at 36–110 days and the animal died or were disposed of at 30–136 days.

The signs noted in both species included reduced water intake, ataxia, intermittent hyperesthesia, tremors, drowsiness and recumbence. No anti-muscarinic signs were reported. Histopathological examination revealed focal necrosis and fatty vacuolation of centrilobular hepatocytes and renal tubular degeneration.

## Springbok

Lindeque and Scheepers (1992) reported that a group of these antelopes, *Antidorcas marsupialis*, in the northern Namibian desert area were forced to graze reasonably exclusively on dense stands of the alien invader, *D. innoxia.* Two adult male animals from this group exhibited abnormal behavior in having an unsteady, jerky gait and a delayed response to visual and auditory stimuli allowing the observers' vehicle to approach to a quarter the distance at which these antelope usually fled. They ascribed this to possible tropane alkaloid toxicity.

In ruminants microbial degradation of some hyoscyamine, scopolamine and related tropanes (most probably by hydrolysis of the ester bond) presumably takes place in the rumen. The toxic dose of hyoscyamine *per os* for cattle appears to be about 2.5 mg/kg in *Datura stramonium* seed. The poisoning was also self-limiting as anorexia

prevented further intake until the levels of alkaloids were reduced to allow normal intestinal function. The signs of intoxication were, however, that of mild muscarinic receptor blockade. Naudé and Kriek (1969), cited by Kellerman *et al*. (2005), also observed mild anti-muscarinic signs with orally administered atropine sulfate at 5 mg/kg. The subcutaneous therapeutic dose of atropine for ruminants (0.05 mg/kg) is, however, the same as for equines. In contrast the toxic oral dose for the horse (a hindgut fermenter) is, *vide supra*, 0.2 mg atropine/kg (equal to 0.1 mg/kg of hyoscyamine). The signs of mainly ataxia and the extended course of intoxication over weeks and even months of goats and sheep produced by El Dirdiri *et al*. (1981) are also not at all reminiscent of muscarinic receptor blockade. Massive doses of both *D. stramonium* leaves or green fruit were needed daily over a period of 12–61 days in sheep and 30–134 days in goats to cause death at an average of 32 days in sheep and 68 days in goats.

*Datura* spp. also contain calystegines (glycosidase inhibitors, *vide supra*) and its possible involvement with *Datura* spp. is mentioned by Burrows and Tyrl (2001). Calystegine $B_2$ was isolated from *D. wrightii* (Nash *et al*., 1993). Although only low levels were found in *D. stramonium* by Dräger *et al*. (1995), it is known that their concentration varies from time to time in the same species (Molyneux R, APHIS, USDA, California, personal communication, 2006). The possibility is thus real that the sheep and goats with the aberrant nervous signs in the chronic experiment reported by El Dirdiri *et al*. (1981), might have succumbed from lysosomal storage disease rather than from chronic muscarinic blockade.

Recently, it was observed that tropane alkaloid levels in the purple-stemmed and green-stemmed varieties of *D. stramonium* (Table 70.1) varied by almost 100% and it is thus possible that the same may apply to the calystegine polyhydroxylated pseudotropine alkaloids. The observation on the abnormal behavior of springbok that were forced to graze *D. innoxia* (Lindeque and Scheepers, 1992) could possibly also be due to this syndrome.

## Pigs

As a hindgut fermenter it is to be expected that this species would be susceptible to tropane alkaloid intoxication. Very little data on this could, however, be obtained. According to Adams (1995), swine are not resistant to ingested material and poisoning occurs more often than in other spp. from eating belladonna, the deadly nightshade plant. The only positive intoxication with signs of typical muscarinic blockade in pigs is reported by Keeler (1984). Subsequent to the speculative linking of arthrogryposis in pigs to *Datura* by Leipold *et al*. (1973), Keeler disproved the speculation by feeding dried *D. stramonium* vegetative material at levels causing typical muscarinic blockade signs to pregnant sows at the two most critical periods of organogenesis. Two groups of animals ($n$ = 4–5 and 5) received for respectively 40 and 10 days at levels of 1.2–1.7 g/kg/day. One animal died at 1.7 g/kg and all showed ataxia, lethargy, docility and drowsiness and pupil dilatation during the treatment period. No arthrogryposis was detected in any of the offspring.

The alkaloid levels of the plant material is not given but calculated from South African data (Table 70.1) the combined levels of hyoscyamine and scopolamine (which are about equal in this species) the toxic dose of alkaloids for the pigs was at an alkaloid level of between 1.8 and 2.6 mg/kg. Worthington *et al*. (1981) on the contrary, fed two groups of pigs ($n$ = 4) with ground *D. stramonium* seed in a ration at alkaloid levels of respectively 1.4 and 2.2 mg/kg/day for 4 days without any signs of intoxication. The pigs, however, found the mixture rather unpalatable. In this publication they, however, refer to litigation where it was claimed that *Datura* seed contamination in corn dust and grits was responsible for 803 deaths in fattening pigs and severe retardation in growth of many more. Similarly, Janssens and de Wilde (1989) fed two groups of pigs ($n$ = 4) for 12 weeks with linseed oil cake contaminated with *Datura stramonium/ferox* seed at a scopolamine level of 2 mg/kg mixed into their grower/finisher ration. The final intake of alkaloid was calculated to have been between 0.05 and 0.06 mg/kg/day. No signs of intoxication were seen but a statistically significant lower intake and weight gain compared to the control group occurred and it took 10 days longer for the test groups to attain slaughter weight. It was concluded that unpalatability at even this rather low alkaloid level contributed greatly to these differences. As no atropine and only scopolamine was detected in this meal, it can be deduced that the seed contamination was probably only that of *D. ferox* (*vide supra*, Table 70.1).

In their publication on the effects of a mixture (92:2) of scopolamine/hyoscyamine in broilers, Kovatsis *et al*. (1993), cite an ILBO report, TNO Nutrition and Food Research, Wageningen (1992) entitled *Toxicity of Datura Alkaloids in Pigs.* It is cited that "pigs fed this mixture at as low as 1.5 mg/kg feed, exhibited marked pupil dilatation and abdominal gas accumulation". An interesting South African case of "head bashing" of the foreheads against the bars above the feeding troughs, in especially adult sows and boars, is described by Spencer *et al*. (2000). The feed was made with maize (corn) contaminated with "*Datura stramonium*" seed. On withdrawal of the contaminated corn the syndrome, suspected to have been due to tropane alkaloid hallucination, ceased. Unfortunately, other typical signs of muscarinic blockade, e.g. mydriasis, were not checked. As the seeds of *D. stramonium* and *D. ferox* look identical (*vide supra*) both could have been involved and it is possible that the pigs had consumed a

diet especially high in scopolamine known to be especially hallucinogenic in man (*vide supra*). The toxic dose of tropane alkaloids for swine is not known but circumstantial evidence indicates that poisoning may certainly be a problem. In addition, milled *Datura* seed in a ration affects its palatability.

## Chickens

The chicken seems to be rather resistant to poisoning. In a well planned and executed trial, Kovatsis *et al*. (1993), investigated the effect of pure scopolamine and hyoscine at a ratio of 98:2 (the ratio at which it occurs in *D. ferox* seed) on broiler chicks. Five groups ($n = 20$, 16 females and 4 males) were exposed over 90 days at levels of 0, 1.5, 15, 75 and 150 mg/kg of feed. Weights were determined weekly, clinical signs were checked daily, clinical chemistry on serum enzymes was done twice. No signs of muscaric receptor blockade or changes of practical significance in these parameters were found except for a significant reduction in weight gain up to day 52 at the higher two doses but not after that. At the highest dose this occurred only during the first 4 weeks. The effect on final weight is, unfortunately, not given.

In a much smaller and shorter-term trial on day-old male chick groups ($n = 5$), Day and Dilworth (1984) fed milled *D. stramonium* seed with an unspecified alkaloid level at 0%, 1%, 3% and 6% admixed in their rations. At 3 weeks there was also a significant drop in weight gain at the two high doses. Similarly, Flunker *et al*. (1987) fed milled *D. stramonium* seed of unknown alkaloid level in their normal rations for 3 weeks at between 0.5% and 3% to six groups ($n = 24$) of day-old chicks and for 14 days at 1%, 2% and 3% to adult White Leghorn hens ($n = 5$). No deleterious effect growth in the chicks and egg production in the hens occurred except that the intake in the latter was decreased at the 3% level. Indications of early embryonic death and malformed embryos in chicken eggs inoculated into the yolk sac with a 92:2 mixture of scopolamine and hyoscyamine at 10 μg or more (Magras *et al*., 1993) does not appear to be of any practical significance. Steyn (1934) cites Sinclair (1898) that ostrich poults are susceptible to *Datura stramonium* seed poisoning. They became sleepy, staggered about before coma set in. This phenomenon has never been reported again during the past century. In practice, poultry will most probably only be exposed to feed contaminated with *Datura* seed. Chickens do not appear to be susceptible to acute tropane alkaloid poisoning. However, the negative effect on weight gain in broilers and possibly egg production in hens and perhaps unpalatability ascribed to milled *Datura* seed as also observed in pigs, might well be of significance in the poultry industry.

# TREATMENT

Impaction colic in equines due to ileus, must be treated symptomatically and according to rectal findings, the extent and severity of the usual signs of colic (pawing, sweating and rolling), reduced or absence of borborygmi, whether nasogastric intubation reveals reflux, if clinicopathological findings are within normal limits and fluid obtained by abdominocentesis reveal any abnormalities (Naudé *et al*., 2005; Gerber *et al*., 2006).

Therapy should include water by nasogastric tube, laxatives *per os* ($MgSO_4$ or technical oil), cisapride *per os*, polyionic fluid by intravenous infusion at a rapid rate (4 ml/kg/h) and an analgesic such as flunixin when required. This should be continued until all signs of discomfort are absent and rectal examination reveals resolution of the impaction.

The use of reversible AChE inhibitors as specific pharmacological antagonists to overcome the muscarinic blockade is of paramount importance in the horse. Neostigmine is generally used in veterinary medicine for this purpose. However according to Brown and Taylor (2001) physostigmine (eserine), a tertiary natural alkaloid is regarded as the drug of choice to treat tropane alkaloid intoxication in man. It rapidly abolishes delirium and coma and should seriously be considered as a cholinergic in especially the horse. It is, in contrast to neostigmine (a quaternary alkaloid), not ionized and consequently readily passes the blood–brain barrier (Brown and Taylor, 2001). It might also just overcome the recalcitrant, perceived gut receptor blockage. As it is rapidly metabolized in man, repeated doses at short intervals (1–2 h) are necessary. It is, however, a very potent drug with a narrow therapeutic index and should be used with circumspection. Adams *et al*. (1984) found in their study on the effect of drugs on the myoelectrical and mechanical activity of the jejunum and pelvic flexure of the equine colon, that whilst neostigmine increased the propulsive activity of the pelvic flexure and regularly resulted in defecation, it had no effect on the motility of the jejunum.

Of the natural cholinomimetic alkaloids, only arecoline and pilocarpine are not ionized and as arecoline also acts at nicotinic sites (Brown and Taylor, 2001) pilocarpine could also be considered as a pharmacological antagonist that might also cross these barriers.

# FUTURE RESEARCH DIRECTIONS

## Horses

Reasonable data are available on the effects of atropine (and thus *l*-hyoscyamine) in this species. However, no

data are available on pure scopolamine and its contribution to the impaction colic syndrome seen with *Datura* spp. As this intoxication possibly occurs more frequently than expected with contaminated hay, the use of pharmacological antagonist drugs that cross tissue barriers, e.g. physostigmine and other tertiary cholinomimetic alkaloids should be thoroughly investigated in the treatment of the resultant intractable, and frequently fatal, impaction colic. It would appear from the work of Galey *et al*. (1996) that with early acute intoxication the tropane residues in urine will be in the mg/ml range. This should be followed up as a matter of priority for diagnosis.

### Cattle

There is a lack of data in relation to the acute oral toxic doses of both atropine (thus hyoscyamine) and of scopolamine in bovines. This species appears to be susceptible. Being continuously exposed to grain contamination with *Datura* seed, the chronic effect of sub-toxic doses on feed intake, growth and production of both beef and dairy animals should be explored. The possibility of adaptation of ruminal microorganism to detoxify tropane alkaloids should be further investigated.

### Sheep and Goats

These two species are very different in reaction to many drugs and the actual acute oral toxic doses of both atropine and scopolamine should be determined. They both, however, appear to be rather refractory to acute anti-muscarinic tropane intoxication. The possibility that calystegines had been the cause of peculiar chronic syndrome described by El Dirdiri *et al*. (1981), should be followed up.

### Swine

There are definite indications that this hindgut fermenter of fiber (like the horse) is probably rather susceptible to acute intoxication. As another species very frequently being fed grain contaminated with *Datura* seed, the oral acute toxic dose of both atropine and scopolamine should also be determined here as well as the effect of chronic exposure at sub-toxic levels on feed intake, growth and production.

### General

The non-tropanyl alkaloid meteloidine (Figure 70.4.2) occurs haphazardly in various *Datura* spp. at levels sometimes comparable with hyoscyamine and/or scopolamine. No toxicity or pharmacological data on it appears to be available and its possible contributory role in tropane alkaloid poisoning should be determined.

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## REFERENCES

Adams HR (1995) Cholinergic pharmacology autonomic drugs. In *Veterinary Pharmacology and Therapeutics*, 7th edn, Adams HR (ed.). Iowa State University Press, Ames, IA.

Adams HR (2001) Cholinergic pharmacology: autonomic drugs. In *Veterinary Pharmacology and Therapeutics*, 8th edn, Adams HR (ed.). Iowa State University Press, Ames, IA.

Adams SB, Lamar CH, Masty J (1984) Motility of the distal portion of the jejunum and pelvic flexure in ponies: effect of six drugs. *Am J Vet Res* **45**: 795–9.

Barney GB, Wilson BJ (1963) A rare toxicity syndrome in ponies. *Vet Med* **48**: 19–21.

Beards SC, Kraus P, Lipman J (1994) Paralytic ileus as a complication of atropine therapy following severe organophosphate poisoning. *Anaesthesia* **49**: 791–3.

Bernheim F, Bernheim MLC (1938) The hydrolysis of homatropine and atropine by various tissues. *J Pharmacol Exp Ther* **64**: 209–15.

Brown JH, Taylor P (2001) Muscarinic receptor agonists and antagonists. In *Goodman and Gilman's the Pharmacological Basis of Therapeutics*, 10th edn, Hardman JG, Limbird LE, Gilman AG (eds). McGraw-Hill, New York.

Budavari S (1996) *The Merck Index*, 12th edn. Merck and co, Whitehouse Station, NJ.

Burrows GE, Tyrl RJ (2001) *Toxic Plants of North America*. Iowa State University Press, Ames, IA.

Case AA (1956) Nightshade poisoning. *Southwest Vet* **9**: 140–3.

Day EJ, Dilworth BC (1984) Toxicity of jimson weed seed and cocoa shell meal to broilers. *Poult Sci* **63**: 466–8.

Dräger B (2004) Chemistry and biology of calystegines. *Nat Prod Rep* **21**: 211–23.

Dräger B, Van Almsick A, Mrachatz B (1995) Distribution of calystegines in several Solanaceae. *Planta Med* **61**, 577–579.

Ducharme NG, Fubini SL (1983) Gastrointestinal complications associated with the use of atropine in horses. *J Am Vet Med Assoc* **182**: 229–31.

El Dirdiri NI, Wasfi IA, Adam SEI, Edds GT (1981) Toxicity of *Datura stramonium* to sheep and goats. *Vet Hum Toxicol* **23:** 241–6.

Everist SL (1974) *Poisonous Plants of Australia*. Angus and Robertson, London and Sydney.

Flunker LK, Damron BL, Sundlof SF (1987) Jimsonweed seed contamination of broiler chicks and White Leghorn hen diets. *Nutr Rep Int* **36:** 551–6.

Galey FD, Holstege DM, Francis T, Hyde W, Jack R (1996) Residues of *Datura* species in horses. In *Proceedings of the 11th International Conference of Racing Analysts and Veterinarians,* Auer DE, Houghton E (eds)., Queensland, Australia, pp. 333–7.

Gerber R, Naudé TW, De Kock SS (2006) Confirmed *Datura* poisoning in a horse most probably due to *D. ferox* in contaminated tef hay. *J SA Vet Assoc* **77**: 107–112.

Henderson L (2001) Alien weeds and invasive plants. A complete guide to declared weeds and invaders in South Africa. *Plant Protection Research Handbook*, No.12, Agricultural Research Council.

Henderson M, Anderson JG (1966) Common weeds in South Africa. *Botanical Survey Memoir*, No. 37, Department of Agricultural Technical Services, Republic of South Africa.

Janssens G, De Wilde R (1989) Toxicity of thornapple (*Datura stramonium* and/or *D. ferox*) seed present in pig feed. *Vlaam Diergeneesk Tijdschr* **58**: 84–6.

Keeler RF (1984) Absence of arthrogryposis in newborn Hampshire pigs from sows ingesting toxic levels of jimsonweed during gestation. *Vet Hum Toxicol* **23**: 50–9.

Kellerman TS, Coetzer JAW, Naudé TW, Botha CJ (2005) *Plant Poisonings and Mycotoxicoses of Livestock in Southern Africa,* 2nd edn. Oxford University Press, Cape Town.

Ketchum JS, Sidell FS (1997) Chapter 11. Incapacitating agents. In: Sidell FR, Takafuji ET, Franz DR. In *Medical Aspects of Chemical and Biological Warfare.* Washington: Office of the Surgeon General at TMM Publications.

Kovatsis A, Flaskos J, Nikolaidis E, Kotsaki-Kovatsi VP, Papaioannou N, Tsafaris F (1993) Toxicity study of the main alkaloids of *Datura ferox* in broilers. *Food Chem Toxic* **31**: 841–5.

Krantz Jr JC, Forest JW, Heisse CK (1954) Contribution to the pharmacology of apoatropine and its methyl bromide. *Proc Soc Exp Biol Med* **86**: 511–12.

Leipold HW, Oehme FW, Cook JE (1973) Congenital arthrogryposis associated with ingestion of jimsonweed by pregnant sows. *J Am Vet Med Assoc* **162**: 1059–60.

Lindeque M, Scheepers JL (1992) Use of *Datura innoxia* by ungulates in the Hoanib river, Namibia. *South Afr J Wildl Res* **22**: 45–8.

Magras IN, Kotsaki-Kovatsi VP, Kovatsis A (1993) Teratogenic effects of a mixture of scopolamine and hyoscyamine in chick embryos. *Vet Hum Toxicol* **35**: 434–5.

Milks HJ (1949) *Practical Veterinary Pharmacology, Materia Medica and Therapeutics,* 6th edn. Alex Eger, Chicago.

Nash RJ, Rothschild M, Porter EA, Watson AA, Waigh RD, Waterman PG (1993) Calystegines in *Solanum* and *Datura* species and the death's-head moth (*Acherontia atropus*). *Phytochemistry* **34**: 1281–3.

Naudé TW, Gerber R, Smith RJ, Botha CJ (2005) *Datura* contamination of hay as the suspected cause of an extensive outbreak of impaction colic in horses. *J South Afr Vet Assoc* **76**: 107–12.

Nelson PD, Mercer HD, Essig HW, Minyard JP (1982) Jimson weed seed toxicity in cattle. *Vet Hum Toxicol* **24**: 321–5.

Ofukany L, Frantova E, Turon J (1983) Mass poisoning of cattle with *Datura stramonium*. *Veterinarstvi* **33**: 316–17.

Pearson EG, Rierbold TW (1989) Comparison of bronchodilators in alleviating clinical signs in horses with chronic obstructive pulmonary disease. *J Am Vet Med Assoc* **194**: 1287–91.

Roberts MC, Argenzio A (1986) Effects of amitraz, several opiate derivatives and anticholinergic agents on intestinal transit in ponies. *Equine Vet J* **18**: 256–60.

Schulman ML, Bolton LA (1998) *Datura* seed intoxication in two horses. *J South Afr Vet Assoc* **69**: 27–9.

Spencer BT, Neser JA, du Plessis EC, Moolman GC (2000) Acute deaths with cardiomyopathy and nervous signs in pigs. *Proceedings of the 16th International Pig Veterinary Society Congress,* Melbourne, Australia, 17–20 September, p. 162.

Steyn DG (1934) *The Toxicology of Plants in South Africa.* Central News Agency, South Africa.

Wada S, Yoshimitsu T, Koga N, Yamada H, Oguri K, Yoshimura H (1991) Metabolism *in vivo* of the tropane alkaloid, scopolamine, in several mammalian species. *Xenobiotica* **21**: 1289–300.

Watt JM, Breyer-Brandwijk MG (1962) *The Medicinal and Poisonous Plants of Southern and Eastern Africa,* 2nd edn. ES Livingstone Ltd, Edinburgh and London.

Williams S, Scott P (1984) The toxicity of *Datura stramonium* (thorn apple) to horses. *New Zeal Vet J* **32**: 47.

Worthington TR, Nelson EP, Bryant MJ (1984) Toxicity of thorn apple (*Datura stramonium* L) seeds in the pig. *Vet Rec* **108**: 208–21.

CHAPTER

# 71

# Fescue toxicosis

*Dennis J. Blodgett*

## INTRODUCTION

A hardy tall fescue grass (*Festuca arundinacea* Schreber) was obtained from a Kentucky farm in 1931 and was first marketed commercially in 1943 (Ball, 1984). Today, this variety of tall fescue, Kentucky 31, occupies more than 20 million hectares in the United States, primarily in the southeastern region of the United States (Stuedemann and Seman, 2005). It is a cool season grass that grows well in the fall producing substantial forage for the winter months. By 1948, fescue foot problems had been described in New Zealand and in 1950, the same syndrome was reported in the United Sates (Bacon, 1995). In 1973, it was noticed that cattle on a Kentucky 31 pasture were very unthrifty compared to cattle on an adjoining non-fescue pasture (Schmidt and Osborn, 1993). This observation initiated an investigation that revealed a fungus growing within the plant (endo = within; phyte = plant) (Bacon *et al.*, 1977). Today, it is believed that >95% of tall fescue pastures containing mostly the Kentucky 31 variety are infected by the endophyte (Yates and Powell, 1988). Endophyte-infected (E+) fescue is estimated to cause more than 1 billion dollars of loss yearly in livestock production in the United States (Oliver, 1997).

## BACKGROUND

### Endophyte name

Bacon *et al.* (1977) initially discovered the endophyte and based on earlier morphologic research classified it as *Epichloe typhina*. As is common for many microbial organisms, it was later renamed as *Acremonium coenophialum* (Morgan-Jones & Gams) (Morgan-Jones and Gams, 1982). Further molecular phylogenetic evaluation has generated the latest name of *Neotyphodium coenophialum* (Morgan-Jones & Gams) Glenn, Bacon & Hanlin comb. nov. (Glenn *et al.*, 1996). A similar endophyte, *Neotyphodium lolii*, is found in perennial ryegrass. Endophytes are not uncommon in a number of forage grasses.

### Mutualism

The tall fescue plant and the endophyte enjoy a mutualistic relationship (Thompson *et al.*, 2001). Each benefits the other. The plant supplies a comforting and safe internal environment for the endophyte along with all the required nutrients. In return, the endophyte lives in intercellular spaces in the plant without disrupting cells of the plant. The endophyte generates multiple toxins that are distributed throughout the plant. The toxins make the plant more resistant to drought, insects, parasitic nematodes, fungi and herbivores. The endophyte invades seed heads of the plant and is able to continue its relationship with the next generation of fescue through its contamination of the seed. Endophyte infections cannot be transferred naturally to a non-endophyte-infected variety of tall fescue. However, E+ varieties of fescue are more hardy and persistent than non-endophyte fescue plants and thereby, may eventually take over a pasture. Endophyte-infected plants produce more forage and seeds than do non-infected plants.

### Toxins secreted

Multiple classes of toxins are produced by *N. coenophialum* including ergot alkaloids, peramine and loline alkaloids (Porter, 1995). Peramine and loline alkaloids are primarily

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insect deterrents. Loline alkaloids belong to an aminopyrrolizidine group of alkaloids, but are not known to cause hepatic problems similar to pyrrolizidine alkaloids in other plant species. The loline alkaloids found in fescue include loline, *n*-acetylloline, *n*-formylloline, *n*-acetylnorloline and *n*-methylloline (Porter, 1994).

## Ergovaline

### *General toxicity*

Among the ergot alkaloids found in tall fescue, ergovaline is by far the most prevalent ergopeptide (Porter, 1995). It accounts for approximately 90% of the ergopeptide alkaloid content of tall fescue (Lyons *et al*., 1986). Estimates of the minimum threshold toxic concentrations of ergovaline in fescue to cause clinical problems range from 50 to 500 ppb (Moubarak *et al*., 1993; Tor-Agbidye *et al*., 2001; Aldrich-Markham *et al*., 2003). Minimal concentrations depend somewhat on the type of syndrome being considered, the species affected and environmental temperatures. Species susceptibility from most to least susceptible would be: horses (i.e. pregnant mares) > cattle > sheep (Aldrich-Markham *et al*., 2003). In general, ergovaline concentrations greater than 200 ppb dry weight are toxic for cattle and horses (Rottinghaus *et al*., 1991; Blodgett, 2001). Ergovaline is a peptide (i.e. peptine) alkaloid with a lysergic acid structure combined with three amino acids. It differs in structure from ergotamine by one amino acid. Other ergot alkaloids found in fescue are lysergic acid amides (e.g. ergonovine) and clavine alkaloids (Porter, 1995).

### *Reason for primary toxin*

The ergopeptide alkaloids are believed to be the major toxins responsible for the multiple fescue toxicosis syndromes seen (Porter and Thompson, 1992). Since ergovaline is the most prominent ergopeptide alkaloid, it probably dictates the likelihood of a toxicosis. Ergovaline is difficult to purify and synthesize, so little direct fescue research has been done with pure ergovaline to prove that it is the primary toxin in fescue. There are multiple reasons to believe that it is the primary toxin, however. An experiment with synthetic ergovaline in sheep reproduced most clinical signs of the summer slump syndrome seen in cattle (Gadberry *et al*., 2003). Ergovaline injected intraperitoneally into cattle for 3 days produced increased rectal temperatures and respiratory rates observed with summer slump (Spiers *et al*., 2005). Ergotamine and bromocryptine, other ergot alkaloids, are able to reproduce fescue toxicosis syndromes (Blodgett, 2001). Ergovaline is very potent as a vasoconstrictor in *in vitro* models (Oliver *et al*., 1998). Ergovaline is a strong prolactin inhibitor, whereas ergonovine, a lysergic acid amide in fescue, is unable to produce typical signs of fescue toxicosis or lower prolactin concentrations in cattle (Oliver *et al*., 1994). Antibodies against ergot alkaloids are able to ameliorate some clinical features of fescue toxicosis in cattle and mice (Hill *et al*., 1994; Rice *et al*., 1998). New novel endophyte varieties of fescue that lack ergovaline, but not other types of fescue toxins, do not cause fescue toxicosis (Roberts and Andrae, 2004). Syndromes similar to fescue toxicosis are seen when ergotized grains infected by *Claviceps purpurea* are ingested by livestock (Thompson *et al*., 2001). Finally, dopamine $D_2$ antagonists, which stimulate prolactin secretion, are able to alleviate most of the toxic effects of fescue (Lipham *et al*., 1989; Cross, 1997).

### *Seasonal variation*

The concentration of ergot alkaloids in fescue pastures varies with the season (Rottinghaus *et al*., 1991). Ergovaline is low in the spring, reaches peak concentrations in seed heads during the summer months, decreases somewhat during the early fall and rebounds with fall regrowth. Seed heads contain the highest concentration of ergovaline. Stockpiled fescue pasture has more ergot alkaloids early in the winter than late in the winter (Roberts and Andrae, 2004). Drought and rainy conditions tend to increase ergovaline concentrations (Arechavaleta *et al*., 1992; Aldrich-Markham *et al*., 2003). Fertilization of fescue pastures with nitrogen- and phosphorous-based fertilizers or poultry litter also increases ergot alkaloid concentrations (Stuedemann and Seman, 2005). Since ergovaline is really a mycotoxin and mycotoxin production and persistence is dependent on multiple seasonal and environmental conditions, concentrations of ergovaline in tall fescue would be expected to vary from season to season and year to year (Lyons *et al*., 1986).

# PHARMACOKINETICS/ TOXICOKINETICS

## Absorption

Little research has been devoted to *in vivo* absorption of ergot alkaloids in livestock. After cannulation of ruminal, gastric and mesenteric veins of sheep consuming E+ fescue, ergot alkaloids were only detected in ruminal veins (Hill, 2005). However, *in vitro* tissue specimens of rumen, reticulum and omasum are all capable of ergot alkaloid transport. Ergovaline is hard to detect in rumen fluid and is metabolized by rumen microbes to lysergic acid. Other researchers have found 50–60% of ingested ergovaline in abomasal contents, but minimal remaining amounts of ergovaline in ileal contents or feces (Oliver, 1997). Whether or not ergovaline is absorbed in the live animal quickly and effectively prior to metabolism by rumen microbes is not known for sure.

## Distribution/metabolism

Ergovaline is only in ppb or low ppm in E+ tall fescue, so the dilution factor in the body and sensitivity of various methods make evaluation of the concentration in any organ extremely difficult. Therefore, little is known about the distribution of ergovaline and other ergot alkaloids in the body. Intravenous injection of several ergopeptides in calves documented a distribution and tissue equilibrium phase in serum lasting approximately 1 h (Moubarak *et al.*, 1996). This was followed by an elimination phase with a half-life of approximately 20–30 min. It is known that multiple systems in the body may show some effects of ergot alkaloid consumption including the cardiovascular system, central nervous system and abdominal fat. Ergovaline is believed to be metabolized in the liver by the cytochrome P450 3A4 subfamily of enzymes (Moubarak and Rosenkrans, 2000; Moubarak *et al.*, 2003). Cytochrome P450 3A4 is responsible for adding one or two hydroxyl groups to the peptide ergolene ring to make it more hydrophilic for excretion (Moubarak and Rosenkrans, 2000). This enzyme family is inducible in rats treated with dexamethasone, but is not induced in rats with prior ergot alkaloid exposure (Moubarak *et al.*, 2003). However, sheep fed E+ fescue had increased hepatic activity of mixed function oxidases (Zanzalari *et al.*, 1989). The indole ring of ergopeptide alkaloids may also undergo eventual oxidation (Moubarak and Rosenkrans, 2000). Whether or not other groups of enzymes are involved in metabolizing or conjugating ergovaline is unsure.

## Excretion

Metabolites of ergot alkaloids have been measured indirectly with lysergol antibodies in bile and urine of cattle (Stuedemann *et al.*, 1998). Ninety-six percent of the metabolites in cattle are found in urine. Ergot alkaloids are detected in urine within 12 h of exposure to E+ fescue and are maximal within 24 h. After removal from a fescue pasture, ergot metabolites are gone within 48 h from the urine of cattle. Similarly, post-term mares when removed from E + fescue pastures show signs of impending parturition within 48 h (Schmidt and Osborn, 1993).

# MECHANISMS OF ACTION

## Dopaminergic agonist

### Prolactin inhibition

Ergovaline is a dopamine $D_2$ receptor agonist (Oliver, 1997). Dopamine agonists have a negative feedback mechanism on prolactin secretion by the pituitary. Prolactin inhibition is one of the most consistent problems in multiple species experiencing fescue toxicosis. Ergopeptine alkaloids have 10-fold greater potency for dopamine $D_2$ receptor binding than ergoline alkaloids (Larson, 1997).

#### *Lactation suppression*

One of the roles of prolactin is induction of mammary gland growth and milk production. Lack of prolactin is often associated with agalactia at parturition in many species including the horse and pig. However, ruminant species have a placental lactogen that can overcome the lack of prolactin stimulation of the mammary gland at birth (Cross, 1997). Nevertheless, lower prolactin concentrations in dairy and beef cows consuming E+ fescue may decrease milk production by approximately 50% after the perinatal period is past (Schmidt and Osborn, 1993; Thompson *et al.*, 2001).

#### *Effect on other reproductive hormones*

Prolactin is involved in maintaining corpus luteal function and gonadotropin secretion (Porter and Thompson, 1992). Altered luteal function in heifers grazing E+ fescue results in reduced circulating progesterone. Fescue consumption has been associated with low progesterone production in mares and cows and high estradiol concentrations in mares (Cross, 1997; Thompson *et al.*, 2001). These imbalances of reproductive hormones lead to early pregnancy problems in cattle and late pregnancy problems in horses. Ergopeptide potency for prolactin inhibition has been correlated with inhibition of ovum implantation in rats (Fluckiger *et al.*, 1976). Ergovaline is intermediate among the ergopeptides in its ability to inhibit implantation, but is approximately 2-fold more potent than ergotamine.

#### *Effect on hypothalamic thermoregulatory center*

Another role of dopamine or prolactin is control of the thermoregulatory center in the hypothalamus (Strickland *et al.*, 1993). Diminished prolactin or dopamine receptor perturbation causes the thermoregulatory center to deregulate and cause hyperthermia or hypothermia in the animal. Deregulation is more likely when environmental temperatures are outside of the thermoneutral range of the animal (Spiers *et al.*, 2005). Fescue foot problems are more likely at temperatures less than 8ºC (Tor-Agbidye *et al.*, 2001), whereas summer slump problems are more apparent when temperatures exceed 31ºC (Schmidt and Osborn, 1993; Spiers *et al.*, 2005). Metoclopramide, a dopamine $D_2$ antagonist, decreases body temperature in steers grazing E+ fescue (Lipham *et al.*, 1989).

#### *Effect on lipogenesis*

Prolactin also has a role in lipogenesis through control of metabolism of cholesterol and triglycerides by the liver (Strickland *et al.*, 1993). Cattle suffering from summer slump traditionally have low serum cholesterol and triglyceride concentrations (Oliver, 1997). Low serum cholesterol is also commonly found in cattle herds with abdominal fat necrosis (Schmidt and Osborn, 1993). Necrotic abdominal fat is lower in ether-extractable material, but has a much higher cholesterol content of the ether-extractable fraction. Lipolysis in cattle experiencing fescue toxicosis is decreased (Thompson *et al.*, 2001). Metoclopramide, a dopamine antagonist and prolactin enhancer, increases serum cholesterol levels in steers grazing E+ fescue pastures (Lipham *et al.*, 1989). Serum triglycerides and cholesterol are also reduced by alpha-1 adrenergic receptor antagonists and alpha-2 adrenergic agonists, which are both physiologic effects of ergot alkaloids (Oliver, 1997).

#### *Effect on winter hair loss*

Change in photoperiod with the end of winter and the start of spring normally will increase prolactin production and stimulate shedding of long winter hair in favor of new spring hair coat growth (Thompson *et al.*, 2001). Associated with a decrease of prolactin, cattle experiencing fescue toxicosis often fail to shed some or all of their long winter hair. This long hair may become bleached out by the summer sun and contributes to an unthrifty appearance.

#### *Effect on immunity*

Prolactin is considered an immunomodulator (Strickland *et al.*, 1993). Impaired immune function from E+ fescue seed was noted in mice and rats, whereas cattle were not affected. However, numerous other E+ fescue studies in cattle cite decreased antibody response, reduced globulins or increased morbidity and mortality (Thompson *et al.*, 2001). In contrast, Rice *et al.* (1997) noted increased humoral immunity in cattle fed E+ fescue.

### *Miscellaneous neurologic effects*

Other miscellaneous neurologic effects include a negative effect of dopamine agonists on feed intake. Use of a dopamine antagonist, metoclopramide, increased feed intake in lambs fed E+ fescue without an effect on body temperature (Thompson *et al.*, 2001). Nervous behavior in cattle fed E+ fescue might be related to the ability of ergopeptide alkaloids to release dopamine in *in vitro* synaptosomal preparations (Larson, 1997).

## Alpha-2 adrenergic agonist

### *Vasoconstriction*

#### *Gangrene of extremities*

Ergovaline acts as an alpha-2 adrenergic agonist on blood vessels, especially arterioles (Oliver, 1997). The persistent vasoconstriction of peripheral arterioles in the back legs of cattle consuming fescue is believed to be responsible for thickening of the smooth muscle wall of the arterioles seen with fescue foot problems (Thompson *et al.*, 2001). Chronic exposure of cattle to E+ fescue makes their alpha-2 adrenergic receptors more reactive to ergot alkaloids (Oliver, 1997).

#### *Decreased heat loss*

Constriction of blood vessels in the skin of cattle also contributes to hyperthermia during the summer months. In addition to the thermoregulatory effects of dopamine/prolactin on the hypothalamus, the decreased dissipation of heat through the skin of cattle is believed to contribute to the higher body temperature observed when summer temperatures are at or above 30ºC (Thompson *et al.*, 2001).

### *Serum enzyme decrease*

Multiple serum enzyme decreases in cattle have been associated with inhibition of adenyl cyclase levels due to alpha-2 adrenergic activity of ergovaline (Oliver, 1997). Ergovaline inhibits cyclic AMP production via the alpha-2a adrenergic receptor (Larson, 1997). Besides the aforementioned decrease in serum cholesterol and triglycerides, there are also decreases in alkaline phosphatase, gamma glutamyltransferase, aspartate aminotransferase, alanine aminotransferase, creatinine kinase, lipase and lactic dehydrogenase (Oliver, 1997). Potentially, the inhibition of ATPase in brain and kidney (Moubarak *et al.*, 1993) may be mediated by the same mechanism.

### *Oxidative stress*

A few studies have noted oxidative stress effects in cattle grazing E+ fescue pastures (Oliver, 1997). Alpha-2 adrenergic receptor agonists have the capability of depleting glutathione, a peptide integral in defending against oxidative stressors. Lakritz *et al.* (2002) noted reduced glutathione concentrations in whole blood samples from cattle exposed to heat stress and E+ fescue.

### *Renal-related effects*

Alpha-2 adrenergic receptor agonists decrease antidiuretic hormone release by the pituitary (Oliver, 1997). This causes an inability of cattle to concentrate their urine. Ergot alkaloids also block aldosterone production in the

adrenals (Oliver, 1997). Decreased aldosterone and antidiuretic hormone promote diuresis and cattle coming to feedlots from E+ fescue pastures have a reputation for producing a muddy wallow in their pens. Aldosterone action could also be compromised by lowered kidney sodium/potassium ATPase activity seen with ergot alkaloid exposure (Oliver, 1997).

## Serotonergic agonist

Ergot alkaloids also act on serotonergic-2 receptors (Oliver, 2005). Ergovaline is an agonist at serotonergic-2 receptors of uterine and umbilical arteries (Dyer, 1993). This serotonergic activity in blood vessels causes persistent vasoconstriction *in vitro*. Serotonergic activity of ergot alkaloids might also be important in the enhanced mitogenesis of vascular smooth muscle, hypothalamic thermoregulatory center effects, pulmonary vasoconstriction and brochoconstriction, and appetite suppression seen with fescue toxicosis (Oliver, 1997, 2005).

# TOXICITY

## Cattle

### *Summer slump*

Summer slump or summer syndrome is the most common and costly syndrome seen in cattle with fescue toxicosis. Although it is most dramatic during the summer when environmental temperatures reach above 31°C, the problem can occur during any time of the year (Schmidt and Osborn, 1993; Stuedemann and Seman, 2005). Slump refers to the fact that cattle just "ain't doing right." Cattle have an unthrifty appearance with rough hair coats that often haven't shed from the winter. The sun may bleach out the hair coats. Because of their high body temperatures, cattle spend more time in the shade or watering holes during the day and less time consuming forage. Beef cows consuming E+ fescue produce approximately 50% less milk for their calves, which results in lower weaning weights (Schmidt and Osborn, 1993). At weaning time, calves raised on E+ fescue pasture may be 30–40 kg lighter than similar calves on endophyte free forage (Schmidt and Osborn, 1993). Other potential clinical signs include nervousness, increased salivation, increased rate of respiration, delayed puberty and reduced conception rates (Schmidt and Osborn, 1993). The reduced conception rates are thought to occur in cattle during the early embryonic period and are not associated with late term abortions or stillborn calves (Thompson *et al*., 2001). Although sudden deaths during hot summer months have been reported, negligible mortality is associated with summer slump.

### *Fescue foot*

Fescue foot refers to a syndrome seen in cattle during the late fall or winter months when dietary ergovaline concentrations exceed 400 ppb (Tor-Agbidye *et al*., 2001). It is not nearly as common as summer slump. Peripheral vasoconstriction from cold environmental temperature is additive to the vasoconstrictive properties of ergot alkaloids. Additional factors that explain why fescue foot appears as opposed to summer slump are not known. Considerations could reflect a concentration difference of ergovaline in the fescue, a difference in concentration of other vasoconstrictive ergot alkaloids or a host of multifactorial concomitant conditions. Vasoconstriction tends to be more severe in rear legs than front legs. The twitch of the tail and sometimes the tips of the ears are also affected. Vasoconstriction of the back legs is between the coronary band of the hooves and the fetlock area. Areas proximal to the vasoconstriction may be congested. Areas distal to the vasoconstriction undergo gangrenous necrosis with hooves potentially being sloughed. Cattle have visibly swollen rear legs with a shifting rear leg lameness, muscle tremors, rough hair coats, knuckling of the pastern and arching of the back (Spiers *et al*., 2005). Later on, they may become prostrate.

### *Lipomatosis*

Lipomatosis is a syndrome of fat necrosis affecting abdominal fat stores in mature cattle (Schmidt and Osborn, 1993). If mesenteric fat surrounding intestines is involved, scanty feces, bloat or intestinal obstruction may result. Perirenal fat may also be affected without causing significant clinical problems. Fat in the pelvic canal may become necrotic and hardened and cause dystocia problems. Although the hardened fat may be detected sometimes by rectal palpation, the discovery is often made at necropsy as an additional finding unrelated to the cause of death of the animal. The incidence of fat necrosis in cowherds is highest in those herds with the lowest concentrations of serum cholesterol (Stuedemann and Seman, 2005).

## Sheep and goats

Sheep may be affected by fescue and have a syndrome very similar to summer slump in cattle. Ewes grazing fescue have decreased milk production and increased early embryonic mortality (Schmidt and Osborn, 1993; Thompson *et al*., 2001). Weight gains and skin temperature in young lambs may be decreased (Gadberry *et al*., 2003). Fescue foot problems are possible in sheep with threshold

dietary concentrations of 500 ppb ergovaline and environmental temperatures of 7.8ºC (Tor-Agbidye *et al.*, 2001). Additionally, ergot alkaloids are known to cause tongue necrosis in sheep along with reproductive infertility problems (Thompson *et al.*, 2001). Goats may experience lipomatosis (Smith *et al.*, 2004).

## Horses

### *Reproductive term dysfunction*

Reproductive problems in late term mares are the most common fescue problem for horses (Cross, 1997; Blodgett, 2001). Mares are most susceptible in late gestation after day 300 of gestation. Failure to remove mares from fescue pasture or hay during the last month of gestation may cause the mare to prolong her pregnancy by 20–27 days (Cross, 1997; Blodgett, 2001). During this extra gestation length, the foal becomes ganglier. Even though the foal spends more time *in utero*, it is typically dysmature with unerupted incisor teeth and overgrown hooves. Early abortion problems are possible but not common. Lack of prolactin in the mare along with decreased progesterone and increased estradiol levels cause multiple problems (Cross, 1997; Blodgett, 2001). The mare does not prepare well for parturition and may experience dystocia problems with tearing of the vagina, which can result in death or rebreeding problems. The chorioallantois may precede the foal through the birth canal and be seen as a "red bag" presentation. Retained placentas are also more common with fescue toxicosis. The placenta doesn't rupture at the cervical star and the placenta may be thick and edematous. If the foal is able to break out of the edematous placenta and not suffocate, it often faces an agalactic mare with minimal colostral antibodies for passive transfer. Foal death associated with dystocia problems or failure of passive transfer is common.

### *Infertility problems*

Early embryonic death losses in mares similar to that experienced by cows are not commonly recognized. Fertility of mares consuming fescue pastures or hay tends to be fairly high. One study in mares noted trends of increased early embryonic mortality (Brendemuehl *et al.*, 1994); whereas another study found no loss in pregnancies between days 21 and 300 of gestation, but lower progestogen concentrations between days 90 and 120 (Brendemuehl *et al.*, 1996).

## Camelids (llamas and alpacas)

Little research has been done with fescue toxicosis in camelid species. Llamas and alpacas have hyperthermia problems in summer months. Because of the ability of fescue to directly affect the thermoregulatory center in the hypothalamus and decrease dermal heat loss via vasoconstriction, fescue should be considered a potential contributor to the heat stroke syndrome seen in llamas and alpacas.

# TREATMENT

## Non-specific treatment

Cattle experiencing summer slump toxicosis are generally unthrifty and are normally just treated by removal from fescue forage or by dilution of the ergovaline content of their overall diet. This is accomplished by feeding supplemental non-fescue forage or concentrates. Clovers or Bermuda grass are often over sown into fescue pastures and aid in dilution for a few years (Thompson *et al.*, 2001). Establishing shade or water holes will help to cool hyperthermic animals. Gangrenous problems with fescue foot are treated with broad-spectrum antibiotics to diminish secondary invaders.

## Specific treatment

A dopamine $D_2$ antagonist has been developed to treat agalactia and prolonged gestation problems in mares (Cross, 1997). The generic name of the drug is domperidone and it is marketed as an oral gel called Equidone®. It is commonly given orally at 1.1 mg/kg once a day during the week before anticipated parturition if the mare shows no signs of milk production. Agalactia in a mare that has already foaled can be treated twice a day with the same dose as above until milk flow resumes.

## Prevention

### *Domperidone*

Domperidone can be given once a day to mares kept on fescue during the last 10–14 days before expected parturition (Cross, 1997). The dose is the same as for treatment above. Removal of the mare from fescue pasture and hay during the last month of gestation will also prevent a foaling problem.

## Novel or non-endophyte fescue

Endophyte-infected tall fescue pastures can be replanted with other grasses, non-endophyte fescue or new novel varieties of endophyte-infected fescue. Renovation requires a non-selective herbicide (e.g. paraquat, glyphosate) to

kill off the pasture grasses and planting with a smother crop for a season before spraying again with a herbicide and replanting with another pasture grass (Roberts and Andrae, 2004). Estimated costs of renovation are approximately $450/hectare (Fribourg and Waller, 2005). Non-endophyte fescue has been used, but it is not very drought, insect, nematode or herbivore resistant. Stand persistence is therefore not good. Novel endophyte varieties have been developed by infecting endophyte free varieties of fescue with endophytic strains that produce the peramine of tall fescue, but minimal or no ergot alkaloids (Roberts and Andrae, 2004; Fribourg and Waller, 2005). Several varieties have shown promise including MaxQ® and ARK Plus®. The novel varieties have insect and drought resistance without adversely affecting cattle, horses or sheep.

### Ammoniation of hay

Ammoniation of hay will degrade the ergovaline content of hay and make it safe to feed (Thompson *et al.*, 2001). Ammoniation requires enclosing the hay in an air-tight tent structure and pumping anhydrous ammonia gas into the tent for a period of time. This process, although effective, is time intensive and costly and generally has not been well accepted.

### Feed supplements

Several different types of feed additives have been investigated as ways to ameliorate fescue toxicosis. A glucomannan product (FEB-200®) from yeast cell walls that purport to bind ergovaline in the gastrointestinal tract and prevent its absorption improved cattle performance (Fribourg and Waller, 2005). Another feed supplement from seaweed, Tasco®, will ameliorate some oxidative effects of tall fescue causing immunosuppression in cattle (Fike *et al.*, 2001; Saker *et al.*, 2001). Just supplementing livestock with concentrates will decrease the overall dose of ergovaline from fescue.

### Pasture considerations

Pastures might be made less toxic by dilution with other grasses or legumes like Bermuda grass or clover, cutting seed heads from the pasture during the most toxic part of the summer, increasing the grazing pressure during the summer to avoid seed heads, or moving cattle to a non-fescue pasture during the warmest summer months (Stuedemann and Seman, 2005). Ergovaline content of stockpiled fescue also decreases by midwinter.

## CONCLUDING REMARKS

Tall fescue toxicosis is the major grass-induced forage problem in the United States (Oliver, 1997). Since the problem is really a mycotoxin (i.e. secondary metabolite of an endophytic fungus), the magnitude of the problem varies every year. Sometimes a lot of ergovaline is produced in a particular fescue pasture and in other years very little is produced and the nutrient rewards of fescue can be very profitable. Researchers are attacking the problem from two different angles. One attempt is to replace tall fescue with a novel endophytic fescue that is not toxic for animals, but still is drought and insect resistant. This has been accomplished, but the costs of pasture renovation are still considerable and some hilly terrain is not suitable for renovation. The other approach has been to find an antidote or preventative for the fescue problem. Domperidone is a suitable treatment or preventative for pregnant mares, but residue concerns will likely prevent its use in the near future for food producing animals. Other possible preventatives for food animals are still being investigated and include adsorbent feed supplements and vaccines.

## REFERENCES


Aldrich-Markham S, Pirelli G, Craig AM (2003) *Endophyte Toxins in Grass Seed Fields and Straw: Effects on Livestock*. Publication EM 8598. Report of Oregon State University Extension Service, Corvallis, OR.

Arechavaleta M, Bacon CW, Plattner RD, Hoveland CS, Radcliffe DE (1992) Accumulation of ergopeptide alkaloids in symbiotic tall fescue grown under deficits of soil water and nitrogen fertilizer. *Appl Environ Microbiol* **58**: 857–61.

Bacon CW (1995) Toxic endophyte-infected tall fescue and range grasses: historic perspectives. *J Anim Sci* **73**: 861–70.

Bacon CW, Porter JK, Robbins JD, Luttrell ES (1977) *Epichloe typhina* from toxic tall fescue grasses. *Appl Environ Microbiol* **34**: 576–81.

Ball DM (1984) An overview of fescue toxicity research. *Agri-Pract* **5**(6): 31–6.

Blodgett DJ (2001) Fescue toxicosis. In *The Veterinary Clinics of North America: Equine Practice*, vol. 17(3), Galey FD (ed.). W.B. Saunders Co., Philadelphia, PA, pp. 567–77.

Brendemuehl JP, Boosinger TR, Pugh DG, Shelby RA (1994) Influence of endophyte-infected tall fescue on cyclicity, pregnancy rate and early embryonic loss in the mare. *Theriogenology* **42**: 489–500.

Brendemuehl JP, Carson RL, Wenzel JGW, Boosinger TR, Shelby RA (1996) Effects of grazing endophyte-infected tall fescue on eCG and progestogen concentrations from gestation days 21 to 300 in the mare. *Theriogenology* **46**: 85–95.

Cross DL (1997) Fescue toxicosis in horses. In *Neotyphodium/Grass Interactions*, Bacon CW, Hill NS (eds). Plenum Press, New York, pp. 289–309.

Dyer DC (1993) Evidence that ergovaline acts on serotonin receptors. *Life Sci* **53**: PL 223–8.

Fike JH, Allen VG, Schmidt RE, Zhang X, Fontenot JP, Bagley CP, Ivy RL, Evans RR, Coelho RW, Wester DB (2001) Tasco-Forage. I.

Influence of a seaweed extract on antioxidant activity in tall fescue and in ruminants. *J Anim Sci* **79**: 1011–21.

Fluckiger E, Marko M, Doepfner W, Niederer W (1976) Effects of ergot alkaloids on the hypothalamic–pituitary axis. *Postgraduate Med J* **52**(Suppl. 1): 57–61.

Fribourg HA, Waller JC (2005) *Neotyphodium* research and applications in the USA, Chapter 1.1. In *Neotyphodium in Cool Season Grasses*, Roberts CA, West CP, Spiers DE (eds). Blackwell Publishing, Ames, IA, pp. 3–22.

Gadberry MS, Denard TM, Spiers DE, Piper EL (2003) Effects of feeding ergovaline on lamb performance in a heat stress environment. *J Anim Sci* **81**: 1538–45.

Glenn AE, Bacon CW, Price R, Hanlin RT (1996) Molecular phylogeny of *Acremonium* and its taxonomic implications. *Mycologia* **88**: 369–83.

Hill NS (2005) Absorption of ergot alkaloids in the ruminant, Chapter 12. In *Neotyphodium in Cool Season Grasses*, Roberts CA, West CP, Spiers DE (eds). Blackwell Publishing, Ames, IA, pp. 271–90.

Hill NS, Thompson FN, Dawe DL, Stuedemann JA (1994) Antibody binding of circulating ergot alkaloids in cattle grazing tall fescue. *Am J Vet Res* **55**: 419–24.

Lakritz J, Leonard MJ, Eichen PA, Rottinghaus GE, Johnson GC, Spiers DE (2002) Whole-blood concentrations of glutathione in cattle exposed to heat stress or a combination of heat stress and endophyte-infected tall fescue toxins in controlled environmental conditions. *Am J Vet Res* **63**: 799–803.

Larson B (1997) *Neotyphodium* toxicoses: an animal cellular/molecular research technique perspective. In *Neotyphodium/Grass Interactions*, Bacon CW, Hill NS (eds). Plenum Press, New York, pp. 347–60.

Lipham LB, Thompson FN, Stuedemann JA, Sartin JL (1989) Effects of metoclopramide on steers grazing endophyte-infected fescue. *J Anim Sci* **67**: 1090–7.

Lyons PC, Plattner RD, Bacon CW (1986) Occurrence of peptide and clavine ergot alkaloids in tall fescue grass. *Science* **232**: 487–9.

Morgan-Jones G, Gams W (1982) Notes on Hyphomycetes, XLI. An endophyte of *Festuca arundinacea* and the anamorph of *Epichloe typhina*, new taxa in one of two new sections of *Acremonium*. *Mycotaxon* **15**: 311–18.

Moubarak AS, Rosenkrans CF (2000) Hepatic metabolism of ergot alkaloids in beef cattle by cytochrome P450. *Biochem Biophys Res Commun* **274**: 746–9.

Moubarak AS, Piper EL, West CP, Johnson ZB (1993) Interaction of purified ergovaline from endophyte-infected tall fescue with synaptosomal ATPase enzyme system. *J Agric Food Chem* **41**: 407–9.

Moubarak AS, Piper EL, Johnson ZB, Flieger M (1996) HPLC method for detection of ergotamine, ergosine, and ergine after intravenous injection of a single dose. *J Agric Food Chem* **44**: 146–8.

Moubarak AS, Rosenkrans CF, Johnson ZB (2003) Modulation of cytochrome P450 metabolism by ergonovine and dihydroergotamine. *Vet Hum Toxicol* **45**: 6–9.

Oliver JW (1997) Physiological manifestations of endophyte toxicosis in ruminant and laboratory species. In *Neotyphodium/Grass Interactions*, Bacon CW, Hill NS (eds). Plenum Press, New York, pp. 311–46.

Oliver JW (2005) Pathophysiologic response to endophyte toxins, Chapter 13. In *Neotyphodium in Cool Season Grasses*, Roberts CA, West CP, Spiers DE (eds). Blackwell Publishing, Ames, IA, pp. 291–304.

Oliver JW, Linnabary RD, Abney LK, van Manen KR, Knoop R, Adair HS (1994) Evaluation of a dosing method for studying ergonovine effects in cattle. *Am J Vet Res* **55**: 173–6.

Oliver JW, Strickland JR, Waller JC, Fribourg HA, Linnabary RD, Abney LK (1998) Endophytic fungal toxin effect on adrenergic receptors in lateral saphenous veins (cranial branch) of cattle grazing tall fescue. *J Anim Sci* **76**: 2853–6.

Porter JK (1994) Chemical constituents of grass endophytes, Chapter 8. In *Biotechnology of Endophytic Fungi of Grasses*, Bacon CW, White Jr JF (eds). CRC Press, Boca Raton, FL, pp. 103–23.

Porter JK (1995) Analysis of endophyte toxins: fescue and other grasses toxic to livestock. *J Anim Sci* **73**: 871–80.

Porter JK, Thompson FN (1992) Effects of fescue toxicosis on reproduction in livestock. *J Anim Sci* **70**: 1594–603.

Rice RL, Blodgett DJ, Schurig GG, Swecker WS, Fontenot JP, Allen VG, Akers RM (1997) Evaluation of humoral immune responses in cattle grazing endophyte-infected or endophyte-free fescue. *Vet Immunol Immunopathol* **59**: 285–91.

Rice RL, Blodgett DJ, Schurig GG, Swecker WS, Thatcher CD, Eversole DE (1998) Oral and parenteral vaccination of mice with protein–ergotamine conjugates and evaluation of protection against fescue toxicosis. *Vet Immunol Immunopathol* **61**: 305–16.

Roberts C, Andrae J (2004) Tall fescue toxicosis and management. Online. *Crop Manage.* doi:10.1094/CM-2004-0427-01-MG.

Rottinghaus GE, Garner GB, Cornell CN, Ellis JL (1991) HPLC method for quantitating ergovaline in endophyte-infested tall fescue: seasonal variation of ergovaline levels in stems with leaf sheaths, leaf blades, and seed heads. *J Agric Food Chem* **39**: 112–15.

Saker KE, Allen VG, Fontenot CP, Bagley RL, Ivy RL, Evans RR, Wester DB (2001) Tasco-forage. II. Monocyte immune cell response and performance of beef steers grazing tall fescue treated with a seaweed extract. *J Anim Sci* **79**: 1022–31.

Schmidt SP, Osborn TG (1993) Effects of endophyte-infected tall fescue on animal performance. *Agric Ecosyst Environ* **44**: 233–62.

Smith GW, Rotstein DS, Brownie CF (2004) Abdominal fat necrosis in a pygmy goat associated with fescue toxicosis. *J Vet Diag Invest* **16**: 356–9.

Spiers DE, Evans TJ, Rottinghaus GE (2005) Interaction between thermal stress and fescue toxicosis: animal models and new perspectives, Chapter 11. In *Neotyphodium in Cool Season Grasses*, Roberts CA, West CP, Spiers DE (eds). Blackwell Publishing, Ames, IA, pp. 243–70.

Strickland JR, Oliver JW, Cross DL (1993) Fescue toxicosis and its impact on animal agriculture. *Vet Hum Toxicol* **35**: 454–64.

Stuedemann JA, Seman DH (2005) Integrating genetics, environment, and management to minimize animal toxicoses, Chapter 14. In *Neotyphodium in Cool Season Grasses*, Roberts CA, West CP, Spiers DE (eds). Blackwell Publishing, Ames, IA, pp. 305–24.

Stuedemann JA, Hill NS, Thompson FN, Fayrer-Hosken RA, Hay WP, Dawe DL, Seman DH, Martin SA (1998) Urinary and biliary excretion of ergot alkaloids from steers that grazed endophyte-infected tall fescue. *J Anim Sci* **76**: 2146–54.

Thompson FN, Stuedemann JA, Hill NS (2001) Anti-quality factors associated with alkaloids in eastern temperate pasture. *J Range Manage* **54**: 474–89.

Tor-Agbidye J, Blythe LL, Craig AM (2001) Correlation of endophyte toxins (ergovaline and lolitrem B) with clinical disease: fescue foot and perennial ryegrass staggers. *Vet Hum Toxicol* **43**: 140–6.

Yates SG, Powell RG (1988) Analysis of ergopeptine alkaloids in endophyte-infected tall fescue. *J Agric Food Chem* **36**: 337–40.

Zanzalari KP, Heitmann RN, McLaren JB, Fribourg HA (1989) Effects of endophyte-infected fescue and cimetidine on respiration rates, rectal temperatures and hepatic mixed function oxidase activity as measured by hepatic antipyrine metabolism in sheep. *J Anim Sci* **67**: 3370–8.

C H A P T E R

# 72

# Mushroom toxins

*Birgit Puschner*

## INTRODUCTION

It is very difficult to confirm exposure to mushroom toxins, and thus, clinical reports of mushroom poisoning are uncommon and there are most likely many unreported cases. Mushroom poisonings of humans and animals can demand extensive effort from clinicians and toxicologists and often involve emotion and publicity. The public expects the toxicology profession to provide guidance and a coherent approach regarding poisoning cases. While it is estimated that very few species are lethal to humans, it is not clear how many of the mushrooms worldwide contain potentially toxic compounds. New species are being discovered continuously and for many species, toxicity data is unavailable. In veterinary medicine, data regarding mushroom toxicity is sparse, however, it is possible that mushroom poisonings are more common in animals than humans as a result of the propensity of accidental exposure.

## BACKGROUND

The frequency of mushroom poisoning is likely to be low because of the lack of methods to confirm exposure to toxic mushrooms. Most cases are diagnosed by positive identification of the suspect mushroom along with the occurrence of consistent clinical signs. In the case of animals, mushroom exposure is even more difficult to prove since animals are often left unattended and a history of ingestion is not available. Between 2002 and 2005, over 700 cases of mushroom intoxication have been registered by the ASPCA Animal Poison Control Center, mainly in dogs, and also in cats. California accounted for 11% of these cases, the highest among all states. California also leads the United States in the number of reported cases of mushroom poisoning in humans, with approximately 2000 cases reported in 2004. Many factors influence the toxicity of mushroom toxins, such as genus and species of mushroom, geographic location where the mushroom is grown, preparation of mushroom prior to ingestion and the individual's susceptibility. While not inclusive of all mushroom toxins, this chapter is organized by the various types of toxins, providing detailed information on their toxic mechanisms, toxicokinetics, diagnostic, and therapeutic approaches with a focus on veterinary medicine.

## HEPATOTOXIC CYCLOPEPTIDES

Worldwide, most fatalities are caused by exposure to cyclopeptides. Three genera, *Amanita*, *Galerina*, and *Lepiota* (Lincoff *et al.*, 1977a) are known to contain hepatotoxic cyclopeptide toxins. The most toxic cyclopeptide-containing mushrooms are *Amanita phalloides*, the ubiquitous Death Cap or Death Angel and *Galerina sulpices*. *A. phalloides* is found throughout North America, commonly in association with oaks and birch, and is the species most frequently resulting in fatalities in humans (Mitchel, 1980; Barbato, 1993). In Eastern Europe, *G. sulpices* is considered the species most commonly associated with human fatalities, followed by *A. phalloides* (Klan, 1993). There are three groups of cyclopeptides, including the amatoxins, phallotoxins, and virotoxins. Amatoxins are bicyclic octapeptides and include the amanitins ($\alpha$-, $\beta$-, $\gamma$-, and $\varepsilon$-amanitins), amanin, amanullin, and proamanullin (Vetter, 1998). Severe poisonings and lethality are mainly attributable to the amanitins. The bicyclic heptapeptides phallotoxins were once thought to be the cause for gastrointestinal clinical signs, however they are no longer believed to exert any

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acute toxicity. Although the amount of research is limited, bicyclic heptapeptides virotoxins are not considered to have toxic effects after oral exposure. Therefore, phallotoxins and virotoxins are not discussed further.

## Pharmacokinetics/toxicokinetics

Definite data on bioavailability for amanitins is lacking, although there are known species differences (Faulstich and Fauser, 1980). In humans, bioavailability of amanitins appears to be much greater than in rodents, dogs, and rabbits. Within animal species, the absorption rate of amanitins is estimated to be much greater in dogs than in mice and rabbits, and rats appear resistant to the toxic effects of amanitins. α-amanitin is taken up by cells in the gastrointestinal tract where the first damaging effects are seen (Gundala *et al.*, 2004). Following systemic absorption, α-amanitin is taken up by the hepatocytes via a sodium-dependent transport system (Kroncke *et al.*, 1986). The α-amanitin has a low volume of distribution, and the renal clearance is high and similar to the creatinine clearance (Faulstich *et al.*, 1985). Following intravenous (i.v.) administration in dogs, it was shown that the plasma half-life of amanitins is short ranging from 25 to 50 min and that amanitins are not detectable in plasma after 4–6 h. There is no known metabolism or plasma protein binding of the α-amanitin. Between 80% and 90% of the administered dose of amanitins are eliminated in urine and up to 7% are eliminated in the bile (Faulstich *et al.*, 1985). After oral ingestion of *A. phalloides* in humans, α- and β-amanitins were detected in plasma up to 36 h after ingestion, and in urine up to 72 h post-exposure (Jaeger *et al.*, 1993). This may partly be due to slow intestinal absorption, entero-hepatic circulation, and reduced renal elimination resulting from nephrotoxicity. Plasma and urine amanitin concentrations do not seem to correlate with the clinical severity or outcome.

Amanitins can be detected in urine well before any clinical sign, whereas routine laboratory tests such as blood CBC (complete blood count) and serum chemistry profiles are unremarkable until liver or kidney damage has occurred. Early recognition of exposure is critical since survival rates are greatly improved with timely therapeutic intervention. Amanitin concentrations in kidneys and liver in people ingesting *A. phalloides* have been detected up to 22 days post-ingestion. The kidneys contain higher concentrations than liver, indicating that toxins are bound to renal tissue.

## Mechanism of action

Amanitins are of greatest significance because, unlike phalloidins, they are heat stable, and are not degraded by the acid environment of the stomach or by freezing (Himmelmann *et al.*, 2001). Therefore, amanitins are toxic by ingestion, whereas phalloidins have only been shown to be toxic when experimentally administered by parenteral routes. Amanitins exert their toxicity by inhibiting nuclear RNA polymerase II (Lindell *et al.*, 1970; Wieland, 1983). The decrease in mRNA and associated decrease in protein synthesis result in cell death. Cells with a high metabolic rate, such as hepatocytes, crypt cells, and proximal convoluted tubules of the kidneys are most commonly affected. While this mechanism is well established, more recent research has suggested that other cellular effects may contribute to the toxicity of amanitins. In mice, apoptosis contributed to amanitin-induced liver failure (Leist *et al.*, 1997) and in pancreatic rat islets, α-amanitin resulted in a dose-dependent insulin releasing and a β-cytotoxic effect (De Carlo *et al.*, 2003). Acute tubular necrosis in the kidneys is believed to be a result of reabsorption of amanitins by renal tubules after glomerular filtration.

The clinical course can be divided in four phases, with the initial phase being the latency period of approximately 8–12 h. The second phase is characterized by severe gastrointestinal signs such as nausea, vomiting, bloody diarrhea, and severe abdominal pain. In dogs, the onset of clinical signs is generally 6–24 h after mushroom ingestion. Beagles that were experimentally given amanitins developed vomiting and diarrhea starting 16 h after sublethal oral exposure. Gastrointestinal signs improved after 60 h (Vogel *et al.*, 1984). The gastroenteric phase is often followed with a lag period of several hours to a few days during which the human or animal will appear to have recovered. During this third phase, close monitoring of liver and kidney function is essential in order to prevent misdiagnosis. The final stage begins approximately 36–84 h after exposure to amanitins. In this stage, fulminant liver, renal, and multiorgan failure may occur. In dogs, significant elevations in serum of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase, and bilirubin are commonly observed (Vogel *et al.*, 1984; Kallet *et al.*, 1988). Coagulopathy, encephalopathy, and coma are common sequelae with liver failure (Vogel *et al.*, 1984). In humans, a combination of the prothrombin index along with serum creatinine concentrations determined between 3 and 10 days after ingestion was most useful as predictors of death (Ganzert *et al.*, 2005). Although no controlled study exists in dogs, both prothrombin (PT) and partial thromboplastin (PTT) times are important liver function parameters and may provide critical information in the assessment of a case (Tegzes and Puschner, 2002). If the animal survives the hepatopathy, renal failure may occur as a consequence of proximal and distal tubular necrosis (Kallet *et al.*, 1988; Tegzes and Puschner, 2002). Clinical signs of renal failure include polyuria, polydypsia, vomiting, and anorexia. Severe hypoglycemia may occur in dogs after the gastrointestinal

phase, and is associated with the breakdown of liver glycogen (Faulstich and Fauser 1980). In a study where dogs were given amanitin toxins or pieces of *A. phalloides* mushrooms in lethal doses, 50% of dogs died from hypoglycemia 1 to 2 days after exposure (Faulstich and Fauser, 1980). In clinical cases, dogs must be monitored closely for hypoglycemia, and treatment may be necessary. Clinical signs of hypoglycemia in dogs and cats include seizures, coma, and death. Finally, it is important to note that not all cases present with the classic four stages. In cases of large exposure to amanitins, or ingestion by puppies, the animal may die within 24 h (Cole, 1993).

## Toxicity

Amanitins are extremely toxic. The i.v. median lethal dose ($LD_{50}$) of α-amanitin in dogs is 0.1 mg/kg body weight (Faulstich and Fauser, 1980). Based on an oral dosing study in dogs, the oral $LD_{50}$ for methyl-γ-amanitin was estimated to be 0.5 mg/kg body weight. In mice and rats, the i.v. $LD_{50}$ of α-amanitin is 0.35 and 3–4 mg/kg body weight, respectively, illustrating significant species differences. Guinea pigs and rabbits are considered about equally sensitive to amanitins as dogs with i.v. $LD_{50}$'s of α-amanitin of 0.1 and 0.2 mg/kg, respectively. In humans, the estimated oral $LD_{50}$ of α-amanitin is 0.1 mg/kg body weight. Considering the average concentration of amanitins per mushroom, one *A. phalloides* has the potential to kill an adult, while it may require two mushrooms to cause death in a dog.

## Treatment

There is no specific antidote for amanitins. Despite the evaluation of numerous treatment options, no specific therapy has proven to be effective. Even with supportive measures, the reported mortality rate from Amanita poisoning in humans is 20–40%, and is often higher in children (Enjalbert *et al.*, 2002). To date, in humans with amanitin poisoning, silibinin, penicillin, and *N*-acetylcysteine (NAC) are most commonly recommended along with decontamination procedures and supportive care. Similar approaches have been used with variable success rates in dogs suffering from amanitin poisoning (Tegzes and Puschner, 2002). Prompt and aggressive measures are required to improve prognosis.

Although the exact mechanisms of silibinin and penicillin are not fully understood, both compounds reduce the uptake of amanitins into hepatocytes. Silibinin (also known as silybin) is the main component of silymarin and provides most of the hepatoprotection that is attributed to milk thistle. Silibinin is also a free radical scavenger, and has immunostimulatory and iron binding properties (Mayer *et al.*, 2005). Experimentally, silibinin was shown to be effective when given twice to dogs at a dose of 50 mg/kg i.v., 5 and 24 h after exposure to *A. phalloides* (Vogel *et al.*, 1984). Dosed dogs had better indices of liver function as assessed by serum elevations of AST, ALT, bilirubin, and prolonged PT. On histopathology, no hepatic lesions were found. The recommended i.v. dose for silibinin in humans is an initial bolus infusion of 5 mg/kg followed by a continuous rate infusion of 20 mg/kg/day for a minimum of 3 days (Karlson-Stiber and Persson, 2003). Side effects of silibinin administration are rare, but include anaphylactic reactions, mild laxative effects, and interactions with certain phase I and phase II metabolism enzymes (Venkataramanan *et al.*, 2000). Recently, the usefulness of penicillin G alone, not in combination with silibinin was shown to be ineffective in humans with amanitin poisoning (Enjalbert *et al.*, 2002). However, mice given 1000 mg/kg of penicillin G intraperitoneally (i.p.) 8 h after exposure to a lethal dose 95 ($LD_{95}$) of amanitin had less morbidity and mortality than control mice (Floersheim, 1972). In dogs, i.v. administration of 1000 mg/kg of penicillin G at 5 h post *A. phalloides* exposure was considered an effective treatment (Floersheim, 1978). Based on recent data, administration of silibinin appears to have greater therapeutic benefit than penicillin G (Enjalbert *et al.*, 2002) at least in humans. Such data is not available for animals. This is also true for the use of antioxidants in amanitin poisoning cases, where information regarding the efficacy has only been published for humans but not animals. The antioxidants evaluated in humans are NAC, cimetidine, and ascorbic acid. Most information is available for NAC, which was shown to be as effective as silibinin in reducing mortality in humans after amanitin poisoning (Enjalbert *et al.*, 2002). In a mouse model, NAC administration was not effective (Schneider *et al.*, 1992). Although efficacy data of NAC administration in dogs with amanitin poisoning is lacking, there is no reason not to include the glutathione precursor in the treatment regimen. Ascorbic acid and cimetidine are known hepatocyte protectors and may be of benefit when managing amanitin poisoning in dogs, but specific data is not available. In contrast, thioctic acid and steroids are no longer recommended in the treatment of amanitin poisoning.

A variety of decontamination procedures have been applied in humans and include hemodialysis, hemoperfusion, activated charcoal, plasmapharesis, forced diuresis, and nasoduodenal suctioning. Controversy still remains about the efficacy of many of these procedures as specific data does not exist. There is general agreement however, that activated charcoal is beneficial in adsorbing toxins within the gastrointestinal tract as well as those that re-enter it due to entero-hepatic recirculation. In dogs, multidose activated charcoal is given every 2–6 h until 2–3 days post-ingestion. Close monitoring, fluid replacement, and supportive care are essential parts of the treatment in amanitin poisonings. As part of vigorous supportive care, i.v. fluids, correction of hypoglycemia and electrolyte

imbalances, vitamin $K_1$, and plasma transfusions should be considered, dependent on the clinical presentation of each poisoned patient. In humans, liver transplantation has been used successfully in patients with fulminant liver failure. At this time, liver transplantation is not an option for animals poisoned with amanitins.

Diagnosis of amanitin toxicosis is aided by identification of amanitin-containing mushrooms in the environment of the animal. Mushroom pieces found in gastric contents can confirm exposure. Accurate mushroom identification will require consultation with an experienced mycologist. Detection of amanitins in biologic specimens is confirmatory, but these tests are not routinely available at diagnostic laboratories. Recently, a liquid chromatography/mass spectrometry method was developed and successfully applied to confirm amanitin poisoning in five humans and a dog (Puschner, personal communication). The method is highly sensitive, specific and rapid, and detects α- and β-amanitins. This is an improvement over the commercially available ELISA that is validated for specimens of human origin and does not detect β-amanitin (Butera *et al.*, 2004), although it is known that some *Amanita* spp. contain only β-amanitin. Recently, a competitive ELISA was constructed that allows for the detection of β-amanitin in human serum and urine, but this assay is not yet available in clinical settings (Abuknesha *et al.*, 2004). Rapid confirmation of amanitins in suspect exposures assists in the early recognition of exposure, while a negative result can prevent unnecessary hospitalization. In a recent study, it was also shown that the newspaper test of Wieland, or Meixner test, should not be used alone to identify amanitin-containing mushrooms (Beuhler *et al.*, 2004). In suspect cases of aminitin poisoning, serum and urine samples should be collected at various time points beginning as early after exposure as possible. In post-mortem presentations, liver and kidney samples are suitable for testing to confirm exposure. The suspect mushroom or vomited gastrointestinal contents should also be saved for further analysis.

Differential diagnoses in dogs and cats with a clinical presentation that involves gastroenteritis and hepatic failure include other toxic ingestions such as blue-green algae, xylitol cocklebur, cycad palm, aflatoxin, gyromitrin, and acetaminophen overdose. The history and geographic environment of the animal can help to eliminate most of the toxicant differentials on the list.

# HYDRAZINES

*Gyromitra* species are members of the false morel family, Helvellaceae and are usually found under conifers, aspens, and sometimes around melting snow banks. The species most commonly associated with poisoning and studied in most detail is *Gyromitra esculenta*, but the toxins have been found in other species of Helvellaceae (Viernstein *et al.*, 1980). These include *Gyromitra gigas*, *Gyromitra fastigiata*, *Gyromitra infula*, *Helvella crispa*, and *Helvella lacunose*. The toxins associated with false morel poisoning are hydrazine analogues. The toxins are heat labile, volatile, and water soluble (Michelot and Toth, 1991). The process of boiling and drying decreases, but does not completely eliminate the toxin concentrations (Pyysalo, 1976). People who eat only a few of the cooked mushrooms may ingest sufficiently detoxified amounts so as to remain symptom free. This has caused misunderstandings among people of the potential lethality of these mushrooms. Animals generally eat raw mushrooms; therefore any exposure to these mushrooms may result in serious morbidity and mortality. Poisoning by some species of *Helvella*, *Verpa*, *Morchella*, *Peziza*, *Disciotis*, and *Sarcosphaera* closely resembles the syndrome caused by gyromitrin. It has been speculated that these mushrooms also contain hydrazines, though analysis has yet to confirm the presence of these toxins (Lincoff and Mitchel, 1977b).

## Pharmacokinetics/toxicokinetics

There is very limited information available. Toxicosis can result after oral and inhalation exposure. Ingestion of gyromitrin (acetaldehyde *N*-methyl *N*-formylhydrazine) containing mushrooms results in the hydrolysis of gyromitrin to *N*-methyl-*N*-formylhydrazine, which is further metabolized to monomethylhydrazine. The degree of hydrolysis is dependent on the pH in the stomach, but it is not complete (Wright *et al.*, 1978). Inhalation of the fumes during the cooking process can also result in poisoning. Once hydrazines reach the liver, they are further metabolized to reactive intermediates, such as methyl cations and free methyl radicals (Gannett *et al.*, 1991).

## Mechanism of action

Gyromitrin is considered a gastrointestinal irritant leading to clinical signs of vomiting, abdominal pain, and diarrhea 6–12 h after ingestion of the poisonous mushroom (Coulet and Guillot, 1982). The principal toxin responsible for convulsions seen in severe cases of gyromitra poisoning is monomethylhydrazine. Monomethylhydrazine inhibits pyridoxal phosphokinase resulting in decreased pyridoxal 5-phosphate concentrations (Lheureux *et al.*, 2005). Depletion of pyridoxal 5-phosphate leads to decreased γ-aminobutyric acid (GABA) synthesis and an increase in glutamic acid concentrations. Because of a study in mice, in which GABA concentrations in the brain were not significantly decreased after methylhydrazine exposure (Maynert and Kaji, 1962) other mechanisms have been proposed. In addition to the gastrointestinal irritation and neurotoxicity, liver and renal failures as well as hemolysis

have been described. *N*-methyl-*N*-formylhydrazine inhibits cytochrome P450 and glutathione metabolizing enzymes (Braun *et al.*, 1979) and can lead to liver necrosis. However, the highly reactive metabolites, such as methyl cations generated in the liver may significantly contribute to the hepatic injury. Furthermore, the hydrazine analogues present in false morels are carcinogenic in laboratory animals (Toth and Gannett, 1994). Only one case report exists in the veterinary literature. A 10-week-old dog vomited 2–3 h after chewing on a mushroom later identified as *G. esculenta* (Bernard, 1979); 6 h post-ingestion, the dog was lethargic, became comatose, and died 30 min later. Histopathologic findings included renal tubulonephrosis, periascinar hepatic degeneration, and erythrophagocytosis.

## Toxicity

In humans, there seems to be great individual variability with regards to the toxicity of false morel poisoning. In mice, the oral $LD_{50}$'s of gyromitrin, *N*-methyl-*N*-formylhydrazine, and monomethylhydrazine are 344, 118, and 33 mg/kg, respectively (Wright *et al.*, 1978). In humans, the estimated lethal dose of gyromitrin is 20–50 mg/kg for adults and 10–30 mg/kg for children (Schmidlin-Meszaros, 1974). Toxicity information for dogs or cats has not been established. Gyromitrin concentrations in fresh *G. esculenta* are estimated from 0.12% to 0.16%.

## Treatment

In humans, most patients develop only mild gastrointestinal symptoms and recover fully within several days after exposure. Management is principally supportive. Early decontamination is often not possible because of the delayed onset of clinical signs. Administration of activated charcoal has been recommended, although efficacy studies have not been performed. Correction of fluid and electrolytes are important measures along with the administration of pyridoxine. The recommended dose in humans is 25 mg/kg i.v. over 15–30 min. The dosing can be repeated but should not exceed more than 20 g/day. While pyridoxine can successfully control seizure activity, it has no benefit in preventing liver injury. In dogs, pyridoxine has been used successfully for non-mushroom toxin-induced seizure activity. It can be used alone or in combination with diazepam. Combination therapy has better efficacy than pyridoxine alone (Villar *et al.*, 1995). The dose for dogs is 75–150 mg/kg body weight, given i.v., during acute phases of seizure activity. Diazepam is given to dogs and cats at 0.5–1.0 mg/kg i.v. to effect. Phenobarbital is not recommended for seizure control because of its cytochrome P450 inducing capability. Administration of folinic acid has been recommended in humans, but controlled studies have not been performed. The cytochrome P450 inhibitor cimetidine and NAC to the prevent hepatic injury should be considered. The dosing is same as that given in acetaminophen-induced hepatic injury. Hemodialysis has been reported in the literature in the treatment of gyromitrin poisoning but its role in removing gyromitrin or its toxic metabolites is not known.

Diagnosis of gyromitrin toxicosis is aided by identification of gyromitrin-containing mushrooms in the environment of the animal. Identification by a mycologist is important to distinguish between the true morels from the false morels. Detection of gyromitrin, hydrazine analogues, or metabolites in biologic specimens is not routinely available and diagnosis is mainly based on clinical clinicopathologic findings, and mushroom identification.

# MUSCARINIC AGENTS

The genera with the highest concentrations of muscarine are in the genera *Inocybe* and *Clitocybe*, though muscarine is also found in lower concentrations in many other genera including *Entoloma* and *Mycena* (Young, 1994). Muscarine was first discovered and characterized in *Amanita muscaria*, but concentrations of muscarine are only about 0.0003% (Eugster and Schleusener, 1969). In comparison, *Inocybe* and *Clitocybe* species have muscarine concentrations between 0.1% and 0.33%. *Inocybe* and *Clitocybe* species have worldwide distributions and are relatively common. *Inocybe* species grow in association with either conifers or broadleafed trees. *Clitocybe* species grow on forest litter or grassland humus. The risk of poisoning remains after cooking because of the heat stability of muscarine. There is one published report of a muscarine-containing mushroom, *Inocybe phaeocomis* poisoning in a dog (Yam *et al.*, 1993).

## Pharmacokinetics/toxicokinetics

There is limited data available. The naturally occurring form of muscarine is the (L)+ form. In general, quarternary ammonium compounds are poorly absorbed after oral exposure. Once absorbed muscarine is quickly distributed throughout the body and clinical signs develop within 30 min to 2 h of ingestion. Because of its quaternary configuration, muscarine does not cross the blood–brain barrier and its cholinergic effects are entirely peripheral. A portion of the ingested muscarine is eliminated unchanged in urine, but detailed toxicokinetic studies have not been performed.

## Mechanism of action

Muscarine acts in the peripheral nervous system, where it competes with acetylcholine at its receptor binding sites. The muscarinic cholinergic receptors that are able to bind

muscarine are found in the heart in both its nodes and muscle fibers, in smooth muscles, and in glands. They do not occur in skeletal muscles. Once bound to the receptor, muscarine mimics the effect of acetylcholine as a neurotransmitter. Toxicity occurs because of uncontrolled stimulation of the receptors. Under normal circumstances, acetylcholinesterase degrades excess acetylcholine into acetate and choline, preventing overstimulation by acetylcholine. Muscarine is not susceptible to inactivation by acetylcholinesterase (Young, 1994) and overstimulation of the receptors occurs. Clinical signs appear within a few hours, and include salivation, lacrimation, vomiting, diarrhea, abdominal pain, miosis, and bradycardia (Stallard and Edes, 1989). Clinical signs in the dog eating *Inocybe phaecocomics* were observed 3 h after exposure and included salivation, diarrhea, vomiting, depression, and collapse (Yam *et al.*, 1993).

## Toxicity

In mice, the i.v. $LD_{50}$ of muscarine is 0.23 mg/kg (Waser, 1961). The lethal dose of muscarine in humans is estimated to be between 180 and 300 mg and the ingestion of a single mushroom containing 0.33% muscarine on a dry weight basis can be lethal (Bresinsky and Besl, 1990a).

## Treatment

Treatment includes early decontamination, administration of activated charcoal, and fluid rehydration. If life-threatening clinical signs are present, atropine should be administered. Atropine acts by competing with both acetylcholine and muscarine at cholinergic muscarinic receptor sites. After giving a test dose to determine its efficacy, atropine can be given repeatedly until symptoms are abolished or until cessation of salivation. Doses in dogs and cats are 0.2–2.0 mg/kg, where one-fourth of the dose is given i.v., and the remainder is given either subcutaneously or intramuscularly. Other criteria for therapeutic endpoint with atropine include ease of respiration and lack of respiratory secretions. Mydriasis is not an indicator of its effectiveness. Because atropine also competes with acetylcholine at the receptors, ongoing treatment must be carefully monitored for its anticholinergic effects. These include tachycardia, gastrointestinal stasis, severe behavioral changes (e.g. delirium), and hyperthermia. The dose of atropine should be reduced or discontinued with these adverse effects.

# ISOXAZOLES

Poisoning in this group is attributed to the heat-stable isoxazoles derivatives, ibotenic acid, and muscimol. These toxins are found in *Amanita pantherina* (panther cap, panther agaric) and *A. muscaria* (fly agaric), the two mushrooms most commonly associated with poisonings in humans in this group (Hall and Hall, 1994). The mushrooms grow from summer to autumn in coniferous and deciduous forests, and are abundant in the Pacific Northwest where they are often found under Douglas fir trees. Other species containing these toxins include *Amanita gemmata*, *Amanita smithiana*, *Amanita strobiliformis*, and *Tricholoma muscarium* (Lincoff and Mitchel, 1977c). There are a few published reports of poisoning in dogs (Naude and Berry, 1997) and cats (Ridgway, 1978) after ingesting *A. pantherina*.

## Pharmacokinetics/toxicokinetics

Definite data on the toxicokinetics of muscimol and ibotenic acid have not been established. Based on the rapid onset of clinical signs after oral exposure to the poisonous mushrooms, rapid absorption of the toxins is suspected. Once absorbed, muscimol and ibotenic acid appear to cross the blood–brain barrier by an active transport system. Ibotenic acid decarboxylates to form muscimol in the stomach, liver, and brain (Nielsen *et al.*, 1985). Muscimol and ibotenic acid can be detected in urine within 1 h of exposure (Ott *et al.*, 1975).

## Mechanism of action

The major toxins in this group are muscimol and ibotenic acid, but other active substances have been identified, although with minor pharmacologic activities. Though both muscimol and ibotenic acid are present in mushrooms, muscimol is further derived from ibotenic acid by spontaneous decarboxylation that can occur during dehydration of the mushroom, during digestion in the stomach, or after absorption in a variety of tissues. Therefore, muscimol is likely the major toxin resulting in clinical signs of poisoning. Muscimol is a potent agonist at $GABA_A$, a partial agonist at $GABA_C$ receptors, and inhibits neuronal and glial GABA uptake. $GABA_A$ and $GABA_C$ receptors are chloride channels that mediate synaptic inhibition, although the receptors differ pharmacologically (Chebib and Johnston, 1999). On activation by muscimol, the membrane permeability for anions increases, usually resulting in a slight, short-lasting hyperpolarization and associated decreased excitability of the receptive neuron. Effects on the CNS are similar to those produced by therapeutic doses of diazepam. Ibotenic acid activates inhibitory glutamate receptors (Cleland, 1996). Interestingly, these ligand-gated ion channels differ from any of the excitatory glutamate receptor families and are much more closely related to the glycine and GABA receptors (Cleland and Selverston, 1998).

Typical clinical signs of muscimol toxicosis begin with 30 min to 2 h after ingestion and have been termed the "pantherine–muscaria" syndrome in humans. In humans, clinical signs include mydriasis, dryness of mouth, ataxia, confusion, euphoria, dizziness, and tiredness. Gastrointestinal signs are not consistently seen in cases of isoxazole poisoning. Full recovery is expected within 1–2 days. In cats, clinical signs have been observed within 15–30 min after ingestion of *A. pantherina* (Ridgway, 1978). After a brief period of sedation, cats experienced a 4-h long state of excitement with pronounced muscle spasms, followed by a deep sleep. Cats are expected to fully recover within 24 h after ingestion, especially if decontamination measures are taken. In dogs, clinical signs observed after ingestion of *A. pantherina* included disorientation, opisthotonus, paresis, seizures, paddling, chewing movements, miosis, vestibular signs, respiratory depression, coma, and death (Hunt and Funk, 1977; Naude and Berry, 1997). Recoveries are recorded within 12–24 h after aggressive supportive care measures including mechanical ventilation during periods of respiratory depression; however death was reported in several dogs. Similar clinical signs were reported in a dog that survived *A. muscaria* poisoning (Martin, 1956).

## Toxicity

In mice, the oral $LD_{50}$ is 22 mg/kg for muscimol and 38 mg/kg for ibotenic acid (Hall and Hall, 1994). In rats, the oral $LD_{50}$ is 45 mg/kg for muscimol and 129 mg/kg for ibotenic acid. The i.p. $LD_{50}$ of muscimol is 2.5 mg/kg in mice and 3.5 mg/kg in rats. In humans, the toxic threshold is estimated to be 6 mg of muscimol and 30–60 mg of ibotenic acid (Halpern, 2004). The concentration of ibotenic acid in *A. muscaria* was estimated at 100 mg/kg fresh, while the concentration of muscimol was below 3 mg/kg fresh weight. Thus, an average size fruit body of *A. muscaria* weighing 60–70 g can contain a toxic concentration. In dogs, toxicity data is not available. However, postmortem examination of puppies indicated that the ingestion of a single *A. pantherina* can be lethal (Hunt and Funk, 1977).

## Treatment

Treatment of exposed animals is mainly symptomatic and supportive. Decontamination measures should be considered in recent exposures, though emetics are only recommended in animals that are not at risk for developing aspiration pneumonia. Specific measures to control seizures are not without complication. Benzodiazepines, as GABA agonists, may potentiate any CNS depression. Therefore, when diazepam is used, CNS and respiratory depression may be severe and prolonged, necessitating the use of mechanical ventilation. As long as ventilation is maintained adequately, the prognosis for recovery is good. Diazepam can be given to dogs and cats at 0.5 mg/kg i.v. to effect, and can be repeated as needed every 10 min for up to three doses. Other drugs to control seizures include phenobarbital and pentobarbital. Phenobarbital is dosed at 6 mg/kg i.v. Pentobarbital is given at 5–15 mg/kg i.v. to effect to dogs and cats. These, too, are agonists at the GABA receptors, and can potentiate CNS and respiratory depression. Careful monitoring of the animal's oxygenation status is vital until the animal is fully awake and alert. Supplemental oxygen can be used if necessary. General supportive measures of unconscious animals include maintaining hydration with i.v. fluids, maintaining the airway free of respiratory secretions, and frequent position changes to prevent decubitus skin ulcerations. In humans, the use of atropine is contraindicated because of the atropine-like clinical presentation in poisonings.

# PSILOCIN AND PSILOCYBIN

Mushrooms that contain psilocybin are commonly referred to as hallucinogenic or magic mushrooms. *Psilocybe*, *Panaeolus*, *Conocybe*, and *Gymnopilus* are the four genera in North America that contain psilocybin (Smolinske, 1994). Many of these mushrooms are coprophilic and grow in fields and animal pastures, particularly in the northwest and southeast United States. The majority of mushrooms contain only psilocybin, but some, such as *Psilocybe cyanescens* contain psilocybin and psilocin. The concentrations of psilocybin and psilocin are influenced by growth conditions, geographic location, storage conditions, and species. Species commonly found in the Pacific Northwest contained between 1.2 and 16.8 mg/kg psilocybin on a dry weight basis. If psilocin was present, concentrations could reach up to 9.6 mg/kg on a dry weight basis (Smolinske, 1994). Psilocin and psilocybin are sensitive to heat. In addition, some of the mushrooms in this group contain other pharmacologically active substances such as serotonin and tryptophan. There is only one published report of hallucinogenic mushroom ingestion in a dog (Kirwan, 1990).

## Pharmacokinetics/toxicokinetics

Psilocybin is a pro-drug and is rapidly dephosphorylated to psilocin. Dephosphorylation can take place in a variety of tissues, but high activity has been identified in kidney and liver of rodents (Horita and Weber, 1961) and in plasma of humans (Grieshaber *et al.*, 2001). However, the

general assumption is that complete conversion of psilocybin to psilocin occurs prior to absorption into the systemic circulation (Laatsch, 1996). In humans, the absolute bioavailability of psilocin liberated from orally administered psilocybin was estimated to be 52.7 ± 20% (Hasler *et al.*, 1997). Once absorbed, psilocin is further metabolized in plasma to 4-hydroxy-tryptophole (4HT) and 4-hydroxyindole-3-acetic acid (4HIAA). Psilocin crosses the blood–brain barrier and concentrates in brain tissue (Horita and Weber, 1961). Psilocin is excreted unchanged or as psilocin–glucuronide in urine and to some extent unchanged via the bile (Hasler *et al.*, 2002).

### Mechanism of action

Psilocin is the pharmacologically active metabolite of psilocybin. Because of its structural similarity to serotonin, psilocin exerts its effects in the CNS by stimulation of serotonin receptors (McKenna *et al.*, 1990). Psilocin primarily activates 5-$HT_{2A}$ receptors, but has affinity for 5-$HT_{1A}$ receptors as well. Activation of 5-$HT_{2A}$ receptors leads to increased cortical activity via glutamatergic excitatory post-synaptic potentials (Aghajanian and Marek, 1997). Activation of 5-$HT_{1A}$ receptors results in the inhibition of pyramidal cell activity (Puig *et al.*, 2005). Additionally, psilocin may also have peripheral effects that involve serotonergic receptors. In humans, psilocin psychoactive effects similar to those produced by LSD are observed within 20–30 min of ingestion and include visual hallucinations, intensified hearing, and incoordination. Other autonomic-mediated effects can include increased heart rate, increased blood pressure, mydriasis, tremors, and increased temperature. The effects can last up to 8 h, but hallucinogenic activity rarely exceeds 1 h. Clinical signs in dogs include ataxia, vocalization, overt aggression, nystagmus, and increased body temperature (Kirwan, 1990). In contrast to the CNS effects after exposure to isoxazoles, there is no subsequent coma. Sedation may be necessary until behavioral signs resolve.

### Toxicity

In many countries psilocybin and psilocin are classified as controlled substances, e.g. in the United States, Great Britain, and Germany. In humans, oral exposure to 10–20 mg of psilocybin can cause mood changes and hallucinations. Information regarding lethal doses in animals are not found in the literature.

### Treatment

The management of hallucinogenic mushroom poisonings is primarily supportive and in most cases, treatment is not necessary. Gastric emptying procedures have not proven beneficial and are not recommended. The effect of activated charcoal in poisonings is not known, but activated charcoal administration can be considered. If severe neurologic signs occur, such as seizures, diazepam is considered the first line medication. Diazepam can be given to dogs and cats at 0.5–1.0 mg/kg i.v. in increments of 5–10 mg, to effect. If diazepam is unsuccessful, subsequent seizures can be controlled with phenobarbital at 6 mg/kg to effect. Additionally, control of body temperature is an important factor of the symptomatic care.

Diagnosis of psilocybin exposure is confirmed by the detection of psilocin and psilocin–glucuronide in urine, serum, and blood. A number of methodologies are available and are routinely used in forensic investigations (Kamata *et al.*, 2003; Albers *et al.*, 2004). Because of the infrequent presentation of hallucinogenic mushroom poisoning in animals, these methodologies are not routinely available at veterinary diagnostic laboratories.

## GASTROINTESTINAL IRRITANTS

This group includes mushrooms that result in gastroenteritis as the primary clinical sign. There are very few reports in the veterinary literature. Genera included are *Agaricus*, *Boletus*, *Chlorophyllum*, *Entoloma*, *Lactarius*, *Omphalotus*, *Rhodophyllus*, *Scleroderma*, and *Tricholoma*. The specific toxins in most have not been identified (Spoerke, 1994). One of the mushrooms in this group commonly reported to cause poisoning is *Chlorophyllum molybdites*. This mushroom is commonly found in the United States except for the colder, northern areas and the principal toxin has not been clearly identified. Clinical signs of nausea, vomiting, and diarrhea appear 1–6 h after ingestion in humans, and there is complete recovery within 24–48 h (Blayney *et al.*, 1980). *Omphalotus olearius*, *Omphalotus subilludens*, and *Lampteromyces japonicus* contain illudin S (Bresinsky and Besl, 1990b). Illudin S is a sesquiterpene with a unique chemical structure. In humans, vomiting and diarrhea occur 1–2.5 h after ingestion (French and Garrettson, 1988). In a pot-bellied pig, death has been reported 5 h after ingesting a fruiting body of *Scleroderma citrinum* (Galey *et al.*, 1990). The pig vomited and collapsed 20 min after exposure and remained weak and recumbent until its death. The toxins in *S. citrinum* have not been characterized. In *Boletus satanas*, lectins may contribute to serious gastroenteritis in man. Lectins, which are storage proteins that may play a role in plant defense, are widely distributed in many species of mushrooms (Wang *et al.*, 1998).

Poisonings by mushrooms of this group are rarely fatal, hence they are likely to be underreported by owners, and

rarely recorded in the literature. After a usually short latent period of 15 min to 2 h after ingestion, an animal may present with vomiting, diarrhea, and abdominal pain. Usually, these clinical signs resolve spontaneously within a few hours, but they may last a day or 2. The clinical signs may resemble any other common cause of gastroenteritis in small animals, including bacterial and viral infections, sudden diet changes or eating spoiled foods, and inflammatory-mediated syndromes such as acute pancreatitis. The diagnostic challenge is that many of the more toxic mushrooms can initially cause these same clinical signs. Cyclopeptides cause vomiting and diarrhea after a lag period similar to that seen with the gastrointestinal irritants. Therefore, any animal presenting with gastrointestinal signs after a known history of mushroom ingestion must be treated thoroughly.

Treatment is entirely non-specific and supportive. Vomiting is a hallmark of poisoning by gastrointestinal irritant mushrooms. Thus, in most cases the stomach has already been emptied and emetics are not necessary. Activated charcoal is thought to adsorb most of the toxins in this group and should be administered orally unless there is protracted vomiting. There are no specific antidotes for the toxins in this group. Treatment should focus on rehydration, correction of serum electrolyte abnormalities, and possibly antiemetics.

## ORELLANINE

There are several cyclopeptide-containing mushrooms that cause renal failure without any hepatic insult. Mushrooms of the genus *Cortinarius* contain the cyclopeptide orellanine (Danel *et al.*, 2001). In North America, there are only a few reported cases in humans, but poisonings occur with much greater frequency in western and central Europe. In humans, symptoms may not appear for 2–20 days after ingestion. Initially, people experience nausea, vomiting, and abdominal pain. This is followed by intense thirst, chills, polyuria or oliguria, and then possibly, anuria. Hemodialysis may be necessary until renal function gradually improves. There are no reports of orellanine poisoning in animals.

## RAMARIA FLAVO-BRUNNESCENS

This mushroom is found in North America, Australia, China, Brazil, and Uruguay and has caused poisoning in cattle and sheep (Kommers and Santos, 1995). *Ramaria flavo-brunnescens* is exclusively found in Eucalyptus woods and thus, the poisoning has been termed "eucalyptus sickness". Clinical signs have been observed as early as 3 days after exposure to the mushrooms, but may be delayed until 6 days post-exposure. Typically, the animals develop anorexia, salivation, diarrhea, and recumbency. This is followed by alteration in keratinization, which becomes apparent by loss of hair and hooves, similar to what is seen in chronic selenosis. The toxins have not been identified, but are most likely volatile and interfere with the incorporation of sulfur-containing amino acids.

## CONCLUDING REMARKS AND FUTURE DIRECTIONS

Overall, the number of reported mushroom poisonings in animals is low, although this is likely a result of the lack of methods to confirm exposure. In humans, most cases are diagnosed by positive identification of the suspect mushroom, which is a very difficult task in veterinary medicine. The chances of obtaining an intact and representative mushroom are slim because animals are often left unattended and a history of ingestion is not available. The development of new analytical techniques to identify mushroom toxins in biological samples of poisoned animals will provide insight into the true frequency of mushroom poisonings. Currently, therapeutic measures are primarily based on both mechanisms of toxicity and clinical signs. Rapid toxin identification would allow for a confirmed, early diagnosis, which is especially important in cases where intensive care is necessary to save lives. In order to develop analytical techniques for specific mushroom toxins, further research is necessary in the area of toxin characterization. Although thousands of mushrooms exist worldwide, only a few have been researched in-depth. It is also important to improve our knowledge of the distribution kinetics and the efficacy of commonly recommended, but poorly evaluated decontamination procedures for many poisonous mushrooms.

## REFERENCES

Abuknesha RA, Maragkou A (2004) A highly sensitive and specific enzyme immunoassay for detection of beta-amanitin in biological fluids. *Anal Bioanal Chem* **379**: 853–60.

Aghajanian GK, Marek GJ (1997) Serotonin induces excitatory postsynaptic potentials in apical dendrites of neocortical pyramidal cells. *Neuropharmacology* **36**: 589–99.

Albers C, Kohler H, Lehr M, *et al.* (2004) Development of a psilocin immunoassay for serum and blood samples. *Int J Legal Med* **118**: 326–31.

Barbato MP (1993) Poisoning from accidental ingestion of mushrooms. *Med J Aust* **158**: 842–7.

Bernard MA (1979) Mushroom poisoning in a dog. *Can Vet J* **20**: 82–3.
Beuhler M, Lee DC, Gerkin R (2004) The Meixner test in the detection of α-amanitin and false positive reactions caused by psilocin and 5-substituted tryptamines. *Ann Emerg Med* **44**: 114–20.
Blayney D, Rosenkranz E, Zettner A (1980) Mushroom poisoning from *Chlorophyllum molybdites*. *West J Med* **132**: 74–7.
Braun R, Greeff U, Netter KJ (1979) Liver injury by the false morel poison gyromitrin. *Toxicology* **12**: 155–63.
Bresinsky A, Besl H (1990a) Muscarine syndrome. In *A Colour Atlas of Poisonous Fungi*, Bresinsky A, Besl H (eds). Wolfe Publishing Ltd, London, pp. 71–3.
Bresinsky A, Besl H (1990b) Gastrointestinal syndrome. In *A Colour Atlas of Poisonous Fungi*, Bresinsky A, Besl H (eds). Wolfe Publishing Ltd, London, pp. 130–76.
Butera R, Locatelli C, Coccini T, *et al.* (2004) Diagnostic accuracy of urinary amanitin in suspected mushroom poisoning: a pilot study. *Clin Toxicol* **42**: 901–12.
Chebib M, Johnston GA (1999) The "ABC" of GABA receptors: a brief review. *Clin Exp Pharmacol Physiol* **26**: 937–40.
Cleland TA (1996) Inhibitory glutamate receptor channels. *Mol Neurobiol* **13**: 97–136.
Cleland TA, Selverston AI (1998) Inhibitory glutamate receptor channels in cultured lobster stomatogastric neurons. *J Neurophysiol* **79**: 3189–96.
Cole FM (1993) A puppy death and *Amanita phalloides*. *Aust Vet Assoc* **70**: 271–2.
Coulet M, Guillot J (1982) Poisoning by Gyromitra: a possible mechanism. *Med Hypotheses* **8**: 325–34.
Danel VC, Saviuc PF, Garon D (2001) Main features of *Cortinarius* spp. poisoning: a literature review. *Toxicon* **39**: 1053–60.
De Carlo E, Milanesi A, Martini C, *et al.* (2003) Effects of *Amanita phalloides* toxins on insulin release: *in vivo* and *in vitro* studies. *Arch Toxicol* **77**: 441–5.
Enjalbert F, Rapior S, Nouguier Soule J, *et al.* (2002) Treatment of amatoxin poisoning: 20-year retrospective analysis. *J Toxicol Clin Toxicol* **40**: 715–57.
Eugster CH, Schleusener E (1969) Stereomere Muscarine kommen in der Natur vor. Gas-chromatographische Trennung der Norbasen 30. Mitteilung über Inhaltsstoffe von Fliegenpilzen. *Helv Chim Acta* **52**: 708–15.
Faulstich H, Fauser U (1980) The course of *Amanita* intoxication in beagle dogs. In *Amanita Toxins and Poisoning*, Faulstich H, Kommerell B, Wieland T (eds). Verlag Gerhard Witzstrock, Baden-Baden, Germany, pp. 115–23.
Faulstich H, Talas A, Wellhoner HH (1985) Toxicokinetics of labeled amatoxins in the dog. *Arch Toxicol* **56**: 190–4.
Floersheim GL (1972) Antidotes to experimental α-amanitin poisoning. *Nat New Biol* **236**: 115–17.
Floersheim GL (1978) Experimental basis for the therapy of Amanita phalloides poisoning. *Schweiz Med Wochenschr* **108**: 185–97.
French AL, Garrettson LK (1988) Poisoning with the North American Jack O'Lantern mushroom, *Omphalotus illudens*. *J Toxicol Clin Toxicol* **26**: 81–8.
Galey FD, Rutherford JJ, Wells K (1990) A case of *Scleroderma citrinum* poisoning in a miniature Chinese pot-bellied pig. *Vet Hum Toxicol* **32**: 329–30.
Gannett PM, Garrett C, Lawson T, *et al.* (1991) Chemical oxidation and metabolism of *N*-methyl-*N*-formylhydrazine. Evidence for diazenium and radical intermediates. *Food Chem Toxicol* **29**: 49–56.
Ganzert M, Felgenhauer N, Zilker T (2005) Indication of liver transplantation following amatoxin intoxication. *J Hepatol* **42**: 202–9.
Grieshaber AF, Moore KA, Levine B (2001) The detection of psilocin in human urine. *J Forensic Sci* **46**: 627–30.
Gundala S, Wells LD, Milliano MT, *et al.* (2004) The hepatocellular bile acid transporter Ntcp facilitates uptake of the lethal mushroom toxin alpha-amanitin. *Arch Toxicol* **78**: 68–73.
Hall AH, Hall PK (1994) Ibotenic acid/muscimol-containing mushrooms. In *Handbook of Mushroom Poisoning – Diagnosis and Treatment*, Spoerke DG, Rumack BH (eds). CRC Press, Boca Raton, FL, pp. 265–78.
Halpern JH (2004) Hallucinogens and dissociative agents naturally growing in the United States. *Pharmacol Ther* **102**: 131–8.
Hasler F, Bourquin D, Brenneisen R, *et al.* (1997) Determination of psilocin and 4-hydroxyindole-3-acetic acid in plasma by HPLC-ECD and pharmacokinetic profiles of oral and intravenous psilocybin in man. *Pharm Acta Helv* **72**: 175–84.
Hasler F, Bourquin D, Brenneisen R, *et al.* (2002) Renal excretion profiles of psilocin following oral administration of psilocybin: a controlled study in man. *J Pharm Biomed Anal* **30**: 331–9.
Himmelmann A, Mang G, Schnorf-Huber S (2001) Lethal ingestion of stored *Amanita phalloides* mushrooms. *Swiss Med Wkly* **131**: 616–17.
Horita A, Weber LJ (1961) The enzymic dephosphorylation and oxidation of psilocybin and psilocin by mammalian tissue homogenisates. *Biochem Pharmacol* **7**: 47–54.
Hunt RS, Funk A (1977) Mushrooms fatal to dogs. *Mycologia* **69**: 432–3.
Jaeger A, Jehl F, Flesch F, *et al.* (1993) Kinetics of amatoxins in human poisoning: therapeutic implications. *J Toxicol Clin Toxicol* **31**: 63–80.
Kallet A, Sousa C, Spangler W (1988) Mushroom (*Amanita phalloides*) toxicity in dogs. *Calif Vet* **42**: 1, 9–11, 22, 47.
Kamata T, Nishikawa M, Katagi M, *et al.* (2003) Optimized glucuronide hydrolysis for the detection of psilocin in human urine samples. *J Chromatogr B Analyt Technol Biomed Life* **796**: 421–7.
Karlson-Stiber C, Persson H (2003) Cytotoxic fungi – an overview. *Toxicon* **42**: 339–49.
Kirwan AP (1990) "Magic mushroom" poisoning in a dog. *Vet Rec* **126**: 149.
Klan J (1993) A review of mushrooms containing amanitins and phalloidines. *Cas Lek Cesk* **132**: 449–51.
Kommers GD, Santos MN (1995) Experimental poisoning of cattle by the mushroom Ramaria flavo-brunnescens (Clavariaceae): a study of the morphology and pathogenesis of lesions in hooves, tail, horns and tongue. *Vet Hum Toxicol* **37**: 297–302.
Kroncke KD, Fricert G, Meier PJ, *et al.* (1986) Alpha-amanitin into hepatocytes. *J Biol Chem* **261**: 12562–7.
Laatsch H (1996) Zur Pharmakologie von Psilocybin und Psilocin. In *Maria Sabina, Botin der heiligen Pilze. Vom traditionellen Schamanentum zur weltweiten Pilzkultur*, Liggenstorfer R, Rätsch C (eds). Nachtschatten Verlag, Solothum, Switzerland, pp. 192–202.
Leist M, Gantner F, Naumann H, *et al.* (1997) Tumor necrosis factor-induced apoptosis during the poisoning of mice with hepatotoxins. *Gastroenterology* **112**: 923–34.
Lheureux P, Penaloza A, Gris M (2005) Pyridoxine in clinical toxicology: a review. *Eur J Emerg Med* **12**: 78–85.
Lincoff G, Mitchel DH (1977a) Cyclopeptide poisoning. In *Toxic and Hallucinogenic Mushroom Poisoning. A Handbook for Physicians and Mushroom Hunters*. Van Nostrand Reinhold Company, New York, pp. 25–48.
Lincoff G, Mitchel DH (1977b) Monomethylhydrazine poisoning. In *Toxic and Hallucinogenic Mushroom Poisoning. A Handbook for Physicians and Mushroom Hunters*. Van Nostrand Reinhold Company, New York, pp. 49–61.
Lincoff G, Mitchel DH (1977c) Ibotenic acid–muscimol poisoning. In *Toxic and Hallucinogenic Mushroom Poisoning. A Handbook for Physicians and Mushroom Hunters*. Van Nostrand Reinhold Company, New York, pp. 77–99.
Lindell TJ, Weinberg F, Morris PW, *et al.* (1970) Specific inhibition of nuclear RNA polymerase II by alpha-amanitin. *Science* **170**: 447–9.
Martin JG (1956) Mycetism (mushroom poisoning) in a dog: case report. *Vet Med* **51**: 227–8.

Mayer KE, Myers RP, Lee SS (2005) Silymarin treatment of viral hepatitis: a systematic review. *J Viral Hepat* **12**: 559–67.

Maynert EW, Kaji HK (1962) On the relationship of brain gamma-aminobutyric acid to convulsions. *J Pharmacol Exp Ther* **137**: 114–21.

McKenna DJ, Repke DB, Lo L, *et al.* (1990) Differential interactions of indolealkylamines with 5-hydroxytryptamine receptor subtypes. *Neuropharmacology* **29**: 193–8.

Michelot D, Toth B (1991) Poisoning by *Gyromitra esculenta* – A review. *J Appl Toxicol* **11**: 235–43.

Mitchel DH (1980) *Amanita* mushroom poisoning. *Ann Rev Med* **31**: 51–7.

Naude TW, Berry WL (1997) Suspected poisoning of puppies by the mushroom *Amanita pantherina*. *J S Afr Vet Assoc* **68**: 154–8.

Nielsen EO, Schousboe A, Hansen SH, *et al.* (1985) Excitatory amino acids: studies on the biochemical and chemical stability of ibotenic acid and related compounds. *J Neurochem* **45**: 725–31.

Ott J, Wheaton PS, Chilton WS (1975) Fate of muscimol in the mouse. *Physiol Chem Phys* **7**: 381–4.

Puig MV, Artigas F, Celada P (2005) Modulation of the activity of pyramidal neurons in rat prefrontal cortex by raphe stimulation *in vivo*: involvement of serotonin and GABA. *Cereb Cortex* **15**: 1–14.

Pyysalo H (1976) Tests for gyromitrin, a poisonous compound in false morel *Gyromitra esculenta*. *Z Lebensm Unters Forsch* **160**: 325–30.

Ridgway RL (1978) Mushroom (*Amanita pantherina*) poisoning. *J Vet Med Assoc* **172**: 681–2.

Schmidlin-Meszaros J (1974) Gyromitrin in Trockenlorcheln (*Gyromitra esculenta sicc.*). *Mitt Geb Lebensm Hyg* **65**: 453–65.

Schneider SM, Michelson EA, Vanscoy G (1992) Failure of *N*-acetylcysteine to reduce alpha amanitin toxicity. *J Appl Toxicol* **12**: 141–2.

Smolinske SC (1994) Psilocybin-containing mushrooms. In *Handbook of Mushroom Poisoning – Diagnosis and Treatment*, Spoerke DG, Rumack BH (eds). CRC Press, Boca Raton, FL, pp. 309–24.

Spoerke DG (1994) Gastrointestinal irritant mushrooms. In *Handbook of Mushroom Poisoning – Diagnosis and Treatment*, Spoerke DG, Rumack BH (eds). CRC Press, Boca Raton, FL, pp. 347–66.

Stallard D, Edes TE (1989) Muscarinic poisoning from medications and mushrooms. A puzzling symptom complex. *Postgrad Med* **85**: 341–5.

Tegzes JH, Puschner B (2002) Amanita mushroom poisoning: efficacy of aggressive treatment of two dogs. *Vet Hum Toxicol* **44**: 96–9.

Toth B, Gannett P (1994) *Gyromitra esculenta* mushroom: a comparative assessment of its carcinogenic potency. *In Vivo* **8**: 999–1002.

Venkataramanan R, Ramachandran V, Komoroski BJ, *et al.* (2000) Milk thistle, a herbal supplement, decreases the activity of CYP3A4 and uridine diphosphoglucuronosyl transferase in human hepatocyte cultures. *Drug Metab Dispos* **28**: 1270–3.

Vetter J (1998) Toxins of Amanita phalloides. *Toxicon* **36**: 13–24.

Viernstein H, Jurenitsch J, Kubelka W (1980) Vergleich des Giftgehaltes der Lorchelarten *Gyromitra gigas, Gyromitra fastigiata* und *Gyromitra esculenta*. *Ernährung/Nutrition* **4**: 392–5.

Villar D, Knight MK, Holding J, *et al.* (1995) Treatment of acute isoniazid overdose in dogs. *Vet Hum Toxicol* **37**: 473–7.

Vogel G, Tuchweber B, Trost W, *et al.* (1984) Protection by silibinin against *Amanita phalloides* intoxication in beagles. *Toxicol Appl Pharm* **73**: 355–62.

Wang HX, Ng TB, Ooi VE (1998) Lectin activity in fruiting bodies of the edible mushroom Tricholoma mongolicum. *Biochem Mol Biol Int* **44**: 135–41.

Waser PG (1961) Chemistry and pharmacology of muscarine, muscarone, and some related compounds. *Pharmacol Rev* **13**: 465–515.

Wieland T (1983) The toxic peptides from Amanita mushrooms. *Int J Pept Protein Res* **22**: 257–76.

Wright AV, Niskanen A, Pyysalo, *et al.* (1978) The toxicity of some *N*-methyl-*N*-formylhydrazones from *Gyromitra esculenta* and related compounds in mouse and microbial tests. *Toxicol Appl Pharmacol* **45**: 429–34.

Yam P, Helfer S, Watling R (1993) Mushroom poisoning in a dog. *Vet Rec* **133**: 24.

Young A (1994) Muscarine-containing mushrooms. In *Handbook of Mushroom Poisoning – Diagnosis and Treatment*, Spoerke DG, Rumack BH (eds). CRC Press, Boca Raton, FL, pp. 289–301.

C H A P T E R

# 73

# Cottonseed toxicity

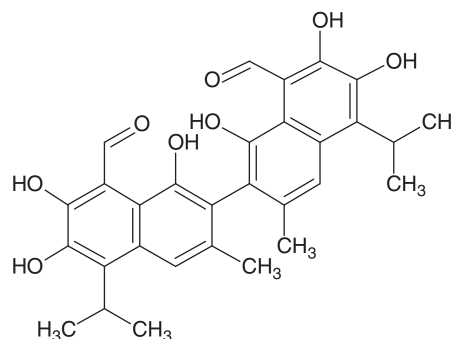
*Steven S. Nicholson*

## INTRODUCTION

Whole cottonseeds are a product of cotton (*Gossypium*) production. They are approximately 20% oil and 20% protein and contain polyphenolic pigments one of which is the toxic component gossypol ($C_{30}H_{30}O_8$). Gossypol is a yellow liquid located within the pigment or oil glands in the cotton plant. In cottonseed these glands appear as brown to black specks within the white matrix of the seed. The content of gossypol in whole cottonseed ranges from 0.02% to 6.64% and is thought to provide resistance to insects (Price *et al.*, 1993). Gossypol is recognized for its toxic effect on cardiac, hepatic, pulmonary, and reproductive systems. Whole cottonseed, cottonseed meal, and cottonseed hulls are important feedstuffs. Cottonseed meal is a 36–41% protein supplement used in beef and dairy rations. Cottonseed hulls are used as a fiber source in cattle rations. The risk of gossypol toxicosis limits the amount of cottonseed meal in the diet of swine, poultry, dogs, and other monogastric animals as well as pre-ruminant calves and lambs. The literature does not describe clinical cases in equids. Adverse effects of gossypol on reproduction in humans and animals may occur in certain situations.

## BACKGROUND

Gossypol is one of the several polyphenolic compounds found in the pigment glands of whole cottonseed. Some gossypol is bound to protein in the stomach contents of ruminants. Cottonseed cake remains after oil is extracted from the seeds. Removal of the oil by commercial processors leaves cottonseed meal containing gossypol in two forms, free, the toxic form, and bound. Methods of extraction of the oil include mechanically extracted cottonseed meal containing 0.06% free gossypol and the expander solvent method cottonseed meal containing 0.14% free gossypol (NCPA, 2005). A solvent only method of extraction produced cottonseed meal with free gossypol content up to 0.6%. Bound gossypol is bound to amino acids in the meal and is considered unavailable for digestion. Some bound gossypol may be digested and absorbed by ruminants.



Gossypol

## PHARMACOKINETICS/ TOXICOKINETICS

Intake of free gossypol at a toxic level for a period of days or weeks by susceptible animals leads to the appearance of toxic effects. Gossypol tends to accumulate in the liver and kidneys. Little or no gossypol was detected in milk from cows fed cottonseed products (Lindsey *et al.*, 1980). Half-life of gossypol in three dairy cows ranged from 40.7 to 67.5 h (Lin *et al.*, 1991). Low-dietary iron leads to increased gossypol absorption and iron added to the diet can reduce absorption (Haschek *et al.*, 1989). Tissue concentrations in

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swine, from highest to lowest, were found in muscle, liver, fat, and red blood cells. In swine, gossypol is metabolized in the liver to various compounds, conjugated as glucuronides and sulfates, and excreted in the feces (Abou-Donia, 1989). In poultry, gossypol reacts with iron in egg yolks to produce an olive green color. Cyclopropenoid fatty acids in cottonseed meal causes egg whites to become pink.

## MECHANISM OF ACTION

Considerable evidence points to oxidative stress, formation of reactive oxygen species, and DNA scission, characteristics of redox cycling by electron transport in biosystems (Kovacic, 2003). Myocardial necrosis, congestive heart failure, and secondary hepatic changes, account for clinical signs and course of the toxicosis. Death may occur suddenly and unexpectedly in some cases.

## TOXICITY

Toxic levels of free gossypol in the diet produce cardiac necrosis and congestive heart failure in swine, dogs, goats, cattle, pre-ruminant calves, and lambs. Dairy cows are susceptible to gossypol toxicosis.

Cottonseed products are seldom fed to horses. Precautions for feeding young horses cottonseed meal include the fact that it is low in lysine and to use meal containing <0.2% gossypol.

High mortality was seen in dairy calves fed cottonseed meal with 250–380 ppm gossypol for the first 10 weeks of life (Holmberg *et al.*, 1988). Gossypol in the diet at 250–300 mg of free gossypol per day for several months apparently caused congestive heart failure in adult goats (East *et al.*, 1989). Holstein calves may develop ascites, diarrhea, and weakness after consuming toxic levels of gossypol in a diet containing cottonseed meal for 70 to perhaps 180 days. In the author's experience (unpublished data) a typical case of gossypol poisoning presents as follows. Holstein calves 3–8 months of age were confined to dry lot and fed up to 3.6–4.5 kg/day of lactating cow ration for 4 months with little or no hay available. The feed contained 600 lb (272 kg) cottonseed meal per ton. The free gossypol content of the cottonseed meal was determined to be 0.6%. Two calves had died a day or so apart from what was thought to be pneumonia because of cough, dyspnea, and blood tinged nasal discharge. Two calves were sick at the time of the farm visit. One had diarrhea, ascites, dyspnea, anemia, and weakness. At necropsy, ascites was marked the liver was swollen, the intestinal tract was edematous, the heart had pale areas within the myocardium, later confirmed as areas of necrosis, and the lungs were edematous. Other causes myocardial necrosis including the plant oleander (*Nerium oleander*), ionophores, excess selenium injection, and white muscle disease were ruled out.

Gossypol poisoning in mature cattle is uncommon but dairy cows can be at risk because they may be fed cottonseed and cottonseed meal during lactation. Dairy cows consuming high levels of cottonseed meal may be at risk of cardiac failure (Jimenez, 1979; Lindsey *et al.*, 1980; Smalley and Bicknell, 1982). High temperature and humidity seem to enhance the risk. Dairy cows tolerated up to 6.2 mg/kg b.w. free gossypol per day for 100 days but 8.8 mg/kg caused red cell fragility, depressed hemoglobin levels, and increased respiratory rate in response to elevated ambient temperatures (Lindsey *et al.*, 1980). Erythrocyte fragility at days 28 and 56 and elevated serum alkaline phosphatase were noted but adverse reproductive effects were not seen in crossbred heifers fed a diet of 30% cottonseed (Calhoun *et al.*, 1991). Consumption of a high gossypol diet for 170 days had no effect on the health of lactating dairy cows, but it increased plasma gossypol concentrations and impaired reproductive performance (Santos *et al.*, 2003). Ammoniation of cottonseed meal to reduce aflatoxin content was thought to have enhanced the toxic effects of gossypol in dairy cattle fed 6–9 lb/day (Smalley and Bicknell, 1982).

Lambs fed a diet containing 900 ppm free gossypol for 3–4 weeks died suddenly or after exhibiting signs of cardiac failure (Morgan *et al.*, 1988). Clinical signs included sudden death and/or chronic dyspnea syndromes. Serum chemistry alterations in the lambs included high serum total lactate dehydrogenase and lactate dehydrogenase liver-specific isoenzyme activities. Serum total creatine kinase activity decreased markedly in lambs of all groups treated with gossypol at 45, 136, or 409 mg/day. All lambs given 409 mg of gossypol per day died before the end of the 30-day study.

In swine, dietary levels of 200–300 ppm free gossypol result in cardiac insufficiency and respiratory distress with mortality rates exceeding 50% (Haschek *et al.*, 1989).

In dogs cardiotoxicity associated with prolonged gossypol intake is similar to that in other species. Four dogs died, with lesions compatible with those described in experimental gossypol poisoning, after eating food sprinkled with cottonseed meal over a period of months. The amount of free gossypol fed was approximately 5.4–5.7 mg/kg/day for an unknown duration (Patton *et al.*, 1985). Rabbits and guinea pigs are susceptible as well.

In chicks, severe cases of perivascular lymphoid aggregate formation, biliary hyperplasia, and hepatic cholestasis were observed in chicks fed 800 and 1600 mg/kg of purified gossypol in feed (Henry *et al.*, 2001). This study also shows that histopathological changes in liver due to gossypol also occur at levels lower than the levels that affect body weight.

In fish, pathological effects of gossypol in tilapia (*Oreochromis* spp.) were decline in body weight, low

hemoglobin and hematocrit levels, abundance of immature red blood, and abnormal spleen morphology (Garcia-Abiado *et al.*, 2004). In rainbow trout feeding diets containing cottonseed meal over a total period of 10 months did not result in differences in growth and mortality compared to the control group ($p > 0.05$). Gossypol was transferred to the eggs and embryo survival increased linearly as gossypol content in the diet was increased (Blom *et al.*, 2001).

Adverse male reproductive effects are of concern since studies have found a decrease in testicular steroidogenesis and spermatogenesis in pubescent rams and bulls (Lin *et al.*, 1981; Kramer *et al.*, 1991). Reversible male infertility has been reported for some animal species and humans (Abou-Donia, 1989). In peripubertal bulls, gossypol (8 mg/kg/day for 56 days) increased sperm abnormalities. The number of sperm with proximal droplets was significantly higher in the gossypol-treated bulls, suggesting testicular degeneration, but the effects were reversible (Hassan *et al.*, 2004).

Extensive damage to the germinal epithelium has been shown in both rams and bulls which were fed diets containing gossypol and is of major concern (Randel *et al.*, 1992). However, cottonseed meal has been and remains an important winter-feeding supplement for beef cattle herds in southwestern United States.

Signs and lesions of gossypol toxicosis include myocardial necrosis, congestive heart failure, centrilobular necrosis of the liver, pulmonary edema, and perhaps mild hemoglobinuria caused by RBC fragility. Mild to moderate anemia may be present. Ascites, diarrhea, and edema of the intestinal tract secondary to congestive heart failure may be seen. Dairy cows and lambs may die suddenly with minimal lesions. Death caused by heart failure could occur several weeks after last consumption of toxic levels of gossypol.

Diagnosis of toxicosis includes history of prolonged dietary exposure, clinical signs, and lesions compatible with acute or chronic heart failure, and elimination of other potential causes.

## TREATMENT

Remove gossypol sources in the diet. Treatment of food animals affected by gossypol toxicosis is impractical. Dogs may benefit from therapy designed to strengthen heart function in congestive heart failure.

## CONCLUDING REMARKS

Pelleting reduced free gossypol by as much as 70% in whole cottonseed and by 48% in cottonseed meal (Barraza *et al.*, 1991). Pelleting represents a mechanism to decrease the toxicity of gossypol in cottonseed. Adding iron sulfate to the diet has a protective effect.

## REFERENCES

Abou-Donia MB (1989) Gossypol. In *Toxicants of Plant Origin, Phenolics*, vol. 4, Cheeke PR (ed.). CRC Press, Boca Raton, FL, pp. 1–22.

Barraza ML, Coppock CE, Brooks KN, Wilks DL, Saunders RG, Latimer Jr GW (1991) Iron sulfate and feed pelleting to detoxify free gossypol in cottonseed diets for dairy cattle. *J Dairy Sci* **74**: 3457–67.

Blom JH, Lee KJ, Rinchard J, Dabrowski K, Ottobre J (2001) Reproductive efficiency and maternal–offspring transfer of gossypol in rainbow trout (*Oncorhynchus mykiss*) fed diets containing cottonseed meal. *J Anim Sci* **79**: 1533–9.

Calhoun MC, Huston JE, Ueckert DN, Baldwin Jr BC, Kuhlmann SW, Engdahl BS (1991) Performance of yearling heifers fed diets containing whole cottonseed. In *Beef Cattle Research in Texas*. Texas Agric Exp Stn Consol Rep, PR-4839.

East NE, Anderson M, Lowenstine IJ (1989) Apparent gossypol induced toxicosis in adult dairy goats. *J Am Vet Assoc* **204**: 642–3, 1994.

Garcia-Abiado MA, Mbahinzireki G, Rinchard J, Lee KJ, Dabrowski K (2004) Effect of diets containing gossypol on blood parameters and spleen structure in tilapia, *Oreochromis* sp., reared in a recirculating system. *J Fish Dis* **27**: 359–68.

Haschek WM, Beasley VR, Buck WB, Finnell JH (1989) Cottonseed meal (gossypol) toxicosis in a swine herd. *J Am Vet Assoc* **195**: 613–15.

Hassan ME, Smith GW, Ott RS, Faulkner DB, Firkins LD, Ehrhart ES, Schaeffer DJ (2004) Reversibility of the reproductive toxicity of gossypol in peripubertal bulls. *Theriogenology* **15**: 1171–9.

Henry MH, Pesti GM, Brown TP (2001) Pathology and histopathology of gossypol toxicity in broiler chicks. *Avian Dis* **45**: 598–604.

Holmberg CA, Weaver LD, Guterbock WM, Genes J, Montgomery P (1988) Pathological and toxicological studies of calves fed a high concentration cottonseed meal diet. *Vet Pathol* **25**: 147–53.

Jimenez AA (1979) Effects of gossypol in milking cow rations. *Feedstuffs* **52**: 28.

Kovacic P (2003) Mechanism of drug and toxic actions of gossypol: focus on reactive oxygen species and electron transfer. *Curr Med Chem* **24**: 2711–18.

Kramer RY, Garner DL, Ericsson SA, Wesen DA, Downing TW, Redelman D (1991) The effects of cottonseed components on testicular development in pubescent rams. *Vet Hum Toxicol* **33**: 11–16.

Lin YC, Murono EP, Osterman J, Nankin HR, Coulsen PB (1981) Gossypol inhibits testicular steroidogenesis. *Fertil Steril* **35**: 563–6.

Lin YC, Nuber DC, Ju Y, Cutler G, Hinchcliff KW, Haibel G (1991) Gossypol pharmacokinetics in mid-lactation Brown Swiss dairy cows. *Vet Res Commun* **15**: 379–85.

Lindsey TO, Hawkins GE, Guthrie LD (1980) Physiological responses of lactating cows to gossypol from cottonseed meal rations. *J Dairy Sci* **63**: 562–73.

Morgan S, Stair EL, Martin T, Edwards WC, Morgan GL (1988) Clinical, clinicopathologic, and toxicologic alterations associated with gossypol toxicosis in feeder lambs. *Am J Vet Res* **49**: 493–99.

NCPA (2005) National Cottonseed Products Association Internet Web, http://www.cottonseed.com/publications/feedproducts guide.asp

Patton CS, Legendre AM, Gompf RE, Walker MA (1985) Heart failure caused by gossypol poisoning in two dogs. *J Am Vet Med Assoc* **187**: 625–7.

Price WD, Lovell RA, McChesney DG (1993) Naturally occurring toxins in feedstuffs: Center for Veterinary Medicine Perspective. *J Anim Sci* **71**: 2556–62.

Randel RD, Chase CC, Wyse SJ (1992) Effects of gossypol and cottonseed products on reproduction in mammals. *J Anim Sci* **70**: 1628–38.

Santos JE, Villasenor M, Robinson PH, DePeters EJ, Holmberg CA (2003) Type of cottonseed and level of gossypol in diets of lactating dairy cows: plasma gossypol, health, and reproductive performance. *J Dairy Sci* **86**: 892–905.

Smalley SA, Bicknell EJ (1982) Gossypol toxicity in dairy cattle. *Compend Contin Educ Pract Vet* **4**: S378–81.

C H A P T E R

# 74

# Toxicity of yew (*Taxus* spp.) alkaloids

*Christina R. Wilson and Stephen B. Hooser*

## INTRODUCTION

Taxines, the principle toxic alkaloids derived from yew (*Taxus* spp.) plants, are responsible for numerous animal deaths each year. They are produced by members of the *Taxus* spp., evergreen trees or shrubs that are commonly used as ornamental landscaping plants. From their leaves and branches numerous taxine alkaloids have been isolated and characterized chemically. However, the degree of toxicity of each individual compound can vary. For the toxic members of the group, their primary mechanism of toxicity appears to be as antagonists of calcium channels in cardiac myocytes. This effect can cause disturbances in electrical conduction and rapid onset of adverse clinical signs often ending in death due to heart failure.

## BACKGROUND

### Plant characteristics

Yews (*Taxus* spp., Taxaceae) are evergreen plants often used for ornamental landscaping in many parts of the United States, Europe, and elsewhere throughout the world. Common varieties in the United States are English Yew (*Taxus baccata*), American Yew (*Taxus canadensis*), Japanese Yew (*Taxus cuspidata*), and Pacific or Western Yew (*Taxus brevifolia*), (Kingsbury, 1964). These plants can be highly toxic and have been implicated in human and animal poisonings. The poisonous taxine alkaloids have been reported to be present in the foliage, bark, and seeds of the plants, but not in the fleshy scarlet aril (berry) (Bryan-Brown, 1932; Kingsbury, 1964).

### Historical references

References to yew toxicity date back over two millennia (Bryan-Brown, 1932). In the first century, B.C.E., Julius Caesar (102–44 B.C.E.) wrote of Catuvolcus, the king of Eburones, who poisoned himself with yew "juice" (Fröhne and Pfänder, 1984). Ancient Celts committed ritual suicides by drinking extracts from yew foliage and used the sap to poison the tips of their arrows during the Gaelic Wars (Foster and Duke, 1990; Hartzell, 1995). Some primitive cultures are reported to have used yew extracts as hunting and fishing aids (Watt and Breyer-Brandwijk, 1962; Hartzell, 1995). During the 18th and 19th centuries, decoctions of yew leaf were documented as having been used as an abortifacient or an emmenagogue by women in Europe and India (Bryan-Brown, 1932; Watt and Breyer-Brandwijk, 1962), however, toxic side effects stemming from these usages may also have occurred.

### Chemical characterization

The first report of preparation of an amorphous, white, non-crystalline powder called, "taxine" was from analysis of yew foliage (*Taxus baccata* L.) for alkaloid content reported in 1856 by Lucas. It was isolated in crystalline form approximately 20 years later by Marmé, a French scientist (Hilger and Brande, 1890), but it wasn't until 1956 that Graf and Boeddeker (1956) discovered that taxine was a mixture of heterogeneous compounds. Further investigations recognized two major types of taxine alkaloids: taxine A and taxine B (Graf, 1956).

By subjecting the taxine extracts to electrophoresis (Graf, 1956), two major bands were noted. The fastest

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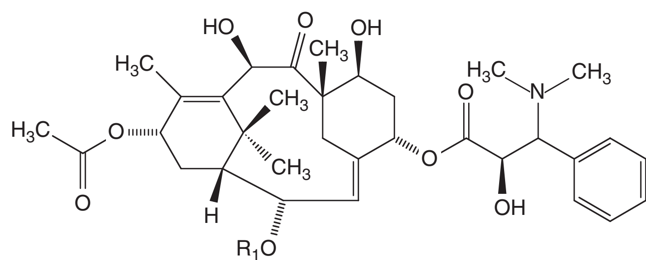
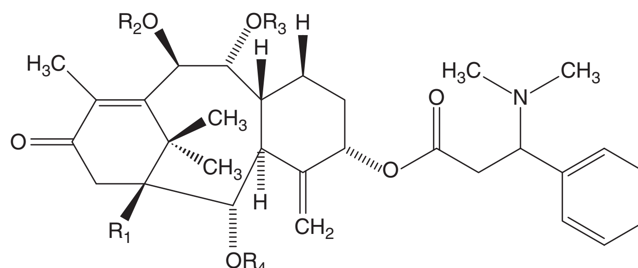
**TABLE 74.1 Physical and chemical properties of taxine alkaloids**

| | Taxines | Molecular formula | Melting point (°C) | Optical rotation | UV max (nm) | IR max ($cm^{-1}$) |
|---|---|---|---|---|---|---|
| 1a | Taxine A | $C_{35}H_{47}NO_{10}$ | 204–206 | $[\alpha]_D^{20} = -140°$ | 220, 255 | 1780, 1250 |
| 1b | 2-Deacetyltaxine A | $C_{33}H_{45}NO_9$ | – | $[\alpha]_D^{20} = -106°$ | 224, 264 | 1734, 1691 |
| 1c | Taxine B | $C_{33}H_{45}NO_8$ | 115 | $[\alpha]_D^{25} = +116°$ | 210, 277 | 3578, 1730 |
| 1d | Isotaxine B | $C_{33}H_{45}NO_8$ | – | – | 282 | – |




| | Taxine alkaloids | $R_1$ |
|---|---|---|
| 1a | Taxine A | Ac |
| 1b | 2-Deacetyltaxine A | H |

**FIGURE 74.1** Structural formulas for taxines A.




| | Taxine alkaloids | $R_1$ | $R_2$ | $R_3$ | $R_4$ |
|---|---|---|---|---|---|
| 2a | Taxine B | OH | Ac | H | H |
| 2b | Isotaxine B | OH | H | Ac | H |
| 2c | 1-Deoxytaxine B | H | Ac | H | H |
| 2d | 1-Deoxyisotaxine B | H | H | Ac | H |

**FIGURE 74.2** Structural formulas for taxines B.

moving band was designated taxine A which comprised approximately 1.3% of the total alkaloid extract. The slower migrating band, taxine B, represented approximately 30% of the total alkaloid fraction extracted from *T. baccata* L. (Graf, 1956; Graf and Bertholdt, 1957). Subsequent analysis elucidated the molecular formula of taxine A as well as its basic physical and chemical properties (Graf and Bertholdt, 1957; Graf *et al.*, 1982; and Table 74.1a and Figure 74.1a). The structural formula of taxine A was reported almost 25 years later (Graf, 1982) and an analog, 2-deacetyltaxine A ($C_{33}H_{45}NO_9$), from the leaves of *T. baccata* (Table 74.1b) in 1994 (Poupat *et al.*, 1994; Figure 74.1b). The structure of taxine B was first reported in 1986 (Graf *et al.*, 1986) which was slightly revised in 1991 (Ettouati *et al.*, 1991; Table 74.1c and Figure 74.2a). Purified taxine fractions from *Taxus* spp. reveal the presence of several taxine B-related compounds. Isotaxine B ($C_{33}H_{45}NO_8$), a structural isomer of taxine B, is present as a major constituent in the alkaloid fractions (Poupat *et al.*, 1994; Jenniskens *et al.*, 1996; Adeline *et al.*, 1997; Potier *et al.*, 1997; Table 74.1d and Figure 74.2b). Present as minor constituents in *Taxus* spp. are 1-deoxytaxine B (Figure 74.2c) and 1-deoxyisotaxine B (Figure 74.2d) (Jenniskens *et al.*, 1996; Potier *et al.*, 1997). Also present as minor constituents, at approximately 2% of the total concentration, are the taxine B pseudoalkaloids 13-deoxo-13α-acetyloxy-taxine B ($C_{35}H_{49}NO_9$), 13-deoxo-13α-acetyloxy-1-deoxytaxine B ($C_{35}H_{49}NO_8$), and 13-deoxo-13α-acetyloxy-1- deoxy- nortaxine B ($C_{34}H_{47}NO_8$) (Appendino *et al.*, 1997). Within the last decade the importance of the antineoplastic drug, taxol, a related member of the taxane diterpenoid family, has spurred the discovery and chemical characterization of over 350 members of this chemical class (Itokawa, 2003).

## PHARMACOKINETICS/ TOXICOKINETICS

For reasons probably related to their acute toxicity and the lack of pharmaceutical uses for the toxic taxine alkaloids, pharmacokinetic studies have not been published. However, extensive pharmacokinetic studies have been reported for the widely used antineoplastic drugs, paclitaxel (isolated from *T. brevifolia*) and docetaxel (synthesized via a taxane precursor from *Taxus bacatta*), which are also members of the taxane diterpenoid family. In studies with these two compounds, it has been found that they are both highly protein bound (>95%) in the serum. In addition, paclitaxel exhibits non-linear kinetics at therapeutic doses while the kinetics of docetaxel are linear. They are both metabolized in the liver by cytochrome P450 enzymes. Work done with docetaxel indicates that this is primarily the result of metabolism by CYP3A4 to pharmacologically inactive oxidation products which are excreted in the bile through a *p*-glycoprotein-dependent

mechanism (Gustafson *et al*., 2003; Baker *et al*., 2006). Less than 10% of the excretion is through the kidneys. Tissue distribution is extensive except for the central nervous system and testes. The elimination half-life for paclitaxel is 5–7 h (two compartment model) or 20 h (three compartment model) while the elimination half-life for docetaxel is 12 h (two compartment model) or 13 h (three compartment model). Liver insufficiency or the co-administration of compounds which modulate P450 activity may influence the activity of these antineoplastic drugs (Brown, 2003) and presumably, the activity of more acutely toxic members of the family such as taxines A and B.

## PHYSIOLOGY/MECHANISM OF ACTION

The earliest investigations of crude extracts of taxine alkaloids published in 1921 described effects that were primarily cardiovascular. When administered by the intraperitoneal or intravenous routes in rabbits and dogs, hypotension and cardiac arrest occurred in both species (Bryan-Brown, 1932). Additionally, when toxicity was severe enough to result in cardiac abnormalities, it was noted that peristaltic contractions of the gastrointestinal (GI) tract ceased.

The first extensive pharmacological research of taxines was reported by Bryan-Brown in 1932. Electrocardiographic analysis of isolated perfused hearts of rabbits and frogs revealed that crude taxine extracts gradually induced bradycardia resulting in diastolic cardiac arrest. More recent investigations have indicated that taxines depress atrioventricular (AV) conduction in a dose-dependent manner in isolated frog heart having the greatest effect on ventricular rate (Smythies *et al*., 1975; Tekol and Kameyama, 1987). In those studies, that effect could not be inhibited by the administration of atropine, vagotomy, or ganglionic/adrenergic blockade (Bryan-Brown, 1932; Vohora, 1972). It was thus concluded that the hypotension induced by taxine extracts was not mediated via the sympathetic or parasympathetic nervous systems, but rather by a direct action on myocardium (Vohora, 1972).

Large differences in the cardiotoxicity of taxine A and taxine B have been reported (Bauereis and Steiert, 1959; Alloatti *et al*., 1996). Administration of taxine B administered either *in vivo* or *in vitro* has shown that taxine B is more cardiotoxic than taxine A (Bauereis and Steiert, 1959; Alloatti *et al*., 1996). In the heart, taxine B causes inotropic effects while eliciting marked changes in AV conduction. In isolated, perfused guinea pig hearts, a 5 μM concentration of taxine B markedly increased AV conduction time and QRS duration (widening of the QRS interval), while 1 μM concentrations (lowest concentration used) significantly reduced heart rate (Alloatti *et al*., 1996). These changes led to AV conduction blocks and complete diastolic cardiac arrest. This marked increase in QRS duration (wide QRS interval) has also been reported in a case of human poisoning by yew ingestion (Matthew *et al*., 1993). Experimental administration of *Taxus* extracts intravenously to pigs also resulted in widening of QRS complexes as evaluated by electrocardiography. In those studies, sodium bicarbonate was not effective in reversing the widening of the QRS interval (Ruha *et al*., 2002). Additionally, taxine B causes a marked reduction in the maximum rate of depolarization of the action potential in isolated papillary muscle and thus, resembles the action of class I antiarrhythmic drugs (e.g. flecainide, procainamide, quinidine) (Bauereis and Steiert, 1959; Tekol, 1985; Alloatti *et al*., 1996). In contrast, taxine A has minimal effects on AV conduction time and QRS duration. Even at the highest concentration used (10 μM), taxine A induced only mild reductions in heart rate (Alloatti *et al*., 1996).

Taxines have lesser effects on other organs. In the few studies reported, crude taxine extracts have adverse effects on involuntary muscle, but not on voluntary muscle (Bryan-Brown, 1932; Vohora, 1972). Uterine contractions, relaxation of the intestines, and contraction of the duodenum and ileum have been noted in experimental animals dosed with yew extracts (Bryan-Brown, 1932; Vohora, 1972). More recently, Tekol and Gögüsten (1999) reported that taxine sulfate inhibits peristaltic movement in rabbit jejunum with a median inhibitory concentration ($IC_{50}$) of $1.86 \times 10^{-5}$ g/ml.

Due to their instability and the lack of purified taxines A and B for experimental use, research delving into the mechanism of action of taxines has frequently involved using crude extracts of taxines from yew. However, because taxine B is present at higher concentrations and is more potent than other taxines, it is assumed that the primary adverse effects of taxines in the following investigations are primarily the result of the activity of taxine B. Investigations of taxine extracts on cardiomyocytes and axons indicated that taxines cause an increase in cytoplasmic calcium by altering both calcium and sodium channel conductances (Smythies *et al*., 1975; Tekol, 1985, 1991; Tekol and Kameyama, 1987). Further electrophysiological investigations demonstrated that taxines are calcium and sodium channel antagonists (Tekol and Kameyama, 1987; Tekol and Gögüsten, 1999). However, recent investigations regarding the cardioselectivity of taxines have provided more conclusive evidence that their mode of toxicity is as calcium channel antagonists (Tekol and Gögüsten, 1999). In those studies, isolated aorta, atrium, and jejunum from rabbits were used to compare the cardioselectivity of taxines to verapamil, a known calcium channel antagonist. From these experiments, Tekol and Gögüsten (1999) concluded that the mechanism of action of taxines is

primarily based on its $Ca^{+2}$-channel antagonistic properties. It is likely that the toxicity of taxines in animals and humans also occurs through this same mechanism.

## TOXICITY

With the exception of the aril, all parts of the yew plant, including the seed within the aril, contain taxine alkaloids and are extremely poisonous. One study in laboratory rodents has indicated that higher toxicity is found in stems compared to leaves (Shanker *et al*., 2002). Although maximal concentrations occur during the winter (Watt and Breyer-Brandwijk, 1962), toxic amounts of taxines remain in the plants throughout the year and are not appreciably decreased by drying (Alden *et al*., 1977). It has been reported that the cardiotoxic taxines A and B are relatively abundant in English Yew (*T. baccata*) and Japanese Yew (*T. cuspidata*), yet only minimal amounts are found in Pacific Yew (*T. brevifolia*) (Tyler, 1960; Suffness, 1995; Itokawa, 2003).

Fatal animal toxicoses have been reported in the United States, Canada, Europe, and Asia (Kingsbury, 1964; Lee *et al*., 2003). The majority of these occur in domestic livestock including cattle (Casteel and Cook, 1985; Panter *et al*., 1993), horses (Karns, 1983; Cope *et al*., 2004; Tiwary *et al*., 2005), sheep (Rae and Binnington, 1995) and goats (Coenen and Bahrs, 1994), but have also been reported in dogs (Evans and Cook, 1991; Taksdal, 1994), a bear (Bacciarini *et al*., 1999), fallow deer in captivity (Wacker, 1983), emus (Fiedler and Perron, 1994), budgies (Shropshire *et al*., 1992), canaries (Arai *et al*., 1992), and experimentally in pigs (Ruha *et al*., 2002). It is interesting to note that yew (*Taxus baccata*) is often eaten by white-tailed deer (*Odocoileus virginianus*) in the United States without apparent adverse effects. This may be due, in part, to increased ruminal detoxification of the taxines present in the yew (Weaver and Brown, 2004). Clinical cases resulting in poisoning are often accidental and are frequently a result of livestock being unwittingly fed clippings from yew (*Taxus* spp.) bushes. Because of the difficulties in obtaining purified, stable taxines in quantities sufficient for mammalian studies, minimum lethal dose ($LD_{min}$) values were, in the past, assessed through the oral administration of yew leaves and branches (Watt and Breyer-Brandwijk, 1962; Clarke and Clarke, 1988). Utilizing these values, and estimating that 1 g of yew leaves contains approximately 5 mg of taxines (Smythies *et al*., 1975; Tekol, 1985; Jenniskens *et al*., 1996; Tekol and Gögüsten, 1999), minimal toxic doses of taxines (mg/kg body weight) in animals can be estimated and are summarized in Table 74.2. The body weights of the animals listed are average values for adult animals only (Spector, 1956). From Table 74.2, it is evident that the minimal toxic dose of taxines varies between species. Comparatively, horses are more sensitive ($LD_{min}$ of 1.0–2.0 mg/kg) and chickens are least sensitive ($LD_{min}$ of 82.5 mg/kg) to yew toxins.

Adverse clinical signs in livestock can vary depending on the amount of yew ingested. However, in most cases of acute poisoning, animals are often found dead 24 h or less after ingestion without demonstrating abnormal behavior or adverse signs of toxicity. In subacute poisonings, which have been reported infrequently, clinical signs may include: ataxia, bradycardia, dyspnea, muscle tremors, recumbency, and convulsions leading to collapse and death (Evers and Link, 1972; Casteel and Cook, 1985; Veatch *et al*., 1988; Evans and Cook, 1991; Tekol, 1991;

**TABLE 74.2 Oral lethal doses of yew leaves in animals**

| Animal | *Estimated average body weights (kg) | $LD_{min}$ of yew leaves (g) | $LD_{min}$ (g yew leaves/kg body weight) | Estimated $LD_{min}$ of taxines (mg/kg body weight) | Reference |
|---|---|---|---|---|---|
| Chicken | 1.82 | 30 | 16.5 | 82.5 | Clarke and Clarke (1988) |
| Cow | 250 | 500 | 2.0 | 10.0 | Clarke and Clarke (1988) |
| Dog | 13 | 30 | 2.3 | 11.5 | Clarke and Clarke (1988) |
| Goat | 40 | 480 | 12.0 | 60.0 | Clarke and Clarke (1988) |
| Horse | 500 | 100–200 | 0.2–0.4 | 1.0–2.0 | Clarke and Clarke (1988) |
| Pig | 102 | 75 | 0.7 | 3.5 | Clarke and Clarke (1988) |
| Sheep | 40 | 100 | 2.5 | 12.5 | Clarke and Clarke (1988) |
| Mouse | 0.025 | NR | NR | 0.5 (s.c.) | Tekol (1991), Tekol and Gögüsten (1999) |
| Rat | 0.25 | NR | NR | 5.0 (s.c.) | Tekol (1991), Tekol and Gögüsten (1999) |
| Human | 80 | 50–100 | 0.6–1.3 | 3.0–6.5 | Watt and Breyer-Brandwijk (1962) |
| Rabbit | 2.5 | 1.75 | 0.7 | 3.5 | Watt and Breyer-Brandwijk (1962) |

NR: data not reported.
s.c.: Rat $LD_{min}$ is based on subcutaneous dose.
*Estimated average body weights of adult animals in Table 74.2 referenced in Spector (1956).

Arai *et al*., 1992). In cases of deliberate yew poisoning in humans, adverse symptoms of toxicity are similar to those reported in animals. Yew ingestion results in dizziness, pupil dilation, nausea, vomiting, diffuse abdominal pain, tachycardia (initially), muscle weakness, and convulsions (Czerwek and Fischer, 1960; Fröhne and Pribilla, 1965; Schulte, 1975; Blyth, 1884). In some cases, these symptoms can proceed to bradycardia, bradypnea, diastolic cardiac standstill, or death (Fröhne and Pribilla, 1965; Schulte, 1975; Blyth, 1884; Sinn and Porterfield, 1991).

A diagnosis of yew poisoning in animals is frequently based on a history of exposure and identification of the yew (*Taxus* spp.) in the digestive tract. It is not uncommon that poisoning is associated with pruning bushes and then feeding the trimmings to the livestock. In some instances, it is difficult to readily obtain this information from the owners. In suspect cases, yew fragments (sometimes visible only by microscopic examination) are often found in the mouth, stomach content, rumen content, and/or small intestine. On occasion, exposure may be indicated in the history, yet gross identification of plant material is unconfirmed. This can be especially true in species that chew their food more thoroughly, such as horses. In these cases, diagnosis of taxine poisoning often requires a more detailed microscopic and/or chemical evaluation of the GI contents (Karns, 1983; Tiwary *et al*., 2005). Chemical analysis of GI contents (particularly stomach/rumen contents) via gas chromatography/mass spectroscopy (GC/MS), liquid chromatography/mass spectroscopy (LC/MS), or thin-layer chromatography (TLC) can be used to confirm the presence or absence of taxine alkaloids in extracts from stomach/rumen contents (Stahr *et al*., 1977; Kite *et al*., 2000; Tiwary *et al*., 2005). Of these techniques, GC/MS and LC/MS are the most sensitive. Currently, the only chemical standards available for these analyses are crude extracts from *Taxus* spp. bushes, not unlike the standards that have been used for the past 100 years.

There are no lesions at post-mortem examination which are pathognomonic in animals that have died due to yew toxicosis. Indeed, neither gross nor microscopic abnormalities (with the exception of large pieces of yew leaves and stems, if they are present in the GI tract) are generally seen (Kingsbury, 1964; Alden *et al*., 1977; Ogden, 1988; Rooks, 1994). An exception to this is a recent case of *Taxus* spp. toxicosis in a horse in which ecchymotic hemorrhages were visible grossly along the endocardial surfaces of the ventricles, and microscopically, mild multifocal necrosis of the myocardium was identified in the ventricular wall and papillary muscles of the heart (Tiwary *et al*., 2005). In subacute poisonings, gastroenteritis may be evident, however the inflammation is probably due to an irritant oil present in the yew and not taxine (Watt and Breyer-Brandwijk, 1962; Kingsbury, 1964; Evans and Cook, 1991). Rarely, other gross changes have been reported at necropsy. These have included moderate to severe rumenitis, superficial hemorrhages in the right ventricular myocardium and right atrium, and mild focal interstitial myocarditis (Ogden, 1988; Panter *et al*., 1993).

## TREATMENT

Death is frequently the first adverse clinical sign in animals that have eaten toxic amounts of yew. In these animals, treatment is unrewarding. However, in instances where known ingestion has recently occurred, it is important to remove the plant material from the GI tract and limit absorption. Rumenotomy, followed by replacement therapy with a mixture of mineral oil, electrolytes, activated charcoal, and alfalfa pellets has been effective in treating some cases of *Taxus* spp. poisoning in ruminants (Casteel and Cook, 1985). There is no specific antidote for taxine poisoning. Atropine or lidocaine has been suggested to be beneficial in alleviating the cardiodepressant effect of taxine (Kingsbury, 1964; Schulte, 1975). However, in experimental animal studies (Bryan-Brown, 1932; Vohora, 1972), and in human cases where the cardiac response to attempted treatment was closely monitored via electrocardiography, classic antiarrhythmic therapy has proven ineffective (Willaert et al., 2002). Additionally in humans, a variety of clinical measures such as the administration of circulatory stimulants, artificial respiration, and cardiac pacemakers have not been able to prevent death from yew intoxication (Fröhne and Pfänder, 1984).

## CONCLUSION

Although much progress has been made during the last few decades in identifying the toxins and active diterpenoid taxanes in plants of the genus *Taxus*, their mechanisms of action, and their physiological effects, yew intoxication in animals remains a fairly frequent, often fatal, and largely preventable cause of livestock losses. Since the earliest indication of ingestion of toxic amounts of yew is death, and since there is no antidote and no effective treatments other than evacuation of the GI tract and prevention of absorption, education of the public as to the inadvisability of feeding yew trimmings to livestock is currently the best deterrent to *Taxus* spp. toxicoses.

## REFERENCES

Adeline MT, Wang XP, Poupat C, Ahond A, Potier P (1997) Evaluation of taxoids from *Taxus spp.*crude extracts by high

performance liquid chromatography. *J Liq Chromatogr Relat Tech* **20**(19): 3135–45.

Alden CL, Fosnaugh CJ, Smith JB, Mohan R (1977) Japanese yew poisoning in large domestic animals in the Midwest. *J Am Vet Med Assoc* **170**(3): 314–16.

Alloatti G, Penna C, Levi RC, Gallo MP, Appendino G, Fenoglio I (1996) Effects of yew alkaloids and related compounds on guinea-pig isolated perfused heart and papillary muscle. *Life Sci* **58**(10): 845–54.

Appendino G, Özen H, Fenoglio I, Gariboldi P, Gabetta B, Bombardelli E (1997) Pseudoalkaloid Taxanes from *Taxus baccata*. *Phytochemistry* **33**(6): 1521–3.

Arai M, Stauber E, Shropshire CM (1992) Evaluation of selected plants for their toxic effects in canaries. *J Am Vet Med Assoc* **200**(9): 1329–31.

Bacciarini LN, Wenker CJ, Muller M, Iten P (1999) Yew (*Taxus baccata*) intoxication in a captive brown bear (*Ursus arctos*). *Eur J Vet Pathol* **5**(1): 29–32.

Baker SD, Sparreboom A, Verweij J (2006) Clinical pharmacokinetics of docetaxel: recent developments. *Clin Pharmacokinet* **45**(3): 235–52.

Bauereis VR, Steiert W (1959) Pharmakologische Eigenschaften von Taxin A und B. *Arzneim Forschung* **9**: 77–9.

Blyth AW (1884) Taxine. In *Poisons: Their Effects and Detection*. Charles Griffin and Co., London, pp. 383–4.

Brown DT (2003) Preclinical and clinical studies of the taxanes. In *Taxus: The Genus Taxus*, Itokawa H, Lee K-H (eds). Taylor & Francis, New York, pp. 387–435.

Bryan-Brown T (1932) The pharmacological actions of taxine. *Q J Pharm Pharmacol* **5**: 205–19.

Casteel SW, Cook WO (1985) Japanese yew poisoning in ruminants.*Mod Vet Prac* **66**: 875–6.

Clarke EGC, Clarke ML (1988) Poisonous plants, Taxaceae. In *Veterinary Toxicology*, 3rd ed. Baillière, Tindall & Cassell Ltd., London, pp. 276–7.

Coenen M, Bahrs F (1994) Fatal yew poisoning in goats as a result of ingestion of foliage from garden prunings. *Deutsche Tierarzliche Wochenschrift* **101**(9): 364–7.

Cope RB, Camp C, Lohr CV (2004) Fatal yew (*Taxus* spp.) poisoning in Willamette Valley, Oregon, horses. *Vet Hum Toxicol* **46**(5): 279–81.

Czerwek H, Fischer W (1960) Tödlicher Vergiftungsfall mit *Taxus baccata*. *Arch Toxikol* **18**: 88–92.

Ettouati B, Ahond A, Poupat C, Potier P (1991) Révision Structurale de la Taxine B, Alcaloïde Majoritaire des Feuilles de L'if D'Europe, *Taxus baccata. J Nat Prod* **54**(5): 1455–8.

Evans KL, Cook JR (1991) Japanese Yew poisoning in a dog. *J Am Animal Hosp Assoc* **27**: 300–2.

Evers RA, Link RP (1972) Yews, *Taxus* species. In *Poisonous Plants of the Midwest and Their Effects on Livestock*, College of Agriculture, University of Illinois at Urbana-Champaign, IL pp.81–2.

Fiedler HH, Perron RM (1994) Yew poisoning in Australian emus (*Dromarius novaehollandiae* LATHAM). *Berliner und Munchener Tierarztliche Wochenshrift* **107**(2): 50–2.

Foster S, Duke JA (1990) American yew. In *Eastern/Central Medicinal Plants.* Houghton Mifflin, Boston, MA, p. 226.

Fröhne D, Pfänder J (1984) Taxaceae, *Taxus baccata* L., yew. In *A Colour Atlas of Poisonous Plants*, 2nd ed. Wolfe Publishing Ltd., London, pp. 223–5.

Fröhne D, Pribilla O (1965) Tödliche Vergiftung mit *Taxus baccata. Arch Toxikol* **21**: 150–62.

Graf E (1956) Zur Chemie des Taxins. *Angew Chem* **68**: 249–50.

Graf E, Bertholdt H (1957) Das amorphe Taxin und das kristallisierte Taxin A. *Pharm Zentralhalle* **96**: 385–95.

Graf E, Boeddeker H (1956) Zur Kenntnis der β-Dimethylaminohydrozimtsäure. *Arch der Pharm und Berichte der Deutsch Pharmaz Ges* **289**: 364–70.

Graf E, Kirkel A, Wolff GJ, Breitmaier E (1982) Die Aufklärung von Taxin A aus *Taxus baccata* L. *Liebigs Ann Chem* 376–81.

Graf E, Weinandy S, Koch B, Breitmaier E (1986) $^{13}$C-NMR-Untersuchung von Taxin B aus *Taxus baccata* L. *Liebigs Ann Chem*, 1147–51.

Gustafson DL, Long ME, Zirrolli JA, Duncan MW, Holden SN, Pierson AS, Eckhardt SG (2003) Analysis of docetaxel pharmacokinetics in humans with the inclusion of later sampling timepoints afforded by the use of a sensitive tandem LCMS assay. *Cancer Chemother Pharmacol* **52**: 159–66.

Hartzell H (1995) Yew and us: a brief history of the yew tree. In *Taxol: Science and Applications*, Suffness M (ed.). CRC Press, Boca Raton, FL, pp. 27–34.

Hilger A, Brande F (1890) Ueber Taxin, das Alkaloïd des Eibenbaumes (*Taxus baccata*). *Berichte Deutsch Chem Ges* **23**: 464–8.

Itokawa H (2003) Taxoids occurring in the genus Taxus. In *Taxus: The Genus Taxus*, Itokawa H, Lee K-H (eds). Taylor & Francis, New York, pp. 35–78.

Jenniskens LHD, van Rozendaal ELM, van Beek TA (1996) Identification of six taxine alkaloids from *Taxus baccata* needles. *J Nat Prod* **59**: 117–23.

Karns PA (1983) Intoxication in horses due to ingestion of Japanese Yew (*Taxus cuspidata*). *Equine Prac* **5**(1): 12–14.

Kingsbury JM (1964) Taxaceae. In *Poisonous Plants of the United States and Canada*. Prentice-Hall, Inc., Englewood Cliffs, NJ, pp. 121–3.

Kite GC, Lawrence TJ, Dauncey EA (2000) Detecting Taxus poisoning in horses using liquid chromatography/mass spectrometry. *Vet Human Toxicol* **42**(3): 151–4.

Lee S, Bae C, Chung B (2003) Yew poisoning in 17 dairy cattle. *J Vet Clin* **20**(3): 406–9.

Lucas H (1856) Ueber ein in den Blätter von *Taxus baccata* L. enhaltenes alkaloid (das Taxin). *Arch der Pharmaz* **135**: 145–9.

Matthew N, Elsner G, Purdy C, Zipes DP (1993) Wide QRS rhythm due to Taxine toxicity. *J Cardiovasc Electrophysiol* **3**: 59–61.

Ogden L (1988) *Taxus* (yews) – a highly toxic plant. *Vet Human Toxicol* **30** (6): 563–4.

Panter KE, Molyneux RJ, Smart RA, Mitchell L, Hansen S (1993) English Yew poisoning in 43 cattle. *J Am Vet Med Assoc* **202**(9): 1476–7.

Potier CR, Poujol H, Ahond A, Mourabit AA, Chiaroni A, Poupat C (1997) Taxoïdes: Nouveaux Analogues du 7-Déshydroxydocétaxel Préparés à partir des Alcaloïdes de I'If. *Tetrahedron* **53**(14): 5169–84.

Poupat C, Ahond A, Potier P (1994) Nouveau Taxoïde Basique Isolé Des Feuilles D'if, *Taxus baccata*: La 2-Désacétyltaxine A. *J Nat Prod* **57**(10): 1468–9.

Rae CA, Binnington BD (1995) Yew poisoning in sheep. *Can Vet J* **36**(7): 446.

Rooks JS (1994) Japanese Yew toxicity. *Vet Med* **89**: 950–1.

Ruha AM, Tanen DA, Graeme KA, Curry SC, Miller MB, Gerkin R, Reagan CG, Brandon TA (2002) Hypertonic sodium bicarbonate for Taxus media-induced cardiac toxicity in swine. *Acad Emerg Med* **9**(3): 179–85.

Schulte T (1975) Tödliche Vergiftung mit Eibennadeln (*Taxus baccata*). *Arch Toxikol* **34**: 153–8.

Shanker K, Pathak NKR, Trivedi VP, Chansuria JPN, Pandey VB (2002) An evaluation of toxicity of *Taxus baccata* Linn. (talispatra) in experimental animals. *J Ethnopharmacol* **79**: 69–73.

Shropshire CM, Stauber E, Arai M (1992) Evaluation of selected plants for acute toxicosis in budgerigars. *J Am Vet Med Assoc* **200**(7): 936–9.

Sinn LE, Porterfield JF (1991) Fatal taxine poisoning from yew leaf ingestion. *J Forensic Sci* **36**(2): 599–601.

Smythies JR, Benington F, Morin RD, Al-Zahid G, Schoepfle G (1975) The action of the alkaloids from yew (*Taxus baccata*) on the action potential in the *Xenopus* medullated axon. *Experentia* **31**: 337–8.

Spector WS (1956) *Handbook of Biological Data*. Wright-Patterson AFB, OH.

Stahr HM, Hyde W, Kiesey J, Ross PF (1977) Alkaloids: extraction and qualitative identification. In *Analytical Methods in Toxicology*. Iowa State University Press, Ames, IA, pp. 178–83.

Suffness M (1995) *Taxol: Science and Applications*. CRC Press, Boca Raton, FL, pp. 7–8, 311–12.

Taksdal T (1994) Diagnoses from the Norwegian State Veterinary Laboratory. *Norsk Veterinaertidsskrift* **106**(4): 305–6.

Tekol Y (1985) Negative chronoptopic and atrioventricular blocking effects of taxine on isolated frog heart and its acute toxicity in mice. *Planta Med* **5**: 357–60.

Tekol Y (1991) acute toxicity of taxine in mice and rats. *Vet Human Toxicol* **33**(4): 337–8.

Tekol Y, Gögüsten B (1999) comparative determination of the cardioselectivity of taxine and verapamil in the isolated aorta, atrium, and jejunum preparations of rabbits. *Arzneim Forschung* **49**(8): 673–8.

Tekol Y, Kameyama M (1987) Elektrophysiologische Untersuchungen über den Wirkungsmechanisms des Eibentoxins Taxin auf das Herz. *Arzneim Forschung* **37**(4): 428–31.

Tiwary AK, Puschner B, Kinde H, Tor ER (2005) Diagnosis of Taxus (yew) poisoning in a horse. *J Vet Diagn Invest* **17**(3): 252–5.

Tyler VE (1960) Note on the occurrence of Taxine in *Taxus brevifolia*. *J Am Pharm Assoc* 683–4.

Veatch JK, Reid FM, Kennedy GA (1988) Differentiating yew poisoning from other toxicoses. *Vet Med* **83**: 298–300.

Vohora SB (1972) Studies on *Taxus baccata*. *Planta Med* **22**(1): 59–65.

Wacker R (1983) Yew poisoning in fallow deer. *Tierarztliche Umschau* **38**(4): 267–8.

Watt JM, Breyer-Brandwijk MG (1962) Taxaceae. In *The Medicinal and Poisonous Plants of Southern and Eastern Africa*. E&S Livingstone, Edinburgh, pp. 1019–22.

Weaver JD, Brown DL (2004) Incubation of European yew (*Taxus baccata*) with white-tailed deer (*Odocoileus virginianus*) rumen fluid reduces taxine A concentrations. *Vet Human Toxicol* **46**(6): 300–2.

Willaert W, Claessens P, Vankelecom B, Vanderheyden M (2002) Intoxication with *Taxus bacata*: Cardiac arrhythmias following yew leaves ingestion. *J Pacing Clin Electrophysiol* **25**(4): 511–2.

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# Part 15

## Mycotoxins

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C H A P T E R

# 75

# Aflatoxins

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## INTRODUCTION

Aflatoxins, on a worldwide scale, are important mycotoxins in human foods and animal feedstuffs (Williams *et al.*, 2004). Aflatoxin contamination causes economic losses of corn, cottonseed, peanuts, sorghum, wheat, rice and other commodities, and economic losses of processed food and feedstuffs. Commodities considered unsafe for human consumption can be incorporated into animal feedstuffs (Coppock and Swanson, 1986). Gourami and Bullerman (1995) are of the opinion that aflatoxicosis in domestic animals is considerably more prevalent than it is diagnosed. Health effects occur in companion animals, livestock, poultry and humans because aflatoxins are potent hepatotoxins, immunosuppressant, and mutagens and carcinogens (Clegg and Bryson, 1962; Allcroft and Lewis, 1963; Eaton and Gallagher, 1994). Aflatoxins are teratogenic (Robens and Richard, 1992). Aflatoxicosis in the human population, especially in areas stricken by poverty and drought and other adverse growing conditions, is an important public health problem (Williams *et al.*, 2004).

Most governmental jurisdictions regulate the levels of aflatoxins allowed in animal feedstuffs and human foods because of their toxicity. Worldwide, aflatoxins because of their prevalence and toxicity are important in public health. Public health concerns center on both primary poisoning from aflatoxins in commodities, food and feedstuffs, and relay poisoning from aflatoxins in milk. The allowable levels of aflatoxins in animal feedstuff and human foods vary with governmental jurisdictions. Regulatory information for aflatoxins is usually posted on government websites.

## BACKGROUND

### Identification of aflatoxins

Aflatoxins, in the late 1950s and early 1960s, were identified as the cause of the mysterious turkey "X" disease in Great Britain (Clegg and Bryson, 1962; Allcroft and Lewis, 1963). Aflatoxins were identified as carcinogenic in rainbow trout (Newberne and Butler, 1969). In the United States, studies on aflatoxins incriminated aflatoxins as the cause of epizootic hepatitis in dogs and as the cause of moldy corn poisoning in pigs (Newberne *et al.*, 1966).

## AFLATOXIN PRODUCTION

### Aflatoxigenic fungi

The most commonly recognized aflatoxigenic fungi are *Aspergillus flavus*, *A. parasiticus* and *A. nomius*. Other fungi reported to produce aflatoxins are *A. bombycis*, *A. ochraceoroseus* and *A. pseudotamari* (Klich *et al.*, 2000; Bennett and Klich, 2003; Mishra and Das, 2003). *Aspergillus flavus* and *A. fumigatus* have also been identified as pathogens in animals and humans (Barton *et al.*, 1992; Drakos *et al.*, 1993; Pepeljnjak *et al.*, 2004). Aflatoxins can be produced in tissues by toxigenic fungi. Assays of cultured *A. flavus* and *A. fumigatus* isolated from tissues have shown these fungi can produce aflatoxins, and chemical analyses of infected tissues have shown aflatoxins to be present (Matsumura and Mori, 1998; Mori *et al.*, 1998; Pepeljnjak *et al.*, 2004).

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Aflatoxins being produced in tissues have not been shown to cause liver lesions typical of aflatoxicosis. Aflatoxigenic fungi infect animals and humans that often are immunocompromised. Systemic aspergillosis by aflatoxigenic fungi was considered to contribute to immunosuppression (Mori *et al.*, 1998).

Aflatoxigenic fungi have worldwide distribution. In temperate and tropical areas these species of *Aspergillus* have ubiquitous distribution and are found in soil used for growing crops (Gourami and Bullerman, 1995). These fungi also have distribution in storage areas, processing facilities and in the distribution systems for manufactured products. The production of aflatoxins is associated with spore production by species of *Aspergillus* (Calvo *et al.*, 2002). Strains of *A. flavus* can vary in aflatoxin capability from non-toxic to highly toxigenic and are more likely to produce more aflatoxin (AF) $B_1$ ($AFB_1$) than $AFG_1$. Strains of *A. parasiticus* generally have less variation in toxigenicity and generally produce $AFB_1$ and varying amounts of $AFB_2$, $AFG_1$ and $AFG_2$. Chemical structures of these mycotoxins are shown in Figure 75.1.

Versicolorin A and sterigmatocystin mycotoxins are intermediates in the synthesis of aflatoxins. Strains of *Aspergillus versicolor* have been reported to convert versicolorin A and sterigmatocystin to $AFB_1$ and $AFB_2$ (Gourami and Bullerman, 1995). Aflatoxin (AF) $M_1$ ($AFM_1$) and $AFM_2$ are the 4-hydroxylated metabolites of $AFB_1$ and $AFG_1$, respectively. These metabolites can occur in aflatoxin contaminated feedstuffs and in the milk and tissues. Chemical structures of $AFM_1$ and $AFM_2$ are shown in Figure 75.2.

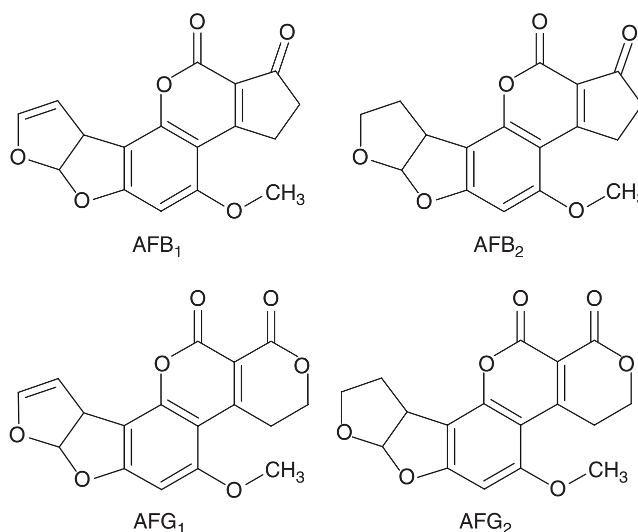


**FIGURE 75.1** Structural formulas of aflatoxins.

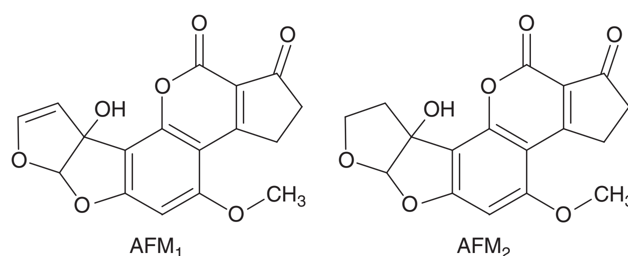


**FIGURE 75.2** Chemical structures of $AFM_1$ and $AFM_2$.

## Aflatoxin production in feedstuffs

All cereal crops can contain aflatoxins. Intensive cropping practices and decreased genetic diversity in cereal crops probably contribute to increased preharvest infections of commodities with fungi that produce aflatoxins (Lillehoj, 1992; Brown *et al.*, 1999). Preharvest contamination of crops with aflatoxins occurs in the temperate and tropical regions. The seeds in growth-stressed plants are the most susceptible to fungal invasion and aflatoxin production. The most common recognized plant stressors are drought, insect damage and timing of irrigation. Postharvest contamination occurs worldwide when conditions in the storage unit exist for the growth of aflatoxigenic fungi.

The primary recognized aflatoxigenic fungi in corn and sorghum are *Aspergillus flavus*, *A. parasiticus* and *A. nomius*. Of these fungi *A. flavus* is the species that has been most studied. *Aspergillus flavus* grows in plant-source debris on the soil (Gourami and Bullerman, 1995). Aflatoxigenic fungi can grow in feedlot manure (Hendrickson and Grant, 1971). Insects spread the spores of aflatoxigenic fungi to plants and the fungi colonize areas of insect damage. The flower and silk in corn can be portals of entry for species of *Aspergillus* (Diener *et al.*, 1987).

Aflatoxin contamination can rapidly occur (Coppock *et al.*, 1989). Lee *et al.* (1986) showed that maturing corn kernels in the ear on the plant inoculated with *A. flavus* had 0.3–2 ppb aflatoxins present at 2 days after inoculation, 950–2800 ppb aflatoxins present at 4 days after inoculation and 3600–4500 ppb aflatoxins present at 7 days after inoculation. The *A. flavus* infection reduced the starch content of the kernel by 12%. Kojic acid was present at 2700 ppb 16 h after inoculation. High moisture corn and sorghum lightly inoculated with *A. flavus* was shown to produce ppm levels of aflatoxins within 48 h (Winn and Lane, 1978). Overmature sweet corn was shown to contain 2.36 ppm $AFB_1$ and 0.21 mg of $AFB_2$ (Hall *et al.*, 1989). Sweet corn has been considered more hazardous than field corn because of its higher free sugar content.

Cottonseed can be a source of aflatoxins in animal diets. Preharvest contamination of cottonseed occurs (Jaimi-Garcia and Cotty, 2003). Insect damage, timing of irrigation or rain, relative humidity around the bolls, stage of

maturity and variety of cotton can be factors in causing preharvest contamination of cottonseed with aflatoxins (Russell *et al.*, 1976; Lillehoj *et al.*, 1987). In stored cottonseed growth of aflatoxigenic fungi may occur when the average moisture level in stored cottonseed is greater than 7–8%. The lipids and proteins in cottonseed enhance aflatoxin production (Mellon and Cotty, 1998; Mellon *et al.*, 2000).

Peanut hay, peanuts and peanut by-products are an important source of mycotoxins (McKenzie *et al.*, 1981; Cullen and Newberne, 1994). Aflatoxins generally are the most concentrated in the seeds. The growth of aflatoxigenic fungi can occur in stored peanuts when moisture exceeds 8% and ambient temperature is above 25°C. Drought-stressed peanuts have decreased native resistance to infection by aflatoxin producing fungi (Wotton and Strange, 1987). Phytoalexin produced by the infected peanut seed increased and inhibited the growth of *A. flavus*, but aflatoxin levels continue to increase for an additional day. Drought-stressed peanut seeds have decreased production of phytoalexin and aflatoxin production in drought-stressed peanut kernels is limited by available moisture.

Distillers by-products can be a source of aflatoxin (Hesseltine, 1984). Corn and other high starch commodities contaminated with aflatoxins can be salvaged by using them for alcohol production. Aflatoxins are not destroyed by the fermentation process. On a dry matter basis, the concentration of aflatoxins in the stillage, compared to aflatoxins in the feedstock, is increased due to the loss of starch. Approximately 40% of the aflatoxins are in the syrup (distillers solubles) fraction and 60% are in the solids fraction. Aflatoxins generally are not found in the alcohol fraction.

## MOISTURE LEVEL OF GRAIN

The most common methods of preventing the growth of aflatoxigenic fungi in dry-stored commodities are by controlling the moisture level. Water available for aflatoxigenic fungi in stored grain is best indicated by equilibrium relative humidity or water activity ($a_w$) of water in the air around the grain (Caddick, 2003). The $a_w$ is equal to % relative humidity/100. Temperature of the grain is important as the $a_w$ increases with temperature. In cereal grains aflatoxigenic fungi generally cannot grow when the $a_w$ is below 0.65. A lower $a_w$ is required for protection of oilseeds. Oilseeds have more available water on the seed surface because less moisture is absorbed by the seed. The greater the oil fraction of the seed the less water that is absorbed into the seed. For most cereal grains, storage moisture of 12% is recommended for a wide range of grain temperatures, and for oilseeds the storage moisture from $<8\%$ to 9% is recommended for wide range of temperatures inside the storage unit.

Measuring moisture with a grain tester provides the average moisture of the kernels. This measurement can misinterpret the storability of grain. If the moisture is heterogeneously distributed among the seeds or pellets hot spots can occur. This phenomenon occurs because a microbial microcosm exists around each high moisture seed and that microcosm can meet the requirements for fungal growth and mycotoxin production. This is the reason why high and low moisture grain should not be blended. In the microcosm with a favorable $a_w$ level, growth of aflatoxigenic fungi can occur. The metabolism of microorganisms in the microcosms produces moisture and some microorganisms can also produce heat. Insects and other pests in grain also produces moisture and hot spots can form because of insect and other pests in stored grain. Monitoring multiple areas of a storage unit is essential for preventing the production of mycotoxins.

Samples for aflatoxin analyses should be placed in moisture-proof bags to prevent desiccation. As soon as possible after sampling, the individual moisture content of at least 100 seeds or pellets should be determined to identify moisture distribution in the kernels or pellets.

## SAMPLING

Aflatoxins in the bulk lot of feedstuffs are generally assumed to be the same as the mycotoxins identified in the sample (Whitaker, 2003). For this reason the samples obtained should be representative of the feedstuffs. A representative sample may be difficult to obtain because the distribution of aflatoxins in the suspect feedstuff can be highly heterogenous. For this reason, a single random sample may not represent the aflatoxin levels in the feedstuffs sampled. A small percentage of the kernels in a lot of wheat, barley, corn, cottonseed and peanuts may be contaminated with very high concentrations of aflatoxins. Sampling error can occur even with a well-designed sampling protocol (Whitaker, 2003). Interpretations of analytical findings should reflect the sampling procedure, and aflatoxicosis cannot be ruled out based on negative chemical analyses of feedstuffs. Another sampling problem is the available feedstuff for laboratory testing may be limited because the majority of the suspect feed has been consumed by livestock or poultry. In these situations feed may be available from the edges of the feed trough, or clumps of feed retained in the feed handing equipment.

Aflatoxin can be present in clumps of feedstuffs (Crockcroft, 1995). These clumps may contain high level of aflatoxins and only a few animals may have consumed feed from this source, or the clumps were mixed with the feed and fed to a large number of animals.

## CHEMISTRY OF AFLATOXINS

Aflatoxins have a difuranocoumarin chemical structure. Approximately 18 aflatoxins have been chemically characterized. Aflatoxins are in two chemical groups, the difurocoumarocyclopentenone series (includes $AFB_1$, $AFB_2$, $AFB_{2A}$, $AFM_1$, $AFM_2$, $AFM_{2A}$ and aflatoxicol) and the difurocoumarolactone series (includes $AFG_1$ and $AFG_2$). The "B" Group fluoresce blue in long wavelength ultraviolet light and the "G" Group fluoresce green. The primary aflatoxins of concern in feedstuffs are $AFB_1$, $AFB_2$, $AFG_1$ and $AFG_2$. Analytical results for aflatoxins generally are the sum of the concentrations of these four toxins. $AFB_1$ is the most potent aflatoxin and this chemical form is generally the most abundant in feedstuffs and foods. The order of toxicity is $AFB_1 > AFG_1 > AFB_2 > AFG_2$. Hydroxylated aflatoxin metabolites are excreted in milk and the important metabolites are $AFM_1$ and $AFM_2$ (Garrido *et al.*, 2003). $AFM_1$ is the toxic metabolite of $AFB_1$ and $AFM_2$ is the hydroxylated form of $AFB_2$. Although $AFM_1$ and $AFM_2$ are commonly associated with milk and other edible animal product, these compounds can also be produced by aflatoxigenic fungi.

The toxicologist generally assumes that the level provided in the analytical findings represents the level of aflatoxins in the substances at issue. The toxicologist should know the sampling and analytical method(s) used and the limitations of these methods before interpreting analytical results. For the analytical results it is important to know the method(s) employed, the reference standards used including internal standards, and an estimate of the percent extracted from the matrix. If this information is not available or is not known to the toxicologists, a risk exists in misinterpreting the qualitative and quantitative results. It is our opinion that chemical methods are the most reliable for testing a wide variety of substances for aflatoxins.

## PHARMACOKINETICS/ TOXICOKINETICS

### Absorption

Aflatoxins are efficiently absorbed by passive diffusion from the gastrointestinal tract and are primarily transferred from the intestine to hepatic portal blood (Hsieh and Wong, 1994). Very little aflatoxins appear to be transferred into the lymphatic system (Kumagai, 1989). Young animals absorb aflatoxin more efficiently than older animals. Rats at 2.5 weeks of age absorbed $AFB_1$ at a rate ~15 times greater than rats 4–5 weeks of age. Cattle were given single oral dose of aflatoxins from rice culture (42% $AFB_1$ and 27% $AFB_2$) in gelatin capsules (Cook *et al.*, 1986). $AFB_1$ and $AFM_1$ were observed in venous blood (jugular vein) 30 min after dosing and reached maximal levels 4–8 h after dosing. The maximal levels of $AFM_1$ occurred before $AFB_1$ and the maximal blood levels of $AFB_1$ generally were higher than $AFM_1$. These findings suggest that aflatoxins are rapidly absorbed from the rumen. Rats have been shown to absorb $AFB_1$ most efficiently from the duodenum and jejunum (Kumagai, 1989). The rate of absorption of $AFB_1$ in rats is dependent on concentration providing evidence that aflatoxins are absorbed by passive diffusion. The rate of $AFB_1$ absorption from the duodenum of rats in diestrus was greater than the rate of absorption of $AFB_1$ from the duodenum in rats in mid-lactation.

### Metabolism and excretion

Biotransformation is important in the toxicology of aflatoxins. The most important organ for biotransformation is the liver, and biotransformation can also occur in the kidney and intestinal tract. The majority of research has focused on the biotransformation of $AFB_1$. With the exception of the $AFB_1$-8,9-epoxide, the biotransformation products are less toxic than $AFB_1$. Cytochrome P450 ($AFB_1$ hydroxylase) has a key role in the biotransformation of $AFB_1$ to $AFB_1$-8,9-epoxide. The formation of $AFB_1$-8,9-epoxide is considered to be the most significant biotransformation pathway because the $AFB_1$-8,9-epoxide forms adducts with DNA, RNA and proteins. Conjugation of $AFB_1$-8,9-epoxide with glutathione is considered to be an important detoxification pathway. Other biotransformation products of $AFB_1$ are $AFQ_1$ which can be metabolized to $AFH_1$. $AFB_1$ is also metabolized to $AFP_1$, $AFM_1$, aflatoxicol and other metabolites. The $AFP_1$, $AFM_1$, $AFQ_1$ and aflatoxicol form glucuronide and sulfate conjugates.

Cattle were given a single oral doses (ranging from 0.2, 0.4, 0.6 and 0.8 mg/kg body weight) of aflatoxins from rice culture (42% $AFB_1$ and 27% $AFB_2$) in gelatin capsules (Cook *et al.*, 1986). $AFM_1$ was identified in rumen contents 2 h after dosing. This observation suggests that $AFM_1$ is formed by the flora and fauna in the rumen. Urine from these cattle contained $AFB_1$ and $AFM_1$. Kuilman *et al.* (1998, 2000) showed that bovine hepatocytes metabolized $AFB_1$ to $AFM_1$ (48% metabolized in 8 h), $AFB_1$ dihydrodiol and minor levels of $AFB_1$ glutathione conjugate. Liver preparations from calves previously treated with β-naphthoflavone had increased *in vitro* transformation of $AFB_1$ to $AFM_1$ when compared to liver preparations from calves that had not been previously treated with β-naphthoflavone (Bodine *et al.*, 1982). Hatch *et al.* (1982) showed that hepatic damage by $AFB_1$ in goats was increased by pretreatment with phenylbutazone and benzoflavones. Hepatic Kupffer and endothelial cells in the rat have been shown to have upregulated activity of $AFB_1$ hydroxylase,

the enzyme that converts $AFB_1$ to $AFM_1$ (Gemechu-Hatewu *et al.*, 1996). The P450 inducers used were phenobarbitol, isosafrole, Aroclor 1254 and 3-methylcholanthrene.

Aflatoxins are excreted in milk, eggs, urine, semen, bile and feces. Excretion of aflatoxins in milk is discussed in following sections (see Residues). Bingham *et al.* (2004) identified $AFM_1$ and AFP in dog urine. $AFM_1$ and aflatoxicol have been shown to be formed by hens and are transferred to the egg (Qureshi *et al.*, 1998). Dogs dosed with 100 μg $AFB_1$/kg body weight excreted $AFM_1$ in the urine and 90% of the $AFM_1$ excreted occurred within 12 h after dosing (Bingham *et al.*, 2004). Humans exposed to dietary aflatoxins excrete $ABM_1$, $AFP_1$ and $AFB_1$-$N^7$-guanine in urine (Groopman *et al.*, 1985). Aflatoxins have been shown to be excreted in boar semen (Picha, 1986). Rats excrete $AFB_1$ into the intestinal tract via bile and the intestinal mucosa also excretes aflatoxins (Kumagai, 1989).

## Residues

Residues of aflatoxins, especially $AFM_1$ can occur in edible animal products. The foodstuffs of greatest public health concern are milk, dairy products, especially those made from casein, and liver and kidney (Applebaum *et al.*, 1982). Aflatoxins can be present in meat. Most governmental jurisdictions regulate the permissible level of aflatoxin in edible animal products.

### *Milk*

Aflatoxins can be present in processed milk products. Issues regarding aflatoxin contamination of fluid milk generally increase when regional contamination of commodities (e.g. corn, sorghum, cottonseed) occurs before harvest (Applebaum *et al.*, 1982). Milk products such as cheese and dried milk also can become directly contaminated with aflatoxins by fungal growth in these substrates. Aflatoxins appear in cow milk within 12–48 h after ingestion (Applebaum *et al.*, 1982; Frobish *et al.*, 1986). The amount of dietary aflatoxins being excreted in milk varies with the high value reported being as 3% and the low value being reported as 0.17% (Applebaum *et al.*, 1982; Stubblefield *et al.*, 1983; Price *et al.*, 1985; Frobish *et al.*, 1986). To compare with other mammalian species, humans excrete 0.09–0.43% of dietary intake in breast milk, sheep excrete 0.112% of the dietary $AFB_1$ as $AFM_1$ in the milk, and aflatoxins in sow milk have been shown to be ~1000-fold less than the aflatoxins in the diet (Zarba *et al.*, 1992; Silvotti *et al.*, 1997; Battacone *et al.*, 2003). The stage of lactation is important for excretion of aflatoxins (Veldman *et al.*, 1992). The percentage of aflatoxins in milk has been reported to increase with increasing milk yields (Frobish *et al.*, 1986). Cows in early lactation can excrete from 3.8% to 6.2% of the dietary $AFB_1$ as $AFM_1$ in the milk, and cows in late lactation can excrete 1.8% to 2.5% of the dietary $AFB_1$ as $AFM_1$. This phenomenon is independent of the level of $AFB_1$ in the diet and appears to be linked to cows having upregulation of $AFB_1$ metabolism during early lactation. The phenomenon of excreting more $AFM_1$ in milk during early lactation must be considered in calculating safe dietary levels of aflatoxins for dairy cattle, and may be of importance in calculating the rations for other milk producing animals. Herd-specific analytical data can be used to calculate the dietary:milk ratio for aflatoxins. Our experience has shown that this approach is accurate to predict aflatoxins in milk after changes have been made to reduce dietary aflatoxins. The dietary threshold for aflatoxins excretion in cow milk appears to be ~15 ppb dietary level or 230 μg $AFB_1$/cow/day. Aflatoxins in milk generally disappear within 24–72 h after all the aflatoxins have been removed from the diet.

Residues of $AFM_1$ in milk have been studied in dairy sheep (Battacone *et al.*, 2003). The ratio of $AFB_1$ in the diet to $AFM_1$ in milk was not affected by the dose of aflatoxins. Sheep receiving a single dose of $AFB_1$ excreted 0.032% of the dose as $AFM_1$ in milk. The mean value was 0.112% of the dietary $AFB_1$ was excreted as $AFM_1$ in the milk.

Aflatoxins are excreted in sow milk. The levels of aflatoxins in sow milk increased over a 25-day lactation period. The sows were fed diets containing 800 ppb $AFB_1$ or a diet containing 400 ppb $AFB_1$ + 400 ppb $AFG_1$ for entire gestation and lactation period (Silvotti *et al.*, 1997). The average ratio of aflatoxins in the diet to aflatoxins in milk was ~1000:1.

The general consensus in the scientific literature is that aflatoxins are stable in milk, and are primary associated with milk protein. Aflatoxins in raw milk slowly decrease by 11–25% in 1–3 days of storage at 5°C. Approximately 75% of aflatoxins in milk are found in the casein (protein) fraction and 25% in the whey fraction of milk. Aflatoxins are also found in butter and the division is ~84% in butter milk and ~16% in butter. Aflatoxins are concentrated in cheese, and the concentration factor varies with the type of cheese and cheese-making procedures. $AFM_1$ is decreased during yogurt manufacturing and a decreasing the pH of yogurt decreases the levels of $AFM_1$ (Govaris *et al.*, 2002).

### *Residues in meat*

Aflatoxins generally are found in liver, kidney and edible parts of the gastrointestinal tract. Aflatoxins are not known to accumulate in the body fat. Two-week-old turkey poults were fed diets containing 50 and 150 ppb $AFB_1$ (Richard *et al.*, 1986). The poults were killed at 11, 13, and 1 or 2 weeks after being fed the 50 and 150 ppb diets for 11 weeks (1 or 2 weeks on aflatoxin free diet). At 50 ppb feeding level for 11 weeks $AFB_1$ was found in liver (0.02–0.09 ng/g), kidney (0.01–0.02 ng/g) and gizzard (0.043–0.162 ng/g), and $AFM_1$ was not shown to be in

**TABLE 75.1 Levels in ng/g or ng/ml of aflatoxins in tissues after steers were fed a diet containing 800 ppb aflatoxins for 17.5 weeks (Richards *et al.*, 1983)**

| Toxin | Liver | Kidney | Muscle | Heart | Lung | Rumen |
|---|---|---|---|---|---|---|
| $AFB_1$ | 0.37 | 0.09 | 0.002 | 0.004 | 0.014 | 13.05 |
| $AFM_1$ | 1.07 | 4.82 | 0.115 | 0.14 | 0.29 | 1.66 |

liver, kidney and gizzard. Feeding 50 ppb aflatoxins for 13 weeks increased the residues of $AFB_1$ and $AFM_1$; for $AFB_1$ liver contained 0.02–0.13 ng/g, kidney contained 0.01–0.34 ng/g and gizzard at trace to 0.113 ng/g; $AFM_1$ in liver was 0.11–0.14 ng/g and kidney contained 0.01–0.07 ng/g. At the 150 ppb feed level, fed for 11 weeks, $AFB_1$ in liver was 0.08–0.13 ng/g, kidney was 0.025–0.08 ng/g and gizzard trace to 0.22 ng/g; $AFM_1$ was 0.03–0.10 ng/g in liver and 0.09–0.13 ng/g in kidney. Aflatoxin $M_1$ was not shown to be present in the gizzard. Breast and thigh muscles did not contain aflatoxins. Birds on the 150 ppm diet for 11 weeks and the control diet (no aflatoxins) for 2 weeks essentially had cleared the aflatoxins from the liver, kidney and gizzard. Steers fed a diet containing 800 ppb aflatoxins for 15 weeks and then placed on an aflatoxin free diet for 2.5 weeks did not have residues of aflatoxin in heart, skeletal muscle, liver and kidney (Richard *et al.*, 1983). Steers fed a diet containing 800 ppb aflatoxins for 17.5 weeks did have residues of aflatoxins (Table 75.1).

## TOXICODYNAMICS

### Mechanisms of action

The metabolism of aflatoxins, unlike many other mycotoxins, is closely linked with toxicity (Eaton and Gallagher, 1994). $AFB_1$ is metabolized in a P450-dependent reaction to $AFB_1$-8,9-epoxide which forms adducts with macromolecules in the cell. The affinity of $AFB_1$-8,9-epoxide in decreasing order for macromolecules is DNA > RNA > protein. The formation of these adducts is considered important in the toxicity of aflatoxins. The DNA adduct is formed with $N^7$-guanine and this adduct is relatively resistant to DNA repair processes. Upregulation of the P450 system increases the toxicity of aflatoxins. Species sensitivity to aflatoxins is linked to the rate of bioactivation and the rate of detoxification. $AFB_1$-8,9-epoxide is primarily detoxified by Phase II synthetic reactions. In mammals glutathione *S*-transferase-mediated conjugation is an important detoxification pathway, and species differences in part account for differences in species susceptibility. Other Phase II pathways that decrease the toxicity of aflatoxins include formation of glucuronide and sulfates.

### Immunotoxic effects

The immunotoxic effects of aflatoxins are of public health and economic importance. The native resistance to disease is reduced and vaccine protection is also impaired. The apparent clinical signs of aflatoxicosis may be limited to increased occurrences and severity of infectious diseases. Recovery from infectious disease may be prolonged and require additional treatments. A clue to immune dysfunction is often infections caused by organisms that generally are not considered to be pathogens. Studies have shown that the immunotoxicity of aflatoxins could be a teratogenic effect of aflatoxins. A literature review is provided for the immunotoxic effects of aflatoxins in different species.

The immunotoxic effects of aflatoxins have been studied in pigs. Treatment with $AFB_1$ has been shown to shorten the incubation period of *Serpulina (Treponema) hyodysenteriae* in pigs (Joens *et al.*, 1981). Pigs given $AFB_1$ and infected with *S. hyodysenteriae* also had a longer period of diarrhea. Feeding weanling piglets a diet containing 140 or 280 ppb aflatoxins (~70% $AFB_1$) for 4 weeks caused an increase in serum γ-globulin (Marin *et al.*, 2002). At the 280 ppb level, a reduced immune response to *Mycoplasma agalactiae* was observed. There were indications that interleukin-1β and tissue necrosis factor-α expression was decreased by exposure at the 280 ppb level. Sows were fed diets containing 800 ppb $AFB_1$ or a diet containing 400 ppb $AFB_1$ + 400 ppb $AFG_1$ for entire gestation and lactation period (Silvotti *et al.*, 1997). At 25 days of age the piglets born to the sows were found to have decreased immune functions in lymphoproliferative response to mitogens, monocyte-derived macrophages failed to have induced oxygen bursts and granulocytes had reduced chemotactic response. There is some evidence that *in utero* exposure of piglets to $AFB_1$ interferes with zinc metabolism and low plasma zinc decreases cell-mediated immunity because of a decrease in a zinc-dependent thymic hormone (Mocchegiani *et al.*, 1998).

Aflatoxins have been shown to be immunotoxic in ruminants. Feeding a diet containing 2 ppm aflatoxin (83.4% $AFB_1$, 12.3% $AFB_2$, and $AFG_1$ and $AFG_2$) to lambs for 37 days increased serum γ-globulin, decreased bacteriostatic activity in serum and decreased cellular immunity (Fernandez *et al.*, 2000). Studies done *in vitro* have shown that aflatoxin inhibits thymidine uptake by phytohemagglutinin-stimulated lymphocytes (Bodine *et al.*, 1984). Holstein steers (183 kg) fed a diet containing ~375 ppb aflatoxin for 17.5 weeks were observed to have a decrease in delayed cutaneous hypersensitivity (Richard *et al.*, 1983). Changes were not observed in antibody production (*Brucella abortus* antigen) and lymphoblastogenesis. Cattle were administered 0.3 mg of $AFB_1$ equivalent/kg of body weight for 12–14 days (Brown *et al.*, 1981). Before administration of aflatoxins, the cows were previously infected intramammary with

*Streptococcus agalactiae*, *Staphylococcus aureus* and *S. hyicus*. Clinical evidence of mastitis was not observed, but 3 of 4 cows had increased bacteria in the milk. The California mastitis test scores increased after the last dose of aflatoxins were given. Steers were administered a single oral dose of 0.6 or 0.8 mg $AFB_1$/kg body weight (Thurston *et al.*, 1986). Complement activity and bacteriostatic activity of serum decreased for 48–72 h and then returned to baseline at 7 days. In the same study cattle were administered 0.25 mg $AFB_1$/kg body weight/day for 14 days. The bacteriostatic activity of serum decreased until day 11 and then remained decreased for 29 days.

The immunotoxic effects of aflatoxins have been studied in poultry. Immunotoxic effects in poultry are lymphocytopenia, reduced T-cell counts and reduced native resistance to disease (Pier and Heddleston, 1970). Two-week-old turkey poults were placed on a diet containing 50 and 150 ppb aflatoxins for 11 weeks (Richard *et al.*, 1986). The poults were vaccinated with sheep red blood cells (SRBC) or *Pasteurella multocida* at 4 weeks and the vaccinations repeated at 8 weeks. Poults in the 150 ppb group had an increase in humoral response to *P. multocida* at 4 weeks and a decreased response to SRBC at 8 weeks. Three-week-old chicks were injected intramuscularly with 0.250 mg $AFB_1$ every 2 days for four administrations of $AFB_1$ (Otim *et al.*, 2005). At 3 and 6 weeks of age the chicks were vaccinated with Newcastle virus vaccines. Hemagglutination reaction to Newcastle disease virus was reduced. The immunosuppression of aflatoxins (from cultures of *A. flavus* NRRL 5518) were found to be additive with fowl adenovirus serotype-4 (Shivachandra *et al.*, 2003). The response to dinitrochlorobenzene hypersensitization was also reduced by $AFB_1$. Hens were fed diets containing 0.2, 1.0 and 5.0 ppm $AFB_1$ and fertile eggs were collected (Qureshi *et al.*, 1998). All chicks from hens given aflatoxins had reduced macrophage phagocytic activity and reactive oxygen burst. Antibody titers to SRBC were not changed on the primary vaccination, but the secondary response was depressed in chicks from hens given the diet containing $AFB_1$. A study by Sur and Celik (2003) showed decreased development of the bursa of Fabricius was observed in chicks after the egg was injected with 10 μg $AFB_1$ at day 7 of incubation.

## Reproductive and teratogenic effects

Reproductive and possible teratogenic effects have been reported for aflatoxins. Aflatoxins have been shown to be disruptive of sperm production and fertility in mice. An intraperitoneal dose of 50 μg of $AFB_1$/kg body weight/day (estimated to be equivalent to ~330 ppm in diet) was given to male mice for varying intervals (Agnes and Akbarsha, 2003). At 35 days, fertility testing showed a decrease in litter size, and tissue examination showed a decrease in spermatozoa numbers present in the caudal epididymis. When the numbers of spermatozoa decreased, forward mobility of spermatozoa was decreased and abnormal spermatozoa were observed. Sows were fed a diet containing 800 ppb $AFB_1$ or 400 ppb $AFB_1$ + 400 ppb $AFG_1$ from day 60 of pregnancy to day 28 of lactation (Mocchegiani *et al.*, 1998). Pigs born to the sows on the 800 ppb $AFB_1$ diet had reduced birth weights. Reduced birth weights was more pronounced in piglets born to sows consuming the diet containing 400 ppb $AFB_1$ + 400 ppb $AFG_1$. Pregnant rats were administered 0.3 mg of $AFB_1$/kg body weight on gestation days 11–14 or gestation days 15–18 (Kihara *et al.*, 2000). The offspring of these rats showed that prenatal exposure to $AFB_1$ caused a delay in early response development, decreased learning ability and impaired locomotor coordination.

# PATHOLOGY

## Clinical pathology

Acute aflatoxicosis causes changes in clinicopathology parameters. The activities of liver enzymes in serum are increased and the increased activity is indicative of hepatic damage. Once released into serum the activities of hepatic enzymes decrease as metabolic processes remove the hepatic enzyme from serum. Increased activity of hepatic enzymes in serum includes γ-glutamyl transpeptidase (transferase) (GGT), aspartate aminotransferase (AST, previously identified as SGOT), alkaline phosphates (ALP), sorbitol dehydrogenase (SDH), ornithine carbamyl transferase (OCT) and isocitric dehydrogenase (ICD). The activities of hepatic enzymes in serum that have been reported to be the most consistently elevated in aflatoxicosis are GGT, AST and SDH. The elevated activity of ALP is variable in aflatoxicosis. The timing of hepatic enzyme determinations in terms of the pathogenesis aflatoxicosis is important and enzymatic activity must be interpreted in context of the temporal aspect of aflatoxicosis. The activity of GGT has a slower temporal decrease than AST.

The clinicopathology of aflatoxicosis has been studied in cattle, sheep, goats, pigs, dogs and horses. Steers were given single oral doses (0.2, 0.4, 0.6 and 0.8 mg/kg body weight) of aflatoxins (Cook *et al.*, 1986). Increase in the serum activity of SDH and AST and not ALP was observed. The increase started at 8 h after dosing and reached maximal activity at 24–48 h and then rapidly decreased. Brucato *et al.* (1986) administered a single oral dose of 1 mg $AFB_1$/kg body weight to calves. Serum AST and GGT activities were increased within 2 days. After 2 days, the AST activity rapidly decreased whereas the GGT activity was elevated for 3 weeks. Aflatoxicosis from consuming high levels of aflatoxins in a concentrate has occurred in

cattle with an increase in serum activity of AST and no increase in GGT activity (Crockcroft, 1995). Feedlot steers fed a diet containing 600 ppb $AFB_1$ for 155 days had an increase in AST, ALP and SDH (Helferich *et al.*, 1986). Steers fed diets containing 60 and 300 ppm $AFB_1$ did not have an increase in AST, ALP and SDH. Holstein calves (70 kg) were fed a diet containing 5 ppm aflatoxins for 3 weeks (Wyatt *et al.*, 1985). Serum activity of lactic acid dehydrogenase (LDH) decreased, ALP peaked on treatment day 7 and then decreased, and AST increased to study day 14 and then plateaued. Holstein steers (183 kg) fed a diet containing ~375 ppb aflatoxin for 17.5 weeks were observed to have an increase in SDH and a decrease in AST activities (Richard *et al.*, 1983). Vaid *et al.* (1981) reported chronic aflatoxicosis in cattle consuming $AFB_1$ (0.11 ppm) contaminated groundnut cake. Hepatic enzymes in serum with increased activity were ALP and AST in 1 of 4 animals sampled. Timing of the blood sampling in terms of aflatoxicosis was not given, and these animals had histopathology consistent with aflatoxicosis. A study by Lynch *et al.* (1970) in calves suggested that the threshold dose for $AFB_1$ to increase ALP was 0.1 mg/kg body weight fed for 6 weeks. Goats given 3 mg of $AFB_1$/kg body weight by intramuscular injections had increased serum activity of AST which peaked at 32–40 h and then decreased (Hatch *et al.*, 1982; Clark *et al.*, 1984). Wethers had elevated serum activity of AST and GGT after consuming groundnut cake containing 750 ppb $AFB_1$ (Suliman *et al.*, 1987). Growing barrows were fed a diet containing 1, 2, 3 or 4 ppm aflatoxins for 28 days (Harvey *et al.*, 1988). On day 14, serum activity of AST and GGT increased in pigs fed the 4 ppm diet, on day 21 ALP increased in barrows fed the 4 ppm diet and AST increased in all pigs fed aflatoxins, and on day 28 ALP and activity was increased in pigs fed aflatoxins, GGT was increased in pigs fed the 3 and 4 ppm diets and serum AST activity was increased in pigs fed the 4 ppm diet. Dogs (1.5 years old) administered intravenously 15 doses of 0.05 mg $AFB_1$/kg body weight for 15 days had increased serum activity of ALP and ICD (Chaffee *et al.*, 1969). The clinicopathology of aflatoxicosis has been studied in horses. Weanling ponies were administered $AFB_1$ at 0.0, 0.5, 1.0 and 2.0 mg/kg body weight (Bortell *et al.*, 1983). Serum activity of GGT was increased in all ponies administered $AFB_1$. The GGT activity increased until day 3 and then decreased. Serum activity of alanine aminotransferase (ALT) was not increased. Ponies given 4, 5, 6 and 7.4 mg $AFB_1$/kg body had an increase in serum ALT and the activity of ALT increased until the ponies died at 33–46 h after dosing (Bortell *et al.*, 1983).

Serum chemistry is useful in the diagnosis of aflatoxicosis. Prothrombin time is generally increased and frank hemorrhage can occur. Serum bilirubin levels are also increased and photosensitization can occur. The changes in hematologic parameters generally are due to hemoconcentration and blood loss.

## Macroscopic and microscopic pathology

The pathology of aflatoxicosis has been described in several species. Edema including anasarca and edema fluid in the peritoneal and thoracic cavities can be observed (Osweiler and Trampel, 1985; Bastianello *et al.*, 1987; Coppock *et al.*, 1989; Hall *et al.*, 1989). The gall bladder, bile duct, abomasal wall and colonic mesentery may be edematous. There may be edema of the omentum near the stomach or abomasum and in the colonic mesentery. Gall bladder edema may be marked in cattle and pigs. Edema of the brain has been reported for horses (Angsubhakorn *et al.*, 1981).

Hemorrhage into the gastrointestinal tract, into body cavities and on body organs has been reported. Hemorrhage is due to decreased production of clotting factors by the liver. Acute aflatoxicosis in cattle, horses, pigs and dogs may result in serious hemorrhages in the gastrointestinal tract, on serosal surfaces, on the epicardium and endocardium, in skeletal muscle, perirenal and in the urinary bladder (Cysewski *et al.*, 1968; Chaffee *et al.*, 1969; Greene and Oehme, 1976; McKenzie *et al.*, 1981; Bortell *et al.*, 1983; Jakhar and Sadana, 2004). Pigs can also have hemorrhage occurring in the pleural and in the peritoneal cavity. The urine may be dark red colored.

Liver and kidney lesions occur. The liver may be swollen, friable and congested. Histologically, the liver may have necrosis in variable patterns. In the rat, monkey and duckling, the necrosis is reported to be periportal, while in cattle, pigs, horses, goats and sheep, the pattern is recorded to be centrilobular (Butler, 1964; Newberne *et al.*, 1966; Cysewski *et al.*, 1968; Samarajeewa *et al.*, 1975; Carlton and Szczech, 1978; Angsubhakorn *et al.*, 1981; Abdelsalam *et al.*, 1989; Coppock *et al.*, 1989; Mathur *et al.*, 1991). In the dog the pattern may be either or both periportal and centrilobular, while in the rabbit the pattern is reported to be midzonal (Newberne *et al.*, 1966; Chaffee *et al.*, 1969; Clark *et al.*, 1980). Hyperplasia of the bile duct cells occurs rapidly in ducklings and may be present in horses, dogs and chickens, and mild bile duct cell hyperplasia may be seen in cattle and pigs (Newberne and Butler, 1969; Ketterer *et al.*, 1975; Coppock *et al.*, 1989). Renal lesions of protein in proximal tubules and glomerular spaces are recorded in horses and rats (Butler, 1964; Newberne and Butler, 1969; Bortell *et al.*, 1983). Hemosiderin deposition in tubule cells occurs in horses (Angsubhakorn *et al.*, 1981). Cardiac myofiber degeneration is recorded in horses and rats (Butler, 1964; Angsubhakorn *et al.*, 1981). Brain lesions of focal malacia in the cerebral hemispheres in horses were recorded in one report (Angsubhakorn *et al.*, 1981), but have not been reported by others or in other species. However, hepatic encephalopathy could occur as a result of liver damage.

Subacute lesions observed at necropsy include firm, pale livers in all species and clear yellow ascites and pleural fluid accumulation especially in pigs and dogs (Newberne

and Butler, 1969). Icterus is usually present. Edema of the mesentery near the colon and perirenal edema may be present in pigs and cattle (Osweiler and Trampel, 1985; Bastianello *et al*., 1987; Coppock *et al*., 1989; Hall *et al*., 1989). Histologic changes include bile duct proliferation, hepatocyte necrosis and early fibrosis of the liver (Newberne and Butler, 1969). Regeneration of hepatocytes may be found in dogs, nodular hyperplasia may be present in turkeys, trout and ducklings (Newberne *et al*., 1966; Newberne and Butler, 1969; Bastianello *et al*., 1987). Variation in the size of hepatocytes, with some megalocytes and binucleate cells is recorded in many reports. Chronic liver lesions are characterized by firm, fibrous pale livers, by nodular hyperplasia and, in some cases, neoplasia. Icterus is present. Pulmonary edema may occur. Pneumonia is reported in calves as likely secondary to edema and the effects of aflatoxin on the immune system. Lesions of photosensitization have been reported in cattle.

## DIFFERENTIAL DIAGNOSIS

The lesions of aflatoxicosis and those of pyrrolizidine alkaloid toxicosis have been observed to be very similar especially in cattle (Loosmore and Markson, 1961). Cattle are generally considered to be more susceptible to pyrrolizidine alkaloids. When aflatoxins and pyrrolizidine alkaloid are at issue, it is important to examine pasture, forage, concentrate feeds and gastrointestinal contents to determine the presence of poisonous plants that may contain pyrrolizidine alkaloids. It is the experience of the authors that domestic animals with signs of aflatoxicosis never fully recover.

## TREATMENT AND OUTCOMES

A specific treatment for aflatoxicosis has not been identified. Aflatoxins from all sources should be removed from the diet. Symptomatic care of the affected animals is recommended. Animals on a low protein diet are more susceptible to aflatoxins (Cullen and Newberne, 1994).

Supplementing diets with choline and methionine, and *N*-acetylcysteine is probably beneficial (Cullen and Newberne, 1994; Valdivia *et al*., 2001). Adding vitamin E to swine diets containing 2.5 ppm aflatoxins did not have a protective effect on the severity of aflatoxicosis (Harvey *et al*., 1994). Increasing dietary zinc did not appear to be of benefit to calves fed a diet containing 5 ppm aflatoxins (Neathery *et al*., 1980). There is some suggestion that selenium provided some protection to Japanese quail fed a diet containing 1 ppm $AFB_1$ (Jakhar and Sadana, 2004). Excessive dietary selenium increases the toxicity of aflatoxins. Pimpukdee *et al*. (2004) found that a proprietary alumino-silicate (feed anti-caking agent) protected chicks from hepatic loss of vitamin A linked to aflatoxins in the diet. Feeding sodium bentonite was considered protective to pigs (Schell *et al*., 1993). Diaz *et al*. (2004) showed that sodium bentonite and MTB-100 (derived from cell wall of yeast) reduced $AFM_1$ in milk.

## AFLATOXIN INTERACTIONS

Multiple mycotoxins can occur when different feed ingredients are blended to manufacture a finished feed. When one mycotoxin is found in feedstuffs, the toxicologist should consider that additional mycotoxins of a different group may be present (Huff *et al*., 1988). The presence of multiple mycotoxins can alter the dose–response curve. Therein, the level of a mycotoxin that is generally considered to be safe can be altered by presence of other mycotoxins. The interactions of mycotoxins may vary between the indicators of effect. The interactions may be additive for one parameter and synergistic for another.

A feeding study in mice showed that a combination of fumonisin $B_1$ ($FB_1$) at 10 ppm and $AFB_1$ at 10 ppb increased feed consumption and decreased weight gains over a 90-day study interval (Casado *et al*., 2001). Liver lesions were considered to be intensified by the combination and this effect increased temporally. $AFB_1$ and $FB_1$ combination were shown to decrease feed conversion and feed consumption in feeder pigs (Dilkin *et al*., 2003). The dietary levels were 30 ppm $FB_1$ and 50 ppb $AFB_1$. Pigs were fed a diet containing aflatoxins produced in rice culture and a diet containing $FB_1$ produced in corn culture, and a diet containing both the rice and corn cultures (Harvey *et al*., 1995). The interactions of aflatoxins and $FB_1$ for immunologic and liver disease parameters were essentially additive. Cultures containing aflatoxins and $T_2$ toxin when fed in combination to Japanese quail and the interactions appeared to be additive (Madheswaran *et al*., 2004). Increased abnormal chicks were observed when ochratoxin A (2 $\mu$g) and $AFB_1$ (0.5 $\mu$g) were injected into eggs on day 3 of incubation (Edrington *et al*., 1995). Calves were fed diets containing ochratoxin A and $AFB_1$ (Patterson *et al*., 1981). The sources of the mycotoxins were naturally contaminated barley or naturally contaminated groundnut meal. The dose of ochratoxin A was 390–540 $\mu$g/kg body weight and the dose of $AFB_1$ was 10–13 $\mu$g/kg body weight. No adverse health effects were observed when the toxins were fed individually or as a mixture likely because of the ability of rumen organisms to degrade the mycotoxins.

Preexisting disease can increase susceptibility to aflatoxins. Osuna *et al.* (1977) concluded that preexisting liver flukes increased the susceptibility of cattle to aflatoxins and the interactive effect was additive. Henry *et al.* (2002) estimated that the presence of hepatitis B surface antigen in serum increased the human susceptibility to aflatoxins by a factor of 30. Shivachandra *et al.* (2003) showed the immunosuppression of aflatoxins were additive with fowl adenovirus serotype-4. Ethanol was found to potentiate $AFB_1$-induced hepatocarcinogenesis (Tanaka *et al.*, 1989).

## CONCLUDING REMARKS AND FUTURE DIRECTIONS

Aflatoxins, on a world scale, are one of the most important groups of mycotoxins (Lanyasunya *et al.*, 2005). The interactive effects of aflatoxins with infectious diseases and other chemical toxins have been demonstrated. Many fungal metabolites have been characterized. Although not classified as mycotoxins, these fungal metabolites should be investigated for interactive effects with aflatoxins.

## REFERENCES


Abdelsalam EB, el-Tayeb AE, Nor Eldin AA, Abdulmagid AM (1989) Aflatoxicosis in fattening sheep. *Vet Rec* **124**: 487–8.

Agnes FA, Akbarsha MA (2003) Spermatotoxic effect of aflatoxin B(1) in the albino mouse. *Food and Chem Toxicol* **41**: 119–30.

Allcroft R, Lewis G (1963) Groundnut toxicity in cattle: experimental poisoning of calves and a report on clinical effects in older cattle. *Vet Rec* **75**: 487–93.

Angsubhakorn S, Poomvises P, Romruen K, Newberne PM (1981) Aflatoxicosis in horses. *J Am Vet Med Assoc* **178**: 274–8.

Applebaum RS, Brackett RE, Wiseman DW, Marth EH (1982) Aflatoxin: toxicity to dairy cattle and occurrences in milk and milk products – a review. *J Food Protect* **45**: 752–77.

Barton JT, Daft BM, Read DH, Kinde H, Bickford AA (1992) Tracheal aspergillosis in 6½-week-old chickens caused by *Aspergillus flavus*. *Avian Dis* **36**: 1081–5.

Bastianello SS, Nesbit JW, Williams MC, Lange AL (1987) Pathological findings in natural outbreak of aflatoxicosis in dogs. *Onderstepoort J Vet Res* **54**: 635–40.

Battacone G, Nudda A, Cannas A, Cappio Borlino A, Bomboi G, Pulina G (2003) Excretion of aflatoxin M1 in milk of dairy ewes treated with different doses of aflatoxin B1. *J Dairy Sci* **86**: 2667–75.

Bennett JW, Klich M (2003) Mycotoxins. *Clin Microbiol Rev* **16**: 497–516.

Bingham AK, Huebner HJ, Phillips TD, Bauer JE (2004) Identification and reduction of urinary aflatoxin metabolites in dogs. *Food Chem Toxicol* **42**: 1851–8.

Bodine AB, O'Dell GD, Janzen JJ, Bishop JR (1982) Effect of β-naphthoflavone on calf liver metabolism of aflatoxin $B_1$. *J Dairy Sci* **65**: 2174–7.

Bodine AB, Fisher SF, Gangjee S (1984) Effect of aflatoxin B1 and major metabolites on phytohemagglutinin-stimulated lymphoblastogenesis of bovine lymphocytes. *J Dairy Sci* **67**: 110–14.

Bortell R, Asquith RL, Edds GT, Simpson CF, Aller WW (1983) Acute experimentally induced aflatoxicosis in the weanling pony. *Am J Vet Res* **44**: 2110–14.

Brown RL, Chen ZY, Cleveland TE, Russin JS (1999) Advances in development of host resistance in corn to aflatoxin contamination by *Aspergillus flavus*. *Phytopathology* **89**: 113–17.

Brown RW, Pier AC, Richard JL, Krogstad RE (1981) Effects of dietary aflatoxin on existing bacterial intramammary infections of dairy cows. *Am J Vet Res* **42**: 927–33.

Brucato M, Sundlof SF, Bell JU, Eadds GT (1986) Aflatoxin $B_1$ toxicosis in dairy calves pretreated with selenium–vitamin E. *Am J Vet Res* **47**: 179–83.

Butler WH (1964) Acute toxicity of aflatoxin $B_1$ in rats. *Br J Cancer* **18**: 756–8.

Caddick L (2003) *Water Activity and Equilibrium Relative Humidity. What Are They and Why Are They Important to Safe Grain Storage?* Stored Grain Research Laboratory, Canberra, Australia.

Calvo AM, Wilson RA, Bok JW, Keller NP (2002) Relationship between secondary metabolism and fungal development. *Microbiol Mol Biol Rev* **66**: 447–59.

Carlton WW, Szczech GM (1978) Mycotoxicosis in laboratory animals. In *Mycotoxic Fungi. Mycotoxins, Mycotoxicoses. An Encyclopedic Handbook*, vol. 2, Wyllie TD, Morehouse LG (eds). Marcel Dekker, New York, pp. 407–10.

Casado JM, Theumer M, Masih DT, Chulze S, Rubinstein HR (2001) Experimental subchronic mycotoxicoses in mice: individual and combined effects of dietary exposure to fumonisins and aflatoxin B1. *Food Chem Toxicol* **39**: 579–86.

Chaffee VW, Edds GT, Himes JA, Neal FC (1969) Aflatoxicosis in dogs. *Am J Vet Res* **30**: 1737–48.

Clark JD, Jain AV, Hatch RC, Mahaffey EA (1980) Experimentally induced chronic aflatoxicosis in rabbits. *Am J Vet Res* **41**: 1841–5.

Clark JD, Hatch RC, Miller DM, Jain AV (1984) Caprine aflatoxicosis: experimental disease and clinical pathologic changes. *Am J Vet Res* **45**: 1132–5.

Clegg FG, Bryson H (1962) An outbreak of poisoning in stored cattle attributed to Brazilian groundnut meal. *Vet Rec* **74**: 992–4.

Cook WO, Richard JL, Osweiler GD, Trampel DW (1986) Clinical and pathologic changes in acute bovine aflatoxicosis: rumen motility and tissue and fluid concentrations of aflatoxins $B_1$ and $M_1$. *Am J Vet Res* **47**: 1187–825.

Coppock RW, Swanson SP (1986) Aflatoxins. In *Current Veterinary Therapy: Food Animal Practice*, 2nd edn, Howard JL (ed.). Saunders, Philadelphia, PA, pp. 363–6.

Coppock RW, Reynolds RD, Buck WB, Jacobsen BJ, Ross SC, Mostrom MS (1989) Acute aflatoxicosis in feeder pigs, resulting from improper storage of corn. *J Am Vet Med Assoc* **195**: 1380–1.

Crockcroft CD (1995) Sudden death in dairy cattle with putative acute aflatoxin B poisoning. *Vet Rec* **136**: 248.

Cullen JM, Newberne PM (1994) Acute hepatotoxicity of aflatoxins. In *The Toxicology of Aflatoxins. Human Health, Veterinary, and Agricultural Significance*, Eaton DL, Groopman JD (eds). Academic Press, Toronto, Ont., pp. 3–26.

Cysewski SJ, Pier AC, Engstrom GW, Richard JL, Dougherty RW, Thurston JR (1968) Clinical pathologic features of acute aflatoxicosis of swine. *Am J Vet Res* **29**: 1577–90.

Diaz DE, Hagler Jr WM, Blackwelder JT, Eve JA, Hopkins BA, Anderson KL, Jones FT, Whitlow LW (2004) Aflatoxin binders II: reduction of aflatoxin M1 in milk by sequestering agents of cows consuming aflatoxin in feed. *Mycopathologia* **157**: 233–41.

Diener UL, Cole RJ, Sanders TH, Payne GA, Lee LS, Klich ML (1987) Epidemiology of aflatoxin formation by *Aspergillus flavus*. *Annu Rev Phytopathol* **25**: 249–70.

Dilkin P, Zorzete P, Mallmann CA, Gomes JDF, Utiyama CE, Oetting LL, Correa B (2003) Toxicological effects of chronic low doses of aflatoxin $B_1$ and fumonisin $B_1$-containing *Fusarium moniliforme* culture material in weaned piglets. *Food Chem Toxicol* **41**: 1345–53.

Drakos PE, Nagler A, Or R, Naparstek E, Kapelushnik J, Engelhard D, Rahav G, Ne'emean D, Slavin S (1993) Invasive fungal sinusitis in patients undergoing bone marrow transplantation. *Bone Marrow Transplant* **12**: 203–8.

Eaton DL, Gallagher EP (1994) Mechanisms of aflatoxin carcinogenesis. *Annu Rev Pharmacol Toxicol* **34**: 135–72.

Edrington TS, Harvey RB, Kubena LF (1995) Toxic effects of aflatoxin $B_1$ and ochratoxin A, alone and in combination, on chicken embryos. *Bull Environ Contam Toxicol* **54**: 331–6.

Fernandez A, Hernandez M, Verde MT, Sanz M (2000) Effect of aflatoxin on performance, hematology, and clinical immunology in lambs. *Can J Vet Res* **64**: 53–8.

Frobish RA, Bradley BD, Wagner DD, Long-Bradley PE, Hairston H (1986) Aflatoxin residues in milk of dairy cattle after ingestion of naturally contaminated grain. *J Food Protect* **49**: 781–5.

Garrido NS, Iha MH, Santos Ortolani MR, Duarte Favaro RM (2003) Occurrence of aflatoxins M(1) and M(2) in milk commercialized in Ribeirao Preto-SP, Brazil. *Food Addit Contam* **20**: 70–3.

Gemechu-Hatewu M, Platt KL, Oesch F, Steinberg P (1996) Distribution and induction of aflatoxin B1-9a-hydroxylase activity in rat liver parenchymal and non-parenchymal cells. *Arch Toxicol* **70**: 553–8.

Gourami H, Bullerman LB (1995) *Aspergillus flavus* and *Aspergillus parasiticus*: aflatoxigenic fungi of concern in foods and feeds: a review. *J Food Protect* **58**: 1395–404.

Govaris A, Roussi V, Koidis PA, Botsoglou NA (2002) Distribution and stability of aflatoxin $M_1$ during production and storage of yoghurt. *Food Addit Contam* **19**: 1043–50.

Greene HJ, Oehme FW (1976) A possible case of equine aflatoxicosis. *Clin Toxicol* **9**: 251–4.

Groopman JD, Donahue PR, Zhu J, Chen J, Wogan GN (1985) Aflatoxin metabolism in humans: detection of metabolites and nucleic acid adducts in urine by affinity chromatography. *Proc Natl Acad Sci USA* **82**: 6492–6.

Hall RF, Harrison LR, Colvin BM (1989) Aflatoxicosis in cattle pastured in a field of sweet corn. *J Am Vet Med Assoc* **194**: 938.

Harvey RB, Huff WE, Kubena LF, Corrier DE, Phillips TD (1988) Progression of aflatoxicosis in growing barrows. *Am J Vet Res* **49**: 482–7.

Harvey RB, Kubena LF, Elissalde MH (1994) Influence of vitamin E on aflatoxicosis in growing swine. *Am J Vet Res* **55**: 572–7.

Harvey RB, Edrington TS, Kubena LF, Elissalde MH, Rottinghaus GE (1995) Influence of aflatoxin and fumonisin $B_1$-containing culture material on growing barrows. *Am J Vet Res* **56**: 1668–72.

Hatch RC, Clark JD, Jain AV, Mahaffey EA, Weiss R (1982) Effect of some enzyme inducers, fluids, and methionine-thiosulfate on induced acute aflatoxicosis in goats. *Am J Vet Res* **43**: 246–51.

Helferich WG, Garrett WN, Hsieh DPH, Baldwin RL (1986) Feedlot performance and tissue residues of cattle consuming diets containing aflatoxins. *J Anim Sci* **62**: 691–6.

Hendrickson DA, Grant DW (1971) Aflatoxin formation in sterilized feedlot manure and fate during simulated water treatment procedures. *Bull Environ Contam Toxicol* **6**: 525–31.

Henry SH, Bosch FX, Bowers JC (2002) Aflatoxin, hepatitis and worldwide liver cancer risks. *Adv Exp Med Biol* **504**: 229–33.

Hesseltine CW (1984) Mycotoxins and alcohol production: a review. *Dev Food Sci* **7**: 153–61.

Hsieh DP, Wong JJ (1994) Pharmacokinetics and excretion of aflatoxins. In *The Toxicology of Aflatoxins. Human Health, Veterinary, and Agricultural Significance*, Eaton DL, Groopman JD (eds). Academic Press, Toronto, Ont., pp. 373–88.

Huff WE, Kubena LF, Harvey RB, Doerr JA (1988) Mycotoxin interactions in poultry and swine. *J Anim Sci* **66**: 2351–5.

Jaimi-Garcia R, Cotty PJ (2003) Aflatoxin contamination of commercial cottonseed in south Texas. *Phytopathology* **93**: 1190–200.

Jakhar KK, Sadana JR (2004) Sequential pathology of experimental aflatoxicosis in quail and the effect of selenium supplementation in modifying the disease process. *Mycopathologia* **157**: 99–109.

Joens LA, Pier AC, Cutlip RC (1981) Effects of aflatoxin consumption on the clinical course of swine dysentery. *Am J Vet Res* **42**: 1170–2.

Ketterer PJ, Williams ES, Blaney BJ, Connole MD (1975) Canine aflatoxicosis. *Aust Vet J* **51**: 355–7.

Kihara T, Matsuo T, Sakamoto M, Yasuda Y, Yamamoto Y (2000) Effects of prenatal aflatoxin $B_1$ exposure on behaviors of rat offspring. *Toxicol Sci* **53**: 392–9.

Klich MA, Mullaney EJ, Daly CB, Cary JW (2000) Molecular and physiological aspects of aflatoxins and sterigmatocystin biosynthesis by *Aspergillus tamarii* and *A. ocjraceoroeus*. *Appl Microbiol Biotechnol* **53**: 605–9.

Kuilman MEM, Maas RFM, Judah DJ, Fink-Gremmels J (1998) Bovine hepatic metabolism of aflatoxin $B_1$. *J Agric Food Chem* **46**: 2707–13.

Kuilman MEM, Maas RFM, Fink-Gremmels (2000) Cytochrome P450 mediated metabolism and cytotoxicity of aflatoxin $B_1$ in bovine hepatocytes. *Toxicol In Vitro* **14**: 321–7.

Kumagai S (1989) Intestinal absorption and excretion of aflatoxin in rats. *Toxicol Appl Pharmacol* **97**: 88–97.

Lanyasunya TP, Wamae LW, Musa HH, Olowofeso O, Lokwaleput IK (2005) The risk of mycotoxins contamination of dairy feeds and milk on smallholder dairy farms in Kenya. *Pakistan J Nutr* **4**: 162–9.

Lee LS, Parrish FW, Jacks TJ (1986) Substrate depletion during formation of aflatoxins and kojic acid on corn inoculated with *Aspergillus flavus*. *Mycopathologia* **93**: 105–7.

Lillehoj EB (1992) Aflatoxin: genetic mobilization agent. In *Handbook of Applied Mycology. Mycotoxins in Ecological Systems*, vol. 5, Bhatnagar D, Lillehoj EB, Arora DK (eds). Marcell Dekker, New York, pp. 1–22.

Lillehoj EB, Wall JH, Bowers EJ (1987) Preharvest aflatoxin contamination: effect of moisture and substrate variation in developing cottonseed and corn kernels. *Appl Environ Microbiol* **53**: 584–6.

Loosmore RM, Markson LM (1961) Poisoning of cattle by Brazilian groundnut meal. *Vet Rec* **73**: 813–14.

Lynch GP, Todd GC, Shalkop WT, Moore LA (1970) Response of dairy calves to aflatoxin-contaminated feed. *J Dairy Sci* **53**: 63–71.

Madheswaran R, Balachandran C, Manohar BM (2004) Influence of dietary cultural material containing aflatoxin and $T_2$ toxin on certain serum biochemical constituents in Japanese quail. *Mycopathologia* **158**: 337–41.

Marin DE, Taranu I, Bunaciu RP, Pascale F, Tudor DS, Avram N, Sarca M, Cureu I, Criste RD, Suta V, Oswald IP (2002) Changes in performance, blood parameters, humoral and cellular immune response in weanling piglets exposed to low doses of aflatoxin. *J Anim Sci* **80**: 1250–7.

Mathur M, Rizvi TA, Nayak NC (1991) Effect of low protein diet on chromic aflatoxin $B_1$-induced liver injury in Rhesus monkey. *Mycopathologia* **113**: 175–9.

Matsumura M, Mori T (1998) Detection of aflatoxins in autopsied materials from a patient infected with *Aspergillus flavus*. *Nippon Ishinkin Gakkai Zasshi* **39**: 167–71.

McKenzie RA, Blaney BJ, Connole MD, Fitzpatrick LA (1981) Acute aflatoxicosis in calves fed peanut hay. *Aust Vet J* **57**: 284–6.

Mellon JE, Cotty PJ (1998) Effects of oilseed storage proteins on aflatoxin production by *Aspergillus flavus*. *J Am Oil Chem Soc* **75**: 1085–9.

Mellon JE, Cotty PJ, Dowd MK (2000) Influence of lipids with and without other cottonseed reserve materials on aflatoxin B(1) production by *Aspergillus flavus*. *J Agric Food Chem* **48**: 3611–15.

Mishra HN, Das C (2003) A review on biological control and metabolism of aflatoxin. *Crit Rev Food Sci Nutr* **43**: 245–64.

Mocchegiani E, Corradi A, Santarelli L, Tibaldi A, DeAngelis E, Borghetti P, Bonomi A, Fabris N, Cabassi E (1998) Zinc, thymic endocrine activity and mitogen responsiveness (PHA) in piglets exposed to maternal aflatoxicosis $B_1$ and $G_1$. *Vet Immunol Immunopathol* **62**: 245–60.

Mori T, Matsumura M, Yamada K, Irie S, Oshimi K, Suda K, Oguri T, Ichinoe M (1998) Systemic aspergillosis caused by an aflatoxin-producing strain of *Aspergillus flavus*. *Med Mycol* **36**: 107–12.

Neathery MW, Moos WH, Wyatt RD, Miller WJ, Gentry RP, George LW (1980) Effects of dietary aflatoxins on performance and zinc metabolism in dairy calves. *J Dairy Sci* **63**: 789–99.

Newberne PM, Butler WH (1969) Acute and chronic effects of aflatoxin on the liver of domestic and laboratory animals: a review. *Cancer Res* **29**: 236–50.

Newberne PM, Russo R, Wogan GN (1966) Acute toxicity of aflatoxin $B_1$ in the dog. *Pathol Vet* **3**: 331–40.

Osuna O, Edds GT, Blankespoor HD (1977) Toxic effects of aflatoxin $B_1$ in male Holstein calves with prior infection by flukes (*Fasciola hepatica*). *Am J Vet Res* **38**: 341–9.

Osweiler GD, Trampel DW (1985) Aflatoxicosis in feedlot cattle. *J Am Vet Med Assoc* **187**: 636–7.

Otim MO, Mukiibi-Muka G, Christensen H, Bisgaard M (2005) Aflatoxicosis, infectious bursal disease and immune response to Newcastle disease vaccination in rural chickens. *Avian Pathol* **34**: 319–23.

Patterson DSP, Shreeve BJ, Roberts BA, Berrett S, Brush PJ, Glancy EM, Krogh P (1981) Effect on calves of barley naturally contaminated with ochratoxin A and groundnut meal contaminated with low concentrations of aflatoxin $B_1$. *Res Vet Sci* **31**: 213–18.

Pepeljnjak S, Slobodnjak Z, Segvic M, Peraica M, Pavlovic M (2004) The ability of fungal isolates from human lung aspergilloma to produce mycotoxins. *Hum Exp Toxicol* **23**: 15–19.

Picha J, Cerovsky J, Pichova D (1986) Fluctuation in the concentration of sex steroids and aflatoxin B1 in the seminal plasma of boars and its relation to sperm production. *Vet Med (Praha)* **31**: 347–57.

Pier AC, Heddleston KL (1970) The effect of aflatoxin on immunity in turkeys. I. Impairment of actively acquired resistance to bacterial challenge. *Avian Dis* **14**: 797–809.

Pimpukdee K, Kubena LF, Bailey CA, Huebner HJ, Afriyie-Gyawu E, Phillips TD (2004) Aflatoxin-induced toxicity and depletion of hepatic vitamin A in young broiler chicks: protection of chicks in the presence of low levels of NovaSil PLUS in the diet. *Poult Sci* **83**: 737–44.

Price RL, Paulson JH, Lough OG, Gingg C, Kurtz AG (1985) Aflatoxin conversion by dairy cattle consuming naturally-contaminated whole cottonseed. *J Food Protect* **48**: 11–15.

Qureshi MA, Brake J, Hamilton PB, Hagler WM, Nesheim S (1998) Dietary exposure of broiler breeders to aflatoxin results in immune dysfunction in progeny chicks. *Poult Sci* **77**: 812–19.

Richard JL, Pier AC, Stubblefield RD, Shotwell OL, Lyon RL, Cutlip RC (1983) Effect of feeding corn naturally contaminated with aflatoxin on feed efficiency, on physiologic, immunologic, and pathologic changes, and on tissue residues in steers. *Am J Vet Res* **44**: 1294–9.

Richard JL, Stubblefield RD, Lyon RL, Peden WM, Tuurston JR, Rimler RB (1986) Distribution and clearance of aflatoxins $B_1$ and $M_1$ in turkeys fed diets containing 50 and 150 ppb aflatoxin from naturally contaminated corn. *Avian Dis* **30**: 788–93.

Robens JF, Richard JL (1992) Aflatoxins in animal and human health. *Rev Environ Contam Toxicol* **127**: 69–94.

Russell TE, Watson TF, Ryan GF (1976) Field accumulation of aflatoxin in cottonseed as influenced by irrigation termination dates and pink bollworm infestation. *Appl Environ Microbiol* **31**: 711–13.

Samarajeewa U, Arseculeratne SN, Tennekoon GE (1975) Spontaneous and experimental aflatoxicosis in goats. *Res Vet Sci* **19**: 269–77.

Schell TC, Lindemann MD, Kornegay ET, Blodgett DJ (1993) Effects of feeding aflatoxin-contaminated diets with and without clay to weanling and growing pigs on performance, liver function and mineral metabolism. *J Anim Sci*, 1209–18.

Shivachandra SB, Sah RL, Singh SD, Kataria JM, Manimaran K (2003) Immunosuppression in broiler chicks fed aflatoxin and inoculated with fowl adenovirus serotype-4 (FAV-4) associated with hydropericardium syndrome. *Vet Res Commun* **27**: 39–51.

Silvotti L, Petterino C, Bonomi A, Cabassi E (1997) Immunotoxicological effects on piglets of feeding sows diets containing aflatoxins. *Vet Rec* **141**: 469–72.

Stubblefield RD, Pier AC, Richard JL, Shotwell OL (1983) Fate of aflatoxins in tissues, fluids and excrements from cows dosed orally with aflatoxin $B_1$. *Am J Vet Res* **44**: 1750–2.

Suliman HB, Mohamed AF, Awadelsied NA, Shommein AM (1987) Acute mycotoxicosis in sheep: field cases. *Vet Hum Toxicol* **29**: 241–3.

Sur E, Celik I (2003) Effects of aflatoxin $B_1$ on the development of the bursa of Fabricius and blood lymphocytes acid phosphatase of the chicken. *Br Poult Sci* **44**: 558–66.

Tanaka T, Nishikawa A, Iwata H, Mori Y, Hara A, Hirono I, Mori H (1989) Enhancing effect of ethanol on aflatoxin B1-induced hepatocarcinogenesis in male ACI/N rats. *Jpn J Cancer Res* **80**: 526–30.

Thurston JR, Cook W, Driftmier K, Richard JL, Sacks JM (1986) Decreased complement and bacteriostatic activities in the sera of cattle given single or multiple doses of aflatoxin. *Am J Vet Res* **47**: 846–9.

Vaid J, Dawra RK, Sharma OP, Negi SS (1981) Chronic aflatoxicosis in cattle. *Vet Hum Toxicol* **23**: 436–8.

Valdivia AG, Martinez A, Damian FJ, Quezada T, Ortiz R, Martinez C, Llamas J, Rodriguez ML, Yamamoto L, Jaramillo F, Loarca-Pina MG, Reyes JL (2001) Efficacy of *N*-acetylcysteine to reduce the effects of aflatoxin B1 intoxication in broiler chickens. *Poult Sci* **80**: 727–34.

Veldman A, Meijs JAC, Borggreve GJ, Heeres-van der Tol JJ (1992) Carry-over of aflatoxin from cows' food to milk. *Anim Prod* **55**: 163–8.

Whitaker TB (2003) Detecting mycotoxins in agricultural commodities. *Mol Biotechnol* **23**: 61–71.

Williams JH, Phillips TD, Jolly PE, Stile JK, Jolly CM, Aggarwal D (2004) Human aflatoxicosis in developing countries: a review of toxicology, exposure, potential health consequences, and interventions. *Am J Clin Nutr* **80**: 1106–22.

Winn RT, Lane GT (1978) Aflatoxin production on high moisture corn and sorghum with a limited incubation. *J Dairy Sci* **61**: 762–4.

Wotton HR, Strange RN (1987) Increased susceptibility and reduced phytoalexin accumulation in drought-stress peanut kernels challenged with *Aspergillus flavus*. *Appl Environ Microbiol* **53**: 270–3.

Wyatt RD, Neathery MW, Moos WH, Miller WJ, Gentry RP, Ware GO (1985) Effects of dietary aflatoxin and zinc on enzymes and other blood constituents in dairy calves. *J Dairy Sci* **68**: 437–42.

Zarba A, Wild CP, Hall AJ, Montesano R, Hudson GJ, Groopman JD (1992) Aflatoxin $M_1$ in breast milk from the Gambia west Africa quantified by combined monoclonal antibody immunoaffinity chromatography and HPLC. *Carcinogenesis* **13**: 891–4.

C H A P T E R

# 76

# Trichothecenes

*Michelle S. Mostrom and Merl F. Raisbeck*

## INTRODUCTION

While hundreds of fungal metabolites have been discovered that are potentially toxic in animals, five mycotoxins are generally recognized as an agricultural problem in North America: deoxynivalenol (DON), fumonisin, zearalenone, aflatoxin, and ochratoxin. This chapter focuses on DON and the other trichothecenes.

More than 180 trichothecene mycotoxins have been recognized in the past 40 years (Grove, 1988, 2000). These fungal metabolites are a group of sesquiterpenoids characterized by a tetracyclic 12,13–epoxytrichothec-9-ene skeleton and a variable number of acetoxy or hydroxyl group substitutions. The epoxy group at C-12 and C-13 is considered essential for toxicity (Figure 76.1). Trichothecenes can be broadly divided into two groups, macrocyclic and non-macrocyclic trichothecenes, based on the presence of a macrocyclic ring linking C-4 and C-15 with diesters (roridin series) and triesters (verrucarin series). *Fusarium* molds or fungi are the most economically important source of trichothecene mycotoxins. The genus includes many field fungi capable of infecting wheat, corn, barley, oats, and forages. *Fusarium* is most common in temperate climates, but contamination of grains is reported worldwide (Placinta *et al*., 1999; JECFA, 2001; CAST, 2003).

Trichothecenes are potent inhibitors of protein synthesis and are toxic to molds, bacteria, plants, and animals. The hallmark clinical sign of trichothecene toxicosis in animals is feed refusal, which has led to speculation that animals may not voluntarily consume enough contaminated ration to cause marked poisoning; however, when the only available feedstuffs are contaminated with trichothecenes, poisoning may result. Clinical signs include emesis, feed refusal and weight loss, immunomodulation, coagulopathy and hemorrhage, and cellular necrosis of mitotically active tissues such as intestinal mucosa, skin, bone marrow, spleen, testis, and ovary (CAST, 2003). Trichothecenes have caused lethality in horses (Rodricks and Eppley, 1974), man (Joffe, 1974), and cattle (Hsu *et al*., 1972), but most recent scientific interest has focused on subclinical syndromes including nutritional impairment (Rotter *et al*., 1996), loss of production from contaminated feeds, and immunosuppression (Bondy and Pestka, 2000; Pestka and Smolinski, 2005).

Some of the confusion about clinical effects of trichothecenes results from the discrepancy between controlled experiments and field studies, and from the variable toxicity of contaminated feedstuffs. Studies using experimental animals and purified trichothecenes often suggest a dose–response relationship for a particular clinical effect that is not supported by field observations. Some naturally

| Trichothecene | R1 | R2 | R3 | R4 | R5 |
|---|---|---|---|---|---|
| T-2 toxin | OH | OAc | OAc | H | $OCOCH_2CH(CH_3)_2$ |
| HT-2 toxin | OH | OH | OAc | H | $OCOCH_2CH(CH_3)_2$ |
| Diacetoxyscirpenol | OH | OAc | OAc | H | H |
| Neosolaniol | OH | OAc | OAc | H | OH |
| Calonectrin | OAc | H | OAc | H | H |

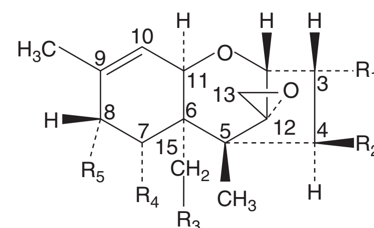


**FIGURE 76.1** Chemical structure of Type A trichothecenes. Substitutions R1 through R5 are given above.

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contaminated grains are more toxic than can be accounted for by their known mycotoxin content. Controlled experiments using identical methods and similar test animals may yield marked variation, both qualitatively and quantitatively, in response to different batches of naturally contaminated feed. It seems likely that spontaneous trichothecene mycotoxicoses in livestock are complicated by the presence of unidentified mycotoxins or additional fungal metabolites in the ration. Diagnosis may also be frustrated by the difficulty of obtaining a representative feed sample to test and appropriate analytical methodology to identify fungal metabolites.

## BACKGROUND

Mold-infected grains have been associated with ill health in livestock and man for over a 100 years. In European Russia and Eastern Siberia, "scabby grains" and "moldy hay" have long been recognized as toxic, and in Japan, a red mold disease or "Akakabi-byo" of wheat caused gastroenteritis in man (Saito and Ohtsubo, 1974). Alimentary toxic aleukia (ATA) of human beings and a similar condition (stachybotryotoxicosis) of horses were associated with overwintered grains and hay during the 1930s and 1940s in the former USSR. Delayed harvest resulted in overwintering of grain, and the cold, wet conditions resulted in the growth of mold on overwintered grain and hay. These molds were later identified as *Fusarium sporotrichioides*, *Fusarium poae*, and *Stachybotrys alternans* in grain and hay, respectively, and several trichothecene mycotoxins were isolated. The primary toxin associated with ATA was identified as 4β,15-diacetoxy-3α-hydroxy-8α-[3-methylbutyryloxy]-12,13-epoxytrichothec-9-ene or T-2 toxin. A brief description of ATA is informative as it illustrates the classical effects of potent trichothecenes, mainly epithelial irritation or dermonecrosis, gastrointestinal irritation, and immunosuppression (Joffe, 1974). Clinically, ATA involved four stages. Stage one occurred shortly after ingestion of toxic grains. Symptoms included a burning sensation of the mouth, tongue, esophagus, and stomach and inflammation of the gastrointestinal mucosa accompanied by vomiting, diarrhea, salivation, dizziness, and tachycardia. This stage lasted from 3 to 9 days, and coincided with the initial clinical appearance of leukopenia. Stage two was termed the latent or leukopenic stage because the victim felt normal and could function normally, but major changes were occurring in the hematopoietic system including progressive leukopenia with granulocytopenia, and a relative lymphocytosis. Anemia, icterus, and lowered immune resistance to infections were typical of this stage and some reports describe abnormalities in the central and autonomic nervous functions. Stage two lasted from 2 to 8 weeks, with eventual recovery if exposure stopped. If victims continued to consume contaminated grain, the syndrome progressed to a third stage. In stage three, leukopenia worsened and thrombocytopenia and decreased fibrinogen resulted in anemia and petechial hemorrhages on the skin of the trunk, lateral surfaces of the arms, the thighs, face, and head. Nasal, gastric, and intestinal hemorrhages were noted. Necrotic lesions could appear in the throat, gums, buccal mucosa, larynx, and vocal cords with secondary bacterial infections. Lymph node enlargement was observed, and death from stenosis of the glottis was reported. If the victim survived, stage four consisted of a convalescent period of 2 or more months while bone marrow recovered. The clinical signs of ATA in humans have been reproduced in cats given repeated doses of T-2 toxin (Lutsky *et al.*, 1978).

The fungus usually involved in scabby grain blights was identified as *Gibberella zeae* (Schwabe) or *Fusarium graminearum*, its asexual or conidial phase (reviewed by Marasas *et al.*, 1984). A trichothecene, chemically described as 3,7,15-trihydroxy-12,13-epoxytrichothec-9-en-8-one, was isolated from barley (Morooka *et al.*, 1972) and field corn infected with *F. graminearum* (Vesonder *et al.*, 1973) and given the trivial name, vomitoxin. Vomitoxin, also known as Rd toxin, 4-deoxynivalenol, or DON, was demonstrated to be the *Fusarium* toxin responsible for feed refusal and emesis in monogastric animals fed contaminated corn (Vesonder *et al.*, 1976). Subsequently, an acetylated form of DON, 3-acetoxy-7,15-dihydroxy-12,13-epoxytrichothec-9-en-8-one (3-ADON) was isolated from a *Fusarium* culture that was more toxic than DON in male DDY mice (Yoshizawa and Morooka, 1973).

### Sources

Trichothecenes are produced by several genera of fungi, including *Fusarium*, *Stachybotrys*, *Myrothecium*, *Trichothecium*, *Trichoderma*, *Cephalosporium*, *Cylindrocarpon*, *Verticimonosporium*, and *Phomopsis* (Scott, 1989). Trichothecene mycotoxins (baccharinoids) have also been isolated from Brazilian plants, notably *Baccharis* spp.; however, all medically and economically important sources to date have been fungal, especially *Fusarium. Fusarium* is a major agricultural plant pathogen of temperate growing regions, where it causes *Fusarium* head blight in wheat, barley, triticale, and other grains. *F. graminearum* has an optimum temperature range for growth of 26–28°C at a water activity ($a_W$) greater than 0.88. *Fusarium culmorum* grows optimally at 21°C when $a_W > 0.87$. While increased rainfall will increase *Fusarium* head blight, the incidence of blight is primarily affected by moisture at anthesis when the temperature is in the optimum range (Miller, 2002). Moisture at silk emergence and wet weather later

| Trichothecene | R1 | R2 | R3 | R4 |
|---|---|---|---|---|
| Nivalenol | OH | OH | OH | OH |
| Deoxynivalenol | OH | H | OH | OH |
| Fusarenon-X | OH | OAc | OH | OH |
| Diacetylnivalenol | OH | OAc | OAc | OH |
| 3-Acetyldeoxynivalenol | OAc | H | OH | OH |
| 15-Acetyldeoxynivalenol | OH | H | OAc | OH |

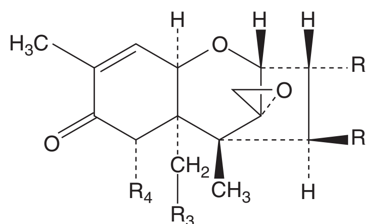


**FIGURE 76.2** Chemical structure of Type B trichothecenes. Substitutions R1 through R4 are given above.

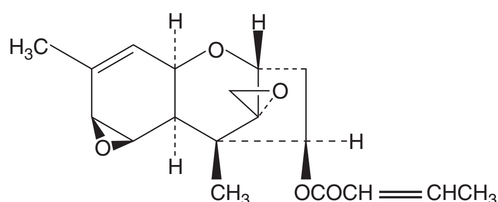


**FIGURE 76.3** Chemical structure of Type C trichothecenes (crotocin).

in the season increase *Gibberella* or pink ear rot caused by *F. graminearum* in corn.

*Fusarium* species vary in toxigenic potential by strain, which in turn varies with geographic location. Mycotoxin production by *Fusarium* fungi is heavily dependent on oxygen, environmental pH, osmotic tension, and sometimes temperature. For example, DON is produced under conditions of low oxygen tension, whereas zearalenone (a non-trichothecene, estrogenic mycotoxin) production by the same fungi requires oxygen saturation, usually occurring after field crops senesce (Miller, 2002). Unusually cool weather conditions in late summer and early fall, coupled with heavy rainfall in the upper Mid-west of the United States can result in widespread, severe *Fusarium* infestation, and mycotoxin production. For example, the moldy corn epidemic in Wisconsin during 1962 and 1963 that lead to the isolation of toxigenic fungi and the discovery of several important Type A trichothecenes, including T-2 toxin occurred in such conditions (Bamburg *et al.*, 1968) and again in 1992, climatic conditions produced a bumper crop of various toxigenic *Fusarium*, *F. sporotrichioides*, *F. poae*, and *F. graminearum* in Wisconsin corn samples and the isolation of several trichothecenes (DON, T-2 toxin, HT-2 toxin, diacetoxyscirpenol (DAS), neosolaniol, and T-2 tetraol) and zearalenone (Park *et al.*, 1996).

Trichothecenes can be chemically classified into four types based on substitutions at five positions of the trichothecene skeleton, including Type A which includes T-2 toxin and HT-2 toxin (Figure 76.1); Type B including nivalenol and DON (Figure 76.2); Type C including crotocin (Figure 76.3); and Type D or macrocyclics (Figure 76.4). Type A trichothecenes include some of the most toxic trichothecenes, T-2 toxin, its deacetylated metabolite, HT-2 toxin, and DAS (or anguidine). These are most commonly produced for research in *F. sporotrichioides* and *F. poae* cultures. Both of these *Fusarium* spp. produce toxic metabolites in light or darkness and at low temperatures ($<-2$°C), however, sharp fluctuations in temperature increases the toxicity of extracts. In addition to ATA, moldy grains produced under low temperature conditions have been associated with moldy corn toxicosis (hemorrhagic disease), moldy bean hull intoxication, moldy cereal emesis, fusariotoxicosis, and dendrodochiotoxicosis (myrotheciotoxicosis) (Smalley and Strong, 1974).

The Type B trichothecenes are characterized by a keto group at C-8 and hydroxyl group at C-7. These are common natural field contaminants of grains and include DON and its acetylated derivatives, nivalenol ($3\alpha,4\beta,7\alpha$, 15-tetrahydroxy-12,13-epoxytrichothec-9-en-8-one), and fusarenon-X (4-acetylnivalenol) produced by *F. culmorum* and *F. graminearum*, and other, closely related fungi. They are less toxic than the other classes of trichothecenes without the C-8 keto substitution, such as T-2 toxin, DAS, and the macrocyclic trichothecenes.

*F. graminearum* most commonly occurs in southern China, North America, and eastern Europe, and *F. culmorum* more commonly occurs in western Europe. In North America, *F. graminearum* characteristically produces DON, which can co-occur with another 8-ketotrichothecene, 15-acetyldeoxynivalenol (15-ADON), but in Asia *F. graminearum* almost always produces DON with 3-ADON (Miller *et al.*, 1991). Although not a trichothecene, the estrogenic mycotoxin zearalenone is often found together with DON in North America. Other strains of *F. graminearum*, mostly of Japanese origin, produced nivalenol and fusarenon-X, zearalenone, and butenolide. *F. culmorum* strains from the Netherlands produced predominately DON and 3-ADON, while strains of *F. crookwellense*, collected from four continents, produced 4,15-diacetylnivalenol regardless of the source. More serious adverse effects could be underestimated from moldy corn if only DON, and no acetylated DON or additional trichothecenes, are assayed.

The Type C trichothecenes typically have a second epoxide ring at C-7,8 that are neither produced by *Fusarium*, nor are these trichothecenes associated with adverse effects in livestock. Crotocin (or antibiotic T) is a Type C trichothecene produced by *Cephalosporium crotocinigenum* and

| Trichothecene | R |
|---|---|
| Roridin A | —C(=O)CH(OH)CH($CH_3$)$CH_2CH_2$OC(CHOH)<br>CH=CHCH=CHC(=O)— |

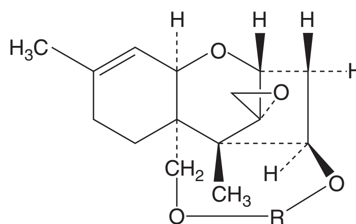


**FIGURE 76.4** Chemical structure of Type D trichothecenes and one example substitution.

*Trichothecium roseum* and has low toxicity in mice (Cole and Cox, 1981). In contrast with Type C trichothecenes, the Type D trichothecenes are potent, cytotoxic compounds with a macrocyclic ring linking C-4 and C-15 on the trichothecene skeleton. The genus *Myrothecium*, including *M. roridum*, *M. leucotrichum*, and *M. verrucaria*, can produce verrucarins and roridins that are diesters of the trichothecene verrucarol and acutely toxic to mammalian cell lines *in vitro* and to a wide range of animals with *in vivo* exposure (Mantle, 1991). No consistent naturally occurring toxicoses have been attributed to the *Myrothecium* toxins (Mantle, 1991).

While *Fusarium* does not produce Type D trichothecenes, the fungus *S. alternans* Bonorden (synonyms *S. atra* Corda and *S. chartarum* (Ehrenberg ex Link) Hughes), which grows worldwide on cellulosic vegetation and particularly on mildewed wet straw, produces macrocyclic trichothecene mycotoxins (including satratoxins G and H, verrucarin J, and roridin E) that are stable, highly toxic, and cause characteristic cytotoxic effects (Bata *et al.*, 1985). Although horses are the most sensitive species, stachybotryotoxicosis has been described in numerous animals, including zoo animals, in many countries of Europe, South Africa, and India.

In North America, the trichothecenes produced by *Fusarium* are the major contaminates in cereal crops. Optimal production of T-2 toxin by *F. sporotrichioides* in grains occurs at 6–12°C (as compared with zearalenone production at an optimal 19–20°C). Park *et al.* (1996) noted that trichothecene concentrations were increased in corn kept in the field and harvested later in cold winter temperatures (grain moistures often greater than 40%), but zearalenone levels were highest in samples collected in the early fall. The co-occurrence of T-2 toxin, DON, and zearalenone in field corn also occurs quickly after hail damage in the late summer or fall as hail damage allows fungi to invade the forming seed. Delayed harvests under wet conditions affect other crops. In the United States upper Mid-West, a humid, wet fall allowed field molds, such as *Alternaria*, *F. graminearum*, and *Phomopsis*, to infect soybeans and produce mycotoxins that include HT-2, DAS, DON, and zearalenone (Jacobsen *et al.*, 1995).

In unusually wet, cool periods, *Fusarium* mycotoxins not only occur in the grain, but also in the vegetative part of the plant. *Fusarium* infection of immature corn generally results in higher trichothecene and zearalenone concentrations in the cob, as compared with the mycotoxin concentrations in the kernels. Thus, avoiding incorporation of the cob in animal feed can potentially reduce exposure to mycotoxins. Usually, grain stored at moisture concentrations less than $0.70a_W$ or 14.5% moisture by weight is not susceptible to fungal growth or mycotoxin production.

Mycotoxin production occurs in hay, green feed, straw, and silage. Hay baled wet or stored in higher moisture conditions (>20%) is subject to molding. Fungal growth in silage usually takes place on the front edges of silage in bunker silos or where silage is not adequately packed and aerobic conditions exist. *Fusarium* occurrence increases with the practice of no till farming and utilizing corn in crop rotations. Hay, straw, or silage from *Fusarium*-contaminated wheat, barley, oats, and corn can contain high levels of trichothecenes, particularly DON and acetylated metabolites. Straw and hay contamination with DON may be as high as 100–150 mg DON/kg (dry weight basis) in wet years (Mostrom *et al.*, 2005).

Of the trichothecenes, DON is probably the most commonly detected in cereal grains throughout the world (Rotter *et al.*, 1996; CAST, 2003). This mycotoxin is resistant to milling and processing and readily enters the animal feed and human food chains. Foodborne trichothecene contamination, in particular DON, has been linked to acute human toxicoses in China, India, and Japan, but little information is available regarding potential health effects from chronic exposure (Bhat *et al.*, 1989; Kuiper-Goodman, 1994; JECFA, 2001).

## TOXICOKINETICS

Trichothecenes undergo all four basic reactions in xenobiotic metabolism. Phase I hydrolysis and oxidation and phase II glucuronide conjugation occur in the body tissues, while reduction of the 12,13-epoxide is thought to occur through microbial action in the gastrointestinal tract; although T-2 toxin is the only trichothecene for

which all four basic reactions or pathways occur simultaneously in the same animal (Swanson and Corley, 1989). The ability to remove the epoxide oxygen (deepoxidation) is an important step in the detoxification of trichothecenes. Orally administered trichothecenes do not accumulate to a significant extent in the body and are rapidly excreted within a few days in urine and feces (bile) (Swanson and Corley, 1989).

Understanding the toxicokinetics of trichothecenes is important for understanding potential effects in animals. Swine are especially sensitive to DON and kinetic parameters have been studied related to intravenous and acute and chronic oral DON exposures (Coppock *et al.*, 1985a; Prelusky *et al.*, 1988, 1990; Prelusky and Trenholm, 1991; Goyarts and Dänicke, 2006). Following an intravenous dose of DON at 1 mg/kg body weight in swine, the mycotoxin distributed rapidly to all tissues and body fluids and declined to negligible levels in all tissues sampled except urine and bile within 24 h (Prelusky and Trenholm, 1991). DON can be detected very rapidly (less than 2.5 min) in the cerebral spinal fluid following intravenous administration in swine; following oral administration of DON in pigs, the plasma DON concentrations correlated closely with cerebral spinal fluid DON levels (Prelusky *et al.*, 1990). Extensive DON tissue accumulation was not detected after dosage indicating that accumulation of edible tissue residues in swine is unlikely at low level DON exposure (Prelusky and Trenholm, 1991). Coppock *et al.* (1985a) reported no detectable residues of DON in skeletal muscle 24 h after intravenous DON administration in pigs. The reported half-life of DON in pigs after an intravenous injection of DON at 0.5 mg/kg body weight ranged between 2.08 and 3.65 h, suggesting that 97% of DON given would be eliminated in 10.1–18.3 h (Coppock *et al.*, 1985a). Following a lower intravenous dose of DON in pigs (0.053 mg/kg body weight), serum DON concentrations decreased biphasically with terminal elimination half-lives ($t_{1/2}\beta$) of between 4.2 and 33.6 h (Goyarts and Dänicke, 2006).

After oral exposure, DON is rapidly and nearly completely absorbed in the stomach and proximal small intestine of pigs (Dänicke *et al.*, 2004a). Pigs dosed with DON at 5.7 mg DON/kg diet chronically for 4 weeks or with one single oral acute exposure (one feeding) had quick absorption of greater than 50% of the DON administered that was highly distributed, with an apparent volume of distribution ($V_d$) higher than total body water, and serum elimination half-lives of 6.3 and 5.3 h in the chronic and acute DON-fed pigs, respectively (Goyarts and Dänicke, 2006). A total of 97% of the DON dose (five elimination half-lives) would be eliminated in 31.5 and 26.5 h after feeding DON chronically or in one single, acute exposure, respectively. The majority of DON ingested from dietary exposure was eliminated in the urine and feces, with urine being the main excretory route of DON in an unmetabolized form. Similar results were reported by Prelusky *et al.* (1988) who observed that after intravenous DON administration (0.30 mg/kg) in swine DON was rapidly cleared primarily unchanged in the urine, with minor elimination in bile. The metabolite deepoxy-DON was found in pigs fed the DON-contaminated diet chronically or for a period of longer than 4 weeks (Goyarts and Dänicke, 2006). Eriksen *et al.* (2003) fed pigs a commercial diet containing 3-ADON at 2.5 mg/kg for 2.5 days. Following ingestion, DON was quickly detected in the plasma (indicating deacetylation), with approximately 42% present as a glucuronide conjugate. DON was excreted mainly in urine, with a smaller amount excreted in feces. Deepoxy-DON constituted 52% of the 3-ADON metabolites detected in feces. The deepoxy-DON metabolite increased in concentration from the distal small intestine to the rectum indicating that deepoxidation occurs in the hindgut, which would have minimal influence on toxicity because of major absorbance of DON in the upper gastrointestinal tract.

In contrast with the poor metabolism of DON by swine, the rumen is capable of extensive metabolism of DON and other trichothecenes. The major metabolite of oral DON in ruminants is 3α,7α,15-trihydroxytrichothec-9, 12-diene-8-one (deepoxy-deoxynivalenol or DOM-1). Deepoxidation of DON to DOM-1 is considered a deactivation step resulting in a non-cytotoxic compound; thus, ruminal metabolism serves a protective function. Because biotransformation occurs in the rumen, little parent compound is available for absorption (Prelusky *et al.*, 1986a; He *et al.*, 1992). Côté *et al.* (1986) reported cows fed high concentrations of DON (66 mg/kg diet for 5 days) excreted approximately 20% of the DON fed in urine and feces as unconjugated DOM-1 (96%) and DON (4%). Preliminary data from the study suggested to the authors that a portion of the remaining 80% of the dose was excreted as glucuronide conjugates of DON and DOM-1 in urine. Dänicke *et al.* (2005) utilized six cannulated dairy cows to evaluate DON and zearalenone metabolism and the effects of *Fusarium*-contaminated wheat on nutrient utilization. The cows were fed a 50:50 mixture of grass silage and contaminated wheat (3.1 mg DON/kg and 0.186 mg zearalenone/kg total ration) providing approximately 25 mg DON/cow/day intake or 0.04 mg DON/kg body weight/day for 4 weeks. The ration was restrictively fed and no feed refusal was observed. Only a small fraction (about 4–28%) of DON was recovered at the duodenum in the form of DON and DOM-1. DOM-1 accounted for 89% of duodenal flow of this mycotoxin. The authors concluded that extensive ruminal metabolism or absorption by rumen mucosa resulted in low DON recovery in the duodenum. No changes were detected in pH or volatile fatty acid concentrations in rumen fluid from cows fed control wheat versus *Fusarium*-contaminated wheat. In contrast, the ammonia concentrations in rumen fluid

were significantly higher from 90 min to 5 h after feeding the *Fusarium*-contaminated wheat, as compared to control wheat. Additionally, the flow of utilizable protein and microbial protein were reduced at the duodenum, suggesting altered ruminal protein utilization could be either the result of mycotoxins in the rumen or from *Fusarium* damage to the structure (cell wall) of wheat grain.

Prelusky *et al*. (1986a) reported that sheep dosed intravenously with DON rapidly excreted two major metabolites of DON as conjugated DON and conjugated DOM-1 in urine, with elimination half-lives of 2.2 and 3.1 h, respectively. Total recovery was 66.5% of the dose, with 63% in the urine and 3.5% in the bile made up of primarily conjugated DOM-1. This suggests the predominant route for elimination in sheep is biotransformation prior to excretion.

Poultry have a greater tolerance to trichothecenes than monogastric mammals because of poor absorption following oral exposure, extensive metabolism, and rapid elimination from the body (Prelusky *et al*., 1986b; Gauvreau, 1991). Oral administration of DON in turkeys (*Meleagris gallopava*) revealed that 0.96% of the dose was absorbed from the gastrointestinal tract with rapid excretion of DON and its metabolites in urine and excreta (Gauvreau, 1991). The terminal elimination half-life in turkeys following an intravenous dose of DON was about 44 min. Tissue residues of DON and/or metabolites declined rapidly to trace levels.

Lactating cows or laying hens consuming elevated concentrations of DON (>5 mg/kg) transfer minimal concentrations of DON to the milk or eggs, respectively. Charmley *et al*. (1993) fed 0.59, 42, and 104 mg DON/head/day from contaminated corn and wheat for 10 weeks in a lactation study and found no detectable residues (<1 ng/ml) of DON or DOM-1 in milk. Prelusky *et al*. (1984) evaluated the absorption and distribution of a single large oral dose, 920 mg of DON via rumen intubation, given to each of two lactating cows. Maximum blood levels occurred 4.7 and 3.5 h after DON administration and were 200 and 90 ng/ml serum (DON and conjugates, respectively). By 24 h post-dosing only trace levels (<2 ng DON/ml serum) were detected. Free and conjugated DON were detected in cow's milk at low levels (<4 ng/ml) with an estimated 0.0001% of administered dose excreted in milk. Following a 5-day oral exposure trial to high concentrations of DON (~66 mg/kg in the diet), lactating dairy cows excreted unconjugated DOM-1 in the milk at concentrations up to 26 ng/ml (Côté *et al*., 1986). No DON was detected (detection limit of 10 μg DON/kg tissue) in eggs or tissues of Leghorn chicks and laying hens and broiler chickens fed a ration containing 4–5 mg DON/kg for 28–160 days (El-Banna *et al*., 1983). Valenta and Dänicke (2005) did not detect DON, DOM-1, or glucuronide conjugates of these compounds in the yolk or albumen of laying hens fed a maize diet containing 11.9 mg DON/kg dry matter for 16 weeks.

T-2 toxin metabolism has been studied in laboratory and agricultural animals. Metabolism of T-2 toxin generally results in reduced toxicity with deacetylation of T-2 toxin to HT-2 (at C-4), to 4-deacetylneosolaniol, and to T-2 tetraol. T-2 toxin is considered 1.5–1.7 times as toxic as HT-2, which is 4.8 times as toxic as T-2 tetraol (Ueno *et al*., 1973). Human and bovine liver homogenates are capable of deacetylating T-2 toxin *in vitro* to HT-2 toxin (Ellison and Kotsonis, 1974). T-2 toxin and metabolites in swine can be eliminated as glucuronide conjugates into bile and undergo deconjugation in the intestinal tract by microbial action and enterohepatic recirculation (Corley *et al*., 1985).

After receiving three daily oral doses of 180 mg T-2 toxin/day (equivalent to dietary levels of 31–35 mg T-2 toxin/kg), a lactating Jersey cow (375 kg) was orally dosed with 156.9 mg of tritium-labeled T-2 toxin (Yoshizawa *et al*., 1981). The cow showed a good appetite, but milk and urine production decreased by 38% and 50%, respectively, during the experimental period. Plasma concentrations peaked at 8 h after the tritium-T-2 toxin dose, at 64 ppb (ng/g) T-2 toxin equivalents, and by 72 h, almost all radioactivity had been eliminated in the urine and feces, in a ratio of 3:7, respectively. About 0.2% of the tritium-T-2 toxin dose was transmitted into the milk with the maximum level of radioactivity in milk at 16 h post-tritium dose of 37 ppb (T-2-toxin equivalents). The cow was killed and tissues were analyzed for T-2 toxin. T-2 toxin was rapidly metabolized and little T-2 toxin accumulated in organ tissues (muscle, liver, kidney, fat, heart, bile, ovaries, and mammary gland), but bile and liver contained higher tritium residues than whole blood. The authors considered that a large amount of the absorbed toxin and its metabolites were eliminated via the bile into the intestinal tract. The delayed elimination of large amounts of radioactivity in the feces indicated that T-2 toxin and its metabolites probably recirculate in the enterohepatic system.

A pregnant Holstein cow was intubated with 182 mg of purified T-2 toxin daily (equivalent to about 0.5 mg T-2 toxin/kg body weight) for 15 consecutive days (Robison *et al*., 1979). Milk samples collected on days 2, 5, 10, and 12 contained T-2 toxin ranging from 10 to 160 ng/g. The dose given to this cow corresponds to an unusually high feed concentration of 50 mg T-2 toxin/kg; therefore, the authors considered it unlikely that T-2 toxin would be detectable in milk at T-2 toxin concentrations found naturally in feeds. The authors fed a sow (170 kg) T-2 toxin at 12 mg/kg diet (equivalent to 0.5 mg T-2 toxin/kg body weight/day) for 220 days. Six days post-parturition a milk sample was analyzed for T-2 toxin and contained 76 ng/g. Glávits and Ványi (1995) described a "perinatal form of T-2 toxicosis" in swine in Hungary. The authors reported T-2 toxin was excreted in the milk of sows causing lesions, characteristic of T-2, in organs of suckling pigs, including

degeneration and necrosis of cells in the bone marrow, and death.

## Microbial metabolism

In the ruminant, significant metabolism of trichothecenes occurs in the rumen and gastrointestinal tract prior to absorption. King *et al*. (1984) noted almost complete transformation of DON to a deepoxidation product when DON was incubated *in vitro* with rumen fluid for a 24-h period. Swanson *et al.* (1987) reported DON was partially converted to deepoxidated DON (DOM-1) by rumen microbes. Rumen microbes, in particular protozoa, appear to be active in the deacetylation of the trichothecenes T-2 toxin and DAS to HT-2 and monoacetoxyscirpenol (Kiessling *et al*., 1984). The authors reported no effect of ovine rumen fluid on DON metabolism; however, the incubation only lasted 3 h. Several rumen bacteria with esterase activity (*Butyrivibrio fibrisolvens*, *Selenomonas ruminantium*, and *Anaerobivrio lipolytica*) degraded T-2 toxin to HT-2 toxin and T-2 triol, but *B. fibrisolvens* also produced neosolaniol (Westlake *et al*., 1987a, b). The authors suggested that at least two different *B. fibrisolvens* enzymes, differing in their specificity for side chains, were responsible for T-2 toxin degradation.

Microbes in the large intestines of chickens are capable of complete DON transformation *in vitro* to a deepoxy metabolite, whereas no metabolism of DON was reported with the *in vitro* incubation of swine large intestinal contents (He *et al*., 1992). In contrast, Hedman and Pettersson (1997) reported that five of six pigs (weighing about 18 kg) fed nivalenol at 2.5 or 5.0 mg/kg in the diet for 1 week excreted a deepoxidated nivalenol in feces. After 3 weeks on the nivalenol diet, the sixth pig acquired the ability to deepoxidate nivalenol. Additionally, DON was deepoxidated during *in vitro* incubation with microbes that had deepoxidated nivalenol. No deepoxidated nivalenol was detected in feces after feeding a diet of 2.5 or 5 mg nivalenol/kg diet to broiler chickens for 3 weeks, although an unidentified metabolite was found (Hedman and Pettersson, 1997). The ability of microbes to acquire the capability to deepoxidate or detoxify trichothecenes may account for the time course of clinical signs associated with trichothecene ingestion. Microbial adaptation may require a period of several weeks during which the host animal is exposed to more of the toxic parent trichothecene. The rate and location of deepoxidation of trichothecenes prior to absorption is important in the development of toxic effects. Formation of deepoxides higher in the gastrointestinal would reduce the potential toxicity of the trichothecene. Biotransformation of DON is inhibited by low pH *in vitro*, with a pH of 5.2 completely inhibiting DON metabolism either by inactivating the microorganisms or specifically inhibiting the deepoxidation process of DON (He *et al*., 1992). This may have major implications in the metabolism of DON by ruminants on higher grain diets causing production of organic acids that reduce the rumen pH. As can be seen by the foregoing, the apparent resistance of ruminants to trichothecenes is due to ruminal metabolism.

# MECHANISM OF ACTION AND TOXICITY

Trichothecenes have multiple effects on eukaryotic cells, including inhibition of protein, RNA, and DNA synthesis, alteration of membrane structure and mitochondrial function, stimulation of lipid peroxidation, induction of programmed cell death or apoptosis, and activation of cytokines and chemokines (Table 76.1). It is believed that

**TABLE 76.1 Trichothecene effects on eukaryotic cell functions**

| Effect | References |
|---|---|
| Inhibit protein synthesis | Ehrlich and Daigle (1987), McLaughlin *et al*. (1977) |
| Inhibit RNA and DNA synthesis | Rosenstein and Lafarge-Frayssinet (1983), Thompson and Wannemacher (1986) |
| Stimulate lipid peroxidation | Rizzo *et al*. (1994), Vila *et al*. (2002) |
| Alter cellular membrane function | Bunner and Morris (1988) |
| Inhibit mitochondrial and electron transport chain function | Pace *et al*. (1988) |
| Induce apoptosis | Pestka *et al.* (1994), Shinozuka *et al*. (1998), Islam *et al*. (1998) |
| Activate MAPKs | Zhou *et al*. (2005) |
| Modulate immune responses | Corrier (1991), Bondy and Pestka (2000) |
| Alter neurotransmitters | Prelusky *et al.* (1992), Swamy *et al.* (2004) |
| Induce gene expression of numerous chemokines and cytokines | Azcona-Olivera *et al*. (1995), Zhou *et al*. (1997), Moon and Pestka (2002), Ji *et al*. (1998), Pestka *et al.* (2005) |

the primary effect of trichothecenes is inhibition of protein synthesis as all of the other reported effects might be secondary to decreased protein synthesis (Rocha *et al.*, 2005).

## Protein synthesis inhibition

Trichothecenes bind to ribosomes in eukaryotic cells, in particular to the 60S ribosomal subunits, and interfere with peptidyl transferase activity (McLaughlin *et al.*, 1977). Inhibition of protein synthesis requires an intact 9,10 double bond and the C-12,13 epoxide. Trichothecenes can be divided into two groups based on their site of action on protein synthesis, either preferential inhibition of initiation or inhibition of elongation or termination. Trichothecenes with hydroxyl and acetyl substitutions at both C-3 and C-4, such as T-2 toxin, DAS, scirpentriol, and verrucarin A, predominantly inhibit initiation, and compounds such as trichodermin, crotocol, crotocin, and verrucarol inhibit elongation or termination (McLaughlin *et al.*, 1977). The cytotoxicity of DON, a trichothecene with a keto group at C-8 and a hydroxyl group at C-7, results from protein synthesis inhibition at the ribosomal level during the elongation and termination step in mammalian cells (Ueno, 1983; Ehrlich and Daigle, 1987). In an *in vivo* study, Robbana-Barnat *et al.* (1988) dosed Balb-c mice with DON by intraperitoneal injection (4, 10, 20, and 80 mg DON/kg body weight) followed by an injection of radio-labeled leucine, killed the mice 5 h post-dosage, and determined leucine incorporation into proteins. Mice dosed intraperitoneally at 10 mg DON/kg body weight or more had marked inhibition of protein synthesis in cardiac tissue. Mice fed a diet with 10 mg DON/kg ration for several weeks exhibited cardiac lesions, primarily foci of calcified pericarditis, which suggested a preferential effect on cardiac tissue in the mice. In an *in vivo* low dose DON study in pigs, protein synthesis (using the "flooding dose technique" with radio-labeled phenylalanine as tracer and expressed as fractional synthesis rate) was significantly reduced in kidneys, spleen, and ileum of pigs exposed orally to 5.7 mg DON/kg diet for about 4 weeks (Dänicke *et al.*, 2006). Protein synthesis of the liver, skeletal and cardiac muscle, mesenteric lymph nodes, duodenum, jejunum, pancreas, and lung was not significantly affected by oral DON exposure.

Trichothecenes inhibit both RNA and DNA synthesis. Using hepatoma cells and phytohemagglutinin-stimulated lymphocytes in an *in vitro* culture, Rosenstein and Lafarge-Frayssinet (1983) reported T-2 toxin inhibited DNA synthesis. DON was demonstrated to inhibit DNA synthesis in splenic lymphocytes and human peripheral blood lymphocytes (Mekhancha-Dahel *et al.*, 1990). Thompson and Wannemacher (1986) reported trichothecenes strongly inhibited RNA synthesis in HeLa cells and had only slight inhibitory effects on Vero cells. The inhibition of nucleic acid synthesis is generally considered secondary to protein synthesis. T-2 toxin inhibited mitochondrial protein synthesis and electron transport action in rat liver cells and *in vivo*, although high doses were used in the studies (Pace, 1983; Pace *et al.*, 1988).

## Lipid peroxidation

T-2 toxin is thought to increase production of oxygen radicals, overwhelming the scavenging system for oxygen radicals and resulting in cell injury. Rizzo *et al.* (1994) administered a single oral dose of DON or T-2 toxin at 28 or 3.6 mg/kg body weight, respectively, to male Wistar rats on antioxidant deficient diets. Liver peroxides, as measured by thiobarbituric-acid-reactive substances, increased 21% and 268% in rats administered DON or T-2 toxin, respectively. Significant decreases in hepatic glutathione concentration and superoxide dismutase activity occurred in the treated rats, as compared with the controls.

Vila *et al.* (2002) studied the effects of T-2 toxin, given by oral gavage at dosages of 4 and 6.25 mg/kg body weight, on vitamin E status and lipid peroxidation in female CD-1 mice. Lipid peroxidation was evaluated by the plasma and organ content of malondialdehyde (MDA). Mice had a decrease in plasma vitamin E concentration 16 h after toxin treatment, and the concentration remained significantly depressed for up to 48–72 h. The MDA content of liver increased significantly 24–48 h after T-2 toxin administration, as compared with control mice, and returned to the control range after 72 h. These findings suggest that T-2 toxin results in lipid peroxidation in mice and vitamin E is consumed by scavenging free radicals from damage by T-2 toxin.

## Neurotransmitter effects

DON produced two main clinical signs in monogastrics, vomiting, or emesis at higher concentrations and reduced food consumption or feed refusal at lower concentrations (Forsyth *et al.*, 1977). The exact mechanism(s) of action of DON which result in feed refusal is/are not known. DON could act directly via a central mechanism controlling hunger or satiety or possibly peripherally through non-specific mechanisms inducing lethargy or depression. Prelusky *et al.* (1992) evaluated the effects of DON on brain biogenic amine concentrations after administration of a single intravenous dose of 0.25 mg DON/kg body weight to young swine (15–23 kg body weight). Norepinephrine was significantly increased and dopamine significantly decreased in the brain regions of the hypothalamus, frontal cortex, and cerebellum for up to 8 h post-treatment, as compared with control animals. Serotonin or 5-hydroxytryptamine was initially increased 1 h post-treatment in

the hypothalamus and by 8 h post-treatment was significantly decreased in the hypothalamus and frontal cortex, as compared with controls. The authors concluded that changes in neurotransmitters were not consistent with known neurochemical changes associated with chemically induced anorexia, but might be due to other toxicological responses, such as vomiting. Neurotransmitter concentrations were measured in the cortex, hypothalamus, and pons of starter pigs (9.3 kg) fed 21 days of control and *Fusarium*-contaminated feeds (approximately 3.9 and 5.8 mg DON/kg diet) and in 1-day-old male broiler chicks fed *Fusarium*-contaminated feeds (approximately 6.3 and 9.7 mg DON/kg diet) for 56 days (Swamy *et al*., 2004). The pigs on mycotoxin-contaminated feeds had alterations in regional brain neurochemistry, including a linear increase in cortex serotonin concentrations with a linear decrease in hypothalamic tryptophan and norepinephrine concentrations and pons dopamine concentrations. At the end of the broiler study, chicks on mycotoxin-contaminated diets had linear increases of serotonin in the pons and cortex, and norepinephrine and dopamine concentrations in the pons. Perhaps through peripheral action on serotonin-3 receptors, rodents displayed inhibited gastric emptying in a dose-related manner when DON was given orally at 50–1000 $\mu$g/kg body weight (Fioramonti *et al*., 1993). Intestinal propulsion was reduced at the highest dose (1000 $\mu$g/kg). This effect is not common to all trichothecenes because T-2 toxin accelerated gastric emptying under similar experimental conditions (Fioramonti *et al*., 1987).

While the exact role(s) of these neurotransmitters in eating disorders is/are not known, Swamy *et al*. (2004) suggested that stimulation of serotonergic and $\alpha_2$-noradrenergic receptors in the hypothalamus of the brain was important in feeding behavior. The increase in serotonergic neurotransmitter concentrations and decrease in norepinephrine concentrations in the brains of pigs fed mycotoxin-contaminated diets might explain some aspects of feed refusal.

Borison and Goodheart (1989) evaluated neural factors involved in the acute emetic, cardiovascular, and respiratory effects of T-2 toxin injected (intravenously or intraperitoneally) at 2 mg/kg body weight in cats. While the causation of vomiting has been attributed to the stimulation of the chemoreceptor trigger zone in the area postrema of the medulla oblongata, area postrema-ablated cats exhibited vomiting 5 h after T-2 administration, as compared with normal cats that vomited within an hour of T-2 administration, and generally persisted in vomiting until the time of death at about 12 h. The authors concluded that the area postrema was not the sole receptor site of trichothecene action to induce emesis. They hypothesized that a possible mechanism of delayed vomiting from trichothecenes may be similar to that of acute radiation sickness in cats exhibiting emesis and malaise, which is mediated by neural afferent pathways from the abdomen that traverse the vagus nerve and dorsal columns of the spinal cord.

## Immunotoxicity

The molecular basis for cytotoxic effects of trichothecenes and immunosuppression is directly or indirectly related to inhibition of protein synthesis. The most potent immunosuppressive trichothecenes are T-2 toxin, DAS, DON, and fusarenon-X, which are the most potent protein synthesis inhibitors (Corrier, 1991). In addition, the occurrence of other secondary metabolites from *Fusarium* associated with trichothecene biosynthesis, such as substituted neosolaniols and calonectrins, inhibit T- and B-lymphocytes when evaluated in mouse spleen lymphocyte proliferation assays (Bondy *et al*., 1991). The most widely studied trichothecene because of potential acute toxicity and use as a chemical warfare agent is T-2 toxin. T-2 toxin caused necrosis and lymphoid depletion in the thymus, spleen, and lymph nodes of a variety of laboratory animals (Ueno, 1977; Hayes *et al*., 1980; Taylor *et al*., 1989), swine (Weaver *et al*., 1978a), cattle (Osweiler *et al*., 1981), and sheep (Friend *et al*., 1983). Necrosis and thymic depletion have been reported in the thymus, bursa of Fabricius and spleen in chickens treated with T-2 toxin (Hoerr *et al*., 1981). Age of exposure is important, as neonatal animals are more sensitive than older animals. Holladay *et al*. (1993) dosed pregnant mice by oral gavage with 1.2 or 1.5 mg T-2 toxin/kg body weight on gestational days 14–17 and found that lymphocyte progenitor cells appear to be more sensitive than thymocytes to T-2 toxin, and are responsible for fetal thymic atrophy and potential immunosuppression seen with T-2.

Trichothecenes have been shown to both stimulate and impair humoral immunity, cell-mediated immunity, and host resistance in experimental and food animals (reviewed by Corrier, 1991; Pestka and Bondy, 1994; Rotter *et al*., 1996; Bondy and Pestka, 2000; Pestka and Smolinski, 2005). The dose and duration of toxin exposure in relation to the timing of the immune assay determined whether stimulation or suppression resulted. For example, Bottex *et al*. (1990) reported contradictory effects in Swiss mice given intraperitoneal doses of 5.3 or 2.7 mg DAS/kg either prior to (range of −10 to −3 days) or following (range of +3 h to +5 days) intraperitoneal injection of *Salmonella typhimurium*. Mice injected with DAS prior to *Salmonella* infection had a lower mortality rate than control mice given just *Salmonella*. In contrast, mice injected with DAS following infection had a significantly higher mortality than controls given *Salmonella*. Similar results were observed in mice given intraperitoneal injections of DAS either prior to or following antigenic stimulation with sheep erythrocytes. The splenic plaque-forming cell

response against sheep erythrocytes was increased in mice given DAS prior to the antigenic stimulation and significantly inhibited in mice given DAS after antigenic stimulation.

Immunostimulation after small doses of trichothecenes apparently results from induction of immune- and inflammation-associated genes. Trichothecene doses that partially inhibited translation upregulated expression of immune-related genes including proinflammatory cytokines and chemokines, cyclooxygenase 2, and inducible nitric oxide synthase (Azcona-Olivera *et al.*, 1995; Zhou *et al.*, 1997; Ji *et al.*, 1998; Moon and Pestka, 2002; Pestka *et al.*, 2005). DON regulated interleukin (IL) 2, a cytokine considered to be a central growth and death factor for antigen-activated T-cells, and IL-8, a proinflammatory chemokine that affects host-defense induction of trafficking neutrophils across vascular walls (Pestka *et al.*, 2005).

The frequent occurrence of DON in cereals and potential chronic exposures to contaminated feed sources has focused attention on DON immunomodulation in animals, particularly the impact on susceptibility to infections and altered vaccination responses. Tryphonas *et al.* (1986) suggested a no-effect level in Swiss–Webster mice of 0.25–0.50 mg DON/kg diet/day based on *Listeria monocytogenes* challenge, serum $\alpha_2$- and $\beta$-globulins, and splenic lymphocyte proliferation to phytohemagglutinin P. Rotter *et al.* (1994) reported no significant differences in lymphocyte proliferation responses to phytohemagglutinin or pokeweed mitogens between treatments of pigs fed 0, 0.95, 1.78, and 2.85 mg DON/kg diet. The pigs responded well to primary immunization with sheep erythrocytes. However, pigs fed higher concentrations of 1.78 and 2.85 mg DON/kg diet, and pair-fed control pigs developed the maximum titer 7 days later than controls. As the level of DON contamination increased, pigs showed increased albumin concentrations, decreased $\alpha$-globulin concentrations, and increases in the albumin/globulin ratios. Similarly, Accensi *et al.* (2006) observed no significant changes in lymphocyte proliferation to mitogens or in cytokine production of IL-4 and interferon-$\gamma$ in young swine fed low doses of DON (0, 0.28, 0.56, and 0.84 mg/kg diet) for 4 weeks. Øvernes *et al.* (1997) reported a reduction in secondary antibody responses to tetanus toxoid injections in pigs fed 1.8 and 4.7 mg DON/kg feed for about 9 weeks, but no titer reductions to sheep erythrocytes, human serum albumin, paratuberculosis vaccine, or diphtheria toxoid. Peripheral blood lymphocyte responses to phytohemagglutinin were increased in pigs fed 4.7 mg DON/kg feed for 9 weeks.

In a high dose exposure of poultry to DON, Harvey *et al.* (1991) reported a reduction in immune responses to Newcastle vaccination in Leghorn hens fed a ration of naturally DON-contaminated wheat at 18 mg DON/kg feed for 18 weeks. DON fed at increasing concentrations (5.8–13.6 mg DON/kg feed) to day-old turkey poults for 12 weeks reduced contact sensitivity responses to dinitrochlorobenzene (a CD8+ T-lymphocyte cell-mediated response), but DON-fed turkeys generated primary and secondary antibody responses to sheep erythrocytes (a CD4+ T-lymphocyte humoral response) similar to the control group, which was exposed to very low concentrations of DON in the feed (Chowdhury *et al.*, 2005a). In a similar experiment with day-old Pekin male ducklings fed a naturally contaminated ration with DON (6.3–18.6 mg DON/kg feed) for 6 weeks, the cell-mediated response to dinitrochlorobenzene was decreased with no effect on the antibody response to sheep erythrocytes (Chowdhury *et al.*, 2005b). Consumption of contaminated grains by both turkey poults and ducklings resulted in only minor impacts on serum chemistries and hematology, and no effect on body weight gains or feed consumption in the duckling study.

While T-2 toxin exposure in numerous laboratory animal models and other species causes immunosuppression, the specific functions of various cell type affected by the mycotoxin have not been definitively ascertained (Taylor *et al.*, 1989). T-2 toxin given by intraperitoneal administration to mice reduced thymus weights, inhibited antibody synthesis against sheep erythrocytes, and prolonged skin graft rejection times (Rosenstein *et al.* 1979). Rabbits given 0.5 mg T-2 toxin/kg body weight per os for 17 or 28 days had reduced antibody responses to aerosolized *Aspergillus fumigatus* challenge (Niyo *et al.*, 1988). Calves dosed orally with T-2 toxin at 0.6 mg/kg/day for 43 days had depressed mitogen responsiveness to phytohemagglutinin at 1, 8, and 29 days of toxin administration and decreased mitogen responsiveness to concanavalin A and pokeweed mitogens on day 29 of toxin administration (Buening *et al.*, 1982). Phagocytosis of 2-hydroxymethacrylate particles by neutrophils was not altered in pigs fed T-2 toxin for 21 days (Rafai *et al.*, 1995b). The authors reported humoral immunity, as measured by titers against horse globulin, a T-dependent antigen, was significantly decreased in pigs fed 14–21 days of T-2 toxin at 0.5, 1, 2, and 3 mg/kg feed. Additional evidence of impaired T-lymphocyte function was found in the reduction of mitogen-induced lymphocyte proliferation tests with phytohemagglutinin, concanavalin A, and purified horse IgG at 2 and 3 mg T-2 toxin/kg feed. The authors remarked on minor histological changes including a decrease in the size of thymic lobules and lobular cortex of the thymus, a decrease in white pulp of the spleen, and regressive changes (interpreted as depletion of lymphocytes) of the germinal centers of lymph nodes of T-2 treated pigs.

Humoral immune responses to anaplasma vaccine were not different between T-2 treated and control calves, but calves dosed with 0.6 mg T-2 toxin/kg body weight had significantly lower concentrations of $\alpha$-, $\gamma$-, $\beta_1$-,and $\beta_2$-serum protein fractions and elevated albumin/globulin

ratio compared with control calves (Osweiler *et al.*, 1981). Serum IgA and IgM concentrations were significantly lower for the high dose T-2 toxin calves, as compared with controls. Pathological changes included decreases in body and thymus weights of T-2 toxin treated calves. Histologically, thymii from calves dosed with 0.3 mg T-2/kg body weight exhibited cortical thinning with loss of mature lymphocytes and fibrous connective tissue separating individual thymic lobules. The high dose calves had almost complete replacement of normal thymic architecture with fibrous connective tissue and loss of the normal lobular pattern and mature lymphocytes. Calves (158–225 kg) dosed orally with T-2 toxin at 0.5 mg/kg body weight for 28 days had reduced serum IgG, IgM, albumin, globulin, and complement proteins (Mann *et al.*, 1983). Lambs treated orally with a high dose of T-2 toxin (0.6 mg T-2 toxin/kg body weight) daily were leukopenic on day 7 and lypmphopenic on days 7 and 14 (Friend *et al.*, 1983). On day 7 of this study, lambs showed decreased lymphocyte blastogenic responses to concanavalin A and lipopolysaccharide. Mitogen responses improved by day 21 of T-2 exposure. At necropsy no differences in parasite loads were noted among T-2 treated lambs, suggesting that the dosage of T-2 toxin did not reduce immunity to a threshold allowing a fulminating coccidiosis to occur in treated lambs. Lambs showed no significant alteration of peripheral blood lymphocyte blastogenesis to phytohemagglutinin after 34 days of DAS treatment (5 mg/kg diet), as compared with control lambs (Harvey *et al.*, 1995).

### IgA

Increased serum IgA and potential IgA dysregulation and nephropathy have been reported in rodents after high (about 25 mg/kg) dietary DON exposure. Forsell *et al.* (1986) reported dose-dependent increases in serum IgA and decreases in serum IgM in female B6C3F1 mice fed 0.5, 2, 5, 10, and 25 mg DON/kg diet for 8 weeks. Further investigation with B6C3F1 mice showed that feeding 25 mg DON/kg diet for 4 weeks increased serum IgA, and continued DON feeding for 24 weeks increased serum IgA 17-fold over controls (Pestka *et al.*, 1989). Mice exhibited a shift from primarily monomeric IgA to polymeric IgA and increased *in vitro* splenocyte production of IgA from both spontaneous-and lipopolysaccharide-stimulated cultures. The DON-associated increase in serum IgA resulted from stimulation of CD4+ and IgA+ lymphocytes in Peyer's patches and in the spleen (Pestka *et al.*, 1989; Dong *et al.*, 1991). DON fed mice showed marked mesangial IgA accumulation based on immunofluorescence staining of kidney tissue (Pestka *et al.*, 1989). In humans, deposition of IgA in the mesangium and capillary loops of the kidney can lead to a focal, non-progressive glomerulonephritis (Jones *et al.*, 1997).

Elevated serum IgA in animals exposed to oral DON does not appear to be consistent across species. Elevations in serum IgA concentrations were not observed in horses fed DON-contaminated feed (exposure of ~0.06–0.1 mg DON/kg body weight/day) (Johnson *et al.*, 1997; Raymond *et al.*, 2005). Bergsjo *et al.* (1992) did not detect any significant differences in serum IgA concentrations in pigs fed DON-contaminated diets from 0.5 to 4 mg/kg feed for 100 days. The authors stated that variation between individuals in the group and the low number of pigs per group ($n = 8$) might not have revealed small differences. Weaned pigs fed low concentrations of DON (<0.84 mg DON/kg diet) for 4 weeks (Accensi *et al.*, 2006) or fed higher concentrations of DON (at 5.8 mg/kg) for 3 weeks (Swamy *et al.*, 2003) showed no consistent changes in serum IgA. In contrast, Goyarts *et al.* (2005) found that finishing pigs fed DON-contaminated wheat (6.5 mg DON/kg diet) *ad libitum* had a significant increase in serum IgA concentrations, with male pigs significantly higher than females. Significant differences in serum IgA concentrations were not observed in control and DON fed pigs on the restricted feeding regimen (assured similar feed intake at the lowest level for all animals of both control and DON groups).

## Apoptosis

Apoptosis, a form of programmed cell death, has been proposed to explain the loss of lymphocytes and hematopoietic cells during trichothecene poisoning (Pestka *et al.*, 1994; Shinozuka *et al.*, 1998). Apoptosis normally serves as a self-regulating pathway in the immune system that reduces excessive inflammation and prevents autoimmune disease (Dong *et al.*, 2002); however, inappropriate activation by trichothecenes results in dysfunction. Activation of mitogen-activated protein kinases (MAPKs) by satratoxins and other trichothecenes correlated with and preceded apoptosis (Yang *et al.*, 2000). The authors used two myeloid models, RAW 264.7 murine macrophages and U937 human leukemic cells, in a cleavage assay and determined the potency of cytotoxicity to be satratoxin G, roridin A, verrucarin A > T-2 toxin, satratoxin F, H > nivalenol and vomitoxin. Using flow cytometry cell cycle analysis and phenotypic staining to study *in vitro* effects, Pestka *et al.* (1994) demonstrated that DON could either inhibit or enhance apoptosis in T-, B-, and IgA+ cells from murine spleen and Peyer's patch. Apoptosis was dependent on lymphocyte subset, source of tissue, and glucocorticoid induction. Islam *et al.* (1998) demonstrated *in vivo* that T-2 toxin, given by intraperitoneal injection to mice, induced thymic atrophy, DNA fragmentation, and histopathological changes in thymic tissue characteristic of apoptosis, including cell shrinkage and nuclear condensation. Macrophages appeared to be very sensitive

to trichothecenes and could undergo trichothecene-stimulated apoptosis (Zhou *et al.*, 2005). The authors suggested that in addition to trichothecenes binding to the 60S ribosomal subunits and producing translational inhibition, another molecular pathway for trichothecene induction of apoptosis was through triggering a ribotoxic stress response activating MAPKs. MAPKs modulate cell physiological processes, such as cell growth, differentiation, and apoptosis, and are important for signal transduction in immune responses (Dong *et al.*, 2002). For example, T-2 toxin given orally to pregnant rats on day 13 of gestation induced apoptosis in neuroepithelial cells of the fetal rat brain that coincided with expression of oxidative stress-related genes (such as heat shock protein 70 and heme oxygenase) and subsequent activation of MAPKs and caspase-2, which are important factors in cell signaling pathways for apoptosis (Sehata *et al.*, 2004).

## Cell membrane function

At low concentrations (0.4 pg/ml to 4 ng/ml), T-2 toxin altered several cell membrane functions in L-6 myoblasts, including uptake of calcium, rubidium, and glucose, incorporation of thymidine or leucine and tyrosine into DNA or protein, and residual cellular lactate dehydrogenase (Bunner and Morris, 1988). Changes occurred within 10 min of exposure, suggesting to the authors that T-2 toxin directly or indirectly affected glucose, nucleotide, and amino acid transporters and calcium/potassium channel activities independent of protein synthesis inhibition.

## Reproductive toxicity

No studies have indicated that DON is a reproductive toxin at concentrations typical of naturally contaminated feed. Friend *et al.* (1986) reported significant weight reductions in young male and female pigs fed DON-contaminated feeds at 3.7 and 4.2 mg DON/kg (~0.14 and 0.17 mg/kg body weight/day) feed for 7 weeks; however, no significant histological changes were observed in the testis (seminiferous epithelium) or ovary (follicle). In contrast, several studies indicate that T-2 toxin can affect reproduction. Glávits *et al.* (1983) reported in a field case involving a large swine herd infertility in gilts and sows that coincided with the detection of T-2 and HT-2 toxins at 1–2 mg/kg feed. Pathology revealed cystic degeneration of the ovaries and uterine atrophy. Huszenicza *et al.* (2000) evaluated low oral T-2 toxin exposures of 0, 0.3, or 0.9 mg T-2 toxin/day and 9 mg T-2 toxin/day for 3 weeks in ewes and heifers, respectively, on a rich, acidosis-inducing concentrate diet. The results suggested that in ewes and heifers rumen acidosis along with exposure to low oral T-2 toxin intake might delay maturation of the dominant ovarian follicle and ovulation and shorten corpora lutea lifespan (lower plasma progesterone concentrations); although the number of animals in the experiment was small. In an equine study of trotter mares, the horses were given 7 mg purified T-2 toxin/day in oats (~0.01 mg/kg body weight/day) for 32–40 days beginning on estrous cycle day 10 (Juhasz *et al.*, 1997). Skin lesions were noted around the mouth of three horses; however, no adverse effects were noted on the length of the interovulatory interval, luteal, and follicular phases of the estrous cycle, plasma progesterone profiles, or follicular kinetics. Uterine flushing of five mares in the trial yielded three embryos suggesting that T-2 toxin had no detrimental effect on ovarian activity, fertilization, or oviductal transport.

In pregnant rats, T-2 toxin crosses the placenta and is distributed to fetal tissues (Lafarge-Frayssinet *et al.*, 1990). Rousseaux and Schiefer (1987) reported that T-2 toxin caused fetal death at high doses (associated with maternal toxicity), with fetal toxicity primarily in the central nervous system and skeletal system. T-2 toxin administered intravenously at approximately one-third or one-sixth of the $LD_{50}$ (0.41 or 0.21 mg/kg body weight) to sows at the beginning of the third trimester of pregnancy caused vomiting 90 min post-injection with the sows becoming listless and aborting their litters 48–80 h later (Weaver *et al.*, 1978b). In another study, three sows were fed purified T-2 toxin in a standard swine ration at 12 mg/kg diet for up to 220 days, causing clinical signs of repeat breeding, small litters (four piglets) and small (0.37–0.65 kg) piglets, which had no gross or histological lesions attributable to T-2 toxin (Weaver *et al.*, 1978a). The sows did not develop changes in the complete blood count, total protein, or alterations in the bone marrow. These studies used concentrations of T-2 higher than commonly found in feeds screened for visible molds.

Thirty 3-week-old Single Comb White Leghorn hens were fed either purified T-2 toxin or DAS at 2 mg/kg diet for 24 days (Diaz *et al.*, 1994). Egg production dropped about 7% in hens fed either T-2 or DAS on days 13–18 of the study, but recovered to near normal by day 24. Shlosberg *et al.* (1984) reported acute, severe reduction in egg production, feed refusal, depression and recumbency, cyanotic appearance of the comb and wattles, and some blue-green discoloration of droppings in a flock of 8-month-old laying hens. Following delivery of new feed, the mean daily egg production dropped from about 2400 to 150 eggs on day 5 of the new feed (94% drop in production). The feed was changed on day 6 and improvement in clinical signs and normal levels of egg production resumed about 12 days later. Mortality was not changed in the flock; however, necropsies of hens that died after 4 days of the new feed revealed atrophy of the ovaries and abnormally small oviducts. The authors hypothesized that the hens were exposed to a small quantity of highly contaminated trichothecene mycotoxin feed causing direct effects on the female reproductive tract and drop in egg production.

The feed sample analyzed for mycotoxins contained T-2 toxin and HT-2 toxin at 3.5 and 0.7 mg/kg, respectively, which the authors thought might be unrepresentatively low. Brake *et al.* (1999) reported low levels of purified DAS (≤5 mg DAS/kg diet) fed to broiler hens from 67 to 69 weeks of age increased fertility, with little effect on hatchability of fertile eggs. In contrast, DAS fed at 10 or 20 mg/kg diet to broiler males from 25 to 27 weeks of age decreased the hatchability of fertile eggs, which the authors attributed to direct toxic effects on the testes (and obviously on sperm). Reproductive toxicity associated with trichothecenes generally occurs when exposures reach maternally toxic concentrations, but natural trichothecene-contaminated diets can pose a serious risk to reproductive performance of livestock (Francis, 1989).

## Genotoxicity and teratogenesis

Trichothecenes are not mutagenic in bacterial assays, but *in vitro* exposure of Chinese hamster cells to DON at concentrations less than 1 mg/ml caused chromatid breaks (Hsia *et al.*, 1988). A 2-year chronic study in B6C3F1 mice fed diets containing 0, 1, 5, or 10 mg DON/kg diet revealed no consistent toxic effects, though mice fed higher concentrations of DON gained less weight than controls (Iverson *et al.*, 1995). DON did not consistently alter clinical chemistry or hematology parameters. The pathology revealed a reduction in development of pre-neoplastic and neoplastic lesions in the liver that may have resulted from reduced feed intake and reduction and body weights.

When considering the etiology of congenital malformation, the role of maternal toxicity must be evaluated. Maternally toxic doses of trichothecenes can be embryotoxic, with fetal death common in both birds and mammals, generally few frank congenital defects are observed in surviving fetuses, though anomalies in the nervous and skeletal systems have been noted (Francis, 1989). Khera *et al.* (1982) studied the embryotoxicity of DON in pregnant Swiss–Webster mice dosed orally with purified DON at concentrations of 0–15 mg DON/kg body weight for 4 days on gestational days 8 through 11. Mice dosed with 5–15 mg DON/kg body weight apparently resorbed the embryos, but no adverse effects were noticed in the dams given 2.5 mg DON/kg body weight. A number of skeletal malformations were observed in offspring of mice dosed at 1, 2.5, and 5 mg DON/kg body weight, but no adverse effects were reported in offspring of mice dosed at 0.5 mg DON/kg body weight.

DON fed to rabbits on days 0 through 30 of gestation at increasing levels of 0.3–2.0 mg/kg body weight/day caused 100% fetal resorption at 1.8 and 2.0 mg/kg/day and reduced body weight in rabbit does (Khera *et al.*, 1986). Dosages of 0.3 and 0.6 mg DON/kg body weight/day did not produce adverse effects in rabbit fetuses at term and were not maternotoxic. The authors concluded that DON did not produce a teratogenic response in rabbits. A two-generation study of female reproduction and teratology in CD-1 mice fed 0, 1.5, and 3 mg T-2 toxin/kg in a semi-synthetic diet did not reveal any significant differences in major or minor defects among treatment groups (Rousseaux *et al.*, 1986). No long-term reproductive or teratological effects were noted.

Minor malformations described as delayed ossification and un-withdrawn yolk sac were reported in chick embryos from hens fed rations containing DON at 2.5 and 3.1 mg DON/kg diet (Bergsjo *et al.*, 1993a).

## Clinical pathology

Trichothecenes, in particular T-2 toxin, have been associated with hemorrhagic syndromes; however, the effects are not consistently seen in experimental studies across species (Raisbeck *et al.*, 1991). Prolonged activated partial thromboplastin time and decreased hematocrit, leukocyte count, and serum alkaline phosphatase activity were observed in rabbits injected with 0.5 mg T-2 toxin/kg body weight (Gentry and Cooper, 1981). In contrast, rabbits given 2 mg T-2 toxin/kg body weight orally displayed oral lesions, diarrhea, and anorexia but no significant changes in hematological and biochemical parameters. Young mice and rats fed purified T-2 toxin at concentrations of 10–20 mg/kg for 2–4 weeks were relatively resistant to hematopoietic suppression, but developed atrophy of the thymus and thymus-dependent lymphoid tissue and lymphopenia (Hayes and Schiefer, 1982). Lambs dosed orally with 0.6 mg T-2 toxin/kg body weight/day displayed significantly prolonged prothrombin times on day 7 (Friend *et al.*, 1983). Although lambs fed DAS (5 mg/kg diet) for 34 days showed significant decreases in several serum clinical chemistry values (cholinesterase and urea nitrogen), no changes were noted in prothrombin times, white blood count, or other hematological parameters (Harvey *et al.*, 1995). Growing pigs fed purified DAS at 0, 2, 4, 8, and 9 mg/kg diet for up to 9 weeks exhibited decreased feed consumption and weight gain (Weaver *et al.*, 1981). No changes in hemoglobin concentration, total red or white blood cell counts, and terminal bone marrow smears or in serum activities of aspartate or alanine aminotransaminases or lactate dehydrogenase were reported in the pigs. DAS given intravenously to pigs, cattle, and dogs produced moderate-to-severe necrosis of bone marrow hematopoietic elements 8 h post-administration (Coppock *et al.*, 1989). The authors ranked species sensitivity to DAS destruction of bone marrow hematopoietic elements; most sensitive species were pigs and dogs and least sensitive species were cattle.

The clinical signs observed in laboratory animals treated with T-2 toxin and DAS are those of a radiomimetic

poison with damage to the lymph nodes, thymus, spleen, and bone marrow. A single dose of a potent trichothecene releases stored leukocytes into the blood stream but repeated administrations damage the hematopoietic system, depleting the leukocyte population. Lutsky *et al.* (1978) reported a marked depletion of leukocytes, similar to ATA, in cats dosed orally at 0.06–0.10 mg T-2 toxin/kg body weight for 24 days. T-2 toxin given intravenously to calves at 0.25 mg/kg body weight caused a marked decrease in leukocyte and neutrophil counts, with a small increase in serum aspartate aminotransferase and lactate dehydrogenase activities (Gentry *et al.*, 1984). In a study of lambs dosed orally at 0, 0.3, and 0.6 mg T-2 toxin/kg body weight for 21 days, examination of the myeloid:erythroid ratio of sternal bone marrow of the lambs revealed on day 12 that the ratio was significantly increased in 0.6 mg T-2 toxin/kg body weight lambs (Friend *et al.*, 1983). The bone marrows of these lambs appeared hypocellular with degenerating cells and pyknotic nuclei, and cells of the myeloid series predominated with few metarubricytes and more immature erythroid cells. None of the lambs were anemic, no hemorrhage was observed; nor were any significant differences found in hematological parameters. At the end of the 21-day study, the lambs were killed and necropsied. The highest dosed lambs had little body fat and pale bone marrow. In the 0.6 mg T-2 toxin/kg body weight lambs, the splenic white pulp was less cellular than low T-2 dosed and control lambs. The cortices of the mesenteric lymph nodes of T-2 treated lambs were thin, as compared with control lambs, and the lymphocyte population of the medullary and paracortical areas and germinal centers of the mesenteric lymph nodes were markedly depleted.

Starter pigs fed T-2 toxin at 0.5, 1, 2, and 3 mg/kg diet (average daily intakes 0.38, 0.81, 1.24, and 1.42 mg T-2 toxin, respectively) for 3 weeks showed significant decreases in leukocyte counts, and pigs fed 2 and 3 mg T-2 toxin/kg had significant decreases in red blood cell counts, mean corpuscular volume, and hemoglobin concentration on day 21 of the study (Rafai *et al.*, 1995b). The authors suggested that T-2 toxin affects hematopoiesis in young pigs at low concentrations (0.5 mg T-2 toxin/kg diet). In contrast, no effects on leukocyte counts or lymphoid tissue morphology were found in pigs fed 1, 2, 4, or 8 mg T-2 toxin/kg diet for 8 weeks (Weaver *et al.*, 1978a), nor were changes in hematocrit, hemoglobin concentration, or erythrocyte counts found in pigs fed 0.4, 0.8, 1.6, or 3.2 mg T-2 toxin/kg feed for 5 weeks (Friend *et al.*, 1992). However, microcytic, hypochromic anemia was reported in pigs fed higher concentrations of T-2 toxin at 10 mg/kg diet for 21–28 days (Harvey *et al.*, 1990).

Patterson *et al.* (1979) could not reproduce a feed-associated hemorrhagic disease syndrome in weaned calves or piglets by dosing orally with either purified T-2 or DAS (0.1 mg toxin/kg body weight for 36 days to pigs and 0.2 mg toxin/kg body weight for 11 days to calves) or with whole cultures of *F. tricinctum* containing T-2 toxin. While none of the pigs displayed clinical signs of toxicity, the calves dosed with 0.2 mg T-2 toxin/kg body weight did became weak, anorectic, and died with evidence of a prolonged partial thromboplastin time.

Oral lesions were observed in most birds fed T-2 toxin diets, but no significant changes were observed in hemoglobin, hematocrit, and erythrocyte counts in the Leghorn laying hens fed T-2 toxin (0, 0.5, 1, 2, 4, and 8 mg/kg diet) (Chi *et al.*, 1977). Serum activities of alkaline phosphatase and lactate dehydrogenase and uric acid concentrations were elevated in hens fed 8 mg T-2 toxin/kg diet, as compared with controls. In a high dose T-2 toxin study, plasma protein concentrations decreased by 15% after the first week and by 20% after the third week in hens fed T-2 toxin (20 mg/kg diet) for 21 days, as compared with control birds (Wyatt *et al.*, 1975). After receiving T-2 toxin for 3 weeks, treated hens had about 30% lower leukocyte counts than the control hens. No differences were observed in prothrombin times, erythrocyte counts, hematocrit, hemoglobin concentrations, or spleen and heart weight.

No consistent changes in serum enzyme activities have been associated with trichothecenes in animals. Low dietary concentrations of DON (<0.84 mg/kg diet) fed to weanling pigs for 4 weeks caused no significant changes in plasma enzyme activities, glucose, urea, bilirubin, and hematological parameters during the study (Accensi *et al.*, 2006). Controlled studies of T-2 toxin fed to swine resulted in altered serum concentrations of glucose and other parameters associated with decreased feed intake and changes in metabolism. Pigs fed 1, 2, and 3 mg T-2 toxin/kg feed for 3 weeks had significantly lower plasma glucose concentrations, as compared with control pigs and pigs dosed at 0.5 mg T-2 toxin/kg diet (Rafai *et al.*, 1995a). The decrease in plasma glucose was attributed to glucose malabsorption or to enhanced glucose degradation. Schiefer and Beasley (1989) suggested that in acute T-2 toxicosis in pigs, the ability of glucocorticoids to induce enzymes associated with heptaic gluconeogenesis and convert amino acids into glucose is reduced. Following an acute, intravenous toxic dose of DAS in pigs, Coppock *et al.* (1985b) reported the pigs developed hypoglycemia and depletion of liver glycogen stores. The authors attributed changes in blood glucose concentrations to cellular damage of endocrine and exocrine pancreas and adrenal gland by DAS.

## TOXICITY

Trichothecenes are toxic to all animal species that have been tested. The Type D trichothecenes, macrocyclics such as the verrucarins and roridin E, are the most acutely toxic

trichothecenes, followed by the Type A compounds, DAS and T-2 toxin, Type B, nivalenol, and the lowest acute toxicity is associated with the Type C trichothecene, crotocin (Ueno, 1983). Neonatal animals are more susceptible. T-2 toxin, the first trichothecene recognized as a naturally occurring mycotoxin, has been studied extensively because of its relative ease of production and its potential as a chemical warfare agent, but T-2 toxicosis is rare in North America. Oral $LD_{50}$ concentrations for T-2 toxin in laboratory animals did not demonstrate marked species differences in sensitivity, but agricultural species do vary in their sensitivity to the different trichothecene toxins. For example, based on toxicity the species susceptibility to DON are ranked as pig (most sensitive), followed by rodent > dog > cat > poultry > ruminants (least sensitive) (Prelusky *et al.*, 1994).

Diarrhea is fairly common following trichothecene ingestion and altered intestinal absorption of compounds and impaired permeability is caused by morphological and functional damage to intestinal mucosa (Ueno, 1983). Epithelial irritation, particularly associated with the metabolites of *F. sporotrichioides*, is considered the underlying cause of oral and cutaneous inflammatory lesions noted in ATA, stachybotryotoxicosis, and fusariotoxicosis of chickens. Dermal toxicity is common to many trichothecenes and the basis for screening toxicity of trichothecenes using dermal bioassays. The chemical structure of trichothecenes determines their dermal toxicity and their cytotoxicity in cultured cell lines. Macrocyclic trichothecenes and T-2 toxin, HT-2 toxin, and DAS (Type A trichothecenes) caused marked epithelial irritation, while nivalenol and DON (Type B trichothecenes) were weak dermal irritants in a guinea pig dermal bioassay (Ueno, 1984). Topical applications of purified T-2 toxin to various animal species caused similar morphological changes, including hyperemia, swelling, exudation, and necrosis with subsequent formation of scabs that fall off (Pang *et al.*, 1989). Histological changes included vascular dilation, edema, congestion, and infiltration of neutrophils, mononuclear, and mast cells in the dermis with varying degrees of epidermal necrosis. The mechanism by which trichothecenes induced cutaneous damage is unknown. Ueno (1984) suggested that increased vascular permeability has a role in inducing cutaneous injury from trichothecene administration. Release of chemical mediators of inflammation could initiate changes leading to microvascular injury in dermal tissue.

In addition to the direct clinical effects on animals, Goyarts *et al.* (2005) noted in natural DON-contaminated wheat that DON significantly increased metabolizable energy, nitrogen retention digestibility of organic matter, crude protein, crude fat, and crude fiber of the cereal grain. However, positive effects of a DON-contaminated diet did not compensate for adverse effects on feed intake or performance in weight gain in swine. Matthaus *et al.* (2004) reported marked changes in nutrient characteristics in *Fusarium*-contaminated wheat, with increased activities of non-starch polysaccharide hydrolyzing enzymes. This may represent a type of "pre-digestion effect" (Dänicke *et al.*, 2004b). Contrary to the positive findings of DON on feed digestibility, DiCostanzo *et al.* (1994) reviewed data reporting that moldy corn had decreased nutritional value, particularly lowered crude fat, along with energy and crude protein values.

### *Swine*

Clinical effects associated with DON ingestion in pigs include feed refusal, weight loss, poor performance, gastrointestinal irritation (such as diarrhea, colic, and rectal prolapse), squamous hyperplasia of the gastric lining, and possible immunomodulation. The DON dose affecting feed intake and rate of gain in swine studies varied with the age of the pig, source of DON (purified versus natural contamination), and exposure conditions (*ad libitum* versus restrictive feeding and duration of exposure). In a chronic study of the effects of DON on grower (starting weight of about 26 kg) and finisher swine, Goyarts *et al.* (2005) fed DON-contaminated wheat at about 6.5 mg DON/kg diet for 11 weeks in both *ad libitum* and restricted feeding regimens. Swine fed the DON-contaminated diet *ad libitum* showed significant reductions in feed intake (15% less) and weight gain (13% less), but the feed-to-gain ratio was not affected. The lower growth performance in swine fed the DON-contaminated diet was attributed to lower voluntary feed intake because no differences were detected in weight gain for control and DON fed swine on the restricted feeding regimen. Similarly, Rotter *et al.* (1994) attributed depressed growth of growing pigs to reduced voluntary feed intake of DON-contaminated diets. Chavez and Rheaume (1986) observed that pigs fed DON-contaminated diets generally consume small amounts of feed continuously throughout the day. Pigs not only consumed less DON-contaminated feed when fed *ad libitum*, but also required more time to consume an equivalent amount of feed compared with a control group (Goyarts *et al.*, 2005).

Pollmann *et al.* (1985) fed DON-contaminated wheat at increasing concentrations of 0–2.8 mg DON/kg diet to starter pigs (7.7–8.3 kg) and concentrations of 0–4.2 mg DON/kg diet to growing/finishing pigs (60.5 kg). In the starter pigs, feed intake declined when the DON concentration was greater than 1 mg DON/kg diet, which was more noticeable during the first week of exposure. No clinical signs, including vomiting, were observed nor were lesions observed in examined organs. Vomiting was the only adverse clinical sign observed in growers fed a diet of DON at 2.8 mg/kg for 42 days. Feed refusal was observed in growers fed DON at 2.2, 2.8, and 4.2 mg/kg diet. Pigs (25 kg) fed naturally contaminated oats at 2 and 4 mg DON/kg feed experienced a dose-related depression of weight gain in the first 8 weeks of the trial (Bergsjo *et al.*,

1992). The authors determined a no-effect level based on feed intake, weight gain, and feed efficiency in growing pigs (25 kg initial weight) of 0.5 and 1.0 mg DON/kg feed. In contrast, Friend *et al*. (1982) fed pigs a diet of 0.7 mg DON/kg feed and saw an immediate reduction in feed consumption, which gradually recovered to nearly normal feed intake. Significant reductions in feed intake and weight gain occurred in pigs fed corn silage, naturally contaminated with 1.3 mg DON/kg feed (Young *et al*., 1983). However, Chavez (1984) reported no reduction in weight gain or feed consumption in piglets (initial weight 7 kg) fed naturally contaminated wheat at DON concentration <2.5 mg DON/kg diet. Williams *et al*. (1988) observed that naturally DON-contaminated wheat fed to growing pigs inhibited voluntary feed intake linearly with dietary concentrations of DON up to 16 mg DON/kg feed. They also noted vomiting and signs of abdominal distress at dose rates from 0.055 to 0.097 mg DON/kg body weight. These values are close to the minimum oral emetic dose of young swine (9–10 kg) at 0.1 mg DON/kg body weight (Forsyth *et al*., 1977).

Using naturally contaminated oats at 0, 0.7, 1.7, and 3.5 mg DON/kg feed, Bergsjo *et al*. (1993b) examined clinical effects in growing pigs (~21 kg) fed *ad libitum* for 95 days. The daily weight gain of pigs fed 3.5 mg DON/kg feed was 82% of the control pigs over the whole experiment. Significant decreases in body weight gain, slaughter weight, and feed utilization were observed in pigs fed a diet of 3.5 mg DON/kg. The authors concluded that 1.7 mg DON/kg feed, in an otherwise adequate diet which contained only minor traces of other mycotoxins, depressed daily feed intake. Dänicke *et al*. (2004b) reported no significant effects on the performance of finishing pigs when fed DON at concentrations of 0.2, 0.7, 1.2, 2.5, and 3.7 mg/kg diet.

Pigs fed higher DON-contaminated diets (>3.6 mg/kg diet) have significant weight reductions (Friend *et al*., 1986) and reduced feed consumption (Forsyth *et al*., 1977), as compared with controls. Significant reductions in the feed-to-gain ratio in swine were only observed at higher DON and zearalenone concentrations, 9.1 and 11.0 mg DON/kg feed and 1.2 and 2.0 mg zearalenone/kg feed, and not at lower DON concentrations (<0.5, 2.6, 5.0, and 8.3 mg DON/kg feed) when naturally contaminated wheat was fed to growing pigs for 14 weeks (Williams *et al*., 1988). In contrast, Swamy *et al*. (2002) reported a significant decrease of about 30% in feed intake and growth performance, but not in the feed-to-gain ratio, in growing pigs fed naturally contaminated DON at 5.6 mg/kg *ad libitum* for 21 days. In another study with swine, Swamy *et al*. (2003) reported an increase in feed efficiency in pigs fed DON and suggested that pigs adjusted to the reduction in feed intake by improving feed utilization.

Based on reviews of the literature, Dänicke *et al*. (2001) suggested dietary DON concentrations in livestock feeds that ensured no adverse effects on animal health or performance if all production conditions are optimal (Table 76.2). These suggested concentrations of DON in livestock feed are similar to the US Food and Drug Administration (FDA) advisory guidelines for DON in livestock feed. Eriksen and Pettersson (2004) proposed lower guidelines for DON in livestock feed based on risk assessment of data from toxic effects (Table 76.2).

**TABLE 76.2 Recommended values for concentrations of DON in the final ration**

| Animal species/type | DON[a] | DON[b] | DON[c] |
|---|---|---|---|
| Pigs | 1 | 1 | 0.3 |
| Pre-ruminant calf | 2 | | |
| Dairy cow/pregnant cattle | 5 | 2[d] | – |
| Beef cattle | 5 | 5 | – |
| Poultry (laying hens, broilers) | 5 | 5 | 2.5 |

[a]Values for critical concentrations of DON in diets of pigs, ruminants, gallinaceous poultry (mg/kg, 88% dry matter) (Dänicke *et al*., 2001).
[b]Values are in mg/kg for the final ration (FDA advisory guidelines).
[c]Risk assessment guidelines (Eriksen and Pettersson, 2004).
[d]Under the "intended use" dairy cattle would in the "all other animal species".

Sex-related differences have been reported in pigs fed DON-contaminated feed, but the findings are equivocal. Côté *et al*. (1985) reported lower weight gains in castrated male pigs, as compared to female pigs, in the first 4 weeks of feeding DON-contaminated feeds at 3.1 and 5.8 mg DON/kg. Conversely, Goyarts *et al*. (2005) reported that *ad libitum* fed castrated male pigs had significantly higher feed intake and weight gains, as compared with female pigs, during the 11-week exposure to DON-contaminated feed (about 6 mg DON/kg diet) in grower swine; whereas, Bergsjo *et al*. (1992) and Williams *et al*. (1988) reported no interactions between sex and weight gains at any dietary DON concentrations.

Rotter *et al*. (1994) concluded that pigs show a physiobiochemical adaptation response to DON-contaminated diets ranging from 0.95 to 2.85 mg DON/kg. Even at the lowest dose, 0.95 mg DON/kg diet (0.11 mg DON/kg body weight/day), pigs showed a reduction in feed consumption over the first 2 days of the experiment but were able to adapt by the end of 28 days. Overall gains of pigs on DON-contaminated diets were no different than pair-fed controls. This result is consistent with those of Friend *et al*. (1986) and Bergsjo *et al*. (1992) who reported that swine could adapt to dietary DON contamination. Morphological changes in pigs fed the highest DON-contaminated feed were a reduction in thyroid size and a higher degree of folding and thickening of the esophageal region of the stomach (Rotter *et al*., 1994). In a similar study, Øvernes *et al*. (1997) fed growing pigs (25 kg) naturally DON-contaminated oats at 0.6, 1.8, and 4.7 mg DON/kg feed, both restricted and *ad libitum* for about 100 days. No changes in feed intakes were detected in pigs fed DON-contaminated rations *ad libitum*, but feed intakes

were significantly reduced in pigs fed restricted levels of 1.8 and 4.7 mg DON/kg feed. Post-mortem examination of the pigs revealed no differences in the stomach mucosal appearance between the different DON-treatment groups. The authors hypothesized that when DON-contaminated feed is freely available, pigs would reject less feed than when offered large quantities for a limited period. Results of this study in growing pigs indicated that using naturally contaminated oats at levels up to 4.7 mg DON/kg feed might cause slight immunosuppression, but did not represent a serious health problem.

In a study at low T-2 toxin concentrations, Rafai *et al*. (1995a) fed 7-week-old pigs (~9 kg) a diet with 0.5, 1, 2, 3, 4, 5, 10, and 15 mg/kg of purified T-2 toxin for 3 weeks, with average daily intakes by experimental pigs of T-2 toxin between 0.029 and 0.23 mg T-2 toxin/kg body weight/day. No vomiting or diarrhea was observed in any group, but pigs fed 10 and 15 mg T-2 toxin/kg diet became somnolent and huddled as if chilled. Feed consumption was decreased (not significantly) and serum aspartate aminotransferase activity was significantly increased in pigs by the lowest T-2 toxin concentration. Pigs fed 3.0 mg T-2 toxin/kg feed had significant decreases in weight gain, but not feed conversion, for 3 weeks. After 9–14 days on 4, 5, 10, or 15 mg T-2 toxin/kg diet, pigs began to show signs of dermatitis and crusting of the skin and the snout, with inflammation becoming more severe with time. Histological examination of affected skin revealed hyperkeratosis, parakeratosis, acanthosis, and superficial to deep erosions into the subepithelial layer in all groups exposed to T-2 toxin. Pigs at the higher T-2 toxin dosages had involvement of the skin and mucous membranes of the prepuce, tongue, and renal pelvis (urothelium). The authors of this study concluded that the no-effect level of T-2 toxin for performance was probably less than 0.5 mg T-2 toxin/kg diet. In contrast, growing crossbred pigs (18 kg) fed 8.0 mg T-2 toxin/kg diet for 30 days showed reduced feed consumption and weight gain but no changes in clinical appearance, as compared with controls (Harvey *et al*., 1994).

Toxicoses from macrocyclic trichothecenes have occurred in pigs housed on straw litter infected with *Stachybotrys* (Dankó, 1975). The clinical signs included irritation, necrosis, and hemorrhages, and are similar to those described for cattle with stachybotryotoxicosis (see Ruminants).

### *Horses*

Few experimental trichothecene studies using the equine species have been published. Five adult horses (~444 kg) were fed DON-contaminated barley (average range of 36–44 mg DON/kg grain) and consumed approximately 1.27 kg barley/horse/day (44–55 mg DON/horse/day or about 0.099–0.124 mg DON/kg body weight/day) for 40 days (Johnson *et al*., 1997). The horses had access to free-choice pasture grass. No signs of feed refusal were observed in the horses, and though not weighed, all animals appeared to gain weight and improve body condition according to the authors. No remarkable changes were detected in any hematological or serum biochemical parameters. Both serum IgG and IgA decreased in a linear manner through the trial. The authors attributed decreases in serum total protein, globulin, and albumin to changes in the hydration status of the animals and voluntary water consumption during the study. The authors also suggested that gastric microflora detoxified DON prior to absorption. Raymond *et al*. (2003) fed mature, non-exercising mares a blend of naturally contaminated wheat and corn (15.0 mg DON and 0.8 mg 15-ADON/kg diet), with and without 0.2% glucomannan polymer (an adsorbent), for 21 days. Control mares consumed approximately 0.004 mg DON/kg body weight/day, the mycotoxin group 0.029 mg DON/kg body weight/day, and the mycotoxin plus adsorbent group 0.051–0.058 mg DON/kg body weight/day. Feed intake was significantly reduced in the mycotoxin fed groups, as compared with the control group. The authors reported that $\gamma$-glutamyltransferase activities were increased in mares fed the mycotoxin diet on days 7 and 14, but not on day 21 of the trial. Unfortunately, no serum chemistry analyses were undertaken prior to the experiment on day 0 to establish a common baseline for the horses. Additional serum chemistry parameters and hematology were not affected by the diets. In a subsequent study of exercised horses, mares were fed one of three treatments, a control diet, a mycotoxin diet (11.2 mg DON/kg, 0.7 mg 15-acetyldeoxynivalenol/kg diet), and a mycotoxin diet (14.15 mg DON/kg feed, 0.7 mg 15-acetyldeoxynivalenol/kg diet) with 0.2% glucomannan polymer for 21 days (Raymond *et al*., 2005). Feed intake and weight gains were depressed in horses fed the mycotoxin-contaminated diets, as compared with control mares. No effect of diet was seen on hematology or serum chemistries, which included $\gamma$-glutamyltransferase activities, nor were any differences noted in athletic ability measured by time-to-fatigue treadmill step tests. Feeding 2% glucomannan polymer as an adsorbent did not prevent a depression in feed consumption in this study.

Lethal trichothecene mycotoxicoses from *Stachybotrys* have been recognized in field cases involving horses in the former USSR, particularly the Ukraine since the late 1930s (reviewed by Forgacs, 1972; Dankó, 1975; Hintikka, 1978). Stachybotryotoxicosis typically occurred during indoor feeding of horses with *S. alternans*-contaminated straw or hay. The horse is very sensitive to *Stachybotrys* toxins, with 1 mg of pure *Stachybotrys* toxin considered a lethal dose. Soviet authors described two forms of the disease, “typical” or prolonged disease and “atypical” or acute disease, based on the dose and duration of toxin ingested. The typical form, which is further subdivided

into three stages, was more common, and occurred after continuous consumption of sub-lethal quantities of moldy vegetation. The first stage occurred 2 or 3 days following consumption of moldy feed. Irritation, and later necrosis, occurred wherever the toxin(s) contacted tissue, especially the oral, nasal, and ocular mucous membranes. Clinical signs of conjunctivitis, lacrimation, and profuse salivation, along with spontaneous nosebleeds, hemorrhage of mucous membranes, maxillary lymph node swelling, and dysphagia also occurred. The first stage lasted 2–3 days or could persist for a month. The second stage lasted 15–50 days and was characterized primarily by clinicopathological changes; clinical signs were nonspecific. An initial leukocytosis was followed by leukopenia and thrombocytopenia and, in some cases, blood failed to clot. During the third stage, thrombocytopenia and leukopenia worsened, horses became febrile (40–41.5°C), anorexic, and prone to colic. Weight loss, recumbency, and septicemia were common. This stage lasted 1–6 days and usually ended in death.

The atypical form of stachybotryotoxicosis was peracute (1–3 days) and associated with high concentrations of *Stachybotrys* toxins in the feed. This form was characterized by nervous system irritation or depression, cardiac arrhythmias, pulmonary edema, and hemorrhages of serosal and mucosal membranes and muscular tissue, muscle necrosis, and oral ulcers. Post-mortem findings were characterized by hemorrhage and necrosis of tissues (Hintikka, 1978). Hemorrhages occurred on serous and mucous membranes and in the spleen, liver, lungs, brain, spinal cord, lymph nodes, and most notably in muscle tissue. Clearly circumscribed necrotic foci, yellow to brown-gray in color, were seen on oral mucosa, with the entire digestive tract generally appearing hemorrhagic and necrotic.

In addition to toxicoses involving macrocyclic trichothecenes, historical accounts of equine deaths were reported in horses provided moldy feed in northern Japan. Horses developed clinical signs and died after consuming moldy bean hulls, which were commonly used as fodder and bedding especially in the winter and spring (Ishii *et al*., 1971). The clinical signs of affected horses were central nervous system disturbances including convulsions and cyclic movements, depressed respiration, icterus, and bradycardia. Several trichothecenes, including T-2 toxin, DAS, and neosolaniol, were associated with the clinical syndrome, but cause–effect was not established.

### Dogs and cats

Trichothecenes are fairly heat stable compounds and following incorporation into pet food diets, DON remains stable during extrusion and drying (Hughes *et al*., 1999). The authors reported that DON concentrations (from a naturally contaminated wheat source) greater than 4.5 ± 1.7 or 7.7 ± 1.1 mg DON/kg diet reduced feed intake in dogs and cats, respectively. When DON concentrations reached 8–10 mg/kg feed, clinical signs of vomiting and reduced feed intake and body weight occurred in dogs and cats. Dogs that had been previously exposed to DON-contaminated feed preferentially selected uncontaminated dog food; dogs with no previous experience with DON-contaminated dog food consumed equal quantities of DON-contaminated and uncontaminated food.

### Ruminants

During wet growing seasons, DON contamination of cereal grains can be sufficiently high to make grains unsuitable for human food or swine feed. Anderson *et al*. (1996) evaluated the use of naturally scab-infected barley with elevated DON concentrations (about 35 mg/kg) in beef growing/finishing and pregnant heifer rations. The authors fed increasing levels of DON to crossbred steers for 84 days, at 0.9, 3.7, 6.4, and 9.2 mg DON/kg diet, during the growing period and for 100 days during the finishing period at 1.1, 5.0, 8.8, and 12.6 mg DON/kg diet. No significant differences were found in beef performance including feed intake, feed efficiency, rate of gain, or carcass quality. The authors concluded that DON-contaminated feed could be provided up to 9 mg/kg diet during bovine growth and up to 12 mg/kg diet during finishing without adverse effects. In separate study, the authors fed pregnant yearling heifers DON-contaminated barley, 36.8 mg/kg at 3.6 or 5.4 kg/head/day (about 10.2 or 13 mg DON/kg diet, respectively) from mid-gestation through the first 45 days of lactation. No significant changes were noted in feed intake, heifer weight gain, or calf body weights. Calf weight gains were higher for calves nursing heifers fed the DON-contaminated barley during calving and lactation. In a 3-year follow-up of the experimental heifers retained in the herd, all 14 control heifers had been culled for non-pregnancy after 3 years, whereas only 8 vomitoxin-fed heifers had been culled for non-pregnancy suggesting feeding DON had no impact on subsequent reproduction (Anderson, 2006). The authors concluded that barley unsuitable for human food could be utilized in beef rations and fed safely at 10–12 mg DON/kg diet to pregnant cows.

Trenholm *et al*. (1985) reported an initial slight decrease in feed intake of dry cows fed a wheat–oats-contaminated diet with 6.4 mg DON/kg concentrate. The cows returned to normal feed consumption by the end of the sixth week of exposure, with no differences reported in body weight. The only trichothecene mycotoxin apparently tested in the contaminated wheat was DON, so the presence of additional mycotoxins could not be discounted. Charmley *et al*. (1993) fed primparous Holstein cows DON-contaminated wheat and corn at increasing concentrations of 0.07, 4.95, and 12.09 mg DON/kg of dry matter concentrate (daily

DON intake of 0.59, 42, and 104 mg, respectively) for 10 weeks in a lactation study. The total mixed ration was formulated to support 25 kg milk/day and a milk fat level of 3.8%. No changes were observed in total milk output or intake of concentrate or forage. Cows fed 4.9 mg DON/kg concentrate had the lowest milk fat content, which the authors did not attribute to DON intake. Similar results were reported by Ingalls (1996) who fed high-producing dairy cows DON-contaminated barley diets up to 14.6 mg DON/kg concentrate dry matter (about 8.5 ppm in diet, 0.31 mg DON/kg body weight) for 3 weeks and found no significant changes in milk production or feed intake between controls and treated cows.

Oral T-2 toxin dosing studies in calves resulted in more severe clinical signs. Pier *et al*. (1976) dosed young Jersey calves, 35–78 kg, with oral gelatin capsules for 30 days at 0.08, 0.16, 0.32, and 0.64 mg T-2 toxin/kg body weight (one calf per dose level). The authors estimated that the lower T-2 doses were realistic, as a 50 kg calf would have to eat 2 kg of corn containing 2 mg T-2 toxin/kg to mimic the lowest dose of 0.08 mg T-2 toxin/kg feed. All T-2 toxin dosages caused mild enteritis and loose feces in calves. At the higher dosage levels of 0.32 and 0.64 mg/kg, bloody feces were apparent, and the calves became anorexic, dehydrated, and lost body weight. The calves had elevated serum aspartate aminotransferase activities and prolonged prothrombin times. The high dose calf died on day 20 of the trial. Post-mortem lesions in the highest T-2 toxin dosed calves were ruminal ulcers and abomasitis, while the lower dosed calves had mild enteritis. Abomasal ulcers were described in the calf dosed at 0.16 mg T-2 toxin/kg body weight.

Osweiler *et al.* (1981) dosed beef calves (85–200 kg) orally with 0.1, 0.3, and 0.6 mg purified T-2 toxin/kg body weight/day for 6 weeks, and found that 0.3 mg/kg (mimic ~10 mg T-2/kg diet) decreased feed consumption with no outwards signs of clinical disease. A clinically effective dose of 0.6 mg/kg (mimic ~20 mg T-2/kg diet) produced marked differences in body weight gain, as compared with control animals. All calves in the high dose group (0.6 mg T-2 toxin/kg body weight) refused to eat the concentrate portion of their diet starting on the second day of the trial and consumed a limited portion of diet provided throughout the entire 6 weeks of T-2 toxin exposure. The high T-2 toxin dosed calves had intermittent diarrhea, soft tan to dark, semi-fluid feces, and became thin with rough hair coats. Calves dosed with 0.3 mg/kg generally showed a lack of interest in eating the total amount of feed provided. No coagulopathies were observed in the T-2 toxin treated calves. The authors questioned whether cattle would consume T-2-toxin-contaminated feed in the range of 10–20 mg/kg for a sufficient time to produce clinical signs.

Weaver *et al*. (1977) intubated daily a pregnant Holstein cow (third trimester of gestation) with 182 mg of purified T-2 toxin for 15 days. On the fourth day of dosing the cow delivered a bull calf that was also intubated with T-2 toxin at 0.6 mg/kg body weight for 7 consecutive days, and then on alternate days for a total of 16 days treatment. The calf became severely depressed, with hind-quarter ataxia, knuckling of rear feet, and anorexia. Complete blood counts, serum total protein, and several liver enzymes were within the normal range for both cow and calf throughout the treatment. Gross and microscopic lesions were seen only in the cow and included congestion of the small intestine mucosa and a ruminal ulcer. Histological changes were congestion of the lamina proprial blood vessels of the omasum, rumen, jejunum, and ileum, and edema of the submucosa, muscular layers, and serosa of the cecum and colon. The bone marrows of both animals were normal.

In a field case, anorexia, periodic elevated temperatures, abortions in mid-gestation, and a 20% death loss were associated with feeding moldy, high moisture corn to lactating Holstein dairy cows during a 5-month period in late winter (Hsu *et al*., 1972). Post-mortem lesions of extensive hemorrhages on serosal surface of internal viscera were reported. Among the fungi cultured from the corn were *F. tricinctum*, *F roseum*, and *F. moniliforme* and various *Penicillium* spp. A sample of feed taken in late May contained 2 mg T-2 toxin/kg, which the authors estimated as a low concentration because of losses during analysis. The authors assumed other toxins were also present and contributed to clinical toxicity, but were not analyzed.

Stachybotryotoxicosis in cattle occurred mainly as large outbreaks in young animals (Dankó, 1975; Hintikka, 1978). Clinical signs included lethargy, inappetance, bloody diarrhea, and epistaxis. In chronic toxicosis, changes in the hematology appeared to be the initial toxic event. After a leukocytosis of short duration, leukopenia and thrombocytopenia developed. Clinical signs appeared gradually with hematological changes and were similar to those seen in the "typical" form of horses. Cows could abort during the second half of gestation with hemorrhagic lesions apparent in the fetus and fetal membranes. Mortality could be as high as 90–100% in affected cattle. Toxicosis in sheep was similar to cattle. Additionally, sheep exhibited hyperthermia and progressive anemia with marked impairment of the lymphoid system (Schneider *et al.*, 1979) and wool loss with skin hemorrhaging (Hajtós *et al*., 1983).

Dankó (1975) commented that animals preferred to eat *Stachybotrys*-contaminated straw rather than good-quality hay because of a flavor produced by the mold in the straw, but this observation was not supported in other literature. *Stachybotrys* toxins appeared more toxic in an acidic pH (Dankó, 1975). Therefore, ruminants were considered relatively resistant because of salivation and the higher pH environment of the rumen. Feeding a high carbohydrate diet, which lowers rumen pH, could enhance

the toxic effects of *Stachybotrys*. Treatment of animals affected with *Stachybotrys* mycotoxins was generally unrewarding due to severe damage to the lymphoid system. Immunosuppression of both humoral and cellular systems was an important clinical effect from toxins of *Stachybotrys*.

Ovine responses to trichothecene exposure are similar to cattle. Seven crossbred lambs (mean weight of 17.9 kg) between 3 and 6 months of age consumed a DON-contaminated wheat ration (15.6 mg DON/kg) for 28 days with no significant changes in behavior, clinical signs, feed consumption, hematology, or serum clinical chemistries compared to a control group (Harvey *et al.*, 1986). In an experiment using purified DAS, crossbred ewe lambs (average 38 kg) were fed *ad libitum* 5.0 mg DAS/kg diet for 34 days (Harvey *et al.*, 1995). The lambs developed diarrhea during the first week of the study, with a significant decrease in feed intake and weight gains by the end of the exposure period. No dermal or oral lesions were observed in DAS treated lambs, nor were gross lesions observed at necropsy of the lambs. The authors fed lambs a diet contaminated with both aflatoxin at 2.5 mg/kg feed and DAS at 5.0 mg/kg feed and noted a synergistic response in the marked reductions in feed intake and weight gains.

Friend *et al.* (1983) dosed 6–8-week-old male lambs with purified T-2 toxin in gelatin capsules at 0, 0.3, and 0.6 mg/kg body weight for 21 days. All lambs had bouts of diarrhea, but several lambs dosed with 0.6 mg T-2 toxin/kg body weight had prolonged periods of diarrhea, appeared unthrifty, and started fleece-pulling at the end of 3 weeks of treatment. At necropsy, T-2 treated lambs appeared to have smaller and less active thymic tissue, as compared to the control group, and lesions of focal rumenitis with parakeratosis and infiltration by polymorphonuclear cells.

### Poultry

Poultry are not as sensitive to the effects of trichothecenes as swine, but are probably more sensitive than ruminants. Day-old chickens fed DON-contaminated feed at 9 or 18 mg DON/kg diet for 7 weeks had decreased relative and absolute liver weights, but showed no changes in feed intake (Kubena *et al.*, 1985). Chickens fed high DON concentrations in rations (>15 mg DON/kg) exhibited feed refusal and weight loss (Kubena *et al.*, 1987; Kubena and Harvey, 1988). DON contamination of rations up to concentrations of 18 mg/kg feed provided to laying hens did not affect hatchability of eggs (Lun *et al.*, 1986; Bergsjo *et al.*, 1993b). Bergsjo *et al.* (1993a) fed a naturally contaminated oat diet to White Leghorn laying hens at concentrations of 0.12–4.9 mg DON/kg feed for 70 days. Low concentrations of 3-ADON (0–0.63 mg/kg) and zearalenone (trace to 0.55 mg/kg) were detected in the diets. No significant differences in feed intake, weight gain, egg production, body weight at hatching, and viability of the chicks were found between treatment groups.

Purified T-2 toxin added to a commercial laying hen ration at 20 mg T-2 toxin/kg diet for 3 weeks caused raised oral lesions, yellowish-white to gray in color, during the first week of T-2 toxin administration on the sublingual and palatine areas, and then on the tongue and corners of the mouth (Wyatt *et al.*, 1975). Feed consumption by hens decreased by about 25% compared with control diet intake during the first week of T-2 toxin exposure, with the treated laying hens showing a significant weight loss after 1 week of the trial. Egg production of the T-2 toxin treated hens declined by 20% in the second and the third week of exposure; the eggshell thickness was significantly decreased after 3 weeks of T-2 toxin exposure.

In a study of 27-week-old laying hens fed *ad libitum* diets containing purified T-2 toxin at 0 (control), 0.5, 1, 2, 4, and 8 mg/kg feed for 8 weeks, the highest dosage caused a significant decrease in feed consumption, egg production, and shell thickness, as compared with hens fed the control diet (Chi *et al.*, 1977). Fertility and progeny performance were not altered by feeding T-2 toxin, but hatchability of fertile eggs was significantly decreased in hens fed 2 and 8 mg/kg, as compared with hens fed the control diet. Oral lesions, circumscribed and proliferative plaques, were noticed after the second week in hens fed 4 and 8 mg/kg and after the third week in hens fed 0.5 mg/kg. Severity and incidence of lesions were proportional to dietary T-2 toxin concentration. No significant histopathological changes were observed in the liver, heart, kidney, brain, or bone marrow, but necrotic lesions were observed in the gizzard and crop. Diaz *et al.* (1994) fed hens both T-2 toxin and DAS, each at 2 mg/kg feed in the diet. Hens exhibited oral lesions, a significant decrease in feed consumption (decline of about 12%) and egg production (decline of 15%), as compared with controls.

Generally, concentrations of T-2 and HT-2 toxin greater than 1 mg/kg are not common in grains except in wet years in the upper Mid-west of the United States; often, HT-2 toxin is found at higher concentrations than T-2 toxin. Rafai *et al.* (2000) evaluated such relatively low levels (0, 0.2, 0.4, 0.6, 0.8, 1, 2, 3, and 4 mg T-2 toxin/kg diet) of exposure for 7 weeks in day-old Pekin ducklings. Within 2 days after starting the experiment, most ducks fed T-2 toxin developed oral lesions. The dermatotoxic lesions gradually disappeared in the ducks fed less than 3 mg T-2 toxin/kg feed. Ducks fed 3 and 4 mg T-2 toxin/kg feed refused the diet in the first week and from weeks 3 to 7. The feed intakes of ducks fed 0.6–4 mg T-2 toxin/kg feed were generally less than that of the control group. No significant dose-related changes were noted in serum chemistry and hematology. Similar dose-related oral lesions were reported in day-old Muscovy ducks

within 16 h of exposure to T-2 toxin and DAS at 0.25, 0.5, or 1 mg/kg feed (Shlosberg *et al.*, 1986). Decreased body weights, thymus, spleen, and bursa of Fabricius weights were noted in 6-week-old mallards fed 2 mg T-2 toxin/kg feed for 9 days (Neiger *et al.*, 1994). Microscopic lesions were observed in lymphoid organs and included a moderate decrease in thymic cortical lymphocytes.

### *New trichothecene concern*

In North America during the mid-1990s, interest focused on *Stachybotrys chartarum* or black mold found in damp buildings as a possible cause of idiopathic pulmonary hemorrhage in infants (Dearborn *et al.*, 1999). Epidemiological investigations of pulmonary hemorrhage in infants found an association with airborne fungi including *Stachybotrys*. *S. chartarum* produces a variety of secondary fungal metabolites, including simple trichothecenes (trichodermol, trichodermin, and verrucarol), macrocyclic trichothecenes (verrucarins B and J; roridins D, E, isoE, and L-2; the satratoxins F, isoF, G, isoG, H, and isoH), and spirocyclic drimanes that are recognized immunosuppressants (Jarvis, 2003). *S. chartarum* appears to divide into two chemotypes, one produces the macrocyclic trichothecenes and the other produces a class of diterpenoids, the atranones that to date have not been shown to possess significant biological activity (Jarvis, 2003). The characterization of natural products from *Stachybotrys* species and any relationship to clinical disease remains a challenge.

## TREATMENT

The first action is to stop exposure to moldy feed. If using screenings or poor-quality grain, cleaning the grain by removing broken, shriveled kernels and washing the grain can lower mycotoxin contamination. Generally, clinical signs of feed refusal will disappear within 7 days after removal of the contaminated feed. Animals return to production within 14 days.

No specific therapies for trichothecene mycotoxicoses are available. Some trichothecenes undergo enterohepatic recirculation and are excreted in the feces. The use of activated charcoal, which binds toxins within the gastrointestinal tract and prevents toxin reabsorption, plus magnesium sulfate, was beneficial as part of a therapeutic protocol for treatment of acute, T-2 toxicosis in swine (Poppenga *et al.*, 1987). Metoclopramide, given intravenously immediately prior to and following T-2 toxin administration, was only partially successful as an antiemetic (Poppenga *et al.*, 1987). A number of binders, such as clay and zeolitic products, have been suggested for use with trichothecene-contaminated feed to prevent absorption by animals. Their efficacy has not been proven and marked species variations exist. The US Food and Drug Administration has not approved any ingredient for use as a trichothecene mycotoxin binder.

## CONCLUSIONS

Trichothecene mycotoxins occur worldwide however both total concentrations and the particular mix of toxins present vary dramatically with environmental conditions. Proper agricultural practices such as avoiding late harvests, removing overwintered stubble from fields, and avoiding a corn/wheat rotation that favors *Fusarium* growth in residue can reduce trichothecene contamination of grains. Storage of grains at less than 13–14% moisture (less than $0.70a_W$) and hay/straw at less than 20% moisture are important in preventing trichothecenes production. Once produced, trichothecenes are stable compounds and can remain present at toxic concentrations in feed for years. Field reports of trichothecene toxicity in livestock in North America generally involve DON contamination of feed. Acute, high dose exposures are not common. More typically, chronic to sub-chronic exposures to low dose DON-contaminated feed in swine occurs and leads to nutritional impairment, poor production, and possible immune susceptibility to infections.

Due to the vague nature of toxic effects attributed to low concentrations of trichothecenes, a solid link between low level exposure and a specific trichothecene(s) is difficult to establish. Mold identification and determination of fungal spores can indicate feedstuffs of deteriorated quality, but provide no information as to the presence or concentration of mycotoxins that actually cause adverse health effects. Multiple factors, such as nutrition, management, and environmental conditions impact animal health and need to be evaluated with the knowledge of the mycotoxin(s) and concentrations known to cause adverse health effects. Future research evaluating the impact(s) of low level exposure on livestock may clarify the potential impact on immunity.

Trichothecenes are rapidly excreted from animals, and residues in edible tissues, milk, or eggs are likely negligible. In chronic exposures to trichothecenes, once the contaminated feed is removed and exposure stopped, animals generally have an excellent prognosis for recovery (Prelusky *et al.*, 1994).

## REFERENCES

Accensi F, Pinton P, Callu P, Abella-Bourges N, Guelfi J-F, Grosjean F, Oswald IP (2006) Ingestion of low doses of deoxynivalenol does

not affect hematological, biochemical, or immune responses of piglets. *J Anim Sci* **84**: 1935–42.

Anderson V (2006) Personal communication.

Anderson VL, Boland EW, Casper HH (1996) Effects of vomitoxin (deoxynivalenol) from scab infested barley on performance of feedlot and breeding cattle. *J Anim Sci* **74**: 208.

Azcona-Olivera JI, Ouyang Y, Warner RL, Linz JE, Pestka JJ (1995) Induction of cytokine mRNAs in mice after oral exposure to the trichothecene vomitoxin (deoxynivalenol): relationship to toxin distribution and protein synthesis inhibition. *Toxicol Appl Pharmacol* **133**: 109–20.

Bamburg JR, Riggs NV, Strong FM (1968) The structures of toxins from two strains of *Fusarium tricinctum. Tetrahedron* **24**: 3329–36.

Bata A, Harrach B, Ujszaszi K, Kis-Tamas A, Lasztity R (1985) Macrocyclic trichothecene toxins produced by *Stachybotrys atra* strains isolated in Middle Europe. *Appl Environ Microbiol* **49**: 678–81.

Bergsjo B, Matke T, Napstad I (1992) Effects of diets with graded levels of deoxynivalenol on performance of growing pigs. *J Vet Med A* **39**: 752–8.

Bergsjo B, Herstad O, Nafstad I (1993a) Effects of feeding deoxynivalenol-contaminated oats on reproductive performance in White Leghorn hens. *Br Poult Sci* **34**: 147–59.

Bergsjo B, Langseth W, Nafstad I, Jansen JH, Larsen HJS (1993b) The effects of naturally deoxynivalenol-contaminated oats on the clinical condition blood parameters, performance, and carcass composition of growing pigs. *Vet Res Commun* **17**: 283–94.

Bhat RV, Beedu SR, Ramakrishna Y, Munshi KL (1989) Outbreak of trichothecene mycotoxicosis associated with consumption of mould-damages wheat production in Kashmir Valley, India. *Lancet* **8628**: 35–7.

Bondy GS, Pestka JJ (2000) Immunomodulation by fungal toxins. *J Toxicol Environ Health B* **3**: 109–43.

Bondy GS, McCormick SP, Beremand MN, Pestka JJ (1991) Murine lymphocyte proliferation impaired by substituted neosolaniols and calonectrins – *Fusarium* metabolites associated with trichothecene biosynthesis. *Toxicon* **29**: 1107–13.

Borison HL, Goodheart ML (1989) Neural factors in acute emetic, cardiovascular, and respiratory effects of T-2 toxin in cats. *Toxicol Appl Pharmacol* **101**: 399–413.

Bottex C, Martin A, Fontanges R (1990) Action of a mycotoxin (diacetoxyscirpenol) on the immune response of the mouse – interaction with an immunomodulator (OM-89). *Immunopharmacol Immunotoxicol* **12**: 311–25.

Brake J, Hamilton PB, Kittrell RS (1999) Effects of the tricothecene mycotoxin diacetoxyscirpenol on fertility and hatchability of broiler breeders. *Poult Sci* **78**: 1690–4.

Buening GM, Mann DD, Hook B, Osweiler GD (1982) The effect of T-2 toxin on the bovine immune system: cellular factors. *Vet Immunol Immunopathol* **3**: 411–17.

Bunner DL, Morris ER (1988) Alteration of multiple cell membrane functions in L-6 myoblasts by T-2 toxin: an important mechanism of action. *Toxicol Appl Pharmacol* **92**: 113–21.

CAST (2003) *Mycotoxins: Risks in Plant, Animal, and Human Systems*. Task Force Report No. **139**. Council for Agriculture, Science and Technology, Ames, IA.

Charmley E, Trenholm HL, Thompson BK, Vudathala D, Nicholson JWG, Prelusky DB, Charmley LL (1993) Influence of level of deoxynivalenol in the diet of dairy cows on feed intake, milk production, and its composition. *J Dairy Sci* **76**: 3580–7.

Chavez ER (1984) Vomitoxin-contaminated wheat in pig diets: pregnant and lactating gilts and weaners. *Can J Anim Sci* **64**: 717–23.

Chavez ER, Rheaume JA (1986) The significance of the reduced feed consumption observed in growing pigs fed vomitoxin-contaminated diets. *Can J Anim Sci* **66**: 277–87.

Chi MS, Mirocha CJ, Kurtz HJ, Weaver G, Bates F, Shimoda W (1977) Effects of T-2 toxin on reproductive performance and health of laying hens. *Poultry Sci* **56**: 628–37.

Chowdhury SR, Smith TK, Boermans HJ, Woodward B (2005a) Effects of feed-borne *Fusarium* mycotoxins on hematology and immunology of turkeys. *Poultry Sci* **84**: 1698–706.

Chowdhury SR, Smith TK, Boermans HJ, Sefton AE, Downey R, Woodward B (2005b) Effects of feeding blends of grains naturally contaminated with *Fusarium* mycotoxins on performance, metabolism, hematology, and immunocompetence of ducklings. *Poultry Sci* **84**: 1179–85.

Cole RJ, Cox RH (1981) *Handbook of Toxic Funal Metabolites*. Academic Press, New York.

Coppock RW, Swanson SP, Gelberg HB, Koritz GD, Hoffman WE, Buck WB, Vesonder RF (1985a) Preliminary study of the pharmacokinetics and toxicopathy of deoxynivalenol (vomitoxin) in swine. *Am J Vet Res* **46**: 169–74.

Coppock RW, Gelberg HB, Hoffmann WE, Buck WB (1985b) The acute toxicopathy of intravenous diacetoxyscirpenol (anguidine) administered in swine. *Fundam Appl Toxicol* **5**: 1034–49.

Coppock RW, Hoffmann WE, Gelberg HB, Bass D, Buck WB (1989) Hematologic changes induced by intravenous administration of diacetoxyscirpenol in pigs, dogs and calves. *Am J Vet Res* **50**: 411–15.

Corley RA, Swanson SP, Buck WB (1985) Glucuronide conjugates of T-2 toxin and metabolites in swine bile and urine. *J Agric Food Chem* **33**: 1085–9.

Corrier DE (1991) Mycotoxins: mechanisms of immunosuppression. *Vet Immunol Immunopathol* **30**: 73–87.

Côté L-M, Beasley VR, Bratich PM, Swanson SP, Shivaprasad JL, Buck WB (1985) Sex related reduced weight gains in growing swine fed diets containing deoxynivalenol. *J Anim Sci* **61**: 942–50.

Côté L-M, Dahlem AM, Yoshizawa T, Swanson SP, Buck WB (1986) Excretion of deoxynivalenol and its metabolite in milk, urine and feces of lactating dairy cows. *J Dairy Sci* **69**: 2416–23.

Dänicke S, Gareis M, Bauer J (2001) Orientation values for critical concentrations of deoxynivalenol and zearalenone in diets for pigs, ruminants and gallinaceous poultry. *Proc Soc Nutr Physiol* **10**: 171–4.

Dänicke S, Valenta H, Döll S (2004a) On the toxicokinetics and the metabolism of deoxynivalenol (DON) in the pig. *Arch Anim Nutr* **58**: 169–80.

Dänicke S, Valenta H, Klobasa F, Döll S, Ganter M, Flachowsky G (2004b) Effects of graded levels of *Fusarium* toxin contaminated wheat in diets for fattening pigs on growth, performance, nutrient digestibility, deoxynivalenol balance and clinical serum characteristics. *Arch Anim Nutr* **58**: 1–17.

Dänicke S, Matthaus K, Lebzien P, Valenta H, Ueberschar KH, Razzazi-Fazeli E, Bohm J, Flachowsky G (2005) Effects of *Fusarium* toxin-contaminated wheat grain on nutrient turnover, microbial protein synthesis and metabolism of deoxynivalenol and zearalenone in the rumen of dairy cows. *J Anim Physiol Anim Nutr (Berlin)* **89**: 303–15.

Dänicke S, Goyarts T, Döll S, Grove N, Spolder M, Flachowsky G (2006) Effects of the *Fusarium* toxin deoxynivalenol on tissue protein synthesis in pigs. *Toxicol Lett* **165**: 297–311.

Dankó G (1975) Stachybotryotoxicosis and immunosuppression. *Int J Environ Stud* **8**: 209–11.

Dearborn DG, Yike I, Sorenson WG, Miller MJ, Etzel RA (1999) Overview of investigations into pulmonary hemorrhage among infants in Cleveland, Ohio. *Environ Health Perspect* **107**(Suppl. 3): 495–9.

Diaz GJ, Squires EJ, Julian RJ, Boermans HJ (1994) Individual and combined effects of T-2 toxin and DAS in laying hens. *Br Poult Sci* **35**: 393–405.

DiCostanzo A, Johnston L, Felice L, Murphy M (1994) Feeding vomitoxin and mold-contaminated grains to cattle. *Proceedings of the 55th Minnesota Nutrition Conference and Roche Technical Symposium*, University of Minnesota, Bloomington, MN, pp. 193–216.

Dong D, Davis RJ, Flavell RA (2002) MAP kinases in the immune response. *Annu Rev Immunol* **20**: 55–72.

Dong W, Sell JE, Pestka JJ (1991) Quantitative assessment of mesangial immunoglobulin A (IgA) accumulation, elevated circulating IgA immune complexes, and hematuria during vomitoxin-induced IgA nephropathy. *Fundam Appl Toxicol* **17**: 197–207.

Ehrlich KC, Daigle KW (1987) Protein synthesis inhibition by 8-oxo-12,13-epoxytrichothecenes. *Biochim Biophys Acta* **923**: 206–13.

El-Banna AA, Hamilton RMG, Scott PM, Trenholm HL (1983) Nontransmission of deoxynivalenol (vomitoxin) to eggs and meat in chickens fed deoxynivalenol-contaminated diets. *J Agric Food Chem* **31**: 1381–4.

Ellison RA, Kotsonis FN (1974) *In vitro* metabolism of T-2 toxin. *Appl Microbiol* **27**: 423–4.

Eriksen GS, Pettersson H (2004) Toxicological evaluation of trichothecenes in animal feed. *Anim Feed Sci Technol* **114**: 205–39.

Eriksen GS, Pettersson H, Lindberg JE (2003) Absorption, metabolism and excretion of 3-acetyl DON in pigs. *Arch Anim Nutr* **57**: 335–45.

Fioramonti J, Fargease MJ, Bueno L (1987) Action of T-2 toxin on gastrointestinal transit in mice: protective effect of an argillaceous compound. *Toxicol Lett* **36**: 227–32.

Fioramonti J, Dupuy D, Dupuy J, Bueno L (1993) The mycotoxin, deoxynivalenol, delays gastric emptying through serotonin-3 receptors in rodents. *J Pharmacol Exp Ther* **266**: 1255–60.

Forgacs J (1972) Stachybotryotoxicosis. In *Microbial Toxins*, vol. 8, Kadis S, Ciegler A, Ajl SJ (eds). Academic Press, New York, pp. 95–128.

Forsell JH, Witt MF, Tai JH, Jensen R, Pestka JJ (1986) Effects of 8-week exposure of the B6C3F1 mouse to dietary deoxynivalenol (vomitoxin) and zearalenone. *Food Chem Toxicol* **24**: 213–19.

Forsyth DM, Yoshizawa T, Morooka N, Tuite J (1977) Emetic and refusal activity of deoxynivalenol to swine. *Appl Environ Microbiol* **34**: 547–52.

Francis BM (1989) Reproductive toxicology of trichothecenes. In *Trichothecene Mycotoxicosis: Pathophysiologic Effects*, vol. I, Beasley VR (ed.). CRC Press, Inc., Boca Raton, FL, pp. 143–59.

Friend DW, Trenholm HL, Elliot JI, Thompson BK, Hartin KE (1982) Effect of feeding vomitoxin-contaminated wheat to pigs. *Can J Anim Sci* **62**: 1211–22.

Friend DW, Thompson BK, Trenholm HL, Hartin KE, Prelusky DB (1986) Effect of feeding diets containing deoxynivalenol (vomitoxin)-contaminated wheat or corn on the feed consumption, weight gain, organ weight and sexual development of male and female pigs. *Can J Anim Sci* **66**: 765–75.

Friend DW, Thompson BK, Trenholm HL, Boermans HJ, Hartin KE, Panich PL (1992) Toxicity of T-2 toxin and its interaction with deoxynivalenol when fed to young pigs. *Can J Anim Sci* **72**: 703–11.

Friend SCE, Hancock DS, Schiefer HB, Babiuk LA (1983) Experimental T-2 toxicosis in sheep. *Can J Comp Med* **47**: 291–7.

Gauvreau HC (1991) Toxicokinetic, tissue residue and metabolite studies of deoxynivalenol (vomitoxin) in turkeys. MSc Thesis, Simon Fraser University, Vancouver.

Gentry PA, Cooper ML (1981) Effect of *Fusarium* T-2 toxin on hematological and biochemical parameters in the rabbit. *Can J Comp Med* **45**: 400–5.

Gentry PA, Ross ML, Chan PK-C (1984) Effect of T-2 toxin on bovine hematological and serum enzyme parameters. *Vet Hum Toxicol* **26**: 24–8.

Glávits R, Ványi A (1995) More important mycotoxicoses in pigs. Comprehensive clinico-pathological communication. *Magy Állatorvosok Lapja* **50**: 407–20.

Glávits R, Gabriella S, Sándor S, Ványi A, Gajdás GY (1983) Reproductive disorders caused by trichothecene mycotoxins in a large-scale pig herd. *Acta Vet Hung* **31**: 173–80.

Goyarts T, Dänicke S (2006) Bioavailability of the *Fusarium* toxin deoxynivalenol (DON) from naturally contaminated wheat for the pig. *Toxicol Lett* **163**: 171–82.

Goyarts T, Dänicke S, Rothkötte HJ, Spilke J, Tiemann U, Schollenberger M (2005) On the effects of a chronic deoxynivalenol intoxication on performance, haematological and serum parameters of pigs when diets are offered either for *ad libitum* consumption or fed restrictively. *J Vet Med A* **52**: 305–14.

Grove JF (1988) Non-macrocyclic trichothecenes. *Nat Prod Rep* **5**: 187–209.

Grove JF (2000) Non-macrocyclic trichothecenes (Part 2). *Prog Chem Org Nat Prod* **69**: 1–70.

Hajtós I, Harrach B, Szigeti G, Fodor L, Malik G, Varga J (1983) Stachybotryotoxicosis as a predisposing factor of ovine systemic pasteurellosis. *Acta Vet Hung* **31**: 181–8.

Harvey RB, Kubena LF, Corrier DE, Witzel DA, Phillips TD, Heidenbaugh ND (1986) Effects of deoxynivalenol in a wheat ration fed to growing lambs. *Am J Vet Res* **47**: 1630–2.

Harvey RB, Kubena LF, Huff WE, Corrier DE, Rottinghaus GE, Phillips TD (1990) Effects of treatment of growing swine with aflatoxin and T-2 toxin. *Am J Vet Res* **51**: 1688–93.

Harvey RB, Kubena LF, Huff WE, Elissalde MH, Phillips TD (1991) Hematologic and immunologic toxicity of deoxynivalenol (DON)-contaminated diets to growing chickens. *Bull Environ Contam Toxicol* **46**: 410–16.

Harvey RB, Kubena LF, Elissalde MH, Rottinghaus GE, Corrier DE (1994) Administration of ochratoxin A and T-2 toxin to growing swine. *Am J Vet Res* **55**: 1757–61.

Harvey RB, Edrington TS, Kubena LF, Elissalde MH, Corrier DE, Rottinghaus GE (1995) Effect of aflatoxin and diacetoxyscirpenol in ewe lambs. *Bull Environ Contam Toxicol* **54**: 325–30.

Hayes MA, Schiefer HB (1982) Comparative toxicity of dietary T-2 toxin in rats and mice. *J Appl Toxicol* **2**: 207–12.

Hayes MA, Bellamy JEC, Schiefer HB (1980) Subacute toxicity of dietary T-2 toxin in mice, morphological and hematological effects. *Can J Comp Med* **44**: 203–18.

He P, Young LG, Forsberg C (1992) Microbial transformation of deoxynivalenol (vomitoxin). *Appl Environ Microbiol* **58**: 3857–63.

Hedman R, Pettersson H (1997) Transformation of nivalenol by gastrointestinal microbes. *Arch Anim Nutr* **50**: 321–9.

Hintikka E-L (1978) Stachybotryotoxicosis in horses. In *Mycotoxic Fungi, Mycotoxins, Mycotoxicoses. Vol. 2. Mycotoxicoses of Domestic and Laboratory Animals, Poultry, and Aquatic Invertebrates and Vertebrates*, Wyllie TD, Morehouse LG (eds). Marcel Dekker, New York, pp. 181–5.

Hoerr FJ, Carlton WW, Yagen B (1981) Mycotoxicosis caused by a single dose of T-2 toxin or diacetoxyscirpenol in broiler chickens. *Vet Pathol* **5**: 652–64.

Holladay SD, Blaylock BL, Comment CE, Heindel JJ, Luster MI (1993) Fetal thymic atrophy after exposure to T-2 toxin: selectivity for lymphoid progenitor cells. *Toxicol Appl Pharmacol* **121**: 8–14.

Hsia CC, Wu JL, Lu XQ, Li YS (1988) Natural occurrence and clastogenic effects of nivalenol, deoxynivalenol, 3-acetyl-deoxynivalenol, 15-acetyl-deoxynivalenol, and zearalenone in corn from a high-risk area of esophageal cancer. *Cancer Detect Prev* **13**: 79–86.

Hsu I-C, Smalley EB, Strong FM, Ribelin WE (1972) Identification of T-2 toxin in moldy corn associated with a lethal toxicosis in dairy cattle. *Appl Microbiol* **24**: 684–90.

Hughes DM, Gahl MJ, Graham CH, Grieb SL (1999) Overt signs of toxicity to dogs and cats of dietary deoxynivalenol. *J Anim Sci* **77**: 693–700.

Huszenicza G, Fekete S, Szigeti G, Kulcsar M, Febel H, Kellems RO, Nagy P, Cseh S, Veresegyhazy T, Hullar I (2000) Ovarian consequences of low dose peroral *Fusarium* (T-2) toxin in a ewe and heifer model. *Theriogenology* **53**: 1631–9.

Ingalls JR (1996) Influence of deoxynivalenol on feed consumption by dairy cows. *Anim Feed Sci Tech* **60**: 297–300.

Ishii K, Sakai K, Ueno Y, Tsunoda H, Enomoto M (1971) Solaniol, a toxic metabolite of *Fusarium solani*. *Appl Microbiol* **22**: 718–20.

Islam W, Nagase M, Yoshizawa T, Yamauchi K, Sakato N (1998) T-2 toxin induces thymic apoptosis *in vivo* in mice. *Toxicol Appl Pharmacol* **148**: 205–14.

Iverson F, Armstrong C, Nera E, Truelove J, Fernie S, Scott P, Stapley R, Hayward S, Gunner S (1995) Chronic feeding study of deoxynivalenol in B6C3F1 male and female mice. *Teratog Carcinog Mutag* **15**: 283–306.

Jacobsen BJ, Harlin KS, Swanson SP, Lambert RJ, Beasley VR, Sinclair JB, Wei LS (1995) Occurrence of fungi and mycotoxins associated with field mold damaged soybeans in the Midwest. *Plant Dis* **79**: 86–8.

Jarvis BB (2003) *Stachybotrys chartarum*: a fungus for our time. *Phytochemistry* **64**: 53–60.

JECFA (2001) Trichothecenes. In *Safety Evaluation of Certain Mycotoxins in Food*. Joint FAO/WHO Expert Committee on Food Additives, FAO Food and Nutrition Paper 74/WHO Food Additives Series 47. World Health Organization, Geneva, pp. 419–680.

Ji GE, Park SY, Wong SS, Pestka JJ (1998) Modulation of nitric oxide, hydrogen peroxide and cytokine production in a clonal macrophage model by the trichothecene vomitoxin (deoxynivalenol). *Toxicology* **125**: 203–14.

Joffe AZ (1974) Toxicity of *Fusarium poae* and *F. sporotrichioides* and its relation to alimentary toxic aleukia. In *Mycotoxins*, Purchase IFH (ed.). Elsevier, New York, pp. 229–62.

Johnson PJ, Casteel SW, Messer NT (1997) Effect of feeding deoxynivalenol (vomitoxin)-contaminated barley to horses. *J Vet Diagn Invest* **9**: 219–21.

Jones TC, Hunt RD, King NW (1997) *Veterinary Pathology*, 6th edn. Williams & Wilkins, Baltimore, MD, pp. 1125.

Juhasz J, Nagy P, Huszenicza G, Szigeti G, Reiczigel J, Kulcsar M (1997) Long term exposure to T-2 *Fusarium* mycotoxin fails to alter luteal function, follicular activity and embryo recovery in mares. *Equine Vet J Suppl* **25**: 17–21.

Khera KS, Whalen C, Angers G, Vesonder RF, Keuiper-Goodman T (1982) Embryotoxicity of 4-deoxynivalenol (vomitoxin) in mice. *Bull Environ Contam Toxicol* **29**: 487–91.

Khera KS, Whalen C, Angers G (1986) A teratology study on vomitoxin (4-deoxynivalenol) in rabbits. *Food Chem Toxicol* **5**: 421–4.

Kiessling K-H, Pettersson H, Sandholm K, Olsen M (1984) Metabolism of aflatoxin, ochratoxin, zearalenone, and three trichothecenes by intact rumen fluid, rumen protozoa, and rumen bacteria. *Appl Environ Microbiol* **47**: 1070–3.

King RR, McQueen RE, Levesque D, Greenhalgh R (1984) Transformation of deoxynivalenol (vomitoxin) by rumen microorganisms. *J Agric Food Chem* **32**: 1181–3.

Kubena LF, Harvey RB (1988) Response of growing Leghorn chicks to deoxynivalenol-contaminated wheat. *Poult Sci* **67**: 1778–80.

Kubena LF, Swanson SP, Harvey RB, Fletcher OJ, Rowe LD, Phillips TD (1985) Effects of feeding deoxynivalenol (vomitoxin)-contaminated wheat to growing chicks. *Poult Sci* **64**: 1649–55.

Kubena LF, Harvey RB, Corrier DE, Huff WE, Phillips TD (1987) Effects of feeding deoxynivalenol (DON, vomitoxin)-contaminated wheat to female White Leghorn chickens from day old through egg production. *Poult Sci* **66**: 1612–18.

Kuiper-Goodman T (1994) Prevention of human mycotoxicoses through risk assessment and risk management. In *Mycotoxins in Grain: Compounds Other than Aflatoxin*, Miller JD, Trenholm HL (eds). Eagan Press, St. Paul, pp. 439–69.

Lafarge-Frayssinet D, Chakor K, Lafont P, Frayssinet C (1990) Transplacental transfer of T2-toxin: pathological effect. *J Environ Pathol Toxicol Oncol* **10**: 64–8.

Lun AK, Young LG, Moran ET, Hunter DB, Rodriguez JP (1986) Effects of feeding hens a high level of vomitoxin-contaminated corn on performance and tissue residues. *Poult Sci* **65**: 1095–9.

Lutsky I, Mor N, Yagen B, Joffe AZ (1978) The role of T-2 toxin in experimental alimentary toxic aleukia: a toxicity study in cats. *Toxicol Appl Pharmacol* **43**: 111–24.

Mann DD, Buening GM, Hook B, Osweiler GD (1983) Effect of T-2 mycotoxin on bovine serum proteins. *Am J Vet Res* **44**: 1757–9.

Mantle PG (1991) Miscellaneous toxigenic fungi. In *Mycotoxins in Animal Foods*, Smith JE, Henderson RS (eds). CRC Press, Inc., Boca Raton, FL, pp. 141–52.

Marasas WFO, Nelson PE, Toussoun TA (1984) *Toxigenic Fusarium Species*. The Pennsylvania State University Press, University Park, PA.

Matthaus K, Danicke S, Vahjen W, Simon O, Wang J, Valenta H, Meyer K, Strumpf A, Ziesenib H, Flachowsky G (2004) Progression of mycotoxin and nutrient concentrations in wheat after inoculation with *Fusarium culmorum. Arch Anim Nutr* **58**: 19–35.

McLaughlin CS, Vaughan MH, Campbell IM, Wei CM, Stafford ME, Hansen BS (1977) Inhibition of protein synthesis by trichothecenes. In *Mycotoxins in Human and Animal Health*, Rodricks JV, Hesseltine CW, Mehlman MA (eds). Pathotox Publishers, Inc., Park Forest South, IL, pp. 263–75.

Mekhancha-Dahel C, Lafarge-Frayssinet C, Frayssinet C (1990) Immunosuppressive effects of four trichothecene mycotoxins. *Food Addit Contam* **7**: S94–6.

Miller JD (2002) Aspects of the ecology of *Fusarium* toxins in cereals. In *Mycotoxins and Food Safety*, DeVries JW, Trucksess MW, Jackson LS (eds). *Adv Exp Med Biol* **54**: 19–27. Kluwer Academic/ Plenum Publishers, New York.

Miller JD, Greenhalgh R, Wang Y, Lu M (1991) Trichothecene chemotypes of three *Fusarium* species. *Mycologia* **83**: 121–30.

Moon Y, Pestka JJ (2002) Vomitoxin-induced cyclooxygenase-2 gene expression in macrophages mediated by activation of ERK and p38 but not JNK mitogen-activated protein kinases. *Toxicol Sci* **69**: 373–82.

Morooka N, Uratsuji N, Yoshizawa T, Yamamoto H (1972) [Studies on the toxic substances in barley infected with *Fusarium* spp.]. *J Food Hyg Soc (Japan)* **13**: 368–75 (in Japanese).

Mostrom MS, Tacke B, Lardy G (2005) Field corn, hail, and mycotoxins. *Proceedings of the North Central Conference of the American Association of Veterinary Laboratory Diagnosticians*, Fargo, ND.

Neiger RD, Johnson TJ, Hurley DJ, Higgins KF, Rottinghaus GE, Stahr HM (1994) The short-term effect of low concentrations of dietary aflatoxin and T-2 toxin on mallard ducklings. *Avian Dis* **38**: 738–43.

Niyo KA, Richard JL, Niyo Y, Tiffany LH (1988) Pathologic, hematologic, and serologic changes in rabbits given T-2 mycotoxin orally and exposed to aerosols of *Aspergillus fumigatus* conidia. *Am J Vet Res* **49**: 2151–60.

Osweiler GD, Hook BS, Mann DD, Buening GM, Rottinghaus GE (1981) Effects of T-2 toxin in cattle. *Proceedings of the United States Animal Health Association, 85th Annual Meeting*, St. Louis, MO, pp. 214–31.

Øvernes G, Matre T, Sivertsen T, Larsen HJS, Langseth W, Reitan LJ, Jansen JH (1997) Effects of diets with graded levels of naturally deoxynivalenol-contaminated oats on immune response in growing pigs. *J Vet Med A* **44**: 539–50.

Pace JG (1983) Effect of T-2 mycotoxin on the rat liver mitochondria electron transport system. *Toxicon* **21**: 675–80.

Pace JG, Watts MR, Canterbury WJ (1988) T-2 mycotoxin inhibits mitochondrial protein synthesis. *Toxicon* **26**: 77–85.

Pang VF, Schiefer HB, Beasley VR (1989) Effects on the integumentary system. In *Trichothecene Mycotoxicosis: Pathophysiologic Effects*, vol. II, Beasley VR (ed.). CRC Press, Inc., Boca Raton, FL, pp. 123–33.

Park JJ, Smalley EB, Chu FS (1996) Natural occurrence of *Fusarium* mycotoxins in field samples from the 1992 Wisconsin corn crop. *Appl Environ Microbiol* **62**: 1642–8.

Patterson DSP, Matthews JG, Shreeve BJ, Roberts BA, McDonald SM, Hayes AW (1979) The failure of trichothecene mycotoxins and whole cultures of *Fusarium tricinctum* to cause experimental haemorrhagic syndromes in calves and pigs. *Vet Rec* **105**: 252–5.

Pestka JJ, Bondy GS (1994) Immunotoxic effects of mycotoxins. In *Mycotoxins in Grain: Compounds Other than Aflatoxin*, Miller JD, Trenholm HL (eds). Eagan Press, St. Paul, pp. 339–58.

Pestka JJ, Smolinski AT (2005) Deoxynivalenol: toxicology and potential effects on humans. *J Toxicol Environ Health B* **8**: 39–69.

Pestka JJ, Moorman MA, Warner RL (1989) Dysregulation of IgA production and IgA nephropathy induced by the trichothecene vomitoxin. *Food Chem Toxicol* **27**: 361–8.

Pestka JJ, Yan D, King LE (1994) Flow cytometric analysis of the effects of *in vitro* exposure to vomitoxin (deoxynivalenol) on apoptosis in murine T, B and IgA+ cells. *Food Chem Toxicol* **32**: 1125–36.

Pestka JJ, Uzarski RL, Islam Z (2005) Induction of apoptosis and cytokine production in the Jurkat human T cells by deoxynivalenol: role of mitogen-activated protein kinases and comparison to other 8-ketotrichothecenes. *Toxicology* **206**: 207–19.

Pier AC, Cysewski SJ, Richard JL, Baetz AL, Mitchell L (1976) Experimental mycotoxicoses in calves with aflatoxin, ochratoxin, rubratoxin, and T-2 toxin. *Proceedings of the United States Animal Health Association, 80th Annual Meeting*, Miami Beach, FL, pp. 130–48.

Placinta CM, D'Mello JPF, Macdonald AMC (1999) A review of worldwide contamination of cereal grains and animal feeds with *Fusarium* mycotoxins. *Anim Feed Sci Technol* **78**: 21–37.

Pollmann DS, Koch BA, Seitz LM, Mohr HE, Kennedy GA (1985) Deoxynivalenol-contaminated wheat in swine diets. *J Anim Sci* **60**: 239–47.

Poppenga RH, Lundeen GR, Beasley VR (1987) Assessment of a general therapeutic protocol for the treatment of acute T-2 toxicosis in swine. *Vet Hum Toxicol* **29**: 237–9.

Prelusky DB, Trenholm HL (1991) Tissue distribution of deoxynivalenol in swine dosed intravenously. *J Agric Food Chem* **39**: 748–51.

Prelusky DB, Trenholm HL, Lawrence GA, Scott PM (1984) Nontransmission of deoxynivalenol (vomitoxin) to milk following oral administration to dairy cows. *J Environ Sci Health B* **19**: 593–609.

Prelusky DB, Veira DM, Trenholm HL, Hartin KE (1986a) Excretion profiles of the mycotoxin deoxynivalenol, following oral and intravenous administration to sheep. *Fundam Appl Toxicol* **6**: 356–63.

Prelusky DB, Hamilton RMG, Trenholm HL, Miller JD (1986b) Tissue distribution and excretion of radioactivity following administration of 14C-labelled deoxynivalenol to White Leghorn hens. *Fundam Appl Toxicol* **7**: 635–45.

Prelusky DB, Hartin KE, Trenholm HL, Miller JD (1988) Pharmacokinetic fate of 14C-labeled deoxynivalenol in swine. *Fundam Appl Toxicol* **10**: 276–86.

Prelusky DB, Hartin KE, Trenholm HL (1990) Distribution of deoxynivalenol in cerebral spinal fluid following administration to swine and sheep. *J Environ Sci Health B* **25**: 395–413.

Prelusky DB, Yeun JM, Thompson BK, Trenholm HL (1992) Effect of deoxynivalenol on neurotransmitters in discrete regions of swine brain. *Arch Environ Contam Toxicol* **22**: 36–40.

Prelusky DB, Rotter BA, Rotter RG (1994) Toxicology of mycotoxins. In *Mycotoxins in Grain: Compounds Other than Aflatoxin*, Miller JD, Trenholm HL (eds). Eagan Press, St. Paul, pp. 359–403.

Rafai R, Bata A, Vanyi A, Papp Z, Brydl E, Jakab L, Tuboly S, Tury E (1995a) Effect of various levels of T-2 toxin on the clinical status, performance, and metabolism of growing pigs. *Vet Rec* **136**: 485–9.

Rafai R, Tuboly S, Bata A, Tilly P, Vanyi A, Papp Z, Jakab L, and Tury E (1995b) Effect of various levels of T-2 toxin in the immune system of growing pigs. *Vet Rec* **136**: 511–14.

Rafai P, Pettersson H, Bata A, Papp Z, Glavits R, Tuboly S, Vanyi A, Soos P (2000) Effect of dietary T-2 fusariotoxin concentrations on the health and production of white pekin duck broilers. *Poult Sci* **79**: 1548–56.

Raisbeck MF, Rottinghaus GE, Kendall JD (1991) Effects of naturally occurring mycotoxins on ruminants. In *Mycotoxins in Animal Foods*, Smith JE, Henderson RS (eds). CRC Press, Inc., Boca Raton, FL, pp. 647–77.

Raymond SL, Smith TK, Swamy HV (2003) Effects of feeding a blend of grains naturally contaminated with *Fusarium* mycotoxins on feed intake, serum chemistry, and hematology of horses, and the efficacy of a polymeric glucomannan mycotoxin adsorbent. *J Anim Sci* **81**: 2123–30.

Raymond SL, Smith TK, Swamy HV (2005) Effects of feeding a blend of grains naturally contaminated with *Fusarium* mycotoxins on feed intake, metabolism, and indices of athletic performance on exercised horses. *J Anim Sci* **83**: 1267–73.

Rizzo AF, Atroshi F, Ahotupa M, Sankari S, Elovaara E (1994) Protective effect of antioxidants against free radical-mediated lipid peroxidation induced by DON or T-2 toxin. *Zentralbl Veterinarmed A* **41**: 81–90.

Robbana-Barnat S, Lafarge-Frayssinet C, Cohen H, Neish GA, Frayssinet C (1988) Immunosuppressive properties of deoxynivalenol. *Toxicology* **48**: 155–66.

Robison TS, Mirocha CJ, Kurtz HJ, Behrens JC, Chi MS, Weaver GA, Nystrom SD (1979) Transmission of T-2 toxin into bovine and porcine milk. *J Dairy Sci* **62**: 637–41.

Rocha O, Ansari K, Doohan FM (2005) Effects of trichothecene mycotoxins on eukaryotic cells: a review. *Food Addit Contam* **22**: 369–78.

Rodricks JV, Eppley RM (1974) Stachybotrys and stachybotryotoxicosis. In *Mycotoxins*, Purchase IFH (ed.). Elsevier, New York, pp. 181–97.

Rosenstein Y, Lafarge-Frayssinet C (1983) Inhibitory effect of *Fusarium* T-2 toxin on lymphoid DNA and protein synthesis. *Toxicol Appl Pharmacol* **70**: 283–8.

Rosenstein Y, Lafarge-Frayssinet C, Lespinats G, Loisillier F, Lafont P, Frayssinet C (1979) Immunosuppressive activity of *Fusarium* toxins. Effects on antibody synthesis and skin grafts of crude extracts, T-2 toxin and diacetoxyscirpenol. *Immunology* **36**: 111–17.

Rotter BA, Thompson BK, Lessard M, Trenholm HL, Tryphonas H (1994) Influence of low-level exposure to *Fusarium* mycotoxins on selected immunological and hematological parameters in young swine. *Fundam Appl Toxicol* **23**: 117–24.

Rotter BA, Prelusky DB, Pestka JJ (1996) Toxicology of deoxynivalenol (vomitoxin). *J Toxicol Environ Health* **48**: 1–34.

Rousseaux CG, Schiefer HB (1987) Maternal toxicity, embryolethality and abnormal fetal development in CD-1 mice following one oral dose of T-2 toxin. *J Appl Toxicol* **7**: 281–8.

Rousseaux CG, Schiefer HB, Hancock DS (1986) Reproductive and teratological effects of continuous low-level dietary T-2 toxin in female CD-1 mice for two generations. *J Appl Toxicol* **6**: 179–84.

Saito M, Ohtsubo K (1974) Trichothecene toxins of *Fusarium* species. In *Mycotoxins*, Purchase IFH (ed.). Elsevier, New York, pp. 263–81.

Schiefer HB, Beasley VR (1989) Effects on the digestive system and energy metabolism. In *Trichothecene Mycotoxicosis: Pathophysiologic*

*Effects*, vol. II, Beasley VR (ed.). CRC Press, Inc., Boca Raton, FL, pp. 61–89.

Schneider DJ, Marasas WFO, Dale Kuys JC, Kriek NPJ, Van Schalkwyk GC (1979) A field outbreak of suspected stachybotry-otoxicosis in sheep. *J South Afr Vet Assoc* **50**: 73–81.

Scott PM (1989) The natural occurrence of trichothecenes. In *Trichothecene Mycotoxicosis: Pathophysiologic Effects*, vol. I, Beasley VR (ed.). CRC Press, Inc., Boca Raton, FL, pp. 1–26.

Sehata S, Kiyosawa N, Makino T, Atsumi F, Ito K, Yamoto T, Teranishi M, Baba Y, Uetsuka K, Nakayama H, Doi K (2004) Morphological and microarray analysis of T-2 toxin-induced rat fetal brain lesions. *Food Chem Toxicol* **42**: 1727–36.

Shinozuka S, Suzuki M, Noguchi N, Sugimoto T, Uetsuka K, Nakayama H, Doi K (1998) T-2 toxin induced apoptosis in hematopoietic tissues of mice. *Toxicol Pathol* **26**: 674–81.

Shlosberg A, Weisman Y, Handji V (1984) A severe reduction in egg laying in a flock of hens associated with trichothecene mycotoxins in the feed. *Vet Hum Toxicol* **26**: 384–6.

Shlosberg AS, Klinger Y, Malkinson MH (1986) Muscovy ducklings, a particularly sensitive avian bioassay for T-2 toxin and diacetoxyscirpenol. *Avian Dis* **30**: 820–4.

Smalley EB, Strong FM (1974) Toxic trichothecenes. In *Mycotoxins*, Purchase IFH (ed.). Elsevier, New York, pp. 199–228.

Swamy HVLN, Smith TK, MacDonald EJ, Boermans HJ, Squires EJ (2002) Effects of feeding a blend of grains naturally contaminated with *Fusarium* mycotoxins on swine performance, brain regional neurochemistry, and serum chemistry and the efficacy of a polymeric glucomannan mycotoxin adsorbent. *J Anim Sci* **82**: 3257–67.

Swamy HVLN, Smith TK, MacDonald EJ, Karrow NA, Woodward B, Boermans HJ (2003) Effects of feeding a blend of grains naturally contaminated with *Fusarium* mycotoxins on growth and immunological measurements of starter pigs, and the efficacy of a polymeric glucomannan mycotoxin adsorbent. *J Anim Sci* **81**: 2792–803.

Swamy HVLN, Smith TK, MacDonald EJ (2004) Effects of feeding blends of grains naturally contaminated with *Fusarium* mycotoxins on brain regional neurochemistry of starter pigs and broiler chickens. *J Anim Sci* **82**: 2131–9.

Swanson SP, Corley RA (1989) The distribution, metabolism, and excretion of trichothecene mycotoxins. In *Trichothecene Mycotoxicosis: Pathophysiologic Effects*, vol. I, Beasley VR (ed.). CRC Press, Inc., Boca Raton, FL, pp. 37–61.

Swanson SP, Nicoletti J, Rood HD, Buck WB, Côte LM, Yoshizawa T (1987) Metabolism of three trichothecene mycotoxins, T-2 toxin, diacetoxyscirpenol, and deoxynivalenol, by bovine rumen microorganisms. *J Chromatogr* **414**: 335–42.

Taylor MJ, Pang VF, Beasley VR (1989) The immunotoxicity of trichothecene mycotoxins. In *Trichothecene Mycotoxicosis: Pathophysiologic Effects*, vol. II, Beasley VR (ed.). CRC Press, Inc., Boca Raton, FL, pp. 1–37.

Thompson WL, Wannemacher RW (1986) Structure–function relationship of 12,13-epoxytrichothecene mycotoxins in cell culture: comparison of whole animal lethality. *Toxicon* **24**: 985–94.

Trenholm HL, Thompson BK, Hartin KE, Greenhalgh R, McAllister AJ (1985) Ingestion of vomitoxin (deoxynivalenol)-contaminated wheat by nonlactacting dairy cows. *J Dairy Sci* **68**: 1000–5.

Tryphonas H, Iverson F, So Y, Nera EA, McGuire PF, O'Grady L, Clayson DB, Scott PM (1986) Effects of deoxynivalenol (vomitoxin) on the humoral and cellular immunity of mice. *Toxicol Lett* **30**: 137–50.

Ueno Y (1977) Mode of action of trichothecenes. *Pure Appl Chem* **49**: 1737–45.

Ueno Y (1983) General toxicology. In *Trichothecenes – Chemical, Biological, and Toxicological Aspects*, Ueno Y (ed.). Elsevier, New York, pp. 135–46.

Ueno Y (1984) Toxicological features of T-2 toxin and related trichothecenes. *Fundam Appl Toxicol* **4**: S124–32.

Ueno Y, Sato N, Ishii K, Sakai K, Tsunoda H, Enomoto M (1973) Biological and chemical detection of trichothecene mycotoxins in *Fusarium* species. *Appl Microbiol* **25**: 699–704.

Valenta H, Dänicke S (2005) Study on the transmission of deoxynivalenol and de-epoxy-deoxynivalenol into eggs of laying hens using a high-performance liquid chromatography–ultraviolet method with clean-up by immunoaffinity columns. *Mol Nutr Food Res* **49**: 779–85.

Vesonder RF, Ciegler A, Jensen AH (1973) Isolation of the emetic principle from *Fusarium*-infected corn. *Appl Microbiol* **26**: 1008–10.

Vesonder RF, Ciegler A, Jensen AH, Rohwedder WK, Weisleder D (1976) Co-identity of the refusal and emetic principle from *Fusarium*-infected corn. *Appl Environ Microbiol* **31**: 280–5.

Vila B, Jaradat ZW, Marquardt RR, Frohlich AA (2002) Effect of T-2 toxin on *in vivo* lipid peroxidation and vitamin E status in mice. *Food Chem Toxicol* **40**: 479–86.

Weaver GA, Kurtz HJ, Mirocha CJ (1977) The effect of *Fusarium* toxins on food producing animals. *Proceedings of the United States Animal Health Association, 81st Annual Meeting*, Minneapolis, MN, pp. 215–18.

Weaver GA, Kurtz HJ, Bates FY, Chi MS, Mirocha CJ, Behrens JC (1978a) Acute and chronic toxicity of T-2 mycotoxin in swine. *Vet Rec* **103**: 531–5.

Weaver GA, Kurtz HJ, Mirocha CJ, Bates FY, Behrens JC, Robinson TS, Gipp WF (1978b) Mycotoxin-induced abortions in swine. *Can Vet J* **19**: 72–4.

Weaver GA, Kurtz HJ, Bates FY, Mirocha CJ, Behrens JC, Hagler WM (1981) Diacetoxyscirpenol toxicity in pigs. *Res Vet Sci* **31**: 131–5.

Westlake K, Mackie RI, Dutton MF (1987a) T-2 metabolism by ruminal bacteria and its effect on their growth. *Appl Environ Microbiol* **53**: 587–92.

Westlake K, Mackie RI, Dutton MF (1987b) Effects of several mycotoxins on specific growth rate of *Butyrivibrio fibrisolvens* and toxin degradation *in vitro*. *Appl Environ Microbiol* **53**: 613–14.

Williams KC, Blaney BJ, Magee MH (1988) Responses of pigs fed wheat naturally infected with *Fusarium graminearum* and containing the mycotoxins 4-deoxynivalenol and zearalenone. *Aust J Sci Res* **39**: 1095–105.

Wyatt RD, Doerr JA, Hamilton PB, Burmeister HR (1975) Egg production, shell thickness, and other physiological parameters of laying hens affected by T-2 toxin. *Appl Microbiol* **29**: 641–5.

Yang G-H, Jarvis BB, Chung Y-J, Pestka JJ (2000) Apoptosis induction by the satratoxins and other trichothecene mycotoxins: relationship to ERK, p38 MAPK, and SAPK/JNK activation. *Toxicol Appl Pharmacol* **164**: 149–60.

Yoshizawa T, Morooka N (1973) Deoxynivalenol and its monoacetate: new mycotoxins from *Fusarium roseum* and moldy barley. *Agric Biol Chem* **37**: 2933–4.

Yoshizawa T, Mirocha CJ, Swanson SP (1981) Metabolic fate of T-2 toxin in a lactating cow. *Food Cosmet Toxicol* **19**: 31–9.

Young LG, McGirr L, Valli VE, Lumsden JH, Lun A (1983) Vomitoxin in corn fed to young pigs. *J Anim Sci* **57**: 655–64.

Zhou H-R, Yan D, Pestka JJ (1997) Differential cytokine mRNA expression in mice after oral exposure to the trichothecene vomitoxin (deoxynivalenol): dose response and time course. *Toxicol Appl Pharmacol* **144**: 294–305.

Zhou H-R, Islam Z, Pestka JJ (2005) Induction of competing apoptotic and survival signaling pathways in the macrophage by the ribotoxic trichothecene deoxynivalenol. *Toxicol Sci* **87**: 113–22.

C H A P T E R

# 77

# Zearalenone

*Michelle S. Mostrom*

## INTRODUCTION

Zearalenone is a nonsteroidal estrogenic mycotoxin produced by several species of *Fusarium* fungi. The primary producer of zearalenone is *Fusarium graminearum* (teleomorph *Gibberella zeae*). Additional *Fusarium* fungi capable of producing zearalenone include *F. culmorum*, *verticillioides* (*moniliforme*), *sporotrichioides*, *semitectum*, *equiseti*, and *oxysporum*. Contamination of cereal grains by zearalenone has been reported worldwide, primarily in temperate climates. Typically, zearalenone concentrations are low in grain contaminated in the field, but increase under storage conditions with moisture greater than 30–40%. Zearalenone has major effects on reproduction that can lead to hyperestrogenism. Prepubertal swine are the most sensitive species. Typical clinical signs of hyperestrogenism are swelling of the vulva, increase in uterine size and secretions, mammary gland hyperplasia and secretion, prolonged estrus, anestrus, increased incidence of pseudopregnancy, infertility, decreased libido, and secondary complications of rectal and vaginal prolapses, stillbirths and small litters. This chapter describes toxicity of zearalenone and its major metabolites in animals.

## BACKGROUND

Estrogenism in swine was reported in the mid-1920s in the Midwest US (McNutt *et al*., 1928). A condition of swelling and eversion of the vagina in young gilts and swelling of the prepuce in males was associated with consuming moldy corn. Prolapse of the vagina and occasionally the rectum were noted as secondary effects. With replacement of the moldy corn with clean corn, the animals recovered to normal, but if exposure to moldy corn continued, eversion of the uterus, secondary infections, and death occurred. Mirocha *et al*. (1971) noted high death losses in some herds and hypothesized that mortality was related to vaginal and rectal prolapses and subsequent septicemias; however, the presence of additional mycotoxins could not be excluded in causing death. Stob *et al*. (1962) isolated an active metabolite with uterotrophic and anabolic activities from culture of *G. zeae* (*F. graminearum*).

The *Fusarium* compound found in corn with uterotrophic activity was named zearalenone, previously known as F-2, and is chemically described as 6-(10-hydroxy-6-oxo-*trans*-1-undecenyl)-β-resorcylic acid lactone (Figure 77.1). At least seven derivatives of zearalenone have been found that naturally occur in corn. Zearalenone and uterotrophic active derivatives were classified as estrogens in the sense that they produce estrus or cornification of the vagina of adult mice (Mirocha and Christensen, 1974). Zearalenone can be produced on numerous substrates, including wheat, barley, corn, corn silage, rice, sorghum, and occasionally in forages. Production in soybeans is uncommon. Moisture content and the presence of oxygen are critical factors for zearalenone production. In laboratory cultures, *Fusarium* growth occurs during a 3-week period at moisture levels

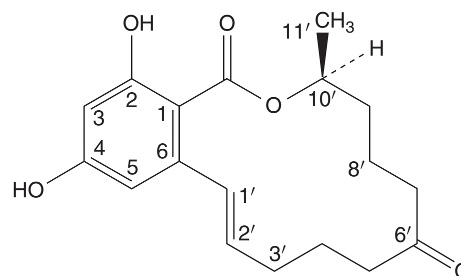


**FIGURE 77.1** Chemical structure of zearalenone.

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over 20% and temperature between 20°C and 25°C. If the fungus is stressed by cool temperatures of 8–15°C for several weeks, zearalenone can be produced. Production of zearalenone in the field is similar to that in the laboratory, with alternating moderate and low temperature weather capable of stimulating zearalenone production. Zearalenone can be produced fairly quickly in the field during wet weather in the late summer or early fall weather following hail damage to corn. Very high concentrations of zearalenone, which can occur naturally in some field samples, generally result from improper storage at high moisture rather than production in the field. Corn stored in a crib and exposed to winter weather was particularly prone to fungal invasion and production of zearalenone. In addition to the co-occurrence of other estrogenic metabolites, such as α- and β-zearalenol, zearalenone is commonly detected in grains with another *Fusarium* mycotoxin deoxynivalenol. Zearalenone is heat stable, but can be partially destroyed during extrusion cooking of cereals (Castells *et al.*, 2005).

## TOXICOKINETICS

Zearalenone is fairly rapidly absorbed following oral exposure (Dailey *et al.*, 1980). Following a single oral dose of 10 mg zearalenone/kg body weight to 15–25 kg pigs, the absorption was approximated to be 80–85% (Biehl *et al.*, 1993). Zearalenone and associated metabolites were found in the plasma of a pig in less than 30 min after initiating feeding with parent compound. Following zearalenone administration, zearalenone can be localized in reproductive tissues (ovary and uterus), adipose tissue, and interstitial cells of the testes (Ueno *et al.*, 1977; Kuiper-Goodman *et al.*, 1987). The reported biological half-life of total plasma zearalenone radioactivity following oral dosage in pigs is 86 h (Biehl *et al.*, 1993). Species differences in zearalenone susceptibility might be related to hepatic biotransformation, with the highest amount of α-zearalenol, which has increased estrogenic activity compared to α-zearalanol and zearalenone produced by pig hepatic microsomes, whereas chicken microsomes produced the highest amounts of β-zearalenol, which has lower estrogenic activity (Malekinejad *et al.*, 2006). Pigs readily conjugated almost all absorbed zearalenone and α -zearalenol through glucuronidation. While the liver plays a major role in glucuronidation, the intestinal mucosa is also active. Zearalenone was reduced to α- and β-zearalenol in sow intestinal mucosa homogenates (duodenum and jejunum) *in vitro* (Olsen *et al.*, 1987). Gastro-intestinal flora can aid in the metabolism of zearalenone. Zearalenone can undergo rumen metabolism, with reduction to mostly α-zearalenol and to a lower amount of β-zearalenol (Kiessling *et al.*, 1984). Whether rumen metabolism will increase or decrease zearalenone toxicity depends on absorption by the gastrointestinal tract, liver metabolism by hydroxysteroid dehydrogenase, and competition at the cytosolic receptor sites in the animal species.

Zearalenone undergoes extensive enterohepatic circulation and biliary excretion in most species. The major route of excretion for most species is through the feces, although rabbits primarily excrete zearalenone in the urine. Most zearalenone administered in a dose is excreted within a 72-h period. Approximately 94% of radio-labeled zearalenone, given orally to White Leghorn laying hens at 10 mg/kg body weight, was eliminated through the excreta within 72 h post-dosing (Dailey *et al.*, 1980). No major retention of radio-labeled activity was found in edible muscle tissue, but lipophilic metabolite(s) were reported in egg yolk (at about 2 mg/kg concentration) 72 h post-dosing.

Concern has focused on potential residue of zearalenone and its metabolites in milk, eggs, and foods and precocious development of sexual characteristics in young female girls (Kuiper-Goodman *et al.*, 1987; JECFA, 2000). Zearalenone and α- and β-zearalenols can be transmitted into the milk of sheep, cows, and pigs administered high doses of zearalenone (Hagler *et al.*, 1980; Mirocha *et al.*, 1981). Hyperestrogenism has been reported in lambs and pigs nursing dams dosed with zearalenone (Hagler *et al.*, 1980; Palyusik *et al.*, 1980). Dairy cows fed rations with purified zearalenone at 50 mg zearalenone/day and 165 mg zearalenone/day for 21 days had no detectable concentrations of zearalenone or α- and β-zearalenol in the milk or plasma (Prelusky *et al.*, 1990). One cow dosed with 544.5 mg zearalenone/day for 21 days had maximum concentrations of 2.5 ng zearalenone/ml and 3.0 ng α-zearalanol/ml in the milk. Cows dosed orally with a 1-day dose of 1.8 or 6 g zearalenone had maximum milk levels on day 2 of 4.0 and 6.1 ng zearalenone/ml, respectively. This research indicates that minimal transmission of zearalanone occurs into milk and only for a short period of time after exposure to high concentrations of zearalenone.

Following intubations of tritiated-zearalenone into the crops of 7-week-old broiler chickens, the greatest accumulation of radioactivity occurred in the liver 30 min post-administration, which became a trace of radioactivity by 48 h post-administration (Mirocha *et al.*, 1982). Only zearalenone was detected in muscle tissue at approximately 4 ppb at 48-h post-administration, indicating the zearalenone residues in edible tissue are minimal.

## MECHANISM OF ACTION

Zearalenone undergoes reduction of the 6′ ketone to a secondary alcohol, which leads to two diastereoisomeric zearalanols (α and β), that are naturally occurring fungal

metabolites. The α-zearalanol metabolite, which is less estrogenic than α-zearalanol but 3 times more estrogenic than zearalenone, is an anabolic growth-promoting compound, zearanol or Ralgro®, used in both cattle and sheep commercially.

Zearalenone and metabolites can interact directly with the cytoplasmic receptor that binds to 17β-estradiol and translocate receptor sites to the nucleus (Katzenellenbogen *et al*., 1979). In the nucleus, stimulation of RNA leads to protein synthesis and clinical signs of estrogenism. Following subcutaneous injection of the compounds, the zearalanols and zearalenone stimulated production of a specific uterine protein and increased uterine weights. Within the resorcylic acids, α-zearalenol exhibited the greatest binding affinity for cytosolic estrogen receptors, while zearalenone and β-zearalenol displayed much lower binding affinities (Fitzpatrick *et al*., 1989). The hydroxylation of zearalenone to α-zearalenol apparently is an activation process, whereas the production of β-zearalenol would be a deactivation process. The relative binding affinity of α-zearalenol was greater in the pig than in the rat or chicken. Interspecies variations in sensitivity to zearalenone in the feed could be related to different metabolites produced and the relative binding affinities of zearalenone and metabolites formed.

Zearalenone can also act on the hypothalamic–hypophysial axis. Using 70-day-old Yorkshire gilts (20–27 kg) fed 1.5–2 mg zearalenone/kg feed for 45–90 days, Rainey *et al*. (1990) determined that prepubertal exposure to zearalenone affected the hypothalamic–hypophysial axis and the luteinizing hormone (LH) surges that lasted for at least 44 days post-exposure. However, zearalenone consumption did not delay the onset of pubertal estrus nor impair conception rates, ovulation rates, or number of fetuses. Slightly older prepubertal gilts (178 days of age and 94 kg) fed 10 mg zearalenone daily for 2 weeks had suppressed mean serum concentrations of LH, but the onset of puberty and subsequent reproduction were not adversely affected (Green *et al*., 1990). Male rats, 70 days old, dosed orally with zearalenone at 20 mg/kg body weight for 35 days had elevated serum prolactin concentrations but showed no changes in serum LH and follicle stimulating hormone concentrations, body and testes weights, or in spermatogonia, spermatocytes, and spermatids (Milano *et al*., 1995). At relatively high concentrations *in vitro*, approximately 400 μM, zearalenone appeared to act directly on interstitial cells of the testes inhibiting steroidogenesis (Fenske and Fink-Gremmels, 1990).

While zearalenone primarily affects reproduction, it may have additional effects. During exposure of *in vitro* cell lines, zearalenone acted as a ligand for human pregnene X receptor (hPXR), which can activate a transcription factor regulating the expression of numerous hepatic drug-metabolizing enzymes, including expression of cytochrome P450 enzymes (Ding *et al*., 2006). This suggests a potential for zearalenone to induce metabolism of drugs.

At natural contamination levels in feeds, zearalenone does not appear to impact the immune response. Results from *in vitro* studies of zearalenone and metabolites α-and β-zearalenol and α- and β-zearalanol and mitogen testing with leukoagglutinin, concanavalin A, and pokeweed revealed that these mycotoxins inhibited mitogen-induced proliferation of both B and T lymphocytes (Forsell and Pestka, 1985). No treatment differences were observed in B6C3F1 mice fed a diet with 10 mg zearalenone/kg diet for 8 weeks and control mice that were tested with a splenic plaque-forming response to sheep erythrocytes and a delayed hypersensitivity response to keyhole hemocyanin (Pestka *et al*., 1987).

## TOXICITY

Zearalenone has low acute toxicity in most species. In most natural conditions, concentrations of zearalenone in feed ingredients are less than 20 mg/kg (ppm) and generally less than 5 mg zearalenone/kg feed (Sundlof and Strickland, 1986). Prepubertal swine are most sensitive to zearalenone, ruminants may exhibit some adverse affects, and poultry appears to be the least sensitive species. Females are more sensitive than males, and cycling female pigs may be more sensitive than pregnant sows. Pregnant swine may abort. Abortions have been associated in field cases with natural *Fusarium* mold exposure, but have not been reproduced with purified zearalenone (Mirocha and Christensen, 1974). Younger male pigs appear to be more sensitive than older males and can undergo atrophy of the testes and enlargement of mammary glands.

### Swine

Gilts fed rations with 0, 3, 6, or 9 mg purified zearalenone/kg feed that started the day after they showed the first estrus were bred at subsequent heat periods (Young and King, 1986a). A majority of gilts fed 6 or 9 mg zearalenone/kg feed became pseudopregnant based on examination of their reproductive tracts or plasma progesterone levels. Gilts fed rations with 3 mg zearalenone/kg feed had no reproductive effects. After removal of zearalenone from the diet, approximately half of the gilts fed 6 or 9 mg zearalenone/kg feed returned to estrus spontaneously. Edwards *et al.* (1987a) reported luteal maintenance and extended inter-estrous intervals in sexually mature gilts fed purified zearalenone at concentrations of 5–10 mg/kg feed from days 5 to 20 of the estrous cycle. Approximately 86% of the retained corpora lutea underwent spontaneous regression and most gilts came into estrus within the next 30 days. In a subsequent study,

Edwards *et al*. (1987b) fed prepubertal gilts a diet with 0 or 10 mg zearalenone/kg feed for 30 days from 145 to 193 days of age, switched the gilts to a control diet, and then exposed the gilts to a mature boar. While treated gilts displayed vulvar swelling during the 30-day feeding period and were delayed in showing first estrus, the proportion of gilts showing estrus with exposure to the boar was similar between treatment and control gilts, and subsequent cycling was not affected when the treated feed was removed. Young *et al*. (1990) reported an increased weaning-to-estrus interval and embryonic mortality (measured as a decreased ratio of fetuses to corpora lutea) and a decreased number of fetuses per sow in second parity sows fed 10 mg zearalenone/kg diet. No reproductive effects were observed in prepubertal gilts fed a ration with 0.5 mg zearalenone/kg feed (Friend *et al*., 1990).

Young gilts (30–35 kg) administered 5 mg of purified zearalenone *per os* daily developed swelling of the vulva on the 4th day of treatment, an approximate daily dose of 0.143–0.167 mg zearalenone/kg body weight (Mirocha and Christensen, 1974). Gilts dosed with 1 mg of purified zearalenone daily for 8 days developed pronounced vulvar swelling. Gilts exposed to higher concentrations of zearalenone may show atrophy of the ovaries along with edema and cellular proliferation of all layers in the uterus. Kuiper-Goodman *et al*. (1987) noted a no adverse effects level for zearalenone in pigs reaching puberty at 0.06 mg zearalenone/kg body weight/day.

Male swine fed a high concentration of zearalenone (30 mg/kg feed) appeared to initially have accelerated maturation of spermatogenesis, which occurred 1.5–2 months earlier than control animals (Ványi and Széky, 1980). Although germinal epithelium damage was limited to several foci initially, with continued zearalenone exposure the damage became widespread with proliferation of the interstitium around seminiferous tubules. Young and King (1986b) fed lower levels of zearalenone in the diet (0, 3, 6, and 9 mg zearalenone/kg feed) to boars from 32 days of age up to 145 or 312 days of age. Feeding up to 9 mg zearalenone/kg feed to the boars did not affect the libido, but the boars fed the highest dose of zearalenone produced lower total and gel-free volumes of semen with lower total motile sperm. Zearalenone does not appear to affect mature boars. No adverse effects in reproductive parameters, including testicular size, libido, sperm motility and morphology, plasma testosterone and 17β-estradiol concentrations, were reported in mature Yorkshire boars fed increasing concentrations of purified zearalenone at 0, 2, 20, and 200 mg/kg ration for 8 weeks (Ruhr *et al*., 1983).

The European Union (2006) guidance values for zearalenone in feedstuff (with a moisture content of 12%) for piglets and gelts is 0.1 mg/kg or ppm, for sows and fattening pigs is 0.25 mg/kg, and for calves, dairy cattle, sheep, and goats is 0.5 mg/kg.

## Ruminants

Several case reports have associated dairy herd health problems and zearalenone in moldy feed. Young dairy heifers, 6–14 months of age, developed slight enlargement of at least one mammary gland quarter while fed moldy corn in a ration (Bloomquist *et al*., 1982). Following a change in the ration, the heifers returned to normal 7 weeks later. Zearalenone contamination of the moldy ration was detected by thin-layer chromatography. Roine *et al*. (1971) reported turbid discharge from the vulva, obvious estrous behavior lasting for 1–2 weeks, and infertility in dairy cows and heifers. Strains of *F. graminearum* and *culmorum* were isolated from the feed that caused an increase in uterine weight in rats and were capable of producing between 3 and 9.5 mg zearalenone/kg feed. Ványi *et al*. (1974) reported a drop in milk production, feed intake, and swelling of the vulva in diary cows exposed to varying concentrations of zearalenone, ranging from 5 to 75 mg zearalenone/kg feed. In an experimental study, 18 cycling heifers were dosed with 0 or 250 mg of purified zearalenone daily through one non-breeding estrous cycle and the next two consecutive estrous cycles during which the heifers were bred (Weaver *et al*., 1986a). The authors calculated that treated heifers were given an average of 250 mg zearalenone/364 kg body weight/day or 0.69 mg zearalenone/kg body weight/day. The control and treated heifers had conception rates of 87% and 62%, respectively, at a statistical probability of $p < 0.065$. Eighteen dairy cows (three cows per group) dosed orally with 0, 31.25, 62.5, 125, 250, and 500 mg of purified zearalenone daily for two consecutive estrous cycles had no changes in serum progesterone concentration, erythrocyte and leukocyte blood counts, packed cell volume, estrous cycle length, clinical health, or sexual behavior (Weaver *et al*., 1986b).

Zearalenone can affect ewe reproduction when ewes are exposed to the mycotoxin prior to mating. Zearalenone, administered orally at concentrations greater than 3 mg/animal/day, given to ewes prior to mating depressed ovulation rates and reduced lambing percentages (Smith *et al*., 1990). Ewes administered a similar range of oral doses of zearalenone (0, 1.5, 3, 6, 12, and 24 mg/ewe/day) for 10 days, starting 5 days after mating, showed no effect of zearalenone exposure after mating on pregnancy rate or embryonic loss. Breeding rams fed a diet containing 12 mg zearalenone/kg feed for 8 weeks had no significant adverse effects on semen volume, concentration, motility, or morphology during the trial and for 6 weeks after zearalenone feeding was ceased (Milano *et al*., 1991).

## Equine

Gimeno and Quintanilla (1983) reported estrogenic signs of edematous vulvas, prolapsed vaginas, oversized uteruses, and internal hemorrhage in mares and severe flaccidity of

genitals in two male horses fed corn screenings for 30 days in a field exposure. All sick animals collapsed with respiratory paralysis and sudden blindness, and died quickly. Analysis for zearalenone in the feed detected 2–3 mg zearalenone/kg diet. While the authors tested for a variety of mycotoxins by thin-layer chromatography, fumonisins in the corn screenings were not determined because they had not been recognized as mycotoxins and a cause of death in horses. Fumonisins would have to be considered as a primary cause of some of the adverse effects, particularly blindness and death, in these horses.

In a study of six cycling trotter mares, Juhász *et al*. (2001) determined that daily oral administration of 7 mg purified zearalenone starting 10 days after ovulation until the subsequent ovulation had no adverse effect on reproduction. Zearalenone had no effect on the length of the interovulatory intervals, luteal and follicular phases of the ovary, and did not significantly affect uterine edema. The authors noted that zearalenone exposure started 10 days after ovulation and the exposure period to zearalenone was short in this study. The dose of purified zearalenone represented a natural contamination of feed of about 1 mg zearalenone/kg feed and ranged between 0.013 and 0.010 mg zearalenone/kg body weight/day for approximately 8–10 days.

### Poultry

Growing female White Leghorn chickens dosed orally once with 15 g zearalenone/kg body weight showed no adverse effects in reproductive tissues (Chi *et al*., 1980). In a second experiment, chickens dosed orally or intramuscularly with increasing concentrations of zearalenone (0–800 mg/kg body weight) for 7 consecutive days had increased oviduct weights with increasing zearalenone doses. Poultry appear to be fairly resistant to the effects of zearalenone.

## TREATMENT

Quick removal of zearalenone contaminated feed from the ration and replacement with clean feed is essential. Generally, 3–7 weeks following removal of the contaminated feed, animals will return to normal reproductive status. No zearalenone mycotoxin binder has been proven to be efficacious in any species of livestock by the US Food and Drug Administration.

## CONCLUSIONS

Maintaining moisture concentrations less than 15–16% in feeds should adequately prevent zearalenone production during storage. When naturally contaminated feed must be used, incorporation into rations for less susceptible species, such as feedlot animals or poultry, should be considered. At naturally occurring concentrations of zearalenone in grains, residues in meat, milk, and eggs are probably not significant.

## REFERENCES


Biehl ML, Prelusky DB, Koritz GD, Hartin KE, Buck WB, Trenholm HL (1993) Biliary excretion and enterohepatic cycling of zearalenone in immature pigs. *Toxicol Appl Pharmacol* **121**: 152–9.

Bloomquist C, Davidson JN, Pearson EG (1982) Zearalenone toxicosis in prepubertal dairy heifers. *J Am Vet Med Assoc* **189**: 164–5.

Castells M, Marin S, Sanchis V, Ramos AJ (2005) Fate of mycotoxins in cereals during extrusion cooking: a review. *Food Addit Contam* **22**: 150–7.

Chi MS, Mirocha CJ, Weaver GA, Kurtz HJ (1980) Effect of zearalenone on female White Leghorn chickens. *Appl Environ Microbiol* **39**: 1026–30.

Dailey RE, Reese RE, Brouwer EA (1980) Metabolism of [14C]zearalenone in laying hens. *J Agric Food Chem* **28**: 286–91.

Ding X, Lichti K, Staudiner JL (2006) The mycoestrogen zearalenone induces CYP3A through activation of the pregnene X receptor. *Toxicol Sci* **91**: 448–55.

Edwards S, Cantley TC, Rottinghaus GE, Osweiler GD, Day BN (1987a) The effects of zearalenone on reproduction in swine. I. The relationship between ingested zearalenone dose and anestrus in nonpregnant, sexually mature gilts. *Theriogenology* **28**: 43–9.

EU Commit. Official Journal of the European Union Commission Recommendation of 17 August 2006. The Commission of European Communities 23.8.2006. Brussels.

Edwards S, Cantley TC, Day BN (1987b) The effects of zearalenone on reproduction in swine. II. The effect on puberty attainment and postweaning rebreeding performance. *Theriogenology* **28**: 51–8.

Fenske M, Fink-Gremmels J (1990) Effects of fungal metabolites on testosterone secretion *in vitro*. *Arch Toxicol* **64**: 72–5.

Fitzpatrick DW, Picken CA, Murphy LC, Buhr MM (1989) Measurement of the relative binding affinity of zearalenone, α-zearalenol and β-zearalenol for uterine and oviduct estrogen receptors in swine, rats and chickens: an indicator of estrogenic potencies. *Comp Biochem Physiol* **94C**: 691–4.

Friend DW, Trenholm HL, Thompson BK, Hartin KE, Fiser PS, Asem EK, Tsang BK (1990) The reproductive efficiency of gilts feds very low levels of zearalenone. *Can J Anim Sci* **70**: 635–45.

Forsell JH, Pestka JJ (1985) Relation of 8-ketotrichothecene and zearalenone analog structure to inhibition of mitogen-induced human lymphocyte blastogenesis. *Appl Environ Microbiol* **50**: 1304–7.

Gimeno A, Quintanilla JA (1983) Analytical and mycological study of a natural outbreak of zearalenone mycotoxicosis in horses. *Proceedings of International Symposium on Mycotoxins*, National Research Centre, Cairo, Egypt, pp. 387–92.

Green ML, Diekman MA, Malayer JR, Scheidt AB, Long GG (1990) Effect of pre-pubertal consumption of zearalenone on puberty and subsequent reproduction of gilts. *J Anim Sci* **68**: 171–8.

Hagler WM, Dankó G, Horvath L, Palyusik M, Microcha CJ (1980) Transmission of zearalenone and its metabolite into ruminant milk. *Acta Vet Acad Sci Hung* **28**: 209–16.

JECFA, Joint FAO/WHO Expert Committee on Food Additives (2000) Zearalenone. In *Safety Evaluation of Certain Food Additives and Contaminants*, *WHO Food Additives Series*, vol. 44, pp. 393–482.

Juhász J, Nagy P, Kulcsár M, Szigeti G, Reiczigel J, Huszenicza G (2001) Effect of low-dose zearalenone exposure on luteal function, follicular activity, and uterine oedema in cycling mares. *Acta Vet Hung* **49**: 211–22.

Katzenellenbogen BS, Katzenellenbogen JA, Mordecai D (1979) Zearalenones: characterization of the estrogenic potencies and receptor interactions of a series of fungal β-resorcylic acid lactones. *Endocrinology* **105**: 33–40.

Kiessling KH, Patterson H, Sandholm K, Olsen M (1984) Metabolism of aflatoxin, ochratoxin, zearalenone, and three trichothecenes by intact rumen fluid, rumen protozoa, and bacteria. *Appl Environ Microbiol* **47**: 1070–3.

Kuiper-Goodman T, Scott PM, Watanabe H (1987) Risk assessment of the mycotoxin zearalenone. *Regul Toxicol Pharmacol* **7**: 253–306.

Malekinejad H, Maas-Bakker R, Fink-Gremmels J (2006) Species differences in the hepatic biotransformation of zearalenone. *Vet J* **172**: 96–102.

McNutt SH, Purwin P, Murray C (1928) Vulvovaginitis in swine. *J Am Vet Med Assoc* **26**: 484–92.

Milano GD, Odriozola E, Lopez TA (1991) Lack of effect of a diet containing zearalenone on spermatogenesis in rams. *Vet Rec* **129**: 33–5.

Milano GD, Becu-Villalobos D, Tapia O (1995) Effects of long-term zearalenone administration on spermatogenesis and serum luteinizing hormone, follicle-stimulating hormone, and prolactin values in male rats. *Am J Vet Res* **56**: 954–8.

Mirocha CJ, Christensen CM (1974) Oestrogenic mycotoxins synthesized by *Fusarium*. In *Mycotoxins*, Purchase IFH (ed.). Elsevier, New York, pp. 129–48.

Mirocha CJ, Pathre SV, Robison TS (1981) Comparative metabolism of zearalenone and transmission into bovine milk. *Food Cosmet Toxicol* **19**: 25–30.

Mirocha CJ, Robison TS, Pawlosky RJ, Allen NK (1982) Distribution and residue determination of [3H]zearalenone in broilers. *Toxicol Appl Pharmacol* **66**: 77–87.

Mirocha CJ, Christensen CM, Nelson GH (1971) F-2 (zearalenone) estrogenic mycotoxin from *Fusarium*. In *Microbial Toxins*, vol. VII, Kadis S, Ciegler A, Ajl SJ (eds). Academic Press, New York, pp. 107–38.

Olsen M, Pettersson H, Sandholm K, Visconti A, Kiessling K-H (1987) Metabolism of zearalenone by sow intestinal mucosa *in vitro*. *Food Chem Toxicol* **25**: 681–3.

Palyusik M, Harrach B, Mirocha CJ, Pathre SV (1980) Transmission of zearalenone and zearalenol into porcine milk. *Acta Vet Acad Sci Hung* **28**: 217–22.

Pestka JJ, Tai JH, Witt MF, Dixon DE, Forsell JH (1987) Suppression of immune response in the B6C3F1 mouse after dietary exposure to the *Fusarium* mycotoxins deoxynivalenol (vomitoxin) and zearalenone. *Food Chem Toxicol* **25**: 297–304.

Prelusky DB, Scott PM, Trenholm HL, Lawrence GA (1990) Minimal transmission of zearalenone to milk of dairy cows. *J Environ Sci Health* **B25**: 87–103.

Rainey MR, Tubbs RC, Bennett LW, Cox NM (1990) Prepubertal exposure to dietary zearalenone alters hypothalamo-hypophysial function but does not impair postpubertal reproductive function of gilts. *J Anim Sci* **68**: 2015–22.

Roine K, Korpinen EL, Kallela K (1971) Mycotoxicosis as a probable cause of infertility in dairy cows. *Nord Vet Med* **23**: 628–33.

Ruhr LP, Osweiler GD, Foley CW (1983) Effect of the estrogenic mycotoxin zearalenone on reproductive potential in the boar. *Am J Vet Res* **44**: 483–5.

Smith JE, di Menna ME, McGowan LT (1990) Reproductive performance of Coopworth ewes following oral doses of zearalenone before and after mating. *J Reprod Fert* **89**: 99–106.

Stob M, Baldwin RS, Tuite J, Amdrews FN, Gillette KG (1962) Isolation of an anabolic, uterotrophic compound from corn infected with *Gibberella zeae*. *Nature* **29**: 1318.

Sundlof SF, Strickland C (1986) Zearalenone and zearanol: potential residue problems in livestock. *Vet Hum Toxicol* **28**: 242–50.

Ueno Y, Ayaki S, Sato N, Ito T (1977) Fate and mode of action of zearalenone. *Ann Nutr Aliment* **31**: 935–48.

Ványi A, Széky A (1980) Fusariotoxicoses. VI. The effect of F-2 toxin (zearalenone) on the spermatogenesis of male swine. *Magy Állatorv Lapja* **35**: 242–6.

Ványi A, Szemerédi G, Szailer ER (1974) Fusariotoxicoses on a cattle farm. *Magy Állatorv Lapja* **29**: 544–6.

Weaver GA, Kurtz HJ, Behrens JC, Robison TS, Sequin BE, Bates FY, Mirocha CJ (1986a) Effect of zearalenone on the fertility of virgin heifers. *Am J Vet Res* **47**: 1395–7.

Weaver GA, Kurtz HJ, Behrens JC, Robison TS, Sequin BE, Bates FY, Mirocha CJ (1986b) Effect of zearalenone on dairy cows. *Am J Vet Res* **47**: 1826–8.

Young LG, King GJ (1986a) Low concentrations of zearalenone in diets of mature gilts. *J Anim Sci* **63**: 1191–6.

Young LG, King GJ (1986b) Low concentrations of zearalenone in diets of boars for a prolonged period of time. *J Anim Sci* **63**: 1197–200.

Young LG, Ping H, King GJ (1990) Effects of feeding zearalenone to sows on rebreeding and pregnancy. *J Anim Sci* **68**: 15–20.

C H A P T E R

# 78

# Fumonisins

*Geoffrey W. Smith*

## INTRODUCTION

Fumonisins ($B_1$ and $B_2$) are a group of naturally occurring mycotoxins produced by the fungus, *Fusarium verticillioides* (formerly *F. moniliforme*). These toxic metabolites that are usually found in corn have been implicated in field cases of porcine pulmonary edema (PPE) (Harrison *et al.*, 1990; Osweiler *et al.*, 1992; Colvin *et al.*, 1993) and equine leukoencephalomalacia (ELEM) (Wilson *et al.*, 1990a). Experimentally, fumonisin has been shown to cause liver damage in multiple species including pigs, horses, cattle, rabbits, and primates (Jaskiewicz *et al.*, 1987; Voss *et al.*, 1989; Haschek *et al.*, 1992; Osweiler *et al.*, 1993; Ross *et al.*, 1993; Gumprecht *et al.*, 1995) as well as species-specific target organ toxicity, such as lung in pigs (Haschek *et al.*, 1992), brain in horses (Ross *et al.*, 1993), kidney in rats, rabbits, and sheep (Voss *et al.*, 1989; Edrington *et al.*, 1995; Gumprecht *et al.*, 1995), and esophagus in rats and pigs (Casteel *et al.*, 1993; Lim *et al.*, 1996). Epidemiologic data has linked ingestion of corn contaminated with *F. verticillioides* to human esophageal cancer (Rheeder *et al.*, 1992), and fumonisins have been shown to be hepatocarcinogenic in rats and mice (Gelderblom *et al.*, 1988; Howard *et al.*, 2001).

## BACKGROUND

### Chemical structure

First isolated in 1988, the fumonisins are a group of structurally related compounds with the terminal carboxy group composed of propane-1,2,3-tricarboxylic acid involved in ester formation with the C-14 and C-15 hydroxy groups. The 20C chain base carries either 2-acetylamino or 2-amino-12,16-dimethyl-3,5,10,14,15, pentahydroxyicosane (Figure 78.1). The structures of $FB_1$ and $FB_2$ have been shown to have the empirical formulas of $C_{34}H_{59}NO_{15}$ and $C_{34}H_{59}NO_{14}$, respectively, with the only difference being the hydroxyl group present at the C-10 position in $FB_1$ (Bezuidenhout *et al.*, 1988). Four additional fumonisin metabolites have been isolated ($B_3$, $B_4$, $A_1$, and $A_2$), but appear to occur in much lower concentrations than $FB_1$

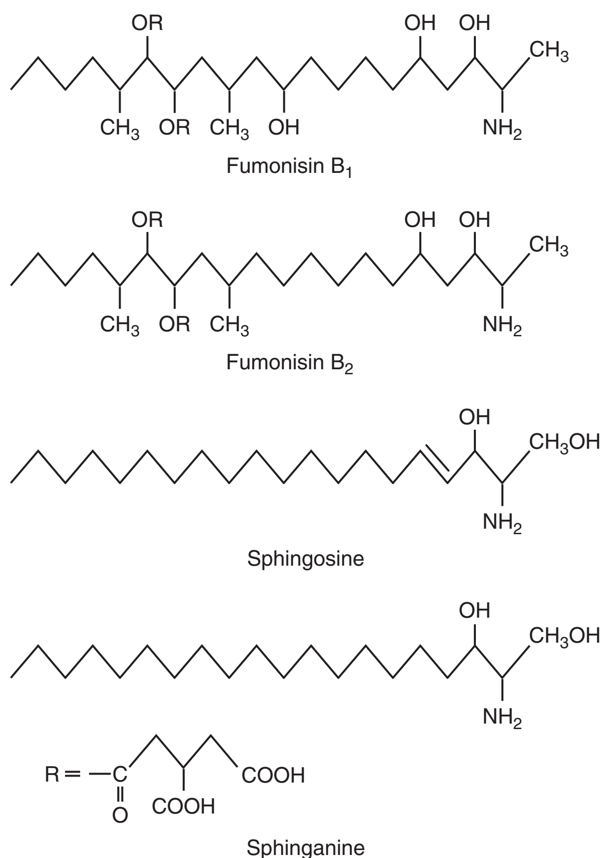


**FIGURE 78.1** The structure of fumonisin $B_1$, fumonisin $B_2$, sphingosine, and sphinganine (modified from Diaz and Boermans, 1994; reprinted with permission from Smith and Constable, 2004).

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or $FB_2$, and are not considered important at this time (Gelderblom *et al.*, 1992).

### Occurrence and distribution

Apart from reports of fumonisin $B_1$ and $B_2$ in "black oats" feed from Brazil (Sydenham *et al.*, 1992), and in New Zealand forage grasses (Mirocha, 1992), the only commodities in which fumonisins have been detected so far are corn and corn-based foods. The occurrence of $FB_1$ in the forage grass was accompanied by mono- and dimethyl esters which may or may not have been artifacts (Mirocha *et al.*, 1992). ELEM has long been associated with the consumption of moldy corn, and has been reported in many areas of the world. However, cases of ELEM that were directly associated with fumonisin-contaminated feed have been reported in South Africa and Egypt (Thiel *et al.*, 1991), the United States (Wilson *et al.*, 1990a; Ross *et al.*, 1991), Brazil (Sydenham *et al.*, 1992), Hungary (Bela and Endre, 1996), Spain (Cerrillo *et al.*, 1996), New Caldonia (Bailly *et al.*, 1996), Mexico (Rosiles *et al.*, 1998), and Iran (Raoofi *et al.*, 2003). Additionally, cases of PPE have been associated with fumonisin-contaminated feeds in the United States (Harrison *et al.*, 1990; Osweiler *et al.*, 1992), Brazil (Sydenham *et al.*, 1992), Hungary (Fazekas *et al.*, 1998), and Thailand (Patchimasiri *et al.*, 1998).

Commercial corn-based human feedstuffs from retail outlets in several countries frequently contain fumonisins (Sydenham *et al.*, 1991; Pittet *et al.*, 1992; Stack and Eppley, 1992). Corn meal and corn grits appear to be the biggest problems, as up to 2.98 μg $FB_1$/g and 0.92 μg $FB_2$/g have been found in corn meal and up to 2.55 μg $FB_1$/g and 1.07 μg $FB_2$/g in corn grits (Sydenham *et al.*, 1991). Only very low incidences and levels of $FB_1$ have been recorded for corn flakes breakfast cereal in Switzerland, the United States, and South Africa (Sydenham *et al.*, 1991; Pittet *et al.*, 1992; Stack *et al.*, 1992). A survey in Maryland showed that nearly 100% of corn-based food products (including corn muffin mixes, corn chips, corn tortillas, corn flakes breakfast cereal, corn starch, infant mixed cereal, and corn grits) purchased in grocery stores contained some level of fumonisins with concentrations ranging up to 7.5 μg/g (Castelo *et al.*, 1998). Corn-based food products purchased in Arizona and Nebraska were also frequently (>85%) contaminated with fumonisins with concentrations up to 5.2 μg/g being reported (Castelo *et al.*, 1998).

## PHARMACOKINETICS/ TOXICOKINETICS

The pharmacokinetics of fumonisin $B_1$ have been examined in several species including rats, pigs, cattle, laying hens, and primates (Shephard *et al.*, 1992, 1995; Prelusky *et al.*, 1994; Vudathala *et al.*, 1994; Prelusky *et al.*, 1995; Richard *et al.*, 1996; Martinez-Larranaga *et al.*, 1999). In general, fumonisin is rapidly absorbed following intravenous or intraperitoneal administration and is eliminated in both the feces and urine. Levels are undetectable by 24 h after dosing in virtually all species and significant concentrations (residues) have not been found in muscle, milk, or eggs. Following oral dosing, very little fumonisin $B_1$ is typically found in the serum of animals indicating low bioavailability.

More specifically, the toxicokinetics of radiolabeled fumonisin $B_1$ were examined after intragastric (0.5 mg fumonisin $B_1$/kg) or intravenous (0.4 mg fumonisin $B_1$/kg) administration to bile-cannulated and non-cannulated pigs (Prelusky *et al.*, 1994). Fumonisin derived radioactivity was not detected in the plasma of pigs dosed intravenously after 180 min in the non-cannulated group, or after 90 min in the cannulated group. Urinary excretion began within 3 h of administration and virtually ended after 8 h, accounting for only a small amount of administered toxin. Fecal excretion of fumonisin persisted for 48 h. The excretion in the intravenously dosed group occurred primarily via the bile, with biliary excretion greatest during the first 4 h, but persisting for 24–36 h.

Plasma radioactivity in intragastrically dosed pigs was first detected 30–45 min after dosing, with maximal activity present between 60 and 90 min. As reflected in plasma and elimination data, systemic bioavailability of the dose ranged from 3% to 6%. Excretion of the fumonisin occurred primarily via feces, with only trace amounts excreted via urine or bile.

At 72 h after administration, tissue radioactivity was highest in the liver, kidney, and large intestine in all groups. Intragastrically dosed groups had 10–20-fold lower tissue concentrations than did intravenously dosed groups, and only intravenously dosed groups had measurable radioactivity in brain, lung, and adrenal. Thus, it seems that liver and kidney are the primary organs of fumonisin metabolism and excretion in the pig, and that enterohepatic circulation prolongs the persistence of fumonisin in the body. The toxicokinetics of fumonisin $B_1$ in horses has not been evaluated.

When pigs were fed fumonisin at daily concentrations between 50 and 500 μg of fumonisin $B_1$ per kg of body weight for the last 5 months before slaughter, no muscle or kidney residues were detected (Liguoro *et al.*, 2004). Fumonisin $B_1$ was not detected in the eggs from laying hens following either intravenous or oral administration (Vudathala *et al.*, 1994). Although negligible concentrations have been shown to cross the mammary barrier (Spotti *et al.*, 2001), the toxin was not detected in milk from cattle that consumed a diet-containing fumonisins (Richard *et al.*, 1996). Therefore, it appears that fumonisin residues in meat, milk, or eggs does not represent a hazard or food safety concern for humans consuming these products.

# MECHANISM OF ACTION

## Sphingolipid alterations

Fumonisins are structurally related to sphingosine, the major long chain base backbone of cellular sphingolipids (Figure 78.1). They are competitive inhibitors of sphinganine and sphingosine *N*-acyltransferase (also known as ceramide synthase), key enzymes in the *de novo* sphingolipid biosynthetic pathway (Figure 78.2). These *N*-acyltransferase enzymes are responsible for catalyzing the acylation of sphinganine and the reutilization of sphingosine derived from sphingolipid turnover. This inhibition by fumonisin has been characterized *in vitro* using liver and brain microsomes, as well as in intact mammalian cells in culture (hepatocytes, neurons, renal cells, and macrophages) (Merrill *et al*., 1995). Fumonisin $B_1$ blocks the incorporation of radiolabeled serine into the sphingoid base backbone of ceramides and complex sphingolipids and prevents the conversion of sphinganine to sphingosine via addition of the 4,5 *trans* double bond, which occurs after acylation of sphinganine. Fumonisin also blocks reacylation of sphingoid bases (primarily sphingosine) released by hydrolysis of more complex sphingolipids (Merrill *et al*., 1995).

Sphingolipids are located in cellular membranes, lipoproteins (especially low-density lipoproteins), and other lipid-rich structures. Complex sphingolipids are critical for the maintenance of membrane structure, particularly microdomains such as caveolae. They also serve as binding sites for extracellular matrix proteins as well as for some microorganisms, microbial toxins, and viruses, and regulate the behavior of growth factor receptors (Merrill and Sweeley, 1996). Complex sphingolipids function as precursors for second messengers that mediate cell responses to growth factors, cytokines (including tumor necrosis factor-$\alpha$), differentiation factors, and 1,25-dihydroxyvitamin $D_3$. Therefore, sphingolipids are involved in the regulation of cell growth, cell-to-cell communication, differentiation, and neoplastic transformation (Hannun and Bell, 1989).

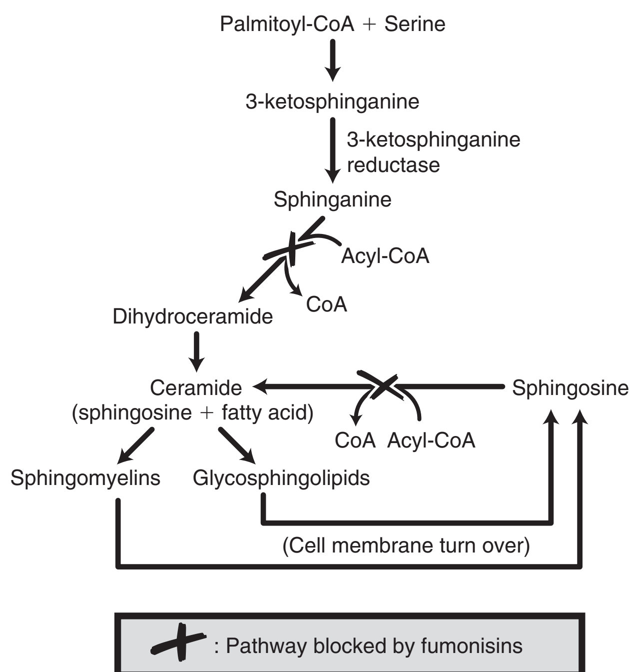



**FIGURE 78.2** The effects of fumonisin on the sphingolipid biosynthetic pathway (modified from Diaz and Boermans, 1994; reprinted with permission from Smith and Constable, 2004).

This enzyme inhibition by fumonisin produces a disruption of sphingolipid metabolism resulting in increased sphinganine and sphingosine along with a decrease in complex sphingolipids in the serum and tissues of animals (Wang *et al*., 1991). These elevations in concentrations of sphinganine and sphingosine have also been observed *in vivo* in several species including pigs, horses, and calves (Riley *et al*., 1993; Goel *et al*., 1996; Smith *et al*., 1999, 2000; Mathur *et al*., 2001). This disruption of sphingolipid metabolism is generally accepted as the probable mechanism of fumonisin toxicity, however only in pigs has the pathophysiology been definitively determined.

PPE has been shown to be a direct result of acute left-sided heart failure related to an increase in plasma and myocardial sphinganine and sphingosine concentrations (Smith *et al*., 1999, 2000; Constable *et al*., 2000). Sphingosine is an important intracellular second messenger that inhibits L-type calcium channels in myocardial cells, thereby decreasing sarcoplasmic reticulum $Ca^{2+}$-induced $Ca^{2+}$-release and cardiac contractility (McDonough *et al*., 1994; Webster *et al*., 1994). As sphingosine concentrations begin to increase in pigs that consume fumonisin, myocardial calcium channels are blocked and contractility begins to decrease (Constable *et al*., 2000; Smith *et al*., 2000). Ultimately, this decrease in cardiac contractility causes acute left ventricular failure and pulmonary edema (Figure 78.3).

The mechanism of ELEM may also be a direct result of fumonisin-induced increases in sphingosine concentrations. A recent study demonstrated that fumonisin administration-induced cardiovascular dysfunction in horses (Smith *et al*., 2002). This study demonstrated an association between neurologic signs, increased serum and myocardial sphingosine concentrations, and cardiovascular depression in fumonisin-treated horses. At necropsy, horses with leukoencephalomalacia have histologic evidence of cerebral edema in the brain. Another study has reported that fumonisin-treated horses also have elevated protein, albumin, and IgG levels in cerebrospinal fluid samples (Foreman *et al*., 2004). Taken together, these findings indicate that fumonisin toxicity in horses is associated with the development of vasogenic cerebral edema as a

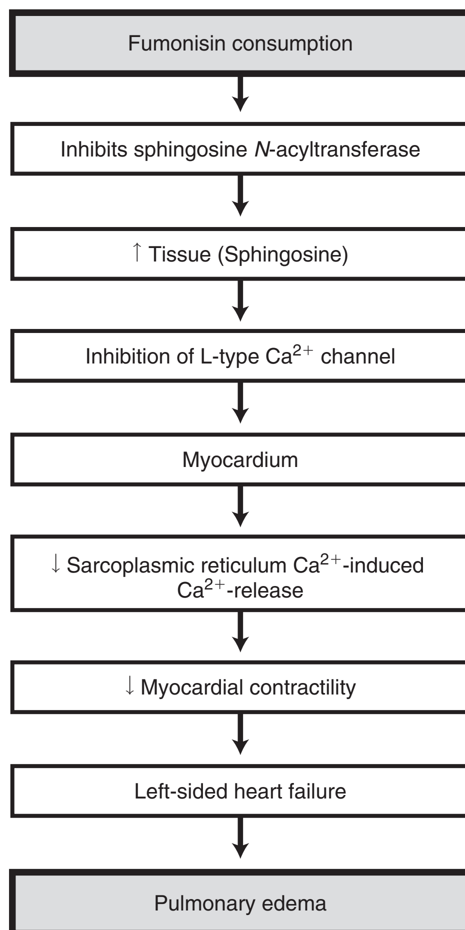



**FIGURE 78.3** Mechanism of fumonisin-induced pulmonary edema in swine. Fumonisin inhibition results in increased tissue sphingosine and sphinganine concentrations. The increased sphingosine concentrations inhibit the L-type $Ca^{2+}$ channels of cardiac myocytes resulting in decreased myocardial contractility. This decrease in contractility results in acute left-sided heart failure and pulmonary edema.

direct result of increased blood–brain barrier permeability. Horses are a species that are dependent on autoregulation of cerebral blood flow when they lower their head to graze. Because of gravitational forces, distal carotid artery pressure can increase tremendously when the animal bends to eat or drink. However, this rise in carotid pressure does not create a significant increase in cerebral blood flow due to the constriction and dilation of cerebral arterioles which maintain normal cerebral blood pressures (Faraci and Heistad, 1990). It has been shown that L-type calcium channels are the primary mediators of vascular tone in these cerebral arterioles (Michelakis *et al*., 1994). Therefore, it has been hypothesized that fumonisin-induced increases in sphingosine concentrations inhibit the calcium channels in cerebral arterioles leading to the inability to maintain normal cerebral blood pressure and vasogenic cerebral edema. This hypothesis will require further research to be proven definitively.

# TOXICITY

Fumonisin has been shown to cause liver damage in multiple species including pigs, horses, cattle, rabbits, and primates (Jaskiewicz *et al*., 1987; Voss *et al*., 1989; Haschek *et al*., 1992; Osweiler *et al*., 1993; Ross *et al*., 1993; Gumprecht *et al*., 1995) as well as species-specific target organ toxicity, such as lung in pigs (Haschek *et al*., 1992), brain in horses (Ross *et al*., 1993), kidney in rats, rabbits, and sheep (Voss *et al*., 1989; Edrington *et al*., 1995; Gumprecht *et al*., 1995), and esophagus in rats and pigs (Casteel *et al*., 1993; Lim *et al*., 1996). This chapter will focus on fumonisin toxicity in pigs and horses since they are the most common clinical poisonings dealt with in veterinary medicine, however cattle and poultry will be briefly discussed.

## Spontaneous and experimental fumonisin toxicosis in swine

In early research prior to the initial isolation and characterization of fumonisins, *F. verticillioides* culture material was reported toxic to swine (Kriek *et al*., 1981). In that experiment, 3 pigs were fed *F. verticillioides* culture material grown on corn. Two of the 3 pigs fed the culture material in this study died within 5 days of pulmonary edema. The third pig was fed culture material for 89 days and was then killed following a period of feed refusal.

The 1989 corn crop in many midwestern and southeastern parts of the United States was heavily infected with *F. verticillioides*, and contaminated screenings fed to animals led to fatal outbreaks of PPE (Harrison *et al*., 1990; Osweiler *et al*., 1992). This syndrome was also reproduced experimentally with contaminated corn screenings and purified fumonisin $B_1$ (Harrison *et al*., 1990; Osweiler *et al*., 1992).

Lung and liver are the major target organs of fumonisin toxicosis in pigs; however, other organs have been reported to be affected. Pigs which ingest fumonisin at concentrations high enough to cause pulmonary edema usually die after about 4 days in field cases (Osweiler, 1992) and after 3–6 days of fumonisin exposure experimentally (Haschek *et al*., 1992; Motelin *et al*., 1994; Gumprecht *et al*., 1998). Pigs which survive chronic exposure to high doses of fumonisin without developing pulmonary edema typically demonstrate hepatic disease with anorexia, weight loss and generalized icterus (Osweiler *et al*., 1992; Colvin *et al*., 1993). Hepatic toxicity occurs at doses significantly

lower than those necessary to cause pulmonary edema (Colvin *et al.*, 1993; Motelin *et al.*, 1994).

## Fumonisins in swine-pulmonary effects

Pulmonary edema (Figure 78.4) has been reported in pigs fed naturally contaminated fumonisin-containing food, fumonisin-containing culture material or following i.v. administration of fumonisin (Harrison *et al.*, 1990; Haschek *et al.*, 1992; Osweiler *et al.*, 1992; Motelin *et al.*, 1994). Reported concentrations of fumonisin required to produce pulmonary edema have been variable, presumably due to variability in susceptibility among exposed animals (Table 78.1). However, other constituents in the diet and analytical detection related to the ability to extract fumonisin from different matrices could account for some of the variability. In addition, some reports have reported only the concentration of fumonisin $B_1$ associated with the development of pulmonary edema, while others have reported both fumonisin $B_1$ and fumonisin $B_2$. Fumonisin $B_2$ usually occurs at about 30% of fumonisin $B_1$ in naturally contaminated corn, and is generally considered to be equitoxic to fumonisin $B_1$ (Ross *et al.*, 1994). Reported doses that induced pulmonary edema in swine include 100 ppm of fumonisin $B_1$ and fumonisin $B_2$ in naturally contaminated corn (Motelin *et al.*, 1994), 16 mg fumonisin $B_1$/kg/day as fumonisin-containing culture material (Colvin *et al.*, 1993), and 20 mg fumonisin $B_1$/kg/day as culture material (Gumprecht *et al.*, 1998). Fumonisin-induced pulmonary edema has also been reported with naturally contaminated corn (330 mg of fumonisin $B_1$ per kg of feed) in Hungary (Fazekas *et al.*, 1998), Brazil (Sydenham *et al.*, 1992), and Thailand (Patchimasiri *et al.*, 1998).

A more recent study has suggested that even lower concentrations of fumonisins may be able to induce pulmonary edema in swine (Zomborszky *et al.*, 2000). Fumonisin $B_1$ was fed added to the feed of weaned pigs at doses of 0, 10, 20, and 40 ppm for 4 weeks as fumonisin-containing culture material (5 pigs per group). Computed tomography of the lungs and magnetic resonance imaging of the brains were performed prior to the study and at 2 and 4 weeks of fumonisin feeding. Histopathology was also done at the time of necropsy (4 weeks). The results of this study showed that all 5 pigs fed fumonisin $B_1$ at 40 ppm developed "severe" pulmonary edema as assessed by computed tomography and histopathology. Two of the 5 pigs fed fumonisin $B_1$ at 20 ppm had "severe" pulmonary edema while 2 other pigs in the group had "mild" edema. In the 10 ppm group, 3 of the 5 pigs were reported to have "mild" pulmonary edema. Magnetic resonance studies of the brain were not able to identify any significant changes during the course of the study in any group.

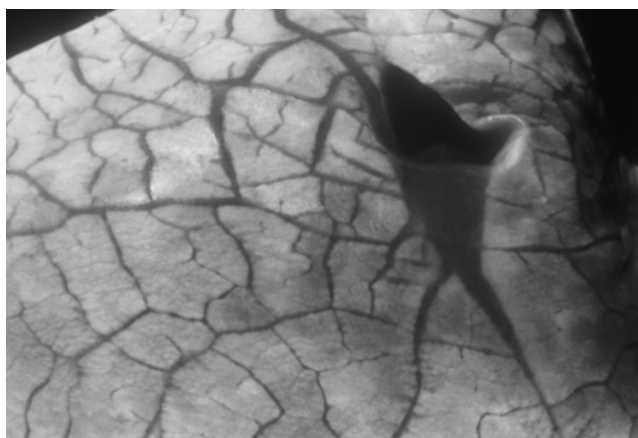


**FIGURE 78.4** Lung from a pig fed fumonisin-containing culture material at a dose of 20 mg fumonisin $B_1$ per kg of body weight for 4 days. Pulmonary edema is characterized by severe widening of the interlobular septa (reprinted with permission from Smith and Constable, 2004). This figure is reproduced in color in the color plate section.

Clinical signs associated with the development of pulmonary edema consistently begin 3–6 days after initiation of exposure to a high concentration of fumonisins. These include dyspnea and open mouthed breathing, increased respiratory rate, cyanosis of skin and mucous membranes, inactivity and sudden death (Osweiler *et al.*, 1992). Pigs usually die within a few hours after the onset of definitive respiratory distress. Histologically, pulmonary edema is present by day 3 of fumonisin exposure (Gumprecht *et al.*, 1998) and is characterized by interstitial edema around airways and vessels, in interlobular and subpleural connective tissues, and in alveolar interstitium (Harrison *et al.*, 1990; Haschek *et al.*, 1992; Osweiler *et al.*, 1992; Gumprecht *et al.*, 1998). Lymphatics are dilated and alveolar edema is often present. Fluid is also present within the thoracic cavity.

In ultrastructural studies using immersion fixed lungs, the endothelium was found to be swollen, vacuolated, and sometimes missing in pigs with pulmonary edema (Haschek *et al.*, 1992). Additional studies using intravascularly perfused lungs (to allow better examination of the vascular system) demonstrated accumulations of fragmented membranous material in the cytocavitary region of endothelial cells (Gumprecht *et al.*, 1998).

## Fumonisins in swine-hepatic effects

Hepatic changes in pigs exposed to fumonisins include elevation of liver associated enzyme activities, altered clinical chemistries, changes in sphingolipid parameters, and morphological alterations. In pigs, hepatic toxicity occurs prior to the development of pulmonary edema,

**TABLE 78.1 Effect of fumonisin in pigs**

| Number of animals | Dose and route | Duration | Toxic effects | References |
|---|---|---|---|---|
| *Experimental studies using purified fumonisin* | | | | |
| 4 pigs | 0.174–0.4 mg $FB_1$ per kg of BW/day, i.v. | 4–8 days | 2 high dose pigs developed pulmonary edema | Harrison *et al*. (1990) |
| 2 pigs | 0.88–1.15 mg $FB_1$ per kg of BW/day, i.v. | 1 pig for 4 days (0.88 mg/kg/day) – 2nd pig for 9 days (1.15 mg/kg/day) | Mild interstitial pulmonary edema histologically in 1 pig; hepatic lesions; pancreatic lesions | Haschek *et al*. (1992) |
| 3 pigs | 4.5–6.6 mg $FB_1$ per kg of BW/day in feed | 5–15 days | 2 of the 3 pigs developed severe pulmonary edema; hepatic lesions and mild pancreatic lesions noted | Haschek *et al*. (1992) |
| 2 pigs | 0.4 mg $FB_1$ per kg of BW/day, i.v. | 12–14 days | Elevated AST, GGT, bilirubin; liver lesions | Osweiler *et al*. (1992) |
| 8 pigs | 10 ppm $FB_1$ added to the diet – fed *ad libitum* | 8 weeks | Decreased weight gain Elevated cholesterol | Rotter *et al*. (1996) |
| 6 pigs | 1 mg $FB_1$ per kg of BW/day, i.v. | 4 days | Decrease in left ventricular contractility and mild pulmonary edema | Smith *et al*. (2000) |
| *Experimental studies using fumonisin-containing culture material* | | | | |
| 6 pigs | Feeds containing 100 and 190 ppm $FB_1$ – fed *ad libitum* | 100 ppm diet fed *ad libitum* for 7 days followed by 190 ppm diet for 83 days | Elevated AST, ALP, GGT; nodular hyperplasia of the liver; histologic esophageal lesions | Casteel *et al*. (1993) |
| 4 pigs | Feed containing 200 ppm of $FB_1$ fed *ad libitum* | up to 43 days | Elevated bilirubin, AST, cholesterol; hepatic lesions; decreased weight gain | Colvin *et al*. (1993) |
| 7 pigs | 16–64 mg $FB_1$ per kg of BW/day as oral gavage | 3–5 days | All pigs developed pulmonary edema within 5 days | Colvin *et al*. (1993) |
| 3 pigs | 4–16 mg $FB_1$ per kg of BW/day as oral gavage | up to 45 days | Severe hepatic disease; icterus; elevated liver enzymes; no pulmonary edema | Colvin *et al*. (1993) |
| 11 pigs | Feeds containing 100, 160, and 190 ppm $FB_1$ – fed *ad libitum* | 6 pigs were fed 100 ppm for 10 days then 190 ppm for up to 83 days; 5 pigs were fed 100 ppm for 5 days and then 160 ppm for up to 205 days | Nodular hyperplasia of the liver; Elevated AST, ALP, GGT, bilirubin<br><br>Right ventricular hypertrophy; medial hypertrophy of the small pulmonary arteries | Casteel *et al*. (1994) |
| 6 pigs | Feed containing 47 ppm $FB_1$ fed *ad libitum* | 28 days | Decreased feed consumption; Elevated AST, GGT, ALP, and creatinine; hepatic and renal lesions; medial hypertrophy of the pulmonary arteries | Harvey *et al*. (1996) |
| 10 pigs | 20 mg of $FB_1$ per kg of BW/day, in feed | 7 days | Pulmonary edema and cardiovascular abnormalities | Smith *et al*. (1996a, b) |
| 2 pigs | 14.5 and 16 mg of $FB_1$ per kg of BW/day, in feed | 4 days | Severe pulmonary edema; renal lesions | Fazekas *et al*. (1998) |
| 24 pigs | 20 mg of $FB_1$ per kg of BW/day, in feed | up to 5 days – some pigs were euthanized each day of the study | All 12 pigs euthanized on days 0, 1, and 2 had no lesions<br>2/5 day 3 pigs and all of the day 4 and 5 pigs had pulmonary edema<br>Elevations in liver enzymes and hepatic lesions seen on day 2 – bile acids first liver parameter to increase | Gumprecht *et al*. (1998) |
| 6 pigs | 20 mg of $FB_1$ per kg of BW/day, in feed | 5–6 days – pigs were euthanized as they developed pulmonary edema | Increased pulmonary artery pressure, pulmonary artery wedge pressure and decreased cardiac output, heart rate, and mean arterial pressure | Smith *et al*. (1999) |

**TABLE 78.1 (Continued)**

| Number of animals | Dose and route | Duration | Toxic effects | Reference |
|---|---|---|---|---|
| 5 pigs | Feed containing 40 ppm $FB_1$ fed *ad libitum* | 4 weeks | No clinical signs – gross pulmonary edema evident at necropsy | Zomborszky *et al.* (2000) |
| 5 pigs | Feed containing 20 ppm $FB_1$ fed *ad libitum* | 4 weeks | 2 of the 5 pigs had gross pulmonary edema – 2 additional pigs had mild evidence of edema present histologically | Zomborszky *et al.* (2000) |
| 4 pigs | Feed containing 20 ppm $FB_1$ fed *ad libitum* | 28 days | 3 of the 4 pigs had mild pulmonary edema evident histologically | Zomborszky *et al.* (2000) |
| 7 pigs | 20 mg of $FB_1$ per kg of BW/day, in feed | 3 days | Decreased left ventricular contractility; heart rate, cardiac output, and mechanical efficiency of the left ventricle | Constable *et al.* (2000) |
| *Experimental studies using naturally contaminated corn screenings* | | | | |
| 6 pigs | 105–155 ppm $FB_1$ in corn screenings fed *ad libitum*) | Up to 28 days | 3 of 6 pigs developed pulmonary edema; liver and pancreatic lesions | Harrison *et al.* (1990) |
| 8 pigs | 92 ppm $FB_1$ in corn screenings fed *ad libitum* | Up to 21 days | 6 of 8 pigs developed pulmonary edema; the remaining 2 pigs were icteric with increased liver enzymes | Osweiler *et al.* (1992) |
| 5 pigs | 175 ppm ($FB_1 + FB_2$) as corn screenings mixed in a complete ration | Up to 14 days | 3 of 5 pigs developed pulmonary edema; hepatotoxicity; decreased weight gain | Motelin *et al.* (1994) |
| 5 pigs | 101 ppm ($FB_1 + FB_2$) as corn screenings mixed in a complete ration | Up to 14 days | Elevated GGT, ALT, AST, ALP, and bilirubin; liver lesions; decreased weight gain | Motelin *et al.* (1994) |
| 5 pigs | 39 ppm ($FB_1 + FB_2$) as corn screenings mixed in a complete ration | 14 days | Histologic liver lesions | Motelin *et al.* (1994) |
| *Reported fumonisin concentrations from naturally occurring outbreaks* | | | | |
| 34 pigs from 2 farms | 105–155 ppm $FB_1$ | Unknown | Lethal pulmonary edema | Harrison *et al.* (1990) |
| 16 pigs from 9 farms | All feed samples associated with pulmonary edema contained $\geqslant$20 ppm $FB_1$ to a maximum of 330 ppm $FB_1$ | Unknown | Lethal pulmonary edema | Osweiler *et al.* (1992) |

and alterations are time and dose dependent (Motelin *et al.*, 1994). Increased activities of serum enzymes such as alkaline phosphatase (ALP), aspartate aminotransferase (AST) and gamma glutamyl transpeptidase (GGT) and concentrations of total bilirubin, bile acids, and cholesterol have been reported as early as 1 day after the initiation of fumonisin exposure (Harrison *et al.*, 1990; Haschek *et al.*, 1992; Osweiler *et al.*, 1992; Colvin *et al.*, 1993; Motelin *et al.*, 1994; Gumprecht *et al.*, 1998). These alterations reflect hepatocyte damage, as well as altered hepatic function.

Morphologic alterations are dose related and progressive with continued ingestion of fumonisins. Following short-term exposure, changes include hepatic cord disorganization, cytoplasmic vacuolation, apoptosis, scattered necrosis, and increased cell proliferation (Harrison *et al.*, 1990; Osweiler *et al.*, 1992; Motelin *et al.*, 1994; Gumprecht *et al.*, 1998). Histologic alterations were observed as early as 2 days after the initiation of treatment with a lethal dose (Gumprecht *et al.*, 1998), and at a concentration as low as 23 ppm when fed for 14 days (Motelin *et al.*, 1994). Long-term fumonisin exposure can result in fibrosis or development of hyperplastic nodules in the liver (Harrison *et al.*, 1990; Casteel *et al.*, 1993). Ultrastructurally, large accumulations of proteinaceous and membranous material were observed in the Space of Disse in pigs that developed fumonisin-induced pulmonary edema (Haschek *et al.*, 1992). Hepatocytes lost microvilli from their sinusoidal face while numerous Kupffer cells contained multilamellar bodies.

## Fumonisins in swine-cardiovascular effects

Fumonisins have been shown to decrease left ventricular contractility, heart rate, cardiac output, mean arterial pressure, arterial and mixed venous blood $O_2$ tensions, and systemic oxygen delivery, while increasing mean pulmonary artery pressure, oxygen consumption, and oxygen extraction ratio in swine (Smith *et al.*, 1996a, b, 1999, 2000; Constable *et al.*, 2000; ). The decrease in cardiac contractility leads to acute left ventricular failure and pulmonary edema in pigs exposed to high concentrations of fumonisin in feed. Chronic exposure to lower levels of fumonisin leads to the development of right ventricular hypertrophy and medial hypertrophy of the small pulmonary arteries in pigs, likely a result of pulmonary hypertension (Casteel *et al.*, 1994).

## Fumonisins in swine-immunologic effects

Fumonisins have also been shown to predispose pigs to respiratory disease. In one case–control study, swine farms with $\geqslant$20 ppm of fumonisin in the feed were at significantly greater risk for pneumonia as compared to farms with low fumonisin concentrations (Bane *et al.*, 1992). As the concentration of fumonisin in the feed increased, the risk of respiratory disease continued to increase. Later it was shown that exposure to fumonisins depressed pulmonary intravascular macrophage function, and pigs exposed to this toxin had decreased pulmonary clearance of blood-borne particulates and bacteria when compared to control animals (Smith *et al.*, 1996c). In a more recent study, exposure to fumonisin exacerbated respiratory disease in a *Pasteurella multocida* challenge model (Halloy *et al.*, 2005). Pigs that were fed 0.5 mg of $FB_1$ per kg of body weight/day for 7 days had delayed growth, increased coughing and more severe lung lesions that control pigs. Therefore, at levels well below those needed to cause hepatic lesions or pulmonary edema, fumonisins are likely to reduce growth rates and increase disease in pigs.

## Fumonisin toxicosis in horses: historical

Several outbreaks of a neurologic disease in horses occurred in the United States in the early 1900s with thousands of deaths reported in several states. The earliest citation of neurologic deaths associated with the feeding of contaminated corn was from Maryland (MacCallum and Buckley, 1902). The condition was commonly referred to as "cerebrospinal meningitis" and presented with fairly characteristic signs. The duration of disease varied from a few hours to a week, and the brains from affected horses had "softened" areas in the cerebrum involving only the white matter. Additional outbreaks were subsequently reported from Kansas, Iowa, Mississippi, and North Carolina. A similar disease, described as "epizootic cerebritis" had been encountered in 1891, however it is not known whether this was associated with corn (Butler, 1902). When feed from an outbreak of "leucoencephalitis" in Kansas was fed to a horse, it died after developing neurologic signs (Butler, 1902). At necropsy, the left cerebral hemisphere was "soft to the touch, and when cut through, the white matter was broken down extensively, nearly the entire hemisphere being involved." Several attempts were made to identify an infectious agent in the brain of affected horses and all were negative (MacCallum and Buckley, 1902). The authors concluded that a toxic etiology was likely.

In central Illinois, more than 5000 horses died during the winter of 1934–1935 from a syndrome referred to as "cornstalk disease" (Graham, 1935). Brain tissue suspensions and filtrates were inoculated into laboratory animals, however no infectious etiology could be identified. Graham then placed 8 horses into a field-containing cornstalks in Rantoul, Illinois. Two of these animals died, 23 and 26 days after being placed in the field (Graham, 1936). These outbreaks were similar to those reported in 1893 and 1914 which had occurred following a summer drought (Graham, 1936). Neurologic deaths with similar lesions were also reported in Iowa during the winter of 1914 and in the spring of 1935 (Schwarte *et al.*, 1937). Histologic examination of the brain revealed no evidence of infectious agents. This "syndrome" was then subsequently reproduced by feeding moldy corn and corn fodder to 5 horses (Schwarte *et al.*, 1937). A similar disease syndrome was identified and confirmed by feeding trials in Egypt (Badiali *et al.*, 1968; Wilson and Maronpot, 1971) and in South Africa (Marasas *et al.*, 1976). At necropsy, these studies were able to consistently demonstrate swelling of the cerebral hemispheres and flattening of the overlying gyri. On coronal sections, there were cavities of varying sizes with liquefactive necrosis of subcortical white matter in one or both cerebral hemispheres (Figure 78.5). There was also scattered multifocal hemorrhages in the surrounding white matter (Marasas *et al.*, 1976; Haliburton *et al.*, 1979). Based on these findings, Marasas and his associates coined the term "equine leukoencephalomalacia" as a distinct clinical and morphologic syndrome in horses associated with the feeding of corn (Marasas *et al.*, 1976).

*F. verticillioides* was later isolated from corn collected from field outbreaks in Egypt, and leukoencephalomalacia was subsequently reproduced in donkeys fed corn inoculated with the fungus (Wilson and Maronpot, 1971). In South Africa however, samples of corn inoculated with *F. verticillioides* produced liver damage and icterus in several horses and donkeys, but not brain lesions (Kellerman *et al.*, 1972). Marasas *et al.* (1976) then produced a batch of culture material using a strain of *F. verticillioides* from an outbreak of leukoencephalomalacia to produce liver damage and neurologic disease in a horse. It was then

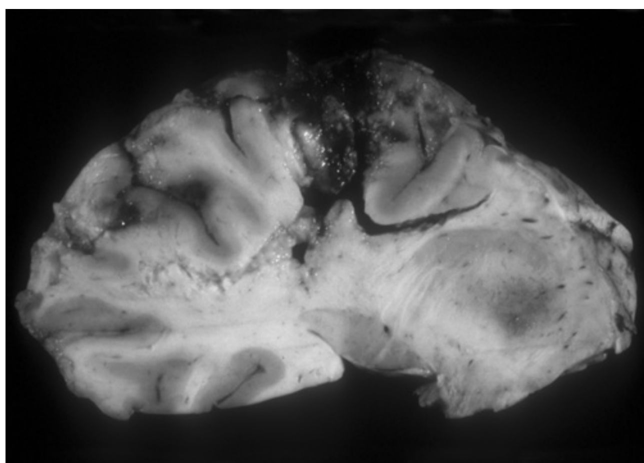


**FIGURE 78.5** A cross-section of a cerebral hemisphere from a horse demonstrating liquefactive necrosis of the white matter typical of ELEM (reprinted with permission from Smith and Constable, 2004). This figure is reproduced in color in the color plate section.

concluded that both hepatic disease and ELEM were manifestations of the same toxicosis, with different clinical syndromes occurring depending on toxin dose and length of exposure. Following the isolation and purification of fumonisin $B_1$ in the late 1980s, ELEM was experimentally induced by the administration of purified toxin (Marasas *et al.*, 1988; Kellerman *et al.*, 1990).

## Spontaneous and experimental fumonisin toxicosis in horses

Since the discovery of fumonisin $B_1$ as the causative agent of ELEM, many more disease outbreaks associated with the feeding of corn have been reported (Wilson *et al.*, 1990a; Ross *et al.*, 1991; Uhlinger, 1991; Binkerd *et al.*, 1993; Christley *et al.*, 1993; Ross *et al.*, 1993; Wilkins *et al.*, 1994; Bailly *et al.*, 1996; Bela and Endre, 1996; Cerrillo *et al.*, 1996; Rosiles *et al.*, 1998). Purified fumonisin $B_1$ has induced ELEM when administered orally (Kellerman *et al.*, 1990) and intravenously (Marasas *et al.*, 1988; Laurent *et al.*, 1989; Foreman *et al.*, 2004). Purified fumonisin $B_2$ has also induced ELEM when given orally (Ross *et al.*, 1994). Fumonisin $B_3$ is suspected to be much less toxic than either $B_1$ or $B_2$ (Ross *et al.*, 1994). Fumonisin $B_1$ is considered to be the primary cause of ELEM however, as fumonisin $B_2$ is usually present in concentrations that are 20–40% of fumonisin $B_1$ (Ross *et al.*, 1991). Although ELEM has occurred in horses eating commercial feedstuffs (Wilson *et al*, 1990b; Ross *et al.*, 1991), the feeding of corn screenings has been more frequently associated with ELEM, because fumonisin concentrations are much higher in screenings than in whole kernels of corn (Binkerd *et al.*, 1993). Fumonisin $B_1$ also appears to survive the pelleting process for equine feeds (Ross *et al.*, 1991).

Leukoencephalomalacia has been reproduced with intravenous administration of fumonisin $B_1$ in three separate studies (Marasas *et al.*, 1988; Laurent *et al.*, 1989; Foreman *et al.*, 2004). Marasas *et al.* (1988) administered 0.125 mg fumonisin $B_1$/kg of body weight, i.v. q24 h which produced ELEM in 9 days. Laurent *et al.* (1989) administered 0.1 mg fumonisin $B_1$/kg of body weight, i.v. q24 h for 16 days followed by 0.2 mg/kg/day for 2 additional days. Leukoencephalomalacia was induced in 18 days. Foreman *et al.* (2004) administered 0.05, 0.1, or 0.2 mg fumonisin $B_1$/kg of body weight i.v. q24 h to 10 horses and all developed neurologic signs and were euthanized between days 4 and 12 of the study. In contrast, horses dosed with 0.01 mg fumonisin $B_1$/kg of body weight for 28 days in this study did not develop neurologic signs. Purified fumonisin $B_1$ has also been administered orally in other studies (1.25 or 2.5 mg fumonisin $B_1$/kg of body weight, p.o., q24 h), producing mild edema of the brain stem and hepatic disease in 11–12 days in 2 horses. In a subsequent study, animals were fed 0.6–4.0 mg fumonisin $B_1$/kg of body weight, p.o., q 24 h for 33 or 35 days, producing hepatotoxicity and neurologic signs starting on days 22 and 24 in two weanling horses (Kellerman *et al.*, 1990).

Doses of fumonisin reported from naturally occurring cases of fumonisin have varied (Table 78.2). One field report calculated that the ingestion of 0.6–2.1 mg fumonisin $B_1$/kg of body weight would induced ELEM in 24–28 days (Wilson *et al.*, 1990b). Another study found that leukoencephalomalacia was associated with ingestion of feed-containing fumonisin $B_1$ concentrations greater than 10 ppm, and concluded that feed with fumonisin $B_1$ concentrations greater than 10 ppm was not safe to be fed to horses (Ross *et al.*, 1991).

## Neurologic and hepatic effects in horses

Several reports have considered ELEM and hepatotoxicity to be two separate syndromes associated with fumonisin toxicity in horses, with the terms "classic neurotoxic syndrome" and "hepatic syndrome" being used (McCue, 1989). However, it appears more likely these are not true "distinct" syndromes but are related to the concentration of fumonisin in the feed, the duration of toxin consumption, and the tolerance of the individual horse to fumonisin. In some outbreaks, horses have died from ELEM while other horses have died from hepatotoxicity, and occasionally individual horses exhibiting both neurologic and hepatic signs have been described. Reported clinical signs associated with hepatic disease include icterus, mucous membrane petechiae, and swelling of the lips or muzzle (Uhlinger, 1991; Ross *et al.*, 1993).

Ross *et al.* (1993) described an experimental study where 1 horse died acutely with "mild encephalopathy

**TABLE 78.2 Effect of fumonisin in horses**

| Number of animals | Dose and route | Duration | Toxic effects | References |
|---|---|---|---|---|
| *Experimental studies using purified fumonisin* | | | | |
| 1 horse | 0.125 mg/kg/day, i.v. | 7 treatments over 9 days | Leukoencephalomalacia<br>Elevated AST, GGT | Marasas *et al*. (1988) |
| 1 horse | 2.5 mg/kg/day, p.o. (by gavage) | 6 doses over 11 days | Severe hepatosis<br>Elevated AST, GGT | Marasas *et al*. (1988) |
| 1 horse | 1.25 mg/kg/day, p.o. (by gavage) | 6 doses over 11 days | Mild hepatosis<br>Elevated AST, GGT | Marasas *et al*. (1988) |
| 1 horse | 0.1 mg/kg/day, i.v. and 0.2 mg/kg/day, i.v. | 0.1 mg/kg dose given for 16 days then 0.2 mg/kg dose given for 2 additional days | Leukoencephalomalacia | Laurent *et al*. (1989) |
| 1 horse | 1.25–4.0 mg/kg, p.o. | 20 doses given over 35 days | Leukoencephalomalacia<br>Elevated AST | Kellerman *et al*. (1990) |
| 1 horse | 1.0–4.0 mg/kg, p.o. | 20 doses given over 33 days | Leukoencephalomalacia<br>Elevated AST, GGT | Kellerman *et al*. (1990) |
| 13 horses | 0.01–0.20 mg/kg, i.v. | Up to 10 days | Leukoencephalomalacia at 0.10 and 0.20 mg/kg – hepatic toxicity only at lower doses | Smith *et al*. (2002), Foreman *et al*. (2004) |
| *Experimental studies using fumonisin-containing culture material* | | | | |
| 2 horses | Diet contained 19 ppm $FB_1$ and was fed *ad libitum* | 27 days | None | Schumacher *et al*. (1995) |
| 2 horses | Diet contained 200 ppm $FB_1$ and was fed *ad libitum* | 12 and 16 days | Leukoencephalomalacia<br>Increased GGT | Schumacher *et al*. (1995) |
| 2 horses | 2 diets containing 65 and 130 ppm $FB_1$ and was fed *ad libitum* | −65 ppm fed for 10 days<br>−130 ppm fed for an additional 17 days | Leukoencephalomalacia<br>Increased GGT | Schumacher *et al*. (1995) |
| *Experimental studies using naturally contaminated corn screenings* | | | | |
| 4 ponies | diet contained 44 ppm $FB_1$ and was fed *ad libitum* | 10–97 days | 2 horses died with ELEM on days 10 and 45<br>–2 horses were normal after 97 days | Wang *et al*. (1992) |
| 4 ponies | Diets contained between 1 and 22 ppm $FB_1$ and were fed *ad libitum* | 238–326 days | ELEM in 2 of 5 horses<br>Moderate to mild hepatosis in all 5 horses | Wilson *et al*. (1992) |
| 5 ponies | Diet contained 8 ppm $FB_1$ and was fed *ad libitum* | 180 days | Mild histologic lesions in the brain and liver at necropsy | Wilson *et al*. (1992) |
| 4 ponies | Diets contained between 1 and 88 ppm $FB_1$ and were fed *ad libitum* | 9–120 days | ELEM in all 4 ponies (day 9–120) – also severe hepatosis in 2 ponies | Ross *et al*. (1993) |
| *Reported fumonisin concentrations from naturally occurring outbreaks* | | | | |
| 18 horses | 37–122 ppm $FB_1$ | Unknown | ELEM confirmed in 14 horses | Wilson *et al*. (1990) |
| 45 horses | 8–126 ppm $FB_1$ | 7–35 days | All cases had confirmed leukoencephalomalacia | Ross *et al*. (1991) |
| 6 horses | 370 ppm $FB_1$ and 105 ppm $FB_2$ | Unknown | 4 horses died with ELEM;<br>2 horses with neurologic signs apparently recovered | Wilkins *et al*. (1994) |
| $100^+$ donkeys | 4–29 ppm $FB_1$ | Unknown | Many donkeys died of neurologic disease – ELEM confirmed in three cases | Rosiles *et al*. (1998) |

and hepatic necrosis" after 9 days of fumonisin exposure whereas 2 other horses died after 75 and 78 days of ELEM. The horse that died on day 9 showed neurologic signs prior to death ("visual impairment, mild ataxia, and slight head tremors") and had histologic evidence of leukoencephalomalacia at necropsy, however his death was primarily attributed to hepatotoxicity. This study lead to a common generalization that high doses of fumonisin were likely to induce hepatotoxicity, whereas lower doses of toxin over a longer period of time were

necessary to induce ELEM (McCue, 1989; Plumlee and Galey, 1994). However in other experimental studies, intravenous administration of fumonisin $B_1$ induced ELEM in 9 days (Marasas *et al.*, 1988) and 18 days (Laurent *et al.*, 1989). In a large study with varying doses of fumonisin, horses treated with higher doses developed leukoencephalomalacia (in 5–8 days), whereas horses that received lower concentrations developed primarily hepatic lesions without any evidence of neurotoxicity (Foreman *et al.*, 2004). Therefore, it can be concluded ELEM results from an acute exposure to feed-containing high concentrations of fumonisin $B_1$, while hepatotoxicity occurs with chronic ingestion of lower levels.

Serum biochemical changes associated with fumonisin toxicity in horses have been predominantly related to hepatotoxicity (increased AST, Wang *et al.*, 1992; increased AST and GGT, Laurent *et al.*, 1989; Kellerman *et al.*, 1990; increased GGT and SDH, Schumacher *et al.*, 1995; increased AST, GGT, and ALP, Ross *et al.*, 1993; increased AST, GGT, ALP, total bilirubin, and bile acids, Wilson *et al.*, 1992; and "elevated liver enzymes," Ross *et al.*, 1994). Serum cholesterol concentrations have not been examined.

The neurologic signs are usually summarized as sudden onset of one or more of the following: frenzy, aimless circling, head pressing, paresis, ataxia, blindness, depression, and hyperexcitability (Ross *et al.*, 1991; Wilson *et al.*, 1992). Other reports have stated that "the disease started with lack of appetite, followed by the disturbance of swallowing and chewing indicating the paralysis of cephalic and pharyngeal muscles. Paralysis of cephalic and cervical muscles spread to the muscles of the extremities and trunk. The animals moved with difficulties, tottering and ataxia developed. Signs of 'blindness' developed in one animal. At the final stage of disease, the affected animals lied down and died" (Bela and Endre, 1996). In a comprehensive study, early neurologic signs included mild proprioceptive abnormalities, including hindlimb ataxia, delayed forelimb placing reactions, and decreased tongue tone and movement (Foreman *et al.*, 2004). These signs progressed over 12–48 h to become more readily apparent. Hindlimb and trunkal ataxia in particular became more apparent with time. A variety of behavioral changes were observed including depression, hyperesthesia, and intermittent dementia. All horses had intact menace and pupillary light responses at the time of death.

Cerebrospinal fluid findings from horses with ELEM include elevations in protein concentration, albumin, and IgG concentrations and increased albumin quotients (Foreman *et al.*, 2004). Cerebrospinal fluid red blood cell, leukocyte, and glucose concentrations along with creatine kinase activity are not altered in horses with neurologic disease. Along with the histopathologic findings, these cerebrospinal fluid changes indicate the presence of a vasogenic cerebral edema in horses with leukoencephalomalacia.

### Fumonisin toxicity in cattle

Adult beef cattle appear relatively resistant to fumonisin. Feeder calves fed a diet-containing fumonisin concentrations up to 148 ppm for 31 days had only mild hepatotoxicity (Osweiler *et al.*, 1993). Although it is tempting to speculate that cattle are able to break down the toxin, it has been shown that fumonisin is poorly metabolized by the rumen. Instead, it is thought that cattle have an increased tolerance to fumonisin because of differences in the mechanism of action. In milk-fed calves treated with purified fumonisin $B_1$, the kidney was the target organ of toxicity (Mathur *et al.*, 2001). However, this study also demonstrated that sphingosine and sphinganine concentrations did not increase in the serum and tissues of calves to the same degree that has been shown in pigs and horses.

When a group of 26 dairy cattle were fed a ration containing 100 ppm of fumonisin for the first 70 days of their lactation period, they had a significant decrease in dry matter intake and a lower milk yield as compared to the control group (Diaz *et al.*, 2000). Milk production averaged 7 kg lower in the group fed the ration-containing fumonisin and there was a 13% decrease in feed intake over the duration of the study period. Therefore, it has been recommended to avoid fumonisin concentrations higher than 30 ppm in the total ration of dairy cattle.

### Fumonisin toxicity in poultry

Fumonisins can be toxic to both chickens and turkeys with concentrations in the feed as low as 100 mg/kg causing decreased body weight gain, diarrhea, and hepatotoxicity (Ledoux *et al.*, 1992; Bermudez *et al.*, 1997). There has also been an association between *F. verticillioides* (the fungus that produces fumonisin) and an acute death syndrome recognized in young chicks called spiking mortality syndrome. It was initially hypothesized that fumonisins were directly cardiotoxic to poultry and were the cause of this syndrome; however, more recent research has suggested moniliformin (another *F. verticillioides* produced mycotoxin) is primarily responsible.

## DIAGNOSIS AND TREATMENT

In addition to pathologic findings in animals, diagnosis of fumonisin toxicosis typically relies on detecting the actual toxin in feed samples. Fungal culture of feeds has little value in diagnosing fumonisin toxicosis because some corn samples contain very high concentrations of toxin with low levels of fungus, while other samples have heavy growths of *Fusarium* fungus with little to no detectable fumonisin. This is partly because the fungus that produces fumonisin

also produces other mycotoxins. Therefore, the definitive diagnosis of fumonisin toxicosis in animals must involve analyzing the feed for the presence of the actual toxin. Many diagnostic laboratories across the world offer assays to detect both fumonisin $B_1$ and $B_2$ in corn and feed samples. The two most commonly used methods for toxin detection are based on high performance liquid chromatography (HPLC) and enzyme-linked immunosorbent assay (ELISA). To date, there are no commercially available assays that detect fumonisin in serum or tissues of animals.

Another assay that may be used more commonly in the future to diagnose fumonisin toxicosis in animals is the sphinganine to sphingosine ratio (Sa:So ratio). Because of the fumonisin-induced disruption of sphingolipid biosynthesis (Wang *et al*., 1992), the Sa:So ratio increases in the serum and tissues of pigs and horses exposed to fumonisin. It has been suggested that this assay could be used to diagnose fumonisin toxicosis when feed analysis is not possible. Sphinganine and sphingosine data may be available from enough pigs and horses in the near future to provide values for normal and affected animals.

To date there have been no treatments described for either ELEM or PPE. Generally, the onset of clinical signs is acute and the progression of disease is rapid for both syndromes. The most important treatment is to identify and remove the source of contaminated feed to prevent other animals from developing clinical signs.

Guidelines for the maximum recommended levels of fumonisins in animal feeds have been published by the FDA Center for Veterinary Medicine (Table 78.3). It is important that livestock producers be aware of these guidelines and have their corn periodically tested for mycotoxins. Research has not yet found effective ways to decrease fumonisin concentrations in animal feedstuffs through processing or feed additives (i.e. binding agents). Corn-containing significant levels of fumonisin should be discarded, diluted with corn-containing lower concentrations of fumonisin, or fed to a less-sensitive species (i.e. ruminants or poultry interested for slaughter).

**TABLE 78.3 Recommended levels for total fumonisins ($B_1$ and $B_2$) in animal feeds**

| Animals | Recommended maximum level of total fumonisins in corn to be used for feed (ppm) | Recommended maximum level of total fumonisin in the ration (ppm) |
|---|---|---|
| Horse[a] | 5 | 1 |
| Swine | 20 | 10 |
| Ruminants[b] | 60 | 30 |
| Poultry[c] | 100 | 50 |
| Ruminant and poultry breeding stock[d] | 30 | 15 |
| Catfish | 20 | 10 |
| Other animals[e] | 10 | 5 |

From the United States Food and Drug Administration, Center for Veterinary Medicine.

[a] Includes donkeys, asses, and zebras.

[b] Cattle, sheep, goats, and other ruminants that are >3 months of age and are being fed for slaughter.

[c] Turkeys, chickens, ducklings, and other poultry being fed for slaughter.

[d] Includes lactating dairy cows, bulls, laying hens, and roosters.

[e] Includes dogs and cats.

# REFERENCES


Badiali L, Abou-Youssef MH, Radwan AI, Hamdy FM, Hildebrandt PK (1968) Moldy corn poisoning as the major cause of an encephalomalacia syndrome in Egyptian equidae. *Am J Vet Res* **29**: 2029–35.

Bailly JD, Raymond I, Le Bars P, Guyomard Y, Abadie J, Le Bars J, Guerre P, Delverdier M, Burgat V (1996) Leucoencephalomalacie des equides cas rapportes au CNITV. *Revue Med Vet* **147**: 787–96.

Bane DP, Neumann EJ, Hall WF, Harlin KS, Slife RL (1992) Relationship between fumonisin contamination of feed and mystery swine disease. *Mycopathology* **117**: 121–4.

Bela F, Endre B (1996) Occurrence of the equine leukoencephalomalacia (ELEM) caused by fumonisin-$B_1$ mycotoxin in Hungary. *Magy Allatorv Lapja* **8**: 484–7.

Bermudez AJ, Ledoux DR, Rottinghaus GE, Bennett GA (1997) The individual and combined effects of the *Fusarium* mycotoxins moniliformin and fumonisin $B_1$ in turkeys. *Avian Dis* **41**, 304–311.

Bezuidenhout SC, Gelderblom WCA, Gorst-Allman CP, Horak RM, Marasas WFO, Spiteller G, Vleggaar R (1988) Structure elucidation of the fumonisins, mycotoxins from *Fusarium moniliforme*. *J Chem Soc Chem Commun* 743–5.

Binkerd KA, Scott DH, Everson RJ, Sullivan JM, Robinson FR (1993) Fumonisin contamination of the 1991 Indiana corn crop and its effects on horses. *J Vet Diagn Invest* **5**: 653–5.

Butler T (1902) Notes on a feeding experiment to produce leucoencephalitis in a horse, with positive results. *Am Vet Rev* **26**: 748–51.

Casteel SW, Turk JR, Cowart RP, Rottinghaus GE (1993) Chronic toxicity of fumonisin in weanling pigs. *J Vet Diagn Invest* **5**: 413–17.

Casteel SW, Turk JR, Rottinghaus GE (1994) Chronic effects of dietary fumonisin on the heart and pulmonary vasculature of swine. *Fundam Appl Toxicol* **23**: 518–24.

Castelo MM, Sumner SS, Bullerman LB (1998) Occurrence of fumonisins in corn-based food products. *J Food Prot* **61**: 704–7.

Cerrillo GN, Rodriguez FS, Gordo LG, de Mendoza-Salcedo MH, Cordero VR (1996) Clinical and pathological aspects of an outbreak of equine leukoencephalomalacia in Spain. *J Vet Med A* **43**: 467–72.

Christley RM, Begg AP, Hutchins DR, Hodgson DR (1993) Leukoencephalomalacia in horses. *Aust Vet J* **70**: 225–6.

Colvin BM, Cooley AJ, Beaver RW (1993) Fumonisin toxicosis in swine: clinical and pathological findings. *J Vet Diagn Invest* **5**: 232–41.

Constable PD, Smith GW, Rottinghaus GE, Haschek WM (2000) Ingestion of fumonisin $B_1$-containing culture material decreases cardiac contractility and mechanical efficiency in swine. *Toxicol Appl Pharmacol* **162**: 151–60.

Diaz GJ, Boermans HJ (1994) Fumonisin toxicosis in domestic animals: a review. *Vet Human Toxicol* **36**: 548–55.

Diaz DE, Hopkins BA, Leonard LM, Hagler WM, Whitlow LW (2000) Effect of fumonisin on lactating dairy cattle. *J Dairy Sci* **83**: 1171.

Edrington TS, Kamps-Holtzapple CA, Harvey RB, Kubena LF, Elissalde MH, Rottinghaus GE (1995) Acute hepatic and renal toxicity in lambs dosed with fumonisin-containing culture material. *J Anim Sci* **72**: 508–15.

Faraci FM, Heistad DD (1990) Regulation of large cerebral arteries and cerebral microvascular pressure. *Circ Res* **66**: 8–17.

Fazekas B, Bajmocy E, Glavits R, Fenyvesi A, Tenyi J (1998) Fumonisin $B_1$ contamination of maize and experimental acute fumonisin toxicosis in pigs. *J Vet Med B* **45**: 171–81.

Foreman JH, Constable PD, Waggoner AL, Levy M, Eppley RM, Smith GW, Tumbleson ME, Haschek WM (2004) Neurologic abnormalities and cerebrospinal fluid changes in horses administered fumonisin B1 intravenously. *J Vet Int Med* **18**: 223–30.

Gelderblom WCA, Jaskiewicz K, Marasas WFO, Thiel PG, Horak RM, Vleggaar R, Kriek NPJ (1988) Fumonisins-novel mycotoxins with cancer-promoting activity produced by *Fusarium moniliforme*. *Appl Environ Micro* **54**: 1806–11.

Gelderblom WCA, Marasas WFO, Vleggaar R, Thiel PG, Cawood ME (1992) Fumonisins: isolation, chemical characterization and biological effects. *Mycopathology* **117**: 11–16.

Goel S, Schumacher J, Lenz SD, Kemppainen BW (1996) Effects of *Fusarium moniliforme* isolates on tissue and serum sphingolipid concentrations in horses. *Vet Hum Toxicol* **38**: 265–70.

Graham R (1935) Results of inoculating laboratory animals with equine brain-tissue suspensions and equine brain-tissue filtrates from spontaneous cases of so-called cornstalk disease. *J Am Vet Med Assoc* **39**: 778–80.

Graham R (1936) Cornstalk disease investigations: toxic encephalitis or non-virus encephalomyelitis of horses. *Vet Med* **31**: 46–50.

Gumprecht LA, Marcucci A, Vesonder RF, Peterson RE, Scott JR, Riley RT, Showker JL, Beasley VR, Haschek WM (1995) Effects of intravenous fumonisin $B_1$ in rabbits: nephrotoxicity and sphingolipid alterations. *Nat Toxins* **3**: 395–403.

Gumprecht LA, Beasley VR, Weigel RM, Parker HM, Tumbleson ME, Bacon CW, Meredith FI, Haschek WM (1998) Development of fumonisin-induced hepatotoxicity and pulmonary edema in orally dosed swine: morphological and biochemical alterations. *Toxicol Pathol* **26**: 777–88.

Haliburton JC, Vesonder RF, Lock TF, Buck WB (1979) Equine leukoencephalomalacia (ELEM): a study of *Fusarium moniliforme* as an etiologic agent. *Vet Hum Toxicol* **21**: 348–51.

Halloy DJ, Gustin PG, Bouhet S, Oswald IP (2005) Oral exposure to culture material extract containing fumonisins predisposes to the development of pneumonitis caused by *Pasteurella multocida*. *Toxicology* **213**: 34–44.

Hannun YA, Bell RM (1989) Functions of sphingolipids and sphingolipid breakdown products in cellular regulation. *Science* **243**: 500–7.

Harrison LR, Colvin BM, Greene JT, Newman LE, Cole Jr JR (1990) Pulmonary edema and hydrothorax in swine produced by fumonisin $B_1$, a toxic metabolite of *Fusarium moniliforme*. *J Vet Diagn Invest* **2**: 217–21.

Harvey RB, Edrington TS, Kubena LF, Elissalde MH, Casper HH, Rottinghaus GE, Turk JR (1996) Effects of dietary fumonisin $B_1$-containing culture material, deoxynivalenol-contaminated wheat, or their combination on growing barrows. *Am J Vet Res* **57**: 1790–4.

Haschek WM, Motelin G, Ness DK, Harlin KS, Hall WF, Vesonder RF, Peterson RE, Beasley VR (1992) Characterization of fumonisin toxicity in orally and intravenously dosed swine. *Mycopathology* **117**: 83–96.

Howard PC, Eppley RM, Stack ME, Warbritton A, Voss KA, Lorentzen RJ, Kovach RM, Bucci TJ (2001) Fumonisin $B_1$ carcinogenicity in a two-year feeding study using F344 rats and B6C3F$_1$ mice. *Environ Health Perspec* **109**(Suppl. 2): 277–82.

Jaskiewicz K, Marasas WFO, Taljaard JJF (1987) Hepatitis in vervet monkeys caused by *Fusarium monliforme*. *J Comp Path* **97**: 281–91.

Kellerman TS, Marasas WFO, Pienaar JG, Naude TW (1972) A mycotoxicosis of equidae caused by *Fusarium moniliforme* sheldon: a preliminary communication. *Onderstepoort J Vet Res* **39**: 205–8.

Kellerman TS, Marasas WFO, Thiel PG, Gelderblom WCA, Cawood M, Coetzer JAW (1990) Leukoencephalomalacia in two horses induced by oral dosing of fumonisin $B_1$. *Onderstepoort J Vet Res* **57**: 269–75.

Kriek NPJ, Kellerman TS, Marasas WFO (1981) A comparative study of the toxicity of *Fusarium verticillioides* (*F. moniliforme*) to horses, primates, pigs, sheep and rats. *Onderstepoort J Vet Res* **48**: 129–31.

Laurent D, Pellegrin F, Kohler F, Costa R, Thevenon J, Lambert C, Huerre M (1989) Fumonisin $B_1$ in equine leucoencephalomalacia pathogenesis. *Microbiol Aliment Nutr* **7**: 285–91.

Ledoux DR, Brown TP, Weibking TS, Rottinghaus GE (1992) Fumonisin toxicity in broiler chicks. *J Vet Diagn Invest* **4**: 330–3.

Liguoro M, Petterino C, Mezzalira G, Tenti S, Ravarotto L (2004) *Vet Hum Toxicol* **46**: 303–5.

Lim CW, Parker HM, Vesonder RF, Haschek WM (1996) Intravenous fumonisin $B_1$ induces cell proliferation and apoptosis in the rat. *Nat Toxins* **4**: 33–41.

MacCallum WG, Buckley SS (1902) Acute epizootic leucoencephalitis in horses. *Am Vet Rev* **26**: 21–36.

Marasas WFO, Kellerman TS, Pienaar JG, Naude TW (1976) Leukoencephalomalacia: a mycotoxicosis of equidae caused by *Fusarium moniliforme* sheldon. *Onderstepoort J Vet Res* **43**: 113–22.

Marasas WFO, Kellerman TS, Gelderblom WCA, Coetzer JAW, Thiel PG, van der Lugt JJ (1988) Leukoencepalomalacia in a horse induced by fumonisin $B_1$ isolated from *Fusarium moniliforme*. *Onderstepoort J Vet Res* **55**: 197–203.

Martinez-Larranaga MR, Anadon A, Diaz MJ, Fernandez-Cruz ML, Martinez MA, Frejo MT, Martinez M, Fernandez R, Anton RM, Morales ME, Tafur M (1999) Toxicokinetics and oral bioavailability of fumonisin $B_1$. *Vet Hum Toxicol* **41**: 357–62.

Mathur S, Constable PD, Eppley RM, Tumbleson ME, Smith GW, Tranquill WJ, Morin DE, Haschek WM (2001) Fumonisin $B_1$ increases serum sphinganine concentration but does not alter serum sphingosine concentration or induced cardiovascular changes in milk-fed calves. *Toxicol Sci* **60**: 379–84.

McCue PM (1989) Equine leukoencephalomalacia. *Comp Contin Educ Pract Vet* **11**: 646–51.

McDonough PM, Yasui K, Betto R, Salviatti G, Glembotski CC, Palade PT, Sabbadini RA (1994) Control of cardiac $Ca^{2+}$ levels: inhibitory actions on sphingosine on $Ca^{2+}$ transients and L-type $Ca^{2+}$ channel conductance. *Circ Res* **75**: 981–9.

Merrill Jr AH, Sweeley CC (1996) Sphingolipids metabolism and cell signaling. In *Biochemistry of Lipids, Lipoproteins, and Membranes*, Vance DE, Vance JE (eds). Elsevier, New York, pp. 43–73.

Merrill Jr AH, Wang E, Schroeder JJ, Smith ER, Yoo HS, Riley RT (1995) *Molecular Approaches to Food Safety. Issues Involving Toxic Microorganisms*, Elklund M, Richards M, Mise K (eds). Alaken Press, Fort Collins, CO, pp. 429–43.

Michelakis E, Tewari K, Simard JM (1994) Calcium channels in smooth muscle cells from cerebral precapillary arterioles activate at more negative potentials than those from basilar artery. *Pflugers Arch* **426**: 459–61.

Mirocha CJ, Mackintosh CG, Mirza UA, Xie W, Xu Y, Chen J (1992) Occurrence of fumonisin in forage grass in New Zealand. *Appl Environ Microbiol* **58**: 3196–8.

Motelin GK, Haschek WM, Ness DK, Hall WF, Harlin KS, Schaeffer DJ, Beasley VR (1994) Temporal and dose–response features in swine fed corn screenings contaminated with fumonisin mycotoxins. *Mycopathology* **126**: 27–40.

Osweiler GD, Ross PF, Wilson TM, Nelson TM, Witte ST, Carson TL, Rice LG, Nelson HA (1992) Characterization of an epizootic of pulmonary edema in swine associated with fumonisins in corn screenings. *J Vet Diagn Invest* **4**: 53–9.

Osweiler GD, Kehrli ME, Stabel JR, Thurston JR, Ross PF, Wilson TM (1993) Effects of fumonisin-contaminated corn screenings on growth and health of feeder calves. *J Anim Sci* **71**: 459–66.

Patchimasiri T, Sailasuta A, Kawtheerakul K (1998) Pathological findings in swine in association with fumonisin contaminated feed. *Thai J Vet Med* **28**: 71–82.

Pittet A, Parisod V, Schellenberg M (1992) Occurrence of fumonisins $B_1$ and $B_2$ in corn-based products from the Swiss market. *J Agric Food Chem* **40**: 1352–4.

Plumlee KH, Galey FG (1994) Neurotoxic mycotoxins: a review of fungal toxins that cause neurological disease in large animals. *J Vet Int Med* **8**: 49–54.

Prelusky DB, Trenholm HL, Savard ME (1994) Pharmacokinetic fate of $^{14}C$-labelled fumonisin $B_1$ in swine. *Nat Toxins* **2**: 73–80.

Prelusky DB, Savard ME, Trenholm HL (1995) Pilot study on the plasma pharmacokinetics of fumonisin $B_1$ in cows following a single dose by oral gavage or intravenous administration. *Nat Toxins* **3**: 384–94.

Raoofi A, Mardjanmehr SH, Khosravi AR, Kojouri GA, Lotfollahzaheh S, Nekoie S, Jafarian M (2003) *J Eq Vet Sci* **23**: 469–70.

Rheeder JP, Marasas WFO, Thiel PG, Sydenham EW, Shepherd GS, van Schalwyk DJ (1992) *Fusarium moniliforme* and fumonisins in corn in relation to human esophageal cancer in Transkei. *Phytopathology* **82**: 353–7.

Richard JL, Meerdink G, Maragos CM, Tumbleson M, Bordson G, Rice LG, Ross PF (1996) Absence of detectable fumonisins in the milk of cows fed *Fusarium proliferatum* (Matusushima) Nirenberg culture material. *Mycopathology* **133**: 123–6.

Riley RT, An NH, Showker JL, Yoo HS, Norred WP, Chamberlain WJ, Wang E, Merrill Jr AH, Motelin G, Beasley VR, Haschek WM (1993) Alteration of tissue and serum sphinganine to sphingosine ratio: an early biomarker of exposure to fumonisin-containing feeds in pigs. *Toxicol Appl Pharmacol* **118**: 105–12.

Rosiles MR, Bautista J, Fuentes VO, Ross F (1998) An outbreak of equine leukoencephalomalacia at Oaxaca, Mexico, associated with fumonisin $B_1$. *J Vet Med A* **45**: 299–302.

Ross PF, Rice LG, Reagor JC, Osweiler GD, Wilson TM, Nelson HA, Owens DL, Plattner RD, Harlin KA, Richard JL, Colvin BM, Banton MI (1991) Fumonisin $B_1$ concentrations in feeds from 45 confirmed equine leukoencephalomalacia cases. *J Vet Diagn Invest* **3**: 238–41.

Ross PF, Ledet AE, Owens DL, Rice LG, Nelson HA, Osweiler GD, Wilson TM (1993) Experimental equine leukoencephalomalacia, toxic hepatitis, and encephalopathy caused by corn naturally contaminated with fumonisins. *J Vet Diagn Invest* **5**: 69–74.

Ross PF, Nelson PE, Owens DL, Rice LG, Nelson HA, Wilson TM (1994) Fumonisin $B_2$ in cultured *Fusarium proliferatum*, M-6104, causes equine leukoencephalomalacia. *J Vet Diagn Invest* **6**: 263–5.

Rotter BA, Prelusky DB, Fortin A, Miller JD, Savard ME (1996) Response of growing swine to dietary exposure to fumonisin $B_1$ during an eight-week period: growth and clinical parameters. *Nat Toxins* **4**: 42–50.

Schumacher J, Mullen J, Shelby R, Lenz S, Ruffin DC, Kemppainen BW (1995) An investigation of the role of *Fusarium moniliforme* in duodenitis/proximal jejunitis of horses. *Vet Human Toxicol* **37**: 39–45.

Schwarte LH, Biester HE, Murray C (1937) A disease of horses caused by feeding moldy corn. *J Am Vet Med Assoc* **43**: 76–85.

Shephard GS, Thiel PG, Syndenham EW, Alberts JF, Gelderblom WCA (1992). Fate of a single dose of the $^{14}C$-labelled mycotoxin, fumonisin $B_1$, in rats. *Toxicon* **30**: 768–70.

Shephard GS, Thiel PG, Syndenham EW, Savard ME (1995) Fate of a single dose of $^{14}C$-labelled fumonisin $B_1$ in Vervet monkeys. *Nat Toxins* **3**: 145–50.

Smith GW, Constable PD (2004) Fumonisin. In *Clinical Veterinary Toxicology*, Plumlee KH (ed.). Mosby, St. Louis, MO, pp. 250–4.

Smith GW, Constable PD, Bacon CW, Meredith FI, Haschek WM (1996a) Cardiovascular effects of fumonisins in swine. *Fundam Appl Toxicol* **31**: 169–72.

Smith GW, Constable PD, Haschek WM (1996b) Cardiovascular responses to short-term fumonisin exposure in swine. *Fundam Appl Toxicol* **33**: 140–8.

Smith GW, Constable PD, Smith AR, Bacon CW, Meredith FI, Wollenberg GK, Haschek WM (1996c) Effects of fumonisin-containing culture material on pulmonary clearance in swine. *Am J Vet Res* **57**: 1233–8.

Smith GW, Constable PD, Tumbleson ME, Rottinghaus GE, Haschek WM (1999) Sequence of cardiovascular changes leading to pulmonary edema in swine fed culture material containing fumonisin. *Am J Vet Res* **60**: 1292–9.

Smith GW, Constable PD, Eppley RM, Tumbleson ME, Gumprecht LA, Haschek-Hock WM (2000) Purified fumonisin $B_1$ decreases cardiovascular function but does not alter pulmonary capillary permeability in swine. *Toxicol Sci* **56**: 240–9.

Smith GW, Constable PD, Foreman JH, Eppley RM, Waggoner AL, Tumbleson ME, Haschek WM (2002) Cardiovascular changes associated with intravenous administration of fumonisin $B_1$ in horses. *Am J Vet Res* **63**: 538–45.

Spotti M, Caloni F, Fracchiolla L, Pompa G, Vigo D, Maffeo G (2001) Fumonisin $B_1$ carry-over into milk in the isolated perfused bovine udder. *Vet Hum Toxicol* **43**: 109–11.

Stack ME, Eppley RM (1992) Liquid chromotographic determination of fumonisins $B_1$ and $B_2$ in corn and corn products. *J Assoc Off Anal Chem* **75**: 834–7.

Sydenham EW, Shephard GS, Thiel PG, Marasas WFO, Stockenstrom S (1991) Fumonisin contamination of commercial corn-based human foodstuffs. *J Agric Food Chem* **39**: 2014–18.

Sydenham EW, Marasas WFO, Shephard GS, Thiel PG, Hirooka EY (1992) Fumonisin concentrations in Brazilian feeds associated with field outbreaks of confirmed and suspected animal mycotoxicoses. *J Agric Food Chem* **40**: 994–7.

Thiel PG, Shephard GS, Sydenham EW, Marasas WFO, Nelson PE, Wilson TM (1991) Levels of fumonisin $B_1$ and $B_2$ in feeds associated with confirmed cases of equine leukoencephalomalacia. *J Agric Food Chem* **39**: 109–11.

Uhlinger C (1991) Clinical and epidemiologic features of an epizootic of equine leukoencephalomalacia. *J Am Vet Med Assoc* **198**: 126–8.

Voss KA, Norred WP, Plattner RD, Bacon CW (1989) Hepatotoxicity and renal toxicity of corn samples associated with field cases of equine leukoencephalomalacia. *Food Chem Toxicol* **27**: 89–96.

Vudathala DK, Prelusky DB, Ayroud M, Trenholm HL, Miller JD (1994) Pharmacokinetic fate and pathological effects of 14C-fumonisin $B_1$ in laying hens. *Nat Toxins* **2**: 81–8.

Wang E, Norred WP, Bacon CW, Riley RT, Merrill Jr AH (1991) Inhibition of sphingosine biosynthesis by fumonisins. *J Biol Chem* **266**: 14486–90.

Wang E, Ross PF, Wilson TM, Riley RT, Merrill Jr AH (1992) Increases in serum sphingosine and sphinganine and decreases in complex sphingolipids in ponies given feed containing fumonisins, mycotoxins produced by *Fusarium moniliforme*. *J Nutr* **122**: 1706–16.

Webster R, Sabbadini RA, Paolini P (1994) Sphingosine effects on the contractile behavior of skinned cardiac myocytes. *J Mol Cell Cardiol* **26**: 1273–90.

Wilkins PA, Vaala WE, Zivotofsky D, Twitchell E (1994) A herd outbreak of equine leukoencephalomalacia. *Cornell Vet* **84**: 53–9.

Wilson BJ, Maronpot RR (1971) Causative fungus agent of leucoencephalomalacia in equine animals. *Vet Rec* **88**: 484–6.

Wilson TM, Ross PF, Rice LG, Osweiler GD, Nelson HA, Owens DL, Plattner RD, Reggiardo C, Noon TH, Pickrell JW (1990a) Fumonisin $B_1$ levels associated with an epizootic of equine leukoencephalomalacia. *J Vet Diagn Invest* **2**: 213–21.

Wilson TM, Nelson PE, Marasas WFO, Thiel PG, Shephard GS, Sydenham EW, Nelson HA, Ross PF (1990b) A mycological evaluation and *in vivo* toxicity evaluation of feed from 41 farms with equine leukoencephalomalacia. *J Vet Diagn Invest* **2**: 352–4.

Wilson TM, Ross PF, Owens DL, Rice LG, Green SA, Jenkins SJ, Nelson HA (1992) Experimental reproduction of ELEM. A study to determine the minimum toxic dose in ponies. *Mycopathologia* **117:** 115–20.

Zomborszky MK, Vetesi F, Repa I, Kovacs F, Bata A, Horn P, Toth A, Romvari R (2000) Experiment to determine limits of tolerance for fumonisins $B_1$ in weaned piglets. *J Vet Med B* **47**: 277–86.

C H A P T E R

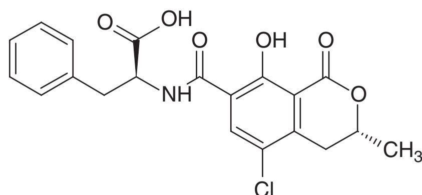
# 79

# Ochratoxins and citrinin

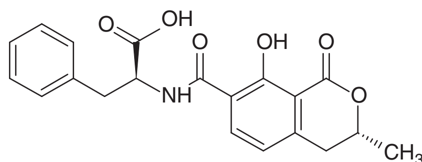
*Ramesh C. Gupta*

## INTRODUCTION

Ochratoxins and citrinin are produced by several species of genera *Aspergillus* and *Penicillium*. The two most common species that produce ochratoxin A (OTA) are *Aspergillus ochraceus* and *Penicillium verrucosum*. These fungi are ubiquitous and the potential for contamination of animal feed and human food is widespread. *Aspergillus* spp. appears to produce ochratoxins at conditions of high humidity and temperature, whereas some *Penicillium* spp. may produce ochratoxins at temperatures as low as 5 °C. OTA has been found in a variety of food/feed, with levels in commodities used as feed ranging up to 27 ppm, and with levels in foodstuffs for human consumption in the range of trace to about 100 ppb. Unlike OTA, the occurrence of ochratoxin B is rare. Chemical structures of OTA and ochratoxin B are shown below. Ochratoxin B lacks chlorine and thereby it is less toxic than OTA.

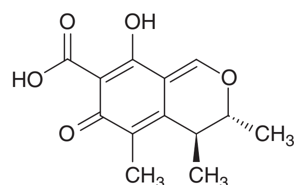


Ochratoxin A



Ochratoxin B

Citrinin was first isolated as a pure compound from a culture of *P. citrinum* in 1931. Later, it was also isolated from *A. ochraceus*, *P. verrucosum*, and related species that contaminate grain. In 1951, yellowish colored rice imported from Thailand to Japan was found to be contaminated with *P. citrinum*, which contained citrinin. Synthesized citrinin is also used in biological research, as it induces mitochondrial permeability pore opening and inhibits respiration by interfering with complex I of the respiratory chain. The structure of citrinin is shown below.



Citrinin

Both OTA and citrinin cause nephropathy in animals and they have also been implicated as the cause of Balkan endemic nephropathy in humans. The literature reveals that OTA has been studied to a greater extent than citrinin, partly because OTA is at least 10 times more toxic than citrinin. This chapter describes in detail the toxicity of ochratoxins and citrinin in animals.

## BACKGROUND

The fungi producing ochratoxins and citrinin are commonly encountered in animal feed and human food around the world. They are encountered with great frequency in the

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Balkan area. Both ochratoxins and citrinin are fungal metabolites. There are two major ochratoxins (A and B). OTA occurs naturally with a greater frequency in a variety of cereal grains (barley, wheat, oats, corn, and beans), peanuts, dried fruits, grapes/raisins, cheese, and other food products. OTA accumulates in the food chain because of its long half-life. Citrinin usually co-occurs with OTA, and commonly contaminates cereal grains, including wheat, barley, oats, corn, and rice. Citrinin also contaminates peanuts and fruits. The levels of OTA and citrinin have been found far lower in human food than in raw animal feed, because during processing and baking of human food citrinin is almost eliminated and OTA is significantly reduced. Compared to OTA, ochratoxin B is rarely found and very less toxic.

Both OTA and citrinin are well-known nephrotoxins. OTA is also carcinogenic to rodents (Creppy *et al.*, 1985) and possesses teratogenic (Arora *et al.*, 1983), immunotoxic (Stormer and Lea, 1995), neurotoxic (Bruinink and Sidler, 1997; Sava *et al.*, 2006), mutagenic (Stetina and Votava, 1986), and genotoxic (Meisner *et al.*, 1983) properties. In humans, exposure to OTA and citrinin has been linked with Balkan endemic nephropathy, a chronic kidney disease associated with tumors of the renal system, which can be fatal. Co-occurrence with OTA of citrinin has been implicated in nephropathy of pigs in Denmark, Sweden, Norway, and Ireland. Citrinin and OTA are also involved in avian nephropathies. Residues of OTA have been detected in the tissues of pigs in slaughterhouses, and it has been shown, under experimental conditions, that residues can still be detected in tissues 1 month after the end of exposure. Due to long half-life of OTA in the feed and biological system, serious concerns have been raised about the animal health, as well as for human consumption of meat. Thus, it appears that both OTA and citrinin seriously affect animal health and economic impact is enormous.

## TOXICOKINETICS

In most animal species, OTA is absorbed from the stomach because of its lipid soluble, non-ionized, and acidic properties ($pK_a = 7.1$) (Kumagai and Aibara, 1982; Kumagai, 1988). Absorption of OTA also takes place in the intestine, is involved in enterohepatic circulation, and its biliary excretion is very efficient. OTA is distributed to various organs, mainly to the kidneys. Liver, muscle, and fat contain lower concentrations. The overall percentage of OTA absorption is found to be 66% in pigs, 56% in rats, 56% in rabbits, and 40% in chickens (Suzuki *et al.*, 1977; Galtier *et al.*, 1981). After a single oral dose, the maximum concentrations of OTA are found within 10–48 h in pigs and rats (Galtier *et al.*, 1979, 1981), 2–4 h in ruminant calves (Sreemannarayana *et al.*, 1988), after 1 h in rabbits, and after 0.33 h in chickens (Galtier *et al.*, 1981). Maximum tissue concentrations in rat tissues occur within 48 h.

The serum half-life of OTA is long and varies widely among species, for example 24–39 h in mice, 55–120 h in rats, 6.7 h in quail, 510 h in Macaca mulata monkeys (Hagelberg *et al.*, 1989), 72–120 h in pigs, 4.1 h in chicken (Galtier *et al.*, 1981), and 840 h in a volunteer (Benford, *et al*. 2001).

In pigs, it has been observed that the kidney is generally the most heavily contaminated tissue with OTA, and that the levels in the blood are about 5-fold greater than in the kidney. Krogh *et al*. (1976) illustrated that if the level of OTA in swine kidney is 12.1 ng/g (resulting from about 1000 ng/g in the feed), its levels would be 7.8 ng/g in the liver, 4.2 ng/g in the muscle, and 2.8 ng/g in the adipose tissue. OTA in ruminants is usually hydrolyzed in the forestomach by protozoans and bacterial enzymes, and consequently little OTA is found in the tissues (Hult *et al*., 1976).

Residue of OTA can be passed in the milk of rats, rabbits, and women, but very little is passed in the milk of ruminants because of its metabolism by the ruminal microflora. The major routes of excretion are urine and feces, and excretion is influenced by the extent of the enterohepatic circulation and binding to serum albumin and other macromolecules (Galtier *et al*., 1980; Hult and Fuchs, 1986). The association constant for the binding of OTA to serum albumin is $7.1 \times 10^4$ per mol for pigs, $5.1 \times 10^4$ per mol for chickens, and $4.0 \times 10^4$ per mol for rats (Galtier *et al*., 1981).

In various tissues of all species that are examined, OTA is hydrolyzed to ochratoxin *alpha*, which is the major metabolite. This detoxication process takes place in cecum of rats and is facilitated by bacterial microflora (Galtier, 1978). The enzymes responsible for hydrolysis to ochratoxin *alpha* in cows and rodents are carboxypeptidase A and chymotrypsin. Suzuki *et al*. (1977) demonstrated that the rat tissue homogenates of the duodenum, ileum, and pancreas also have a high activity to catalyze this reaction. Activity of these enzymes in liver and kidney is low. Studies in mice suggest that OTA circulates from the liver into the bile and into the intestine, where it is hydrolyzed to ochratoxin *alpha* (Moroi *et al*., 1985). About 25–27% of OTA, given either i.p. or orally to rats, was found as ochratoxin *alpha* in the urine. Its presence in the urine can be explained by reabsorption from the intestine. A similar mechanism of intestinal reabsorption of ochratoxin *alpha* has been suggested to occur in ruminant calves (Sreemannarayana *et al*., 1988). For details of biotransformation of OTA, refer to an extensive review by Benford *et al*. (2001).

From animal studies, it is clear that OTA has a high degree of bioavailability, low plasma clearance rate, and long tissue half-life. All metabolites of OTA are less toxic than the parent compound.

## MECHANISM OF ACTION

OTA produces a variety of toxic effects in several organs, and therefore multiple mechanisms are involved. In addition to nephrotoxicity, OTA disrupts blood coagulation (Gupta *et al*., 1979) and glucose metabolism (Pitout, 1968). OTA is immunotoxic (Stormer and Lea, 1995), teratogenic (Arora *et al*., 1983), and carcinogenic (Creppy *et al*., 1985). A brief description of mechanisms involved in common toxic effects is given below.

### *Protein synthesis*

A dose-related inhibition of protein synthesis was found in mice given OTA intraperitoneally at a dose $\geqslant$1 mg/kg body weight. The degree of inhibition of protein synthesis 5 h after administration of OTA at 1 mg/kg dose was 26% in liver, 68% in kidney, and 75% in spleen as compared with controls (Creppy *et al*., 1984).

### *Nephrotoxicity*

Both OTA and citrinin are potent nephrotoxins. At high doses, OTA affects both renal function and morphology, as indicated by increased weight, urine volume, blood urea nitrogen, urinary glucose, and proteinuria (Hatey and Galtier, 1977). The last two findings indicate that the site of reabsorption (i.e. the proximal convoluted tubules) is damaged. OTA specifically causes defect of the organic anion transport mechanism located on the brush border of the proximal convoluted tubules and basolateral membranes. OTA also adversely affects the organic ion transport system by which OTA enters proximal tubular cells. The middle (S2) and terminal (S3) segments of the proximal tubule of the isolated nephron were found to be the most sensitive to the toxic effects of OTA (0.05 mmol/l), as shown by a significant decrease in cellular ATP and a dose-related decrease in mitochondrial ATP content (Jung and Endou, 1989).

Mitochondrial dysfunction has been shown to be involved in the development of OTA-induced toxicity in proximal renal tubule cells (Aleo *et al*., 1991). OTA toxicity is associated with inhibition of both protein and RNA synthesis (Dirheimer and Creppy, 1991). OTA is known to interfere with the charging of tRNA with amino acids. OTA treatment can increase oxidative stress in peripheral organs. Administration of OTA to rats (1 mg/kg) resulted in a 22% decrease in $\alpha$-tocopherol plasma levels and a 5-fold increase in the expression of the oxidative stress responsive protein heme oxygenase-1, specifically in the kidney (Gautier *et al*., 2001).

### *Neurotoxicity*

Although toxic effects of OTA on the CNS have not yet been fully characterized, evidence strongly suggests that OTA has potential for neurotoxicity. Bruinink *et al*. (1998) demonstrated that OTA is neurotoxic and may affect selected structures of the brain. This mycotoxin has complex multiple mechanisms of action that include evocation of oxidative stress, bioenergetic compromise, inhibition of protein synthesis, production of DNA single-stranded breaks, and formation of OTA–DNA adducts (Sava *et al*., 2006). These authors found that administration of OTA in mice, at a single dose (3.5 mg/kg) that is approximately 10% of the reported $LD_{50}$, caused widespread oxidative injury in six discrete brain regions.

### *Immunotoxicity*

There is ample evidence, resulting from studies conducted in several animal species, that under certain conditions of treatment, OTA can produce defects in the structure and/or function of elements comprising the immune system (Pohland *et al*., 1992). The size of the mouse thymus was reduced to 33% that of controls after four i.p. injections of OTA at 20 mg/kg body weight on alternate days, a dose which caused minimal nephrotoxicity. Bone marrow depression was found to be dose related, significantly decreased marrow cellularity, including a reduction in bone marrow macrophage–granulocyte progenitors, a decreased number of hematopoietic stem cells, a significant decrease in erythropoiesis, and increased phagocytosis by macrophages (Boorman *et al*., 1984). The effects of OTA on the bone marrow and lymphatic cell population may reflect the sensitivity of these cells to the inhibition of protein synthesis induced by OTA. These effects on the structural components of the immune system indicated that OTA is likely to have an effect on immune function.

In chickens fed diets containing OTA at a concentration of 2–4 mg/kg for 20 days, the lymphoid cell population of immune organs was decreased (Dwivedi and Burns, 1984a), and IgA and IgM in lymphoid tissues and serum were decreased (Dwivedi and Burns, 1984b). Complement activity was slightly affected in birds fed diets containing 2 mg/kg for 5–6 weeks (Campbell *et al*., 1983). Immune suppression was observed in chickens fed diets containing OTA at 0.05 or 2 mg/kg for 21 days. Treated animals showed reduced total serum protein, lymphocyte counts, and weights of the thymus, bursa of fabricus, and spleen (Singh *et al*., 1990).

### *Carcinogenicity*

The exact mechanism by which OTA induces carcinogenicity is unknown, although both genotoxic and non-genotoxic modes of actions have been proposed.

## TOXICITY

The toxic effects of OTA have been studied extensively in a number of domestic, companion, and experimental

animals. Toxic effects of OTA have also been studied in humans. Overall toxicity of OTA is greatly influenced by species, sex, and route of administration. Based on acute toxicity data, dogs and pigs are the most sensitive species and rats and mice the least sensitive. Oral $LD_{50}$ values (expressed as mg/kg body weight) of OTA are reported to be 46–58 in mouse, 20–30 in rat, 3.9 in neonate rat, 0.2 in dog, 1 in pig, and 3.3 in chicken (Harwig *et al.*, 1983). $LD_{50}$ values via i.p. route are reported to be 22–40 in mouse and 20–30 in rat; and with i.v. route, 26–34 in mouse and 13 mg/kg in rat. It causes renal toxicity, nephropathy, and immune suppression in several animal species. The acute $LD_{50}$ (expressed as mg/kg body weight) of citrinin is reported to be 50 (oral) and 67 (s.c. or i.p.) in rat, 35–58 in mouse, and 19 (i.p. or i.v.) in rabbit. Citrinin causes kidney damage and mild liver damage in the form of fatty infiltration. Other toxic effects include vasodilatation, constriction of the bronchi, and increased muscular tone.

All the animals studied so far have been found susceptible to orally administered OTA with a varying degree of response. It is important to mention that at higher doses OTA causes alterations in kidneys and also in other organs and tissue, but renal lesions can be found at an exposure level that is identical to those occurring environmentally. Ochratoxin B is rarely found as a natural contaminant and is much less toxic than OTA. The other ochratoxins have never been encountered in natural products.

Weanling Fischer 344/N rats of both sexes receiving OTA by gavage in maize oil at a dose of 0.06, 0.12, 0.25, 0.5, or 1 mg/kg body weight/day for 5 days/week for 91 days, showed growth retardation and a reduced relative kidney weight in males at the two higher doses. Karyomegaly of dose-related severity was observed in the proximal tubules at all doses. Milder renal changes consisting of tubular atrophy were seen at a dose of 1, 4, or 16 mg/kg body weight/day on 5 days/week for a total of 12 doses over 16 days. Rats receiving the highest dose had diarrhea and nasal discharge and died before the end of the study. Increased relative weights of kidneys, heart, and brain, thymus atrophy, forestomach necrosis and/or hyperplasia, and hemorrhage of adrenal glands were seen at the two higher doses. Bone marrow hyperplasia and nephropathy were seen at all doses, involving renal tubular degenerative and regenerative changes (NTP, 1989).

OTA has been shown to produce nephrotoxic effects in all animal species examined, with the exception of adult ruminants (Pohland *et al.*, 1992). The nephrotoxic potential of OTA is well documented from all experimental studies, with a feed level of 200 ppb causing nephropathy in pigs and rats. Evidence strongly supports that OTA is involved in porcine nephropathy, which is characterized by degeneration of the proximal tubules, atrophy of the tubular epithelium, interstitial fibrosis in the renal cortex and hyalinized glomeruli. Field cases of OTA-induced nephropathy in farm animals have long been recognized. Benford *et al.* (2001) suggested that the adverse effect at the lowest effective dose in several mammalian species is nephrotoxicity, and this is likely also to be true in humans. Citrinin is also nephrotoxic, but it is 10 times less toxic than OTA.

In a series of experiments, sows were given feed containing OTA at a concentration of 0.2, 1, or 5 mg/kg (equivalent to 0.008, 0.04, and 0.2 mg/kg body weight/day), for a period of 5 days, 8 or 12 weeks, or up to 2 years. Decreased renal function, nephropathy, and reduced renal enzyme activity were observed. Progressive nephropathy but no renal failure was seen in pigs given feed containing 1 mg/kg for 2 years (Krogh *et al.*, 1976; Krogh and Elling, 1977; Elling *et al.*, 1985).

Beagle dogs receiving OTA in capsule form at a dose of 0.1 or 0.2 mg/kg body weight/day for 14 days showed tubular necrosis and ultrastructural changes in the proximal tubules at all doses. Necrosis of lymphoid tissues of the thymus and tonsils was also seen at all doses (Kitchen 1977a, b, c).

In another set of experiments, young beagle dogs were given OTA and citrinin separately and combined for 14 days (Kitchen *et al.*, 1977b). OTA was administered by capsule at 0.1 and 0.2 mg/kg; and citrinin (5 and 10 mg/kg) dissolved in ethanol was given by i.p. injection. Clinical signs of toxicosis with 10 mg/kg citrinin and the higher combined doses included anorexia, retching, tenesmus, weight loss, prostration, and death. Severity of the clinical disease and mortality were increased when the mycotoxins were combined, which indicated synergism. The clinicopathological abnormalities reflected renal damage, cellular and granular casts, ketones, protein, and glucose were in the urine of dogs given large doses of citrinin alone or combined with OTA. In pathological studies, these authors found gross lesions, such as focal peritonitis and intestinal intussusceptions with citrinin. Changes in the kidneys of dogs given OTA were degeneration and necrosis with desquamation of tubular epithelial cells, primarily in the straight segment of the proximal tubules. Dogs given 10 mg/kg citrinin had similar changes in the distal tubules and collecting ducts. Dogs given combined doses of citrinin and OTA had degeneration and necrosis in proximal and distal tubules, and in thin segments and the collecting ducts, and ulceration of the mucosa of the intestine.

In experimental studies, dogs given citrinin showed serous nasal discharge and lacrimation (Carlton *et al.*, 1974; Kitchen *et al.*, 1977a, b). It is important to mention that citrinin is a very strong emetic in dogs, which is a protective mechanism in this species. Therefore, it is very unlikely that dogs will be poisoned by citrinin alone because high amounts of this mycotoxin will induce emesis and feed refusal.

Chicken, turkeys, and ducklings are all susceptible to OTA and it appears that OTA-contaminated feed has

major economic impact on the poultry industry. Feed levels as low as 200 ppb produced renal changes in the course of 3 months in rats and pigs. Field cases of OTA-induced nephropathy are regularly encountered in pigs and poultry. Clinical signs of ochratoxicosis include retarded growth rate, reduction in weight gains, poor feed conversion, reduced egg production, poor egg shell quality and nephrotoxicity/nephropathy, and mortality. Feed refusal has been observed in turkeys.

In chickens, OTA at a dose rate of 3.6 mg/kg can cause 5% mortality. Ochratoxin B at the dose rate of 54 mg/kg causes lowered growth rate, edema of visceral organs, and accumulation of uric acid in kidneys, liver, heart, and spleen. These mycotoxins induce suppression of blood formation in bone marrow, and lymph formation in spleen and bursa of fabricus. Highest toxicity of OTA is found to be in broiler chickens. Broiler chickens given OTA at a dietary concentration of 4 mg/kg for 2 months caused 42% mortality (Gibson *et al.*, 1990). This toxin is involved in reduced growth rate at 5 ppm, high mortality rate at 4–8 ppm, and cessation of egg production at 4 ppm.

In chickens, nephrotoxicity and hepatotoxicity occurs at dietary levels of 250 μg/g of citrinin with liver and kidney enlargements of 11% and 22%, respectively. Necropsy of affected birds revealed the presence of pale and swollen kidneys (Wyatt, 1979). Citrinin is at least 10 times less nephrotoxic than OTA.

Griffiths and Done (1991) described an outbreak of citrinin toxicosis in a herd of cows, which ingested citrus pulp (visibly moldy) pellets that contained 30–40 ppb citrinin. Affected cows showed signs of pruritis, pyrexia, and hemorrhagic syndrome. Signs of the syndrome occurred within 3 days of ingesting the citrus pulp, which was fed for 21 days. Five calves whose dams had been fed citrus pulp were subsequently born with superior prognathism. Older animals were more susceptible to citrinin. The clinical signs, gross pathology, and histology were suggestive of citrinin involvement.

OTA has been well tested for carcinogenicity by oral administration in mice and rats. When OTA was administered in the diet, hepatocellular tumors (designated as well-differentiated trabecular adenomas), renal-cell tumors (renal cystadenomas and solid renal-cell tumors), hepatomas (some exhibiting the trabecular structure), and hyperplastic hepatic nodules were observed in male mice (IARC, 1993). In another study, administration of OTA in the diet-induced hepatocellular carcinomas and adenomas in female mice. Gavage administration of OTA to male and female rats resulted in a dose-related increase in the incidence of renal-cell adenomas and adenocarcinomas. Furthermore, metastasis of the renal-cell tumors was also observed in male and female rats. OTA also increased the incidence and multiplicity of fibroadenomas of the mammary gland in female rats (NTP, 1989, IARC, 1993). In essence, these data suggest that OTA increases the incidence of hepatocellular tumors in mice of each sex and produces renal-cell adenomas in male mice and in rats of each sex. Based on sufficient evidence of carcinogenicity in experimental animals, OTA is classified as a possible carcinogen in humans (Group 2B) (IARC, 1993).

OTA is known to induce teratogenicity in mice, rats, hamsters, and chicken. Details of teratogenic effects are described in Chapter 18. Citrinin has been demonstrated to be mutagenic in hepatocytes (Bailly *et al.*, 2002). There is limited evidence for the carcinogenicity of citrinin to experimental animals.

## TREATMENT

There is no specific antidote for ochratoxin(s) or citrinin toxicity. Recovery is usually slow. Immediate removal of the suspected feed and replacement with clean feed supplemented with increased vitamin levels can be rewarding. Growth of *A. ochraceus* in a common food, such as cereals, can be controlled or minimized by drying them rapidly and thoroughly. Effective approaches to grain storage include fumigation, aeration and cooling, sealed storage, and controlled atmosphere in tropical and subtropical regions where insect damage is a major problem. Citrinin is less of a problem because it is heat unstable. Citrinin is also likely to be destroyed during brewing. Presence of propionic acid destroys citrinin when added as a preservative to protect stored barley destined for animal feed from molding during storage. Currently, highly sophisticated methods are available to detect these mycotoxins at ppb or below levels in food/feed or their products, so as to prevent animal health from toxicosis and economic loss.

## CONCLUSIONS

OTA and citrinin both contaminate a wide range of animal feed and human food. Human risk is less because the levels of these mycotoxins are minimized during processing and baking, but the raw feed remains a potential source for animal poisoning. OTA accumulates and has a long half-life in feed. These mycotoxins have a serious impact on the health of animals, especially, pigs, dogs, and poultry. Pigs and dogs are most sensitive, while rats and mice are least sensitive. In general, females are more sensitive than males. The kidney is a major target organ (as evidenced by functional and morphological changes) for both mycotoxins, but a few other organs are affected as well. In animal studies, OTA has been found to be a nephrotoxin, neurotoxin, immune suppressant, mutagen, carcinogen, and teratogen.

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## REFERENCES

Aleo MD, Wyatt RD, Schnellman RG (1991) Mitochondrial dysfunction is an early event in ochratoxin A but not oosporein toxicity to rats' renal proximal tubules. *Toxicol Appl Pharmacol* **107**: 73–80.

Arora RG, Froler H, Fellner-Feldegg H (1983) Inhibition of ochratoxin A teratogenesis by zearalenone and diethylstilbestrol. *Fd Chem Toxicol* **21**: 779–83.

Bailly JD, Querin A, Bars-Bailly SL, *et al*. (2002) Citrinin production and stability in cheese. *J Fd Protect* **65**: 1317–21.

Benford D, Boyle C, Decant W, *et al*. (2001) Ochratoxin A. *Joint Expert Comm Fd Addit* **47**: 1–125.

Boorman GA, Hong HL, Dieter MP, *et al*. (1984) Myelotoxicity and macrophage alteration in mice exposed to ochratoxin A. *Toxicol Appl Pharmacol* **72**: 304–12.

Bruinink A, Sidler C (1997) The neurotoxic effects of ochratoxin A are reduced by protein binding but are not affected by 1-phenylalanine. *Toxicol Appl Pharmacol* **146**: 173–9.

Bruinink A, Rasonyi T, Sidler C (1998) Differences in neurotoxic effects of ochratoxin A, ochracin and ochratoxin alpha *in vitro*. *Nat Toxins* **6**: 173–7.

Campbell Jr ML, May JD, Huff WE, Doerr JA (1983) Evaluation of immunity of young broiler chickens during simultaneous aflatoxicosis and ochratoxicosis. *Poult Sci* **62**: 2138–44.

Carlton WW, Samsing G, Szczech GM (1974) Citrinin mycotoxicosis in beagle dogs. *Fd Cosmet Toxicol* **12**: 479.

Creppy EE, Röschenthaler R, Dirheimer G (1984) Inhibition of protein synthesis in mice by ochratoxin A and its prevention by phenylalanine. *Fd Cosmet Toxicol* **22**: 883–6.

Creppy EE, Kane A, Dirheimer G, *et al*. (1985) Genotoxicity of ochratoxin A in mice: DNA single-strand break evaluation in spleen, liver, and kidney. *Toxicol Lett* **28**: 29–35.

Dirheimer G, Creppy EE (1991) *Mechanism of action of ochratoxin A. IARC Sci Publ* 171–86.

Dwivedi P, Burns RB (1984a) Pathology of ochratoxicosis A in young broiler chicks. *Res Vet Sci* **36**: 92–103.

Dwivedi P, Burns RB (1984b) Effect of ochratoxin A on immunoglobulins in broiler chicks. *Res Vet Sci* **36**: 117–21.

Elling F, Nielsen JP, Lillehoj EB, *et al*. (1985) Ochratoxin A-induced porcine nephropathy: enzyme and ultrastructure changes after short-term exposure. *Toxicology* **23**: 247–54.

Gautier JC, Holzhaeuser D, Marcovic J, *et al*. (2001) Oxidative damage and stress response from ochratoxin A exposure in rats. *Free Radic Biol Med* **30**: 1089–98.

Galtier P (1978) Contribution of pharmacokinetic studies to mycotoxicology-ochratoxin A. *Vet Sci Commun* **1**: 349–58.

Galtier P, Charpenteau JL, Alvinerie M, Labouche C (1979) The pharmacokinetic profile of ochratoxin A in the rat after oral and intravenous administration. *Drug Metabol Disposit* **7**: 429–34.

Galtier P, Camguilhem R, Bodin G (1980) Evidence for *in vitro* and *in vivo* interaction between ochratoxin A and three acidic drugs. *Fd Cosmet Toxicol* **18**: 493–6.

Galtier P, Alvinerie M, Charpenteau JL (1981) The pharmacokinetic profile of ochratoxin A in pigs, rabbits, and chickens. *Fd Cosmet Toxicol* **19**: 735–8.

Gibson R, Baily C, Kubena L, *et al*. (1990) Impact of L-phenylalanine supplementation on the performance of three-week-old broiler fed diets containing ochratoxin A. I. Effects on body weight, feed conversion, relative organ weight, and mortality. *Poult Sci* **69**: 414–19.

Griffiths IR, Done SH (1991) Citrinin as a possible cause of the pruritus, pyrexia, hemorrhagic syndrome in cattle. *Vet Rec* **129**: 113–17.

Gupta M, Bandopadhyay S, Paul B, Majumder SK (1979) Hematological changes produced in mice by ochratoxin A. *Toxicology* **14**: 95–8.

Hagelberg S, Hult K, Fuchs R (1989) Toxicokinetics of ochratoxin A in several species and its plasma-binding properties. *J Appl Toxicol* **9**: 91–6.

Harwig J, Kuiper-Goodman T, Scott PM (1983) Microbial food toxicants: ochratoxins. In *Handbook of Foodborne Diseases of Biological Origin*, Richcigl M (ed.). CRC Press, Boca Raton, FL, pp. 193–238.

Hatey F, Galtier P (1977) Short-term toxicity of ochratoxin A production by *Aspergillus carbonarious* and *A. niger* isolates and detection using coconut cream agar. *J Fd Mycol* **1**: 67–72.

Hult K, Teiling A, Gatenbeck S (1976) Degradation of ochratoxin A by a ruminant. *Appl Environ Microbiol* **32**: 443–4.

Hult K, Fuchs R (1986) Analysis and dynamics of ochratoxin A in biological systems. In *Mycotoxins and Phycotoxins*, Steyn PS, Vleggaar R (eds). Elsevier Scientific Publication BV, Amsterdam, pp. 365–7.

IARC (1993) Ochratoxin A. *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans*, vol. **56**. International Agency for Research on Cancer, Lyon, France, pp. 489–521.

Jung KY, Endou H (1989) Nephrotoxicity assessment by measuring cellular ATP content. II. Intranephron site of ochratoxin A nephrotoxicity. *Toxicol Appl Pharmacol* **100**: 383–90.

Kitchen DN, Carlton WW, Hinsman EJ (1977a) Ochratoxin A and citrinin induced nephrosis in beagle dogs. III. Terminal renal ultrastructural alterations. *Vet Pathol* **14**: 392–406.

Kitchen DN, Carlton WW, Tuite J (1977b) Ochratoxin A and citrinin induced nephrosis in beagle dogs. I. Clinical and clinicopathological features. *Vet Pathol* **14**: 154–72.

Kitchen DN, Carlton WW, Tuite J (1977c) Ochratoxin A and citrinin induced nephrosis in beagle dogs. II. Pathology. *Vet Pathol* **14**: 261–72.

Krog P, Elling F, Hald B, *et al*. (1976) Experimental avian nephropathy. *Acta Pathol Microbiol Scand A* **84**: 215–21.

Krogh P, Elling F (1977) Mycotoxic nephropathy. *Vet Sci Commun* **1**: 51–63.

Kumagai S (1988) Effects of plasma ochratoxin A and luminal pH on the jejunal absorption of ochratoxin A in rats. *Fd Chem Toxicol* **26**: 753–8.

Kumagai S, Aibara K (1982) Intestinal absorption and secretion of ochratoxin A in the rat. *Toxicol Appl Pharmacol* **64**: 94–102.

Meisner H, Cimbala MA, Hanson RW (1983) Decrease of ` phosphoenolpyruvate carboxykinase RNA and poly(A)RNA level by ochratoxin A. *Arch Biochem Biophys* **223**: 264–70.

Moroi K, Suzuki S, Kuga T, *et al*. (1985) Reduction of ochratoxin A toxicity in mice treated with phenylalanine and phenobarbital. *Toxicol Lett* **25**: 1–5.

National Toxicology Program (NTP) (1989). Technical Report on the toxicology and carcinogenesis studies of ochratoxin A in F344 rats (Gavage studies) (NIH Publication No. 89–2813), National Institutes of Health.

Pitout MJ (1968) The effect of ochratoxin A on glycogen storage in the rat liver. *Toxicol Appl Pharmacol* **13**: 299–306.

Pohland AE, Nesheem S, Friedman L (1992) Ochratoxin A: a review. *Pure Appl Chem* **64**: 1029–46.

Sava V, Reunova O, Velazquez A, *et al*. (2006) Acute neurotoxic effects of the fungal metabolite ochratoxin A. *Neurotoxicology* **27**: 82–92.

Singh GS, Chanhan HV, Jha GJ, Singh KK (1990) Immunosuppression due to chronic ochratoxicosis in broiler chicks. *J Comp Pathol* **103**: 389–410.

Sreemannarayana O, Frohlich AA, Vitti TG (1988). Studies of the tolerance and disposition of ochratoxin A in young calves. *Aim Sci* **66**: 1703–11.

Stetina R, Votava M (1986) Induction of DNA single-stranded breaks and DNA synthesis inhibition by patulin, ochratoxin A, citrinin, and aflatoxin B, in cell lines CHO and AWRF. *Folia Biol* **32**: 128–44.

Stormer FC, Lea T (1995) Effects of ochratoxin A upon early and late events in human T-cell proliferation. *Toxicology* **95**: 45–50.

Suzuki S, Satoh T, Yamazaki M (1977) The pharmacokinetics of ochratoxin A in rats. *Jpn J Pharmacol* **27**: 735–44.

Wyatt RD (1979) *Biological Effects of Mycotoxins (other than ochratoxin) on Poultry. Interaction of Mycotoxins in Animal Production*. National Academy Science, Washington, DC, pp. 87–95.

C H A P T E R

# 80

# Tremorgenic mycotoxins

*Tim J. Evans and Ramesh C. Gupta*

## INTRODUCTION

Fungi belonging to the genera *Penicillium, Aspergillus, Claviceps*, and *Neotyphodium* can produce tremorgenic mycotoxins, which are secondary fungal metabolites that elicit either intermittent or sustained tremors in vertebrate species (Cole and Cox, 1981; Selala *et al.*, 1989; Burrows and Tyrl, 2001). Over 20 mycotoxins containing a tryptophan-derived indole moiety, including: penitrem A, roquefortine, verruculogen, tryptoquivaline, aflatrem, paspalinine, and paxilline, as well as, the paspalitrems and lolitrems (Table 80.1 and Figure 80.1), have demonstrated tremorgenic potential in animals and humans (Selala *et al.*, 1989; Burrows and Tyrl, 2001). Several other fungal metabolites, such as paspaline, paspalacine, and cyclopiazonic acid, are chemically related to these mycotoxins but have been shown to not be tremorgenic (Knaus *et al.*, 1994). Tremorgen-producing fungi grow on a wide variety of foodstuffs, including dairy or grain-containing products intended for human consumption (e.g. cheeses and pastas), stored grains and nuts (e.g. peanuts and walnuts) and a number of forages (e.g. legumes and grasses) consumed by livestock species, and even garbage and compost piles can be sources of tremorgenic mycotoxins (Burrows and Tyrl, 2001; Boysen *et al.*, 2002; Young *et al.*, 2003).

Although there have been several human cases in which tremors and convulsions were attributed to consumption of mold-contaminated food containing penitrem A (Gordon *et al.*, 1993; Lewis *et al.*, 2005), this chapter will focus on the toxicity of tremorgenic mycotoxins in animal species. Dogs, because of their relatively indiscriminate appetite and frequently unsupervised roaming behavior, appear to be very susceptible to intoxication by penitrem A, as well as roquefortine (Boysen *et al.*, 2002; Young *et al.*, 2003). Mycotoxin-associated stagger syndromes in livestock, also described in the literature as "grass staggers" (not to be confused with "grass staggers" or "grass tetany" related to hypomagnesemia), have most frequently occurred following the ingestion of endophyte (*Neotyphodium lolii*) infected perennial ryegrass (*Lolium perenne*) or consumption of Dallisgrass (*Paspalum dilatatum*) or Bahiagrass (*Paspalum notatum*) contaminated by sclerotia of *Claviceps paspali*.

**TABLE 80.1 Representative tremorgenic mycotoxins, associated fungi, and substrates**

| Tremorgenic mycotoxin | Associated fungi | Common substrates |
|---|---|---|
| Penitrem A | *Penicillium crustosum*<br>*Penicillium cyclopium*<br>*Penicillium commune*<br>*Penicillium* spp. | Meat, cereals, nuts, cheeses, eggs, fruits, processed and refrigerated foods, refuse, compost piles |
| Roquefortine | *Penicillium roqueforti* | Same as penitrem A and sometimes concurrently with penitrem A |
| Janthitrems A, B, and C | *Penicillium janthinellum* | Perennial ryegrass |
| Verruculogen | *Penicillium* spp.<br>*Aspergillus* spp. | Soil, seeds, cereal crops |
| Territrems A and B | *Aspergillus terreus* | Cereal grains |
| Aflatrem | *Aspergillus flavus* | Corn |
| Lolitrems A, B, C, and D | *Neotyphodium lolii* | Perennial ryegrass |
| Paxilline | *Neotyphodium lolii* | Perennial ryegrass |
| Lolitriol | *Neotyphodium lolii* | Perennial ryegrass |
| Paspalitrems A, B, and C | *Claviceps paspali*<br>*Claviceps cinerea* | Dallisgrass<br>Bahiagrass<br>*Hilaria* spp.<br>Bermudagrass? |
| Paspalinine | Same as paspalitrems | Same as paspalitrems |

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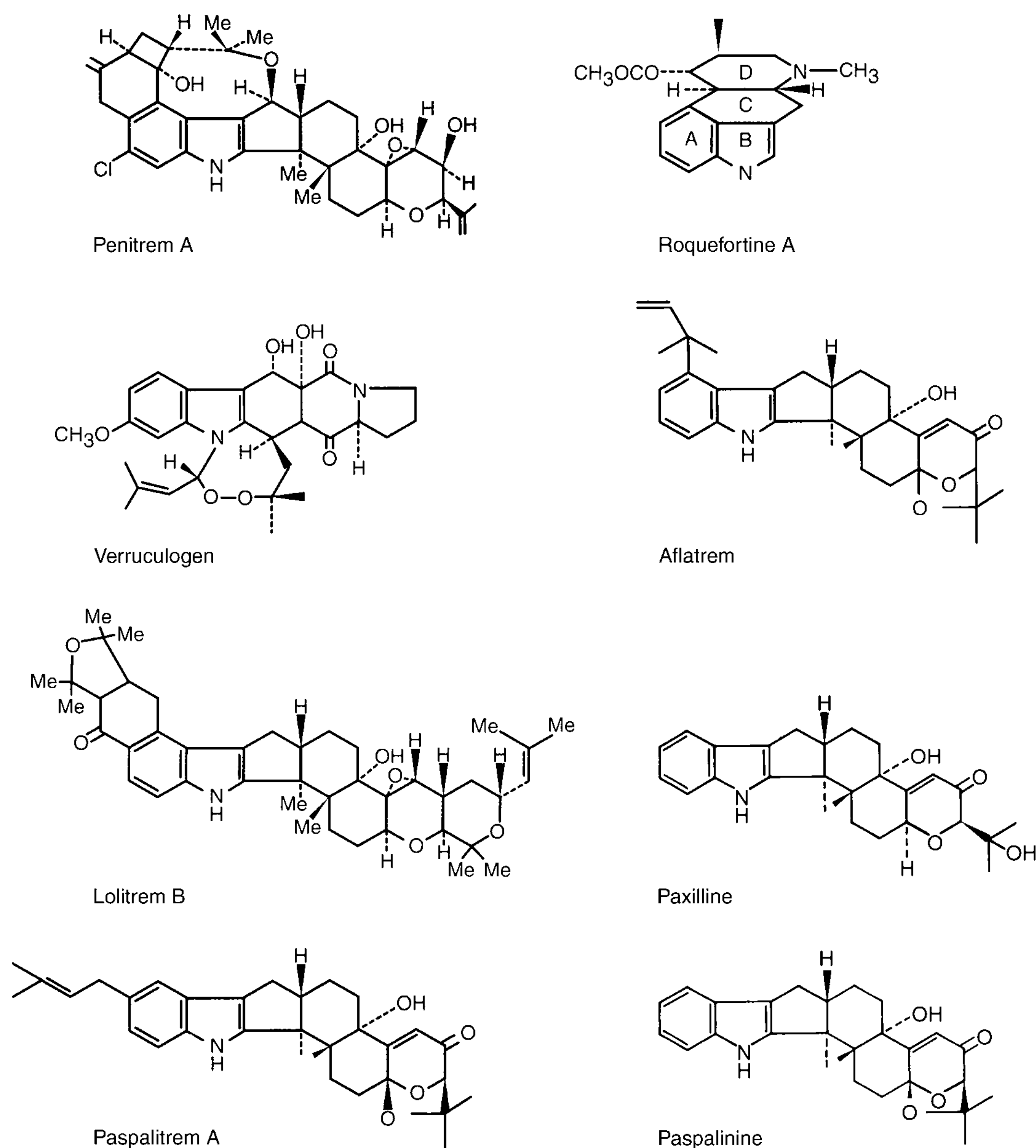


**FIGURE 80.1** The structures of selected indole-diterpene tremorgenic mycotoxins are shown. This figure was adapted, with permission, from Council for Agricultural Science and Technology (CAST). 2003. "Mycotoxins: Risks in Plant, Animal, and Human Systems". Task Force Report 139. CAST, Ames, Iowa and from Cole, R.J. and Cox, R.H. *in* "Handbook of Toxic Fungal Metabolites", Academic Press, New York, 1981 (modifications courtesy of Don Connor and Howard Wilson).

# BACKGROUND

## Penicillium-associated tremorgenic mycotoxins

Although a variety of different fungi synthesize indole–diterpene mycotoxins having specific tremorgenic effects on the central nervous system (CNS), tremorgens produced by *Penicillium* spp. (especially penitrem A and roquefortine) are the most commonly encountered of these mycotoxins. Penitrem A is a potent neurotoxin which causes a syndrome characterized by sustained tremors and, at high doses, convulsions and death in laboratory and farm animals (Wilson, 1971; Arp and Richard, 1981; Peterson and Penny, 1982; Shreeve *et al.*, 1983; Hocking *et al.*, 1988; Breton *et al.*, 1998; Cavanagh *et al.*, 1998). Dogs can be poisoned by eating walnuts and a variety of other unused or discarded foodstuffs infected with *Penicillium* spp. (Boysen *et al.*, 2002; Young *et al.*, 2003), and field cases of poisoning have also been documented in cattle, sheep, and horses

(Cavanagh *et al.*, 1998; Hocking *et al.*, 1988; Boysen *et al.*, 2002; Walter, 2002; Young *et al.*, 2003). *Penicillium crustosum* is an especially common foodborne fungus that causes spoilage in a wide variety of foods, including meat, cereals, nuts, cheese, eggs, fruits, and processed and refrigerated food, and almost all *Penicillium crustosum* isolates produce the mycotoxin penitrem A (Hocking and Pitt, 2003; Rundberget *et al.*, 2004). Roquefortine can also be synthesized by *Penicillium* spp. (Hooser and Talcott, 2006), and, interestingly, *Penicillium crustosum*, as well as several other P*enicillium* spp., can produce penitrem A and roquefortine concurrently following growth and sporulation (Vesonder *et al.*, 1980; Wagener *et al.*, 1980; Kyriakidis *et al.*, 1981; Mantle *et al.*, 1983; Boysen *et al.*, 2002; Young *et al.*, 2003). Three other tremorgenic mycotoxins, janthitrem A, B, and C (molecular weight 601, 585, and 565, respectively) can also be produced by a species of *Penicillium* (i.e. *Penicillium janthinellum*), and these tremorgens have been associated with staggers outbreaks in sheep grazing ryegrass (Gallagher *et al.*, 1980; Burrows and Tyrl, 2001).

## Aspergillus-associated tremorgenic mycotoxins

Verruculogen, another common tremorgenic mycotoxin containing the indole moiety and associated with tremors in mice, rats, and farm animals, is produced by species of *Aspergillus*, as well as *Penicillium* (Gallagher and Latch, 1977). Other tremorgenic mycotoxins isolated from a species of *Aspergillus* (i.e. *Aspergillus terreus*) include territrem A and B, which were previously designated as $C_1$ and $C_2$, respectively (Ling *et al.*, 1979). Although most often associated with the production of aflatoxins, *Aspergillus flavus* can also produce tremorgenic aflatrem and other indole–diterpene mycotoxins (e.g. paspalinine) in contaminated corn (Burrows and Tyrl, 2001).

## Neotyphodium-associated tremorgenic mycotoxins

In Australia, New Zealand, and North America, perennial ryegrass (*Lolium perenne*) infected with the endophyte *Neotyphodium lolii* (formerly *Acremonium lolii*) has been associated with a "grass staggers" syndrome in horses, deer, cattle and, especially, sheep, which is frequently referred to as "perennial ryegrass staggers" and is distinct from annual ryegrass toxicosis (Galey *et al.*, 1991; Cheeke, 1998; Burrows and Tyrl, 2001). *Neotyphodium lolii* concentrates in the caryopsis/seed and the outer, lower leaf sheaths of perennial ryegrass, and the "staggers" syndrome is most often observed after several days of exposure to endophyte-infected ryegrass toward the end of summer when there is shortage of pasture (Galey *et al.*, 1991; Cheeke, 1998). Ergovaline and other ergot alkaloids produced by *Neotyphodium lolii* can cause a clinical syndrome resembling "fescue toxicosis" (Cheeke, 1998). However, the classic neurological signs of perennial ryegrass staggers (e.g. ataxia, muscle rigidity, and convulsive episodes) are indistinguishable from those associated with *Penicillium* and *Aspergillus* species and also result from the adverse effects of neurotoxic indole–diterpene tremorgens, which include lolitrems A, C, and D, lolitrem precursors (e.g. paxilline and lolitriol) and, especially, lolitrem B (Cheeke, 1998; Burrows and Tyrl, 2001).

## Claviceps-associated tremorgenic mycotoxins

While *Claviceps purpurea* is notoriously associated with "classic" ergotism in human and animals and, historically, occasional tremors or convulsions (i.e. nervous ergotism or convulsive ergotism) in livestock (Burrows and Tyrl, 2001; Evans *et al.*, 2004), other species of *Claviceps* produce toxicoses primarily characterized by a tremorgenic "staggers" syndrome (Burrows and Tyrl, 2001). Sheep, horses, and cattle, in particular, can develop a "grass staggers" syndrome several days following the ingestion of mature Dallisgrass (*Paspalum dilatatum*) or Bahiagrass (*Paspalum notatum*) infected with the sclerotia of *Claviceps paspali* (Cheeke, 1998; Burrows and Tyrl, 2001). "Dallisgrass staggers" and "Bahiagrass staggers" have most frequently been observed in the southeastern United States, Central and South America, parts of Europe and South Africa, as well as Australia and New Zealand. Traditionally, the ergot alkaloids produced in large quantities by *Claviceps purpurea* and in much smaller quantities by *Claviceps paspali* were thought to be responsible for the tremors observed in conjunction with exposure to both species of *Claviceps* (Cheeke, 1998). However, it is now understood, at least with respect to the "staggers" syndromes associated with *Claviceps paspali* and, less commonly, *Claviceps cinerea* (fungal infections of *Hilaria* spp. (curly mesquite, curlygrass, galleta, etc.)), that the large concentrations of indole–diterpene tremorgenic mycotoxins (i.e. paspalinine and paspalitrems A, B, and C) found in the sclerotia of these species of *Claviceps* are responsible for the neurotoxicity. As with other "grass staggers", *Claviceps*-related tremorgenic syndromes are characterized by exercise-exacerbated nervousness, "wild" facial expressions, belligerent attitude, tremors, ataxia, convulsions, and occasional deaths attributed primarily to misadventure (Burrows and Tyrl, 2001).

## Bermudagrass staggers

Periodic episodes of tremors have been observed in cattle in the southern United States and Oklahoma and Texas,

as well as horses in California, which have been ingesting mature Bermudagrass (*Cynodon dactylon*) (Cheeke, 1998; Burrows and Tyrl, 2001). The ergot-type alkaloids ergonovine and ergonovinine, which can be produced by several species of *Claviceps*, were isolated in one instance of "Bermudagrass staggers". However, *Claviceps*- or endophyte-associated paspalitrem-type indole alkaloids are suspected of being the primary Bermudagrass tremorgens (Cheeke, 1998; Burrows and Tyrl, 2001). It should be kept in mind that, while the onset and duration of the various "staggers" syndromes might vary somewhat, the tremorgenic neurotoxicities associated with Bermudagrass, perennial ryegrass, Dallisgrass, Bahiagrass, and aflatoxin-infected corn are essentially identical to one another (Cheeke, 1998).

## TOXICOKINETICS

Tremorgenic indole-containing mycotoxins are lipophilic molecules that easily cross the blood–brain barrier and gain the access to the CNS (Patterson *et al.*, 1981). Although there might be some subtle differences between compounds and species of animals, tremorgenic indole–diterpene alkaloids are generally rapidly absorbed from the gastrointestinal tract, and the toxic signs, depending on the specific toxins and the exposed species, are usually seen within several hours (especially penitrem A and roquefortine in dogs) to a few days (frequently lolitrems and paspalitrems in ruminants) following ingestion of contaminated foodstuffs (Burrows and Tyrl, 2001; Hooser and Talcott, 2006). As evidenced by the lack of a cumulative effect following repeated doses, these particular mycotoxins do not appear to accumulate in the body (Peterson and Penny, 1982). Despite some hepatic metabolism, these mycotoxins are usually eliminated mainly through biliary excretion into the feces (Burrows and Tyrl, 2001; Hooser and Talcott, 2006). Young animals are more sensitive than adults to the toxicity of tremorgenic mycotoxins, with 5-month-old lambs being more susceptible than 15-month-old sheep to verruculogen toxicity (Peterson *et al.*, 1982).

## MECHANISM OF ACTION

It should be pointed out that, although related in structure, tremorgenic mycotoxins do not exert toxicity by a single common mechanism, and the exact mechanisms involved in neurotoxicity induced by these tremorgens are yet to be fully elucidated. These mycotoxins work at a different functional level of the nervous system than other mycotoxins having more widespread targets for toxicity associated with the inhibition of basic cellular functions, such as protein synthesis. Several pharmacological/toxicological mechanisms of tremorgenic action have been proposed, and, in general, tremorgenic mycotoxins interfere with excitatory amino acid neurotransmitter release mechanisms (Wilson, 1971; Norris *et al.*, 1980; Selala *et al.*, 1989). In experimental studies, the inhibitory action of these mycotoxins at gamma aminobutyric acid (GABA) receptors was demonstrated (Stern, 1971; Hotujac and Stern, 1974; Hotujac *et al.*, 1976; Selala *et al.*, 1989; Abramson, 1997). Gant *et al.* (1987) studied the effects of four tremorgenic and one nontremorgenic mycotoxins on $GABA_A$ receptor-binding in the rat's brain, and, in essence, with high concentrations, the tremorgenic action of these mycotoxins might, in part, be due to inhibition of $GABA_A$ receptor function.

Among the tremorgenic mycotoxins, penitrem A has been studied the most for the elucidation of its mechanism of action and toxicity. There is convincing evidence suggesting that penitrem A acts on the CNS to induce seizures (Sobotka *et al.*, 1978; Arp and Richard, 1981). Penitrem A was shown to increase the spontaneous release of aspartate and glutamate, as well as GABA (Norris *et al.*, 1980). Glutamate and aspartate are the neurotransmitters of the parallel fibers and the climbing fibers, respectively, which are two major excitatory inputs to Purkinje cells. There is also evidence for a penitrem A-induced partial decrease in glycine levels in the brain in association with pathology related to penitrem A exposure (Catovic *et al.*, 1975). Cavanagh *et al.* (1998) demonstrated that, in rats, penitrem A can cause widespread degeneration of Purkinje cells and foci of necrosis in the cerebellar granular cell layers. These striking lesions are confined to the cerebellum, with no lesions found elsewhere in the brain. Cerebellar Purkinje cells are the primary targets cells for the adverse effects of penitrem A. Penitrem A-induced tremors might also be partly explained by a presynaptic inhibition of inhibitory interneurons.

Verruculogen-induced tremors in rats have been associated with an increased level of excitatory neurotransmitters (glutamate and aspartate) in the lateral ventricle, suggesting subcortical, verruculogen-induced tremorgenic activity. Selala *et al.* (1989) demonstrated that tremorgenic mycotoxins can be partial agonists of GABA. Verruculogen increases spontaneous glutamate and aspartate release *in vivo* in guinea pig ileum preparations. Verruculogen also causes an increase in contractile responses to electrical field stimulation, which has been attributed to enhanced release of acetylcholine from presynaptic nerve terminals.

Although paxilline blocks high-conductance $Ca^{2+}$-activated $K^+$-channels, some related nontremorgenic fungal metabolites, such as paspalinine , have also been shown to inhibit these particular ion channels (Burrows and Tyrl, 2001). Paxilline also inhibits the cerebellar inositol 1,4,5-triphosphate receptor. Paspalinine and the paspalitrems are thought to impair GABA- and glycine-mediated inhibitory pathways (Burrows and Tyrl, 2001).

# TOXICODYNAMICS

## Central effects

Tremorgenic mycotoxins are a group of fungal metabolites which are known to act on the CNS, causing sustained tremors, convulsions, and, occasionally, deaths in animals. In general, the clinical signs and symptoms typically observed during tremorgenic mycotoxicoses include diminished activity and immobility, followed by hyperexcitability, muscle tremor, ataxia, tetanic seizures, and convulsions (Cole and Cox, 1981). These symptoms are reversible if the affected animal is removed from the contaminated feed source, and the incidence of death is generally limited by the emetic effect of tremorgenic mycotoxins. In dogs, the overall clinical picture involves the development of muscle tremors and seizures but can also frequently include vomiting, behavior alterations, hyperthermia, depression, coma, and pulmonary edema.

Depending on the level of tremorgenic mycotoxin exposure, death can occur in some instances within 2–4 h and is usually secondary to respiratory compromise, metabolic acidosis, and hyperthermia. In severe cases, clinical signs of toxicosis can persist for several days, and fine tremors can be seen for a week or more.

The tremors produced by penitrem A are very similar in most species and begin within a few minutes of intraperitoneal (IP) injection and even sooner with IV injection. The tremors can be sustained and lead to both ataxia and episodic spasms (Cavanagh *et al.*, 1998). Larger doses of penitrem A can cause seizures, massive liver necrosis, and death (Hocking *et al.*, 1988). In a histopathological study in rats, Breton *et al.* (1998) revealed that penitrem A induced dose-related injuries in the cerebellum with massive degeneration of Purkinje cells and a significant vacuolization within the molecular layer.

## Peripheral effects

Tremorgenic mycotoxins have also been studied for peripheral effects (McLeay *et al.*, 1999). The tremorgenic mycotoxins, such as penitrem A, paxilline, and lolitrem B can have profound effects on electromyographic (EMG) activity of smooth muscle of the reticulorumen in conscious sheep, with a time course of action similar to their respective characteristic effects on the induction (1–2, 15–20, and 20–30 min) and the duration (1–2, 1–2, and 8–12 h) of tremors. Response to penitrem A revealed a greater sensitivity of smooth muscle than skeletal muscle. The excitatory local effects were partially blocked by atropine, indicating that stimulation of muscarinic cholinergic receptors was involved. Increased local activity may mediate a reflex inhibition of cyclical contractions. However, a nontremorgenic isomer of lolitrem B (31-epilolitrem B) had no effect on the reticulorumen. The intensity and duration of the effects of lolitrem B (up to 12 h) indicate that severe disruption of digestion may occur in animals grazing endophyte-infected pasture.

## Genotoxicity

Five tremorgenic mycotoxins (fumitremorgen B, paxilline, penitrem A, verruculogen, and verrucosidin), which have been associated with molds found in fermented meats, were assessed for genotoxicity (Sabater-Vilar *et al.*, 2003). The mycotoxins were tested in two short-term *in vitro* assays using different genotoxic end points, in different phylogenetic systems, with the use of mammalian-microsome assay and the single-cell gel electrophoresis assay of human lymphocytes. The findings revealed that all of the tested mycotoxins, with the exception of penitrem A, exhibited a certain degree of genotoxicity. Verrucosidin appeared to have the highest toxic potential, testing positive in both assays. Verruculogen tested positive in the *Salmonella*/mammalian-microsome assay, and paxilline and fumitremorgen B caused DNA damage in human lymphocytes.

## Acute toxicity

Acute toxicity data are available for only few of the tremorgenic mycotoxins. It should be noted that the relative doses resulting in lethality in different species might not necessarily be reflective of the relative tremorgenic potentials of these mycotoxins. The $LD_{50}$ of penitrem A in mice is 15–19 mg/kg, IP (Ling *et al.*, 1979). The $LD_{50}$ of verruculogen is reported to be 15.2 mg/kg, IP, and 266 mg/kg, following oral exposure, in the chicken. The corresponding $LD_{50}$ values verruculogen in mice are 2.4 mg/kg, IP, and 127 mg/kg, following oral exposure. The $LD_{50}$ value of territrem A in mice is 15–19 mg/kg, IP (Ling *et al.*, 1979).

## Potential interactions

In previous studies, concurrent production of roquefortine and penitrem A by various species of *Penicillium* (e.g. *Penicillium crustosum, Penicillium cyclopium* Westling, and *Penicillium commune*) was demonstrated in culture extracts (Vesonder *et al.*, 1980; Wagener *et al.*, 1980; Kyriakidis *et al.*, 1981; Mantle *et al.*, 1983). Braselton and Rumler (1996) first reported the concurrent presence of both tremorgenic mycotoxins in naturally occurring field cases of canine intoxication and raised the issue of the potential synergistic interactions between the two tremorgens. In two recent cases, both Boysen *et al.* (2002) and Young *et al.* (2003) diagnosed concurrent intoxication with penitrem A and roquefortine in several dogs. Given that roquefortine and

penitrem A have similar mechanisms of action, there would seem to be a potential for some sort of synergistic interaction between the two tremorgenic mycotoxins or at least a reduction in the amount of ingested contaminated material required for the onset of clinical signs. Likewise, it would seem logical in instances of "grass staggers" that a greater concentration of multiple, potential tremorgenic mycotoxins in contaminated forages would increase the likelihood and, potentially, the severity of observed intoxications.

# CLINICAL ASPECTS OF TREMORGENIC MYCOTOXICOSES

## Diagnosis

Diagnosis of tremorgenic mycotoxin-related intoxication is based on a history of exposure to or consumption of moldy food/feed, clinical signs of tremors and seizures and detection of tremorgenic mycotoxin(s) in the suspected source material, vomitus, gastrointestinal tract contents, or bile. Differential diagnoses should rule out ethylene glycol, strychnine, metaldehyde, methylxanthines, pyrethroids, nicotine, organochlorine insecticides, bromethalin, acetylcholinesterase-inhibiting insecticides, other potential neurotoxicants, and eclampsia in pregnant animals (Hooser and Talcott, 2006). It needs to be pointed out that in field cases of perennial ryegrass staggers, as well as Dallisgrass and Bahiagrass staggers, the onset of clinical signs is most likely related to the intake of the mycotoxins lolitrem B and paspalinine and/or paspalitrems, respectively. The effects of these tremorgenic mycotoxins could potentially be more insidious and longer lasting than those of penitrem A (Burrows and Tyrl, 2001; Hooser and Talcott, 2006). Depending on the laboratory, tremorgenic mycotoxins can generally be quantified using HPLC/MS (Rundberget and Wilkins, 2002), GC/ MS/MS (Braselton and Rumler, 1996), or TLC (Hooser and Talcott, 2006). Analyses for roquefortine and penitrem A are generally more likely to be available to clinicians than analytical procedures for the other tremorgenic mycotoxins.

## Treatment

In a small animal setting the minimum initial database should include a complete blood count, serum biochemical profile, and assessment of acid–base status and urinalysis (Hooser and Talcott, 2006). Depending on the species, the time period since exposure and the presence and severity of clinical signs, suspected poisoned animals should be decontaminated by emesis, gastric lavage, activated charcoal, and/or administration of cathartics. Given that intoxications with tremorgenic mycotoxins can be characterized by convulsions or, conversely, severe depression and coma, care should be taken to not induce vomiting when contraindicated by an increased risk for aspiration pneumonia. Tremors and seizures can be controlled by diazepam or methocarbamol. Animals unresponsive to these medications can be treated with barbiturates. The animal should be periodically assessed for metabolic acidosis, hyperthermia, pulmonary edema, and aspiration pneumonia. With proper initial and ongoing assessment and in the absence of complications, affected animals should recover relatively uneventfully following removal from the source and appropriate decontamination and other therapeutic procedures.

## Prevention

Care should be taken to avoid the presence of moldy feedstuffs in areas where animals have unsupervised and unrestricted access, and potentially contaminated materials should be disposed of properly. Trash receptacles should have securely fitting lids, and animals should not be free to roam in areas where discarded foodstuffs or refuse are stored. Livestock should not be fed overtly mold-contaminated forages or concentrates. With respect to "grass staggers", appropriate stocking rates should be maintained, and management practices should be instituted which ensure that animals have access to young growing plants, especially in pastures dominated by perennial ryegrass, Dallisgrass, Bahiagrass, or Bermudagrass (Burrows and Tyrl, 2001).

# CONCLUSIONS

Tremorgenic mycotoxins containing a tryptophan-derived indole moiety affect many mammalian species, especially sheep, cattle, and dogs. Roquefortine and penitrem A are produced by several species of *Penicillium*, and dogs exposed to these potent tremorgens exhibit tremors and, potentially, severe convulsive episodes and seizures. Several common forages, especially perennial ryegrass, are susceptible to infection by fungi capable of producing indole–diterpene tremorgenic mycotoxins, such as lolitrems and paspalitrems, which cause "grass staggers" in susceptible species. Although multiple mechanisms are involved in the neurotoxicity of tremorgenic mycotoxins, impaired GABA- and glycine-mediated inhibitory pathways and abnormal excitatory neurotransmitter (i.e. glutamate and aspartate) release appear to play major roles in the onset of clinical signs. Removal of the animal from contaminated foodstuffs usually results in recovery, but severely poisoned animals should be properly assessed, their tremors and/or seizures controlled, and appropriate

steps taken to avoid complications. Whenever possible, animals should not have unrestricted and unsupervised access to mold-contaminated foodstuffs.

# REFERENCES


Abramson D (1997) Toxicants of the Genus Penicillium. In *Handbook of Plant and Fungal Toxicants,* Felix JP (ed.). CRC Press, Boca Raton, FL, pp. 303–17.

Arp LH, Richard JL (1981) Experimental intoxication of guinea pigs with multiple doses of the mycotoxin, Penitrem A, *Mycopathologia.* **73**: 109–13.

Boysen SR, Rozanski EA, Chan DL, Grobe TL, Fallon MJ, Rush JE (2002) Tremorgenic mycotoxicosis in four dogs from a single household. *J Am Vet Med Assoc* **221**: 1441–4.

Braselton WE, Rumler PC (1996) MS/MS screen for the tremorgenic mycotoxins roquefortine and penitrem A. *J Vet Diagn Invest* **8**: 515–18.

Breton P, Bizot JC, Bull J, De La Manche I (1998) Brain neurotoxicity of penitrem A: electrophysiological, behavioral and histopathological study. *Toxicon* **36**: 645–55.

Burrows GE, Tyrl RJ (2001) *Toxic Plants of North America*. Iowa State University Press, Ames, IA, pp. 1–1342.

Catovic S, Filipovic N, Stern P (1975) The effect of Penitrem A upon the level of glycine in the CNS. *Bull Scientifique Sect A Yugoslavia* **20**: 284–5.

Cavanagh JB, Holton JL, Nolan CC, Ray DE, Naik JT, Mantle PG (1998) The effects of the tremorgenic mycotoxin penitrem A on the rat cerebellum. *Vet Pathol* **35**: 53–63.

Cheeke PR (1998) *Natural Toxicants in Feeds,* 2nd edn. Interstate Publishers, Inc., Danville, Illinois, pp. 1–479.

Cole RA, Cox RH (1981) *Handbook of Toxic Fungal Metabolites.* Academic Press, New York.

Evans TJ, Rottinghaus GE, Casteel SW (2004) Ergot. In *Clinical Veterinary Toxicology*, Plumlee KH (ed.). Mosby, Inc., St. Louis, MO, pp. 239–43.

Galey FD, Tracy ML, Craigmill AL, Barr BC, Markegard M, Peterson R, O'connor M (1991) Staggers induced by consumption of perennial ryegrass in cattle and sheep from northern California. *J Am Med Assoc* **199**: 466–70.

Gallagher RT, Latch GCM (1977) Production of the tremorgenic mycotoxins verraculogen and fumitremorgen B by *Penicillium piscarium* Westling. *Appl Environ Microbiol* **33**: 730–1.

Gallagher RT, Latch GC, Keogh RG (1980) The janthitrems: fluorescent tremorgenic toxins produced by *Penicillium janthinellum* isolates from ryegrass pastures. *Appl Environ Microbiol* **39**: 272–3.

Gordon KE, Masotti RE, Waddell WR (1993) Tremorgenic encephalopathy: a role of mycotoxins in the production of CNS disease in humans? *Can J Neurol Sci* **20**: 237–9.

Gant DB, Cole RJ, Valdes JJ, Eldefrawi ME, Eldefrawi AT (1987) Action of tremorgenic mycotoxins on GABA/subA/receptor. *Life Sci* **41**: 2207–14.

Hocking AD, Pitt JI (2003) *Foodborne microorganisms of public health significance*. Hocking AD (ed.). AIFST Food Microbiology Group Sydney, pp. 641–74.

Hocking AD, Holds K, Tobin NF (1988) Intoxication by tremorgenic mycotoxin (Penitrem A) in a dog. *Aust Vet J* **65**: 82–5.

Hooser SB, Talcott PA (2006) Mycotoxins. In *Small Animal Toxicology*, 2nd edn, Peterson ME, Talcott PA (eds.). Elsevier Saunders, St. Louis, MO, pp. 888–97.

Hotujac LJ, Stern P (1974) Pharmacological examination of verruculogen induced tremor. *Acta Med Yugoslav* **28**: 223–9.

Hotujac LJ, Muftic RH, Filipovic N (1976) Verruculogen: a new substance for decreasing of GABA levels in CNS. *Pharmacology* **14**: 297–300.

Knaus H-G, McManus OB, Lee SH, Schmalhofer WA, Garcia-Calvo M, Helms LM, Sanchez M, Giangiacomo k, Reuben, JP, Smith III AB, *et al.* (1994) Tremorgenic indole alkaloids potently inhibit smooth muscle high-conductance calcium-activated potassium channels. *Biochemistry* **33**: 5819–28.

Kyriakidis N, Waight ES, Day JB, Martle PG (1981) Novel metabolites from *Penicillium crustosum,* including penitrem E, a tremorgenic mycotoxin. *Appl Environ Microbiol* **42**: 61–2.

Lewis PR, Donohue MB, Hocking AD, Cook L, Granger LV (2005) Tremor syndrome associated with a fungal toxins sequelae of food contamination. *Med J Aust* **182**: 582–4.

Ling KH, Yang CK, Peng FT (1979) Territrems, tremorgenic mycotoxins of *Aspergillus terreus. Appl Environ Microbiol* **37**: 355–7.

Mantle PG, Perera KPWC, Maishman NJ, Mundy GR (1983) Biosynthesis of penitrems and roquefortine by *Penicillium crustosum*. *Appl Environ Microbiol* **45**: 1486–90.

McLeay LM, Smith BL, Munday-Finch SC (1999) Tremorgenic mycotoxins paxilline, penitrem, and lolitrem B, the non-tremorgenic 31-epilolitrem B and electromyographic activity of the reticulum and rumen of sheep. *Res Vet Sci* **66**: 119–27.

Norris PJ, Smith CCT, De Belleroche J, Bradford HF, Mantle PG, Thomas AJ, Penny RH (1980) Actions of tremorgenic fungal toxins on neurotransmitters release. *J Neurochem* **34**: 33–42.

Patterson DS, Shreeve BJ, Roberts BA, MacDonald SM (1981) Verruculogen produced by soil fungi in E. Wales *Appl Environ Microbiol* **42**: 916–17.

Peterson DW, Penny RHC (1982) A comparative study of sheep and pigs given the tremorgenic mycotoxins verruculogen and Penitrem A. *Res Vet Sci* **33**: 1983–7.

Rundberget T, Wilkins AL (2002) Determination of Penicillium mycotoxins in foods and feeds using liquid chromatography-mass spectrometry. *J Chromatogr* A **964**: 189–97.

Rundberget T, Skaar I, Flaoyen A (2004) The presence of Penicllium and Penicillium mycotoxins in food wastes. *Intl J Food Microbiol* **90**: 181–8.

Sabater-Vilar M, Mijmeijer S, Fink-Gremmels J (2003) Genotoxicity assessment of five tremorgenic mycotoxins (fumitremorgen B, paxilline, penitrem A, verruculogen, and verrucosidin) produced by molds isolated from fermented meats. *J Food Prot* **66**: 2123–9.

Selala MI, Daelemans F, Schepens PJC (1989) Fungal tremorgens: the mechanism of action of single nitrogen containing toxin. A hypothesis. *Drug Chem Toxicol* **12**: 237–57.

Shreeve BJ, Patterson DSP, Roberts BA, McDonald SM (1983) Tremorgenic fungal toxins. *Vet Res Commun* **7**: 155–60.

Sobotka TJ, Brodie RE, Spaid SL (1978) Neurobehavioral studies of tremorgenic mycotoxins, verruculogen and penitrem A. *Pharmacology* **16**: 287–94.

Stern P (1971) Pharmacological analysis of the tremor induced by cyclopium toxin. *Yugoslav Physiol Pharmacol* **7**: 187–96.

Vesonder RF, Tjarks L, Rohwedder W, Kleswetter DO (1980) Indole metabolites of *Penicillium cyclopium. Experiencia* **36**: 308.

Wagener RE, Davis ND, Diener UL (1980) Penitrem A and roquefortine production by *Penicillium commune. Appl Environ Microbiol* **39**: 882–7.

Walter SL (2002) Acute penitrem A and roquefortine poisoning in a dog. *Can Vet J* **43**: 372–4.

Wilson BJ (1971) Miscellaneous penicillium toxins. In *Microbial Toxin*, vol. 6, Ciegler A, Kadis S, Ajil SJ (eds). Academic Press, New York, pp. 459–521.

Young KL, Villar D, Carson TL, Imerman PM, Moore RA, Bottoff MR (2003) Tremorgenic mycotoxin with penitrem A and roquefortine in two dogs. *J Am Med Assoc* **222**: 52–3.

C H A P T E R

# 81

# Slaframine

*Geoffrey W. Smith*

## INTRODUCTION

Slaframine is an alkaloidal mycotoxin produced by the fungus *Rhizoctonia leguminicola* that causes profuse salivation (slobbers) in animals. *R. leguminicola* is a common fungal pathogen of red clover (*Trifolium pratense*) and causes a syndrome known as black patch disease in the plant. Ingestion of clover hay containing slaframine causes salivary episodes that last from several hours to over 3 days in ruminants and horses. Although the disease is short term and animals generally recover without treatment, the dramatic clinical signs associated with slaframine ingestion make it readily apparent to livestock owners. Diagnosis can be made by identification of *R. leguminicola* in suspect forage or by the detection of slaframine in plasma samples from exposed animals.

## BACKGROUND

Outbreaks of profuse salivation in cattle were initially reported in the late 1940s and 1950s from agricultural experiment stations in the midwestern United States (O'Dell *et al.*, 1959). Most of these cases were associated with the feeding of second-cutting red clover hay. In 1956, it was first reported that fungal contamination of red clover with *R. leguminicola* was associated with a pasture disease called black patch, which derives its name from the appearance of affected areas in the field and not the characteristic black lesions on the leaves of affected plants (Croom *et al.*, 1995). Although its primary host is red clover, black patch disease has been reported in other legumes including white clover, soybeans, kudzu, cowpea, blue lupine, alsike clover, alfalfa, lespedeza, and milk vetch (Smalley and Sanderson, 1993). However, in most of these cases, infected red clover plants were present in the same areas of the field. Fungal infestations are usually associated with periods of wet weather and high humidity (Croom *et al.*, 1995). Transmission is thought to be primarily seedborne as the fungus overwinters on contaminated hay and can survive at least 2 years on contaminated seed.

## CHEMISTRY AND TOXICOKINETICS

Several laboratories were able to isolate the "slobber-causing" agent in *R. leguminicola*-contaminated red clover (Aust and Broquist, 1965; Rainey *et al.*, 1965) and in 1968 the chemical structure was described as 1-acetoxy-6-aminooctohydroindolizine (Gardiner *et al.*, 1968). The term slaframine became the most commonly used word for describing this compound (Aust *et al.*, 1966). Slaframine is a piperidine or indolizidine alkaloid (Figure 81.1) with the empirical formula $C_{10}H_{10}N_2O_2$ which gives it a molecular weight of 198 Da (Croom *et al.*, 1995).

Slaframine is activated by hepatic microsomal enzymes into a ketoimine (Figure 81.1) that is required to produce clinical signs. In cattle, the onset of salivation is shorter with more direct routes of administration; with intravenous injections producing salivation faster than intraperitoneal or intramuscular routes which are in turn faster than the subcutaneous route (Croom *et al.*, 1995). It has been suggested that slaframine is metabolized in the liver by a microsomal flavoprotein oxidase to the ketoimine metabolite (Guengerich and Aust, 1977) consisting of a quaternary nitrogen separated from an acetate ester by two carbon atoms (Figure 81.1). This structure is very similar to that of acetylcholine, a parasympathetic neurotransmitter.

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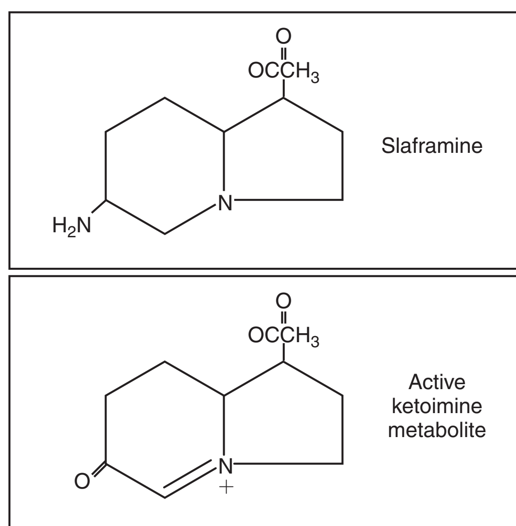



**FIGURE 81.1** The structure of slaframine and the active ketoimine metabolite.

**FIGURE 81.2** A horse showing profuse salivation from ingesting slaframine-contaminated red clover hay (photo courtesy of Dr. Cecil Brownie, North Carolina State University).

## MECHANISM OF ACTION

Pharmacologically, slaframine would be classified as a cholinergic agonist and/or a parasympathomimetic chemical. The majority of the available data indicate that the clinical signs produced by slaframine are due to its high affinity for the $M_3$ muscarinic receptor subtype which is believed to be important in the control of exocrine and endocrine glands (Croom *et al.*, 1995). Early studies demonstrated that the increased salivation associated with slaframine toxicity could be blocked with pre-administration of atropine (Aust, 1970). Additionally, mortality in broiler chicks when slaframine is dosed at the $LD_{50}$ can be significantly decreased by the pre-administration of the muscarinic receptor antagonists atropine (which has affinity for $M_1$, $M_2$, and $M_3$ receptors), and pirenzepine ($M_1$ and $M_2$ selective), but not gallamine which is $M_2$ selective (Croom *et al.*, 1995). The pre-administration of the $M_3$ selective antagonist 4-diphenylacetoxy-*N*-methylpiperidine methiodide (4DAMP) can also block the increase in pancreatic fluid output induced by slaframine (Walker *et al.*, 1994). Furthermore, slaframine has no effect on the cardiovascular system or arterial pressure at doses stimulatory to exocrine glands (Aust *et al.*, 1968), nor does it inhibit blood cholinesterase activity (Crump *et al.*, 1967; Hagler and Croom, 1989).

The stimulation of $M_3$ muscarinic receptors by slaframine produces a profound stimulation of exocrine glands, particularly the salivary gland and pancreas. Steers given an intramuscular administration of purified slaframine had resting salivary flow rates 50–70% greater than saline treated controls (Froetschel *et al.*, 1986). Similar findings documenting increases in saliva production both in cattle and sheep have been reported in other studies as well (Bird *et al.*, 1993; Hibbard *et al.*, 1995). Additional reported physiological effects of slaframine include increased pancreatic enzyme secretion (Aust *et al.*, 1968; Aust, 1970), increased rumen pH and rate of nutrient passage, and decreased ruminal motility in cattle (Croom *et al.*, 1995).

## TOXICITY

Clinical signs of slaframine toxicity are similar in all species. Following exposure to contaminated forages, animals begin to salivate profusely. Experimentally, a single dose of slaframine produces salivation for 6–10 h. However, clinically affected animals can "slobber" for several days, presumably because they have continued access to slaframine-contaminated forages (Figure 81.2). Other clinical signs can include anorexia, diarrhea, frequent urination, and bloat. Decreased milk production can be expected in dairy cattle, likely related to a decrease in feed intake (Crump, 1973). Although slaframine toxicosis has been experimentally induced in a variety of species including swine, poultry, cats, dogs, guinea pigs, and rodents; naturally occurring cases are primarily reported in horses and ruminants (Crump *et al.*, 1967; Sockett *et al.*, 1982; Croom *et al.*, 1995).

Cyanosis and open-mouth breathing have been reported under experimental conditions in sheep, swine, and guinea pigs. Pigs also were observed to vomit, became dyspneic, and collapse with stiffened pelvic limbs (Crump *et al.*, 1967). Only mild salivation was noted in a small chicken fed slaframine, and clinical signs resolved within a few hours. The $LD_{50}$ in day old broiler chicks was estimated at approximately 81.6 mg/kg of body weight (Croom *et al.*,

1995), but higher doses (250–300 mg/kg) were required to produce death in guinea pigs (Crump *et al.*, 1967). Gross lesions in these animals consisted of vascular congestion of the thoracic and abdominal cavities. Pulmonary edema, disruption of the alveolar structure, emphysema, and hepatic centrilobular necrosis were noted histologically. Death in these animals was attributed to suffocation from pulmonary edema and/or emphysema.

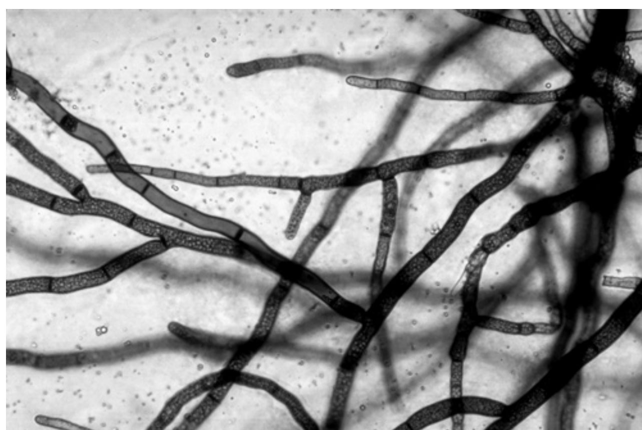
The clinical signs associated with the "slobbers syndrome" as described under field conditions include salivation, lacrimation, feed refusal, bloating, stiff joints, diarrhea, and weight loss. However, very few of these signs have been seen following the administration of purified slaframine under experimental conditions. That has led to speculation that the alkaloid swainsonine might also be involved in producing clinical signs in classic slaframine toxicity (Croom *et al.*, 1995). Swainsonine is another alkaloid produced by *R. leguminicola* and has a similar structure to slaframine. Although it is better known for its association with locoweed toxicity, it is possible that swainsonine is partially responsible for some of the clinical signs classically observed with the "slobbers syndrome."

## DIAGNOSIS AND TREATMENT

Diagnosis of slaframine toxicity is generally made by observation of clinical signs (salivation) in animals consuming legume forage, particularly red clover hay. Further tests can identify the fungus *R. leguminicola* in the hay which usually can be easily isolated in culture (Figure 81.3). Although chemical analysis for slaframine is not usually necessary, chromatographic methods for detecting the toxin in hay, plasma, or milk have been described (Hagler and Croom, 1989; Imerman and Stahr, 1998).

Treatment is not usually indicated as animals usually recover spontaneously when the contaminated hay is removed, however clinical signs may persist for 1–2 days following removal of the toxic forage. In severe cases, atropine may be of benefit to reverse the parasympathomimetic effects of slaframine, however it is unlikely to be completely resolving clinical signs. In guinea pigs, simultaneous administration of atropine and a lethal dose of slaframine prevented clinical signs for 5 h, after which mild salivation was observed. When atropine was given 2–4 h after slaframine administration, profuse salivation was observed, however there was no mortality (Crump *et al.*, 1967). Atropine should be used with caution in ruminants and horses because of possible gastrointestinal side effects. Therefore, treatments other than removing the contaminated hay are not usually recommended in affected animals.

Control of black patch disease remains a major problem for agronomists. Fungicides applied before flowering do not reduce seed infection, and ground sprays at the time of plant growth in the spring and/or immediately after the first hay cutting have not been shown to reduce fungal contamination. Prevention requires selecting *R. leguminicola* varieties that are less susceptible to fungal infection and chemically treating seed prior to planting. Feasible mechanisms to detoxify or degrade the toxin in pasture and/or hay have not been found. Therefore control of slaframine outbreaks rely on completely replacing contaminated forages.



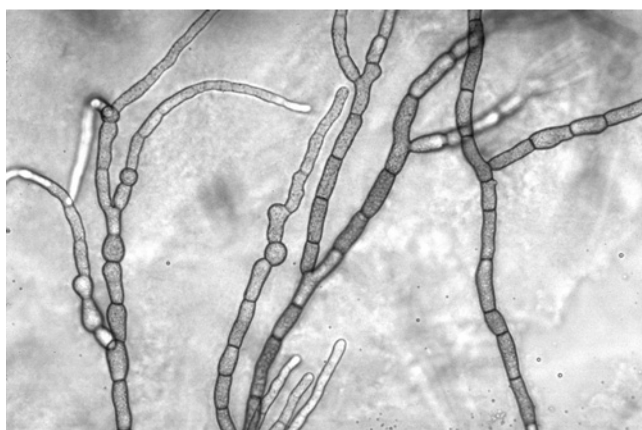


**FIGURE 81.3** Two photomicrographs of *R. leguminicola* mycelia growing in culture – 200× magnification – note the normal difference in pigment color between the two isolates (photos courtesy of Dr. Paul Vincelli and Cheryl Kaiser, University of Kentucky).

## REFERENCES


Aust SD, Broquist HP (1965) Isolation of a parasympathomimetic alkaloid of fungal origin. *Nature* **205**: 204.

Aust SD, Broquist HP, Rinehart Jr KL (1966) Slaframine: structural studies of a parasympathomimetic alkaloid of fungal origin. *J Am Chem Soc* **88**: 2879–80.

Aust SD, Broquist HP, Rinehart Jr KL (1968) Slaframine: a parasympathomimetic from *Rhizoctonia leguminicola*. *Biotech Bioeng* **10**: 403–12.

Aust SD (1970) Effect of slaframine on exocrine gland function. *Biochem Pharmacol* **19**: 427–33.

Bird AR, Croom Jr WJ, Bailey JV, O'Sullivan BM, Hagler Jr WM, Gordon GLR, Martin PR (1993) Tropical pasture hay utilization with slaframine and cottonseed meal: ruminal characteristics and digesta passage in wethers. *J Anim Sci* **71**: 1634–40.

Croom Jr WJ, Hagler Jr WM, Froetschel MA, Johnson AD (1995) The involvement of slaframine and swainsonine in slobbers syndrome: a review. *J Anim Sci* **73**: 1499–505.

Crump MH, Smalley EB, Nichols RE, Rainey DP (1967) Pharmacologic properties of a slobber-inducing mycotoxin from *Rhizoctonia leguminicola*. *Am J Vet Res* **28**: 865–74.

Crump MH (1973) Slaframine (slobber factor) toxicosis. *J Am Vet Med Assoc* **163**: 1300–2.

Froetschel MA, Croom Jr WJ, Hagler Jr WM, Argenzio R, Liacos J, Broquist HP (1986) Effects of slaframine on ruminant digestive function: resting salivary flow and composition in cattle. *J Anim Sci* **62**: 1404–11.

Gardiner RA, Rinehart Jr KL, Snyder JJ, Broquist HP (1968) Slaframine: absolute stereochemistry and a revised structure. *J Am Chem Soc* **90**: 5639–40.

Guengerich FP, Aust SD (1977) Activation of the parasympathomimetic alkaloid slaframine by microsomal and photochemical oxidation. *Mol Pharmacol* **13**: 185–95.

Hagler Jr WM, Croom Jr WJ (1989) Slaframine: occurrence, chemistry, and physiological activity. In *Toxicants of Plant Origin*, vol.1, Cheeke PR (ed.). CRC Press, Boca Raton, FL, pp. 257–79.

Hibbard B, Peters JP, Chester ST, Robinson JA, Kotarski SF, Croom Jr WJ, Hagler Jr WM (1995) The effect of slaframine on salivary output and subacute and acute acidosis in growing beef steers. *J Anim Sci* **73**: 516–25.

Imerman PM, Stahr HM (1998) New, sensitive high-performance liquid chromatography method for the determination of slaframine in plasma and milk. *J Chromatogr A* **815**: 141–5.

O'Dell BL, Reagan WO, Beach TJ (1959) A study of the toxic principle in red clover. *Univ of Missouri Agric Exp Sta Bull* **702**: 1–12.

Rainey DP, Smalley EB, Crump MH, Strong FM (1965) Isolation of salivation factor from *Rhizoctonia leguminicola* on red clover hay. *Nature* **205**: 203–4.

Smalley EB, Sanderson JM (1993) Slaframine (slobber factor). In *Current Veterinary Therapy 3: Food Animal Practice*, Howard JL (ed.). W.B. Saunders Company, Philadelphia, pp. 338–9.

Sockett DC, Baker JC, Stowe CM (1982) Slaframine (*Rhizoctonia leguminicola*) intoxication in horses. *J Am Vet Med Assoc* **181**: 606.

Walker JA, Krehbiel CR, Harmon DL, St. Jean G, Croom Jr WJ, Hagler Jr WM (1994) Effects of slaframine and 4-diphenylacetoxy-*N*-methylpiperidine methiodide (4DAMP) on pancreatic exocrine secretion in the bovine. *Can J Physiol Pharmacol* **72**: 39–44

C H A P T E R

# 82

# Ergot

*Steven S. Nicholson*

## INTRODUCTION

Ergot is a general term that applies to all species of the fungus *Claviceps.* Ergotism refers to the disease conditions associated with ingestion of toxic levels of ergot by animals and humans. Ergot contaminated rye (*Secale cereale*) used as flour was responsible for numerous episodes of human ergotism in Europe during the Middle Ages. The genus *Claviceps* includes very specialized fungi which parasitize the flowers of grasses and cereal grains with no other part of the plant infected. Recently ergot alkaloids produced by *Claviceps cyperi* in nutsedge was described as a toxicant in dairy cattle (Naude *et al*., 2005). This chapter primarily addresses alkaloids produced by *Claviceps purpurea* and the various toxic effects, called ergotism, which they produce in animals and humans. The source of exposure to animals includes ergot infected grains in feeds such as barley, rye, wheat, and oats as well as, infected seeds in forages consumed while grazing or in hay and silage. Clinical syndromes include gangrene of the extremities of cattle and horses, hyperthermia and production loss in cattle, agalactia, and abortion in swine, agalactia, and reproductive effects in mares**.**

Clinical disease associated with *Claviceps* has a parallel in fescue grass toxicosis (see chapter 82) where gangrenous ergotism, hyperthermia, production loss in cattle, and adverse effects on reproduction and lactation in horses are similar (Evans *et al*., 2004). The seeds of fescue grass can be infected with *C. purpurea* but the ergot alkaloids of fescue grass toxicosis are produced by the endophyte fungus *Neotyphodium coenophialum*. Ergot alkaloid induced fescue toxicosis has been studied in much more detail than has ergotism from ingested *C. purpurea* sclerotia.

Ergovaline produced in perennial and annual ryegrasses by the endophyte fungus *Neotyphodium lolii* is associated with clinical toxicosis (Schneider *et al*., 1996; Bourke, 2003).

## BACKGROUND

*C. purpurea* has a host range exceeding 200 species of grasses. It is distributed worldwide in temperate climatic zones. During infection by *C. purpurea*, the ovary of the grass seed or cereal grain is replaced by fungal mycelia. A sticky exudate known as honeydew is produced that contains conidia which can be transferred to infect other seeds. The honeydew hardens to form a hard brown, purple, or black compact mass of fungal tissue called a sclerotium. The ergot bodies or sclerotia contain up to 1.2% dry weight of toxic alkaloids (Burrows and Tyrl, 2001). Sclerotia of most *Claviceps* species are one to four times larger than the host seed and are readily identified in cereal grains. Compared to sclerotia in oats or barley the sclerotia are quite small in grass seeds such as Bahia grass (*Paspalum notatum*). Sclerotia fall to the ground to over winter and later complete the fungal life cycle by germinating and producing ascospores capable of infecting the ovary of developing seeds.

Alkaloids from *C. purpurea* are among the most important natural products used by the pharmaceutical industry. Prior to the industrial cultivation of *C. purpurea* in pure culture, ergot was grown as a crop on rye (*Secale cereale*) under field conditions for use in manufacture of important medicinal drugs. Synthesized ergot alkaloids have a variety of uses in human and veterinary medicine. Chemical structures of some of the ergot alkaloids are shown in Figure 82.1.

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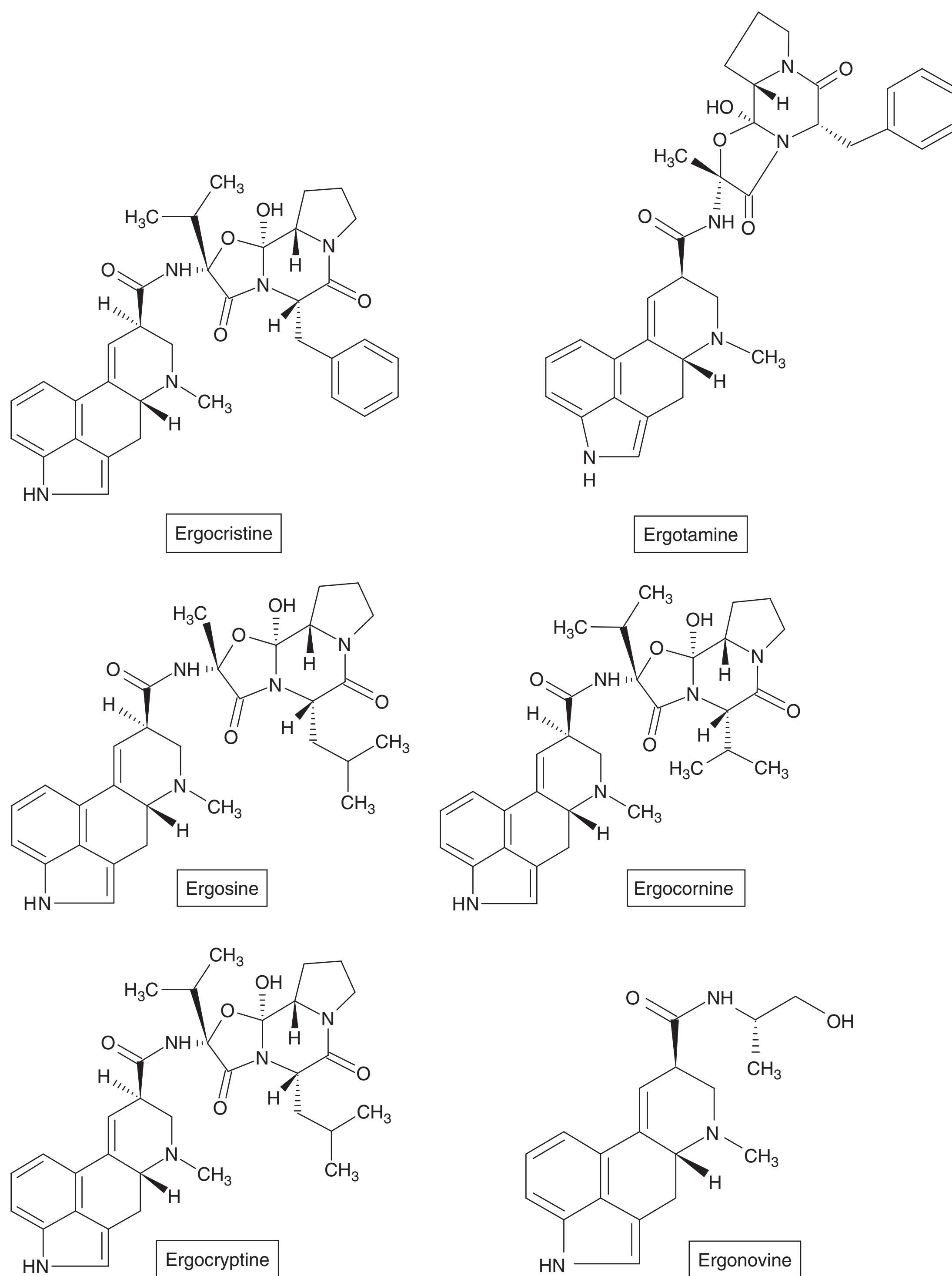


**FIGURE 82.1** Chemical structures of some ergot alkaloids.

## PHARMACOKINETICS/ TOXICOKINETICS

Ergot alkaloids responsible for most clinical signs and lesions are of the ergopeptine class including ergotamine, ergocristine, ergosine, ergocornine, ergocryptine, and ergovaline (Cheeke, 1998; Carson, 1999; Burrows and Tyrl, 2001; Evans *et al*., 2004). Injected intravenously ergopeptine alkaloids are rapidly cleared from the blood by the liver (Cheeke, 1998) and excreted in the bile (Evans *et al*., 2004).

These alkaloids are not secreted in the milk of cows (Cheeke, 1998).

## MECHANISM OF ACTION

Ergot alkaloids have structures similar to the biogenic amines norepinephrine, serotonin, and dopamine. Vasoconstriction is produced by an agonist activity and this effect varies with different vascular beds. Hyperthermia and uterine stimulation are other effects. The alkaloids are antagonistic to dopamine at $D_1$ vasodilatory receptors. Dopaminergic activity at $D_2$ receptors causes inhibition of prolactin secretion (Goldstein *et al.*, 1980). The dopamine antagonist domperidone is an effective treatment in mares suffering agalactia and uterine effects induced by ergot alkaloids in endophyte infected tall fescue grass (Redmond *et al.*, 1993).

## TOXICITY

Expression of clinical toxicoses caused by ergot alkaloids occurs in four forms in livestock. These include cutaneous and gangrenous lesions of the tail and extremities, hyperthermia and production loss, reproductive failure, and a convulsive or nervous form. The latter is apparently uncommon, not well documented, and is perhaps caused by acute ingestion of a large dose of sclerotia. Muscle tremors and hyperexcitability are signs in cattle caused by nonergot compounds produced by *Claviceps paspali* in seeds of Dallis grass.

In countries where cereal grains in commerce are subject to regulatory oversight the presence of ergotized seed at significant levels in food and feedstuffs is not common. Ergotism in cattle caused by sclerotia ingested while grazing remains a sporadic problem in the United States (Burrows and Tyrl, 2001).

The cutaneous and gangrenous form of ergotism is associated with subacute or chronic ingestion of the ergopeptine alkaloids. Cold temperatures enhance the clinical effects. Constriction of small arteries and arterioles leads to necrosis affecting all four limbs, tips of the ears, and distal third of the tail. Distinct lines separating normal tissue from nonviable tissues appear. The odor of rotting flesh may be obvious and affected animals may continue to walk until sloughing of the digits occurs.

Hyperthermia was noted in steers exposed to sunlight and fed 180 mg/kg/body weight of *C. purpurea* even at moderate ambient temperatures and humidity (Bourke, 2003).

Hyperthermia induced by high ambient temperature and humidity in lactating dairy cattle reduces feed intake, milk production, and increases embryonic losses. Ingestion of ergot alkaloids enhances this effect (Al-Tamimi *et al.*, 2003). Hyperthermia and a 30% drop in milk yield occurred in succession in two Holstein dairy herds ($n$ = 240 and $n$ = 150 milking cows, respectively) on the South African Highveld (Naude *et al.*, 2005). Examination of the maize silage from both farms revealed that it was heavily contaminated with nutsedge which contained minute sclerotia, identified as those of *Claviceps cyperi*. This was the first report of bovine ergotism not associated with the grass family Poaceae infected with *C. purpurea* or endophytes. High levels of total ergot alkaloids, predominantly ergocryptine, were found by LC-MS in the silage, as well as in the total mixed ration (115–975 ppb and 65–300 ppb, respectively). The ergot alkaloid content (mainly ergocryptine) of the maize silage on the second affected farm was 875 ppb. A significant 4.6 l decrease in milk production in a herd of Holstein–Friesian dairy cows in Australia was associated with high concentrations of endophyte produced ergovaline (1.6 μg/g) in ryegrass silage (Lean, 2001). Simultaneously, milk somatic cell counts increased significantly over a comparable period and reproductive performance declined. Body condition score and coat condition of cows were adversely affected.

Sheep are susceptible to the vasoconstrictive effects of ergopeptine alkaloids but ergot toxicosis is described as typically mild and not accompanied by gangrenous lesions (Greatorex and Mantle, 1973). Sloughing of the tip of the tongue is said to occur (Burrows and Tyrl, 2001). The effects of ergot alkaloids in swine are primarily those of agalactia, production loss, and an impact on reproduction. Diets containing 0, 1, and 10 g ergot (*C. purpurea*) per kg were fed to 12 pigs in the body weight range of 30–115 kg (Mainka *et al.*, 2005). Tendencies toward reduced feed intake and low rate of weight gain were observed at a feeding level of 4.66 mg total alkaloids per kg diet. At that level protein digestibility was significantly reduced. Birth weight in pigs is reduced, stillborns increased, and neonatal mortality increased due to agalactia in the sow (Lopez *et al.*, 1997).

Agalactia, small, weak foals, stillborn foals, and placental edema occur in pregnant mares ingesting ergot alkaloids in the last month of gestation. This is a common occurrence in mares grazing endophyte infected tall fescue grass in the United States. Similar effects caused by ingestion of *C. purpurea* sclerotia are seldom reported perhaps because heavily infected ergot cereal grains are generally kept out of feed grain commerce. There remains the situation where heavily contaminated cereal grain screenings could be fed to horses. Twelve pregnant mares fed black oat (*Avena strigosa*) during the pre-delivery period experienced agalactia and delivered weak and unviable foals, which showed no suckling reflex and died within a

few hours of birth. *C. purpurea* sclerotia were identified in 0.22% of the examined oat seeds (Copetti *et al*., 2002).

Chickens develop gangrene involving the comb, beak and toes, weight loss and debilitation. Rabbits are extremely sensitive to the hyperthermic effects caused by ergot alkaloids (Burrows and Tyrl, 2001). Ergot alkaloids are embryocidal and abortifacient in laboratory animals (Mantle, 1969).

In dogs and rodents corpora lutea are essential to maintain pregnancy. In these animals ergot alkaloids inhibit prolactin secretion causing loss of corpora lutea and abortion. Ergot alkaloids in oats fed to female mink bred to untreated males caused reduced numbers of kits born compared to controls (Sharma *et al*., 2002). There was a significant effect on kit survivability with no kits surviving in the 12 ppm group. Serum prolactin was significantly depressed in the three ergot alkaloid groups compared to the control group. This study indicated that ingestion of ergot alkaloids at 3 ppm or higher resulted in reproductive toxicity in mink.

## TREATMENT

Removing the source of ergot from the animals' diet is an obvious first step in treatment. The hyperthermic effect in dairy cattle should abate within 1–2 weeks. Animals that have developed gangrene of the extremities would not be expected to recover and should be euthanized.

The dopamine antagonist domperidone is an effective treatment in mares to prevent agalactia and uterine effects induced by ergot alkaloids in endophyte infected tall fescue grass (Redmond *et al*. 1993; Redmond *et al*., 1994).

## CONCLUDING REMARKS

Ergot infection in cereal grains and grasses remains a concern for livestock and horses. Commercial cereal grain screenings and cereal grains produced and fed on the farm, and not inspected, are potential sources of ergotized grain. Exposure while grazing infected grasses may go unnoticed initially, especially if management is not aware of the risk.

## REFERENCES


Al-Tamimi HJ, Rottinghaus GE, Spiers DE, Soain J, Chatman D, Eichen PA, Carson TL (2003) Thermoregulatory response of dairy cows fed ergotized barley during summer heat stress. *J Vet Diagn Invest* **15**: 355–60.

Bourke CA (2003) Evidence that enforced sunlight exposure can cause hyperthermia in cattle ingesting low levels of ergot of rye (*Claviceps purpurea*), when air temperature and humidity conditions are only moderate. *Aust Vet J* **81**: 553–8.

Burrows GE, Tyrl RJ (2001) *Toxic Plants of North America.* Iowa State University Press, Ames, IA.

Carson TL (1999) *Current Veterinary Therapy 4: Food Animal Practice*, Howard JL, Smith RA (eds). W.B. Saunders, Philadelphia, PA.

Cheeke PR (1998) *Natural Toxicants in Feeds, Forages, and Poisonous Plants*. Interstate Publishers, Danville, IL.

Copetti MV, Santurio JM, Boeck AA, Silva RB, Bergermaier LA, Lubeck I, Leal AB, Leal LT, Alves SH, Ferreiro L (2002) Agalactia in mares fed with grain contaminated with *Claviceps purpurea. Mycopathologia* **154**: 199–200.

Evans TJ, Rottinghaus GE, Casteel SW (2004) Ergot. In *Clinical Veterinary Toxicology,* Plumlee KH (ed.). Mosby Inc, St. Louis, MO, pp. 239–43.

Goldstein MJ, Lew JY, Sauter A, Lieberman A (1980) Affinities of ergot compounds for dopamine agonist and dopamine antagonist receptor sites. In *Advances in Biochemical Psychopharmacology*, Raven Press, New York, pp. 75–82.

Greatorex JC, Mantle PG (1973) Experimental ergotism in sheep. *Res Vet Sci* **15**: 337–46.

Lean IJ (2001) Association between feeding perennial ryegrass (Lolium perenne cultivar Grasslands Impact) containing high levels of ergovaline, and health and productivity in a herd of lactating dairy cows. *Aust Vet J* **79**: 262–4.

Lopez TA, Campero CM, Chayer R, Hoyos Mde (1997) Ergotism and photosensitization in swine produced by combined ingestion of *Claviceps purpurea sclerotia* and *Ammi majus* seeds. *J Vet Diagn Invest* **9**: 68–71.

Mainka S, Danike S, Boehme H, Uesberschar KH, Polten S, Huther I (2005) The influence of ergot-contaminated feed on growth and slaughtering performance, nutrient digestibility and carry over of ergot alkaloids in growing-finishing pigs. *Arch Anim Nutr* **59**: 377–95.

Mantle PG (1969) The role of alkaloids in the poisoning of mammals by sclerotia of *Claviceps* spp. *J Stored Prod Res* **5**: 237.

Naude TW, Botha CJ, Vorster JH, Roux C, Van der Linde EJ, Van der Walt SI, Rottinghaus GE, Van Jaarsveld L, Lawerence AN (2005) *Claviceps cyperi*, a new cause of severe ergotism in dairy cattle consuming maize silage and teff hay contaminated with ergotised *Cyperus esculentus* (nut sedge) on the Highveld of South Africa. *Onderstepoort J Vet Res* **72**: 23–37.

Redmond LM, Cross DL, Kennedy SW (1993) Effect of three levels of domperidone on gravid mares grazing endophyte (*Acremonium coenophialum*) infected tall fescue. *J Anim Sci* **71**(Suppl. 1): 16 (Abstract).

Redmond LM, Cross DL, Strickland JR, Kennedy SW (1994) Efficacy of domperidone and sulpiride as treatments for fescue toxicosis in horses. *Am J Vet Res* **55**: 722–9.

Schneider DJ, Miles CO, Garthwaite I, Van Halderen A, Wessels JC, Lategan HJ (1996) First report of field outbreaks of ergot-alkaloid toxicity in South Africa. *Onderstepoort J Vet Res* **2**: 97–108.

Sharma C, Aulerich RJ, Render JA, Reimers T, Rorringhaus GE, Kizilkaya K, Bursian SJ (2002) Reproductive toxicity of ergot alkaloids in mink. *Vet Hum Toxicol* **44**: 324–7.

# Part 16

# Feed and Water Contaminants

C H A P T E R

# 83

# Ionophores

*Meliton N. Novilla*

## INTRODUCTION

Ionophores are compounds that form lipid soluble, dynamically reversible complexes with cations and by this means facilitate specific ionic transport across biological membranes (Pressman, 1976; Reed, 1982; Taylor *et al.*, 1982; Riddell, 2002). There are two major subclasses of ionophores: (1) neutral ionophores, which are highly toxic because they form charged complexes that are capable of perturbing biologic membranes and action potentials and (2) carboxylic ionophores, which form zwitterionic complexes with cations and promote electrically neutral cation exchange diffusion that is tolerated better in intact organisms. The ionophoric activity may alter normal concentration gradients resulting in cellular ion imbalance, pH change, calcium overload, lipid peroxidation, and disruption of plasma membranes. The alteration in the membrane transport of ions is the basis for the metabolic, organic, and functional effects of this class of compounds. Since their pharmacologic activity is dose-related, the usefulness of carboxylic ionophores is based on selective toxicity to protozoan parasites and bacteria and margins of safety in the approved target species. Desirable effects occur when animals are provided feed containing approved dosage ranges, but higher levels may result in adverse effects.

This chapter attempts to provide an overview of ionophores as they relate to veterinary medicine, with emphasis on ionophore-induced toxicity. Large numbers of ionophore safety and toxicity studies have been conducted in support of marketing approval, but results of many studies are unpublished. For this reason, information presented was gleaned from published laboratory and field reports of toxicoses in various species of animals, available reviews, and Freedom of Information summaries obtained from the US Food and Drug Administration (FDA). However, due to space limitations, not all information can be included, hence apologies are extended to laboratory scientists and field researchers whose work has been omitted or inadvertently missed.

## BACKGROUND

Presently, seven carboxylic ionophores (Figure 83.1) are approved for the control of coccidiosis and promotion of growth and feed efficiency in several animals of economic importance. Since their introduction, carboxylic ionophores have played significant roles in livestock and poultry production systems throughout the world. Monensin, first introduced as Coban® in the United States for the control of coccidiosis in chickens in 1971, was later marketed in 1975 as Rumensin® to promote growth and/or increase feed efficiency in cattle. Similarly, lasalocid has been marketed since 1977 as Avatec® for chickens and since 1982 as Bovatec® for cattle. Salinomycin, narasin, and maduramicin were approved for chickens in 1983, 1986, and 1989, respectively while laidlomycin and semduramicin were approved in 1994 for use in cattle and chickens, respectively.

Other benefits of ionophore use include: (1) reduction of coccidial oocyst discharge in ruminants (Stockdale, 1981; Parker *et al.*, 1986), (2) prevention of acute bovine pulmonary edema and emphysema (Hammond *et al.*, 1980; Nagaraja *et al.*, 1982; Nocerini *et al.*, 1985), (3) decreased incidence of bloat (Lowe *et al.*, 1991), (4) prevention of ruminal lactic acidosis (Duffield *et al.*, 2002), and (5) amelioration of ketosis in lactating dairy cows (Heuer *et al.*, 2001). The reduction of deaths in some cattle herds has

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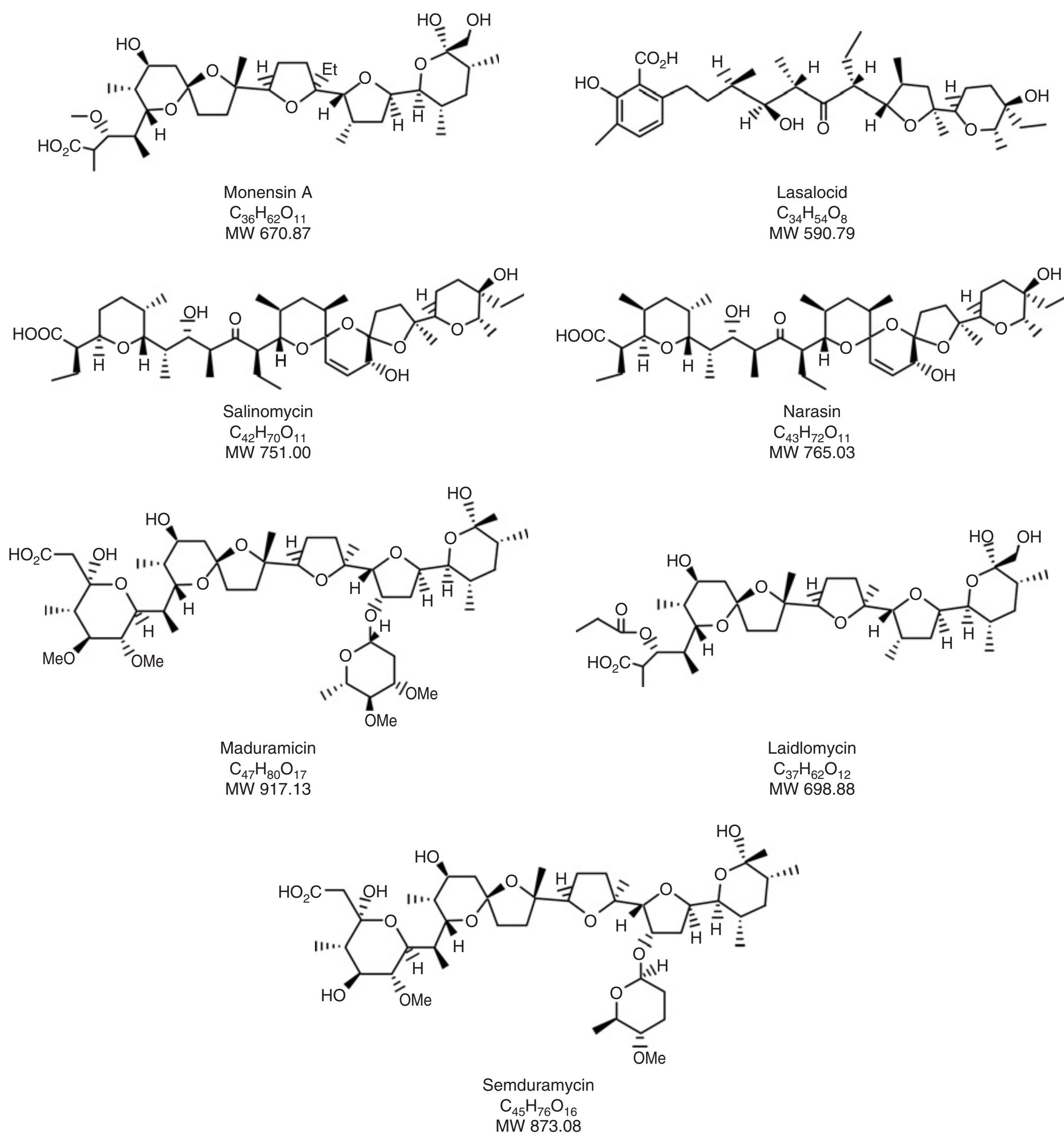


**FIGURE 83.1** Structures of carboxylic ionophores.

been hypothetically related to the reduction of indigestion, metabolic stress, bloat, and enterotoxemia associated with monensin feeding (Black and McQuilken, 1980).

Potential uses of the ionophores are under experimental investigation in many parts of the world and off-label use of some ionophores is known to occur. Monensin has been used for the control of toxoplasmosis in pregnant sheep (Buxton *et al.*, 1988), disseminated visceral coccidiosis in cranes (Carpenter and Novilla 1992), gregarine infections in shrimp (Jones *et al.*, 1994; Fajer-Avila *et al.*, 2005), and diarrheal disease in swine (Kyriakis, 1989; Kyriakis *et al.*, 1993). The use of monensin to improve efficiency of milk production in dairy cattle has recently been approved in the United States (FDA, 2004). Experimental studies on the use of ionophores for the treatment of malaria (Adovelande and Schrevel, 1996; Gumila *et al.*, 1997), lead poisoning (Hamidinia *et al.*, 2002), and as an aid in the potentiation of anti-cancer therapies (Griffin *et al.*, 1993; Shaik *et al.*, 2001) have been conducted. These studies were conducted in cell culture or as challenge studies in laboratory animals and have shown some degree of efficacy. In spite of these encouraging results, there have been no reports of investigational use of ionophores in humans.

# PHARMACOLOGY/ PHARMACOKINETICS

## Pharmacology

Investigational studies were conducted to determine if ionophores had any undesirable pharmacological properties. A series of general pharmacology studies (Table 83.1) conducted with monensin was reviewed recently (Novilla, 2004). Earlier, Hanley and Slack (1982) reviewed the pharmacology of lasalocid. The studies with monensin assessed its effects on the central, peripheral, and autonomic nervous systems and the digestive, respiratory, and cardiovascular systems. The test systems with doses and route of administration and no-observed-effect-level (NOEL) are shown below.

In mice, an oral dose of 10 mg monensin/kg body weight produced no significant effects on general behaviors, coordinating activity in skeletal muscles, electroshock seizures, and acetic acid writhing. However, slight sedation, decreased sensitivity to tactile stimulation, slight depression of muscular coordination, and depressed acetic acid writhing occurred at 30 mg/kg. In cats, immobilized by D-tubocurarine or anesthetized with urethane and $\alpha$-chloralose, an oral dose of 30 mg/kg produced no effect on the electroencephalogram and the spinal reflex. At the same dose in anesthetized cats, the heart rate, electrocardiogram, respiration, and blood pressure responses to epinephrine, acetylcholine, histamine, and contractions of the nictitating membrane in response to electrical stimulation of the cervical sympathetic ganglion were not affected. Charcoal meal transit in mice was not affected by an oral dose of 10 mg/kg while 30 mg/kg significantly depressed the transit rate. Gastric secretion was not affected by an oral dose of 30 mg/kg in rats. In isolated guinea pig ileal preparations, monensin in a bath concentration of $10^{-5}$ g/ml had no antagonistic effects on contractures induced by acetylcholine, histamine, and barium chloride. In the rat anti-carrageenin edema test, monensin given orally at 30 mg/kg did not significantly inhibit carrageenin-induced edema of the hind paw.

The studies targeting specific organ systems of laboratory animals indicated that monensin at a dose of 10 mg/kg orally produced no effect on the central, peripheral, and autonomic nervous systems or the respiratory and digestive systems. However, striated (heart and skeletal) muscle has been identified as the primary target of toxicity in laboratory animals and domestic livestock given large doses of monensin and other ionophores by oral or parenteral routes (Todd *et al.*, 1984; Novilla and Folkerts, 1986; Novilla and Todd, 1991; Van Vleet *et al.*, 1991; Dowling, 1992).

The cardiovascular effects of oral and intravenous (IV) administration of monensin were evaluated in conscious

**TABLE 83.1 General pharmacology studies on monensin**

| Test system | Species | Monensin dose (mg/kg) | Route | NOEL (mg/kg) |
|---|---|---|---|---|
| General behavior | Mouse | 0, 10, and 30 | Oral | 10 |
| Coordinating activity of skeletal muscles | Mouse | 0, 10, and 30 | Oral | 10 |
| Anti-electroshock seizures | Mouse | 0 and 30 | Oral | 30 |
| Analgesic effect | Mouse | 0, 10, and 30 | Oral | 10 |
| Spinal reflex | Cat | 0 and 30 | Oral | 30 |
| Electroencephalogram (D-tubocurarine immobilized) | Cat | 0 and 30 | Oral | 30 |
| Circulatory, respiratory, and autonomic effects | Cat | 0 and 30 | Oral | 30 |
| Charcoal meal transit | Mouse | 0, 10, and 30 | Oral | 10 |
| Gastric secretion | Rat | 0 and 30 | Oral | 30 |
| Isolated ileum | Guinea pig | $1 \times 10^{-5}$ g/ml | *In vitro* | $1 \times 10^{-5}$ g/ml |
| Carragenin-induced edema | Rat | 0 and 30 | Oral | 30 |
| Cardiovascular study | Dog (conscious) | 0, 0.0069, 0.0138, 0.0345, 0.0690, and 0.138 | IV | 0.0345 |
| | | 0, 0.138, 0.345, 0.690, and 1.38 | Oral | 0.345[a] |
| Cardiovascular and respiratory effects | Dog (anesthetized) | 0, 0.00069, 0.0014, 0.0035, 0.0069, 0.014, 0.035, 0.069, 0.14, 0.35, 0.69, and 1.4 | IV | 0.0035 |
| | Pig (anesthetized) | 0, 0.00069, 0.0014, 0.0035, 0.0069, 0.014, 0.035, 0.069, 0.14, 0.35, and 0.69 | IV | 0.0035 |

[a] Based on transient increases in coronary blood flow at doses ≥0.69 mg/kg.

dogs (Holland, 1978). In the oral study, doses of 0, 0.138, 0.345, 0.69, or 1.38 mg monensin/kg body weight in 15 ml of 10% acacia were given by gavage to 4, 4, 6, 6, or 4 dogs, respectively. Effects were limited to increased coronary blood flow at doses ⩾0.69 mg monensin/kg. At the 0.69-mg/kg dose, coronary flow increased from 24 ml/min before dosing to a maximum of 40 ml/min 17.5 min after dosing. At the 1.38-mg/kg dose, coronary flow increased from 18 ml/min predosing to a maximum of 49 ml/min 13 min after dosing. In both cases, coronary blood flow had returned to normal levels by 30 min after dosing. Heart rate and mean blood pressure of monensin-treated dogs did not change significantly from the controls at any dose level. Coronary artery flow did not increase following doses of 0.138 and 0.345 mg monensin/kg. In the IV study, coronary blood flow, blood pressure, and heart rate were measured in each of 6 dogs given total cumulative doses of 0.0069, 0.0138, 0.0345, 0.069, and 0.138 mg monensin/kg body weight. Four control dogs were given dextrose alone. Coronary artery flow significantly increased at 0.069 and 0.138 mg/kg and mean blood pressure increased significantly at the 0.138 mg/kg. Coronary artery flow did not change significantly following doses of ⩽0.0345 mg/kg while heart rate did not change significantly from the controls at any dose level. Mean blood pressure also did not change significantly at dose levels of 0.0069, 0.0345, and 0.069 mg/kg. Based on the transient increases in coronary blood flow observed in these studies, the NOELs were 0.345 and 0.0345 mg monensin/kg following oral and IV dosing, respectively.

The cardiovascular effects of monensin are similar to those observed for lasalocid (De Guzman and Pressman, 1974; Osborne *et al.*, 1977). Hypothetically, based on their inotropic and chronotropic properties, ionophores were considered as potential therapies for human cardiac disease (Pressman, 1976; Saini *et al.*, 1979; Hanley and Slack, 1982).

These pharmacology studies are considered relevant to the overall safety of ionophores as they provide insight into the secondary pharmacological actions and support the margins of safety for exposure in humans during the manufacturing processes or in the mixing or handling of the premixes in preparing feeds for the target species. With regard to the dog cardiovascular studies, monensin-induced increases in coronary blood flow observed at oral doses of 0.69- and 1.38-mg/kg were transient and considered not to be toxicologically important. At these doses, the respective increases of 67% and 172% in blood flow are in the physiological range by virtue of the autoregulatory coronary flow reserve (Cruickshank, 1992; Feliciano and Henning, 1999). Further, the phenomenon was not considered to be deleterious because recognized indicators of cardiovascular toxicity, such as increased blood pressure and/or heart rate, were not observed concurrently in the oral study. If it can be assumed that the relative potency of IV dosing to produce increased blood pressure is 11 times greater than by oral dosing (Holland, 1978), then the equivalent dose of 1.52 mg monensin/kg given orally by capsule would be expected to produce this change. According to Pressman and Fahim (1983), a single oral dose of 2.0 mg/kg in dogs produced increased heart rate and arterial pressure for up to 2 h after dosing, hence the lower dose of 1.38 mg monensin/kg may be considered as the no observed adverse effect level (NOAEL). If one takes 0.69 mg monensin/kg as a pharmacologically significant oral dose and 0.345 mg/kg as a NOEL for humans, then a 60-kg person would require 41.4 mg of monensin to manifest a transient effect on coronary blood flow while 20.7 mg monensin will have no effect (Donoho, 1984).

Human exposure to systemic levels of monensin and other ionophores high enough to produce any toxicological effects is very unlikely. With the use of safety equipment and close adherence to good manufacturing practices (GMPs) and label instructions, personnel manufacturing and handling ionophore products should not be subjected to undue risk. Furthermore, it is highly unlikely for these exposures to occur in humans consuming meat and milk products from animals fed a complete feed formulated with ionophores because residues are extremely low (Novilla, 2004).

## Pharmacokinetics

Tissue residue and metabolism studies were required to be conducted on all marketed ionophores to support human safety. For monensin, several studies with both $^{14}C$-monensin and unlabeled compound have shown that following oral administration in cattle and other species, monensin is rapidly absorbed and extensively metabolized by the liver and that most of the administered monensin and its metabolites are excreted in the bile. Results of biochemical studies on the metabolic fate of monensin in animals have been published (Davison, 1984; Donoho, 1984; Atef *et al.*, 1993). The metabolite pattern for monensin is similar in cattle, chickens, rats, turkeys, sheep, and pigs. Metabolite characterization studies indicated that hydroxylation (*o*-demethylation and oxidation) is the primary metabolic pathway of monensin resulting in low concentrations of a large number of polar metabolites in the feces of $^{14}C$-monensin-dosed animals. The pattern of metabolites in cattle and rats is similar. Based on radiolabeled studies in steers and dairy cows, the liver had the highest total monensin residue following zero withdrawal (Donoho *et al.*, 1978; Herberg *et al.*, 1978; Kennington *et al.*, 1995). Parent monensin and five metabolites were identified in liver, bile, and/or feces from monensin-treated steers and dairy cows. Studies have shown that metabolite M-1 (*o*-desmethyl monensin) was 20 times less active than parent monensin, the marker residue. Hence, the suspected first step in monensin metabolism appears to eliminate most of the biological

activity. Plasma levels of monensin are low and decline rapidly in cattle treated with monensin. In cattle administered $^{14}C$-monensin, residues of radioactivity depleted rapidly from the tissues. In a recent tissue residue study, no monensin was detected in extracted fat, liver, kidney, and muscle tissues from cattle given feed containing 100 ppm to provide a dose of 0.9 mg monensin/kg body weight/day for 28 days (Coyle and Walker, 2005). Feeding of muscle, liver, and other viscera from cattle provided dietary levels of 165-ppm monensin (5 times the approved use level) to rats and dogs for 3 months produced no adverse effects (Gossett *et al.*, 1975). Moreover, monensin does not accumulate in tissues of animals even when given intoxicating doses (Donoho, 1984; Atef *et al.*, 1993). Thus, exposure of humans to residues of monensin in meat or milk from animals treated according to the label instructions will be very limited and will be due primarily to the parent compound. Furthermore, when residues, primarily the parent compound, are present in animal products intended for human consumption, they will be at minimal levels for which a sufficient margin of safety has been demonstrated in the toxicology studies (Novilla, 2004).

Pharmacokinetic studies with other marketed ionophores generated data similar to that of monensin. Results of these studies in the Freedom of Information (FOI) summaries facilitated determination of the safe residue concentration, acceptable daily intake, marker residue and tolerance, and withdrawal time by the US FDA.

## MECHANISM OF ACTION

Carboxylic ionophores are known to form cationic ($Na^+$, $K^+$, $Ca^{2+}$) complexes (Figure 83.2) that enhance their transport across bimolecular lipid membranes (Pressman, 1976; Pressman and Fahim, 1982, 1983; Taylor *et al.*, 1982; Reed, 1982; Reed and Bokoch, 1982; Mollenhauer *et al.*, 1990). However, ion proclivities differ and evidence gathered in various laboratories indicates that carboxylic ionophores may have multiple effects at the cellular level.

In this diagram, monensin, classified as a $Na^+$ selective ionophore, binds to $Na^+$ outside the cell, carries it into the cell, and produces higher intracellular concentrations of $Na^+$. Influx of $Na^+$ is counterbalanced by an efflux of $K^+$ but, since $K^+$ efflux is slow, an initial efflux of proton ($H^+$) occurs, which may result in intracellular alkalosis. With salinomycin and narasin and others that show $K^+$ selectivity, the $K^+$ egress is countered by an initial $H^+$ ingress, which could result in intracellular acidification. Salinomycin and narasin preferentially complex with $K^+$ over $Na^+$ at a ratio of 4:1. An ionophore-mediated rise in intracellular $Na^+$ is known to increase the intracellular levels of $Ca^{2+}$ due to an ATPase-driven exchange mechanism at the cell membrane

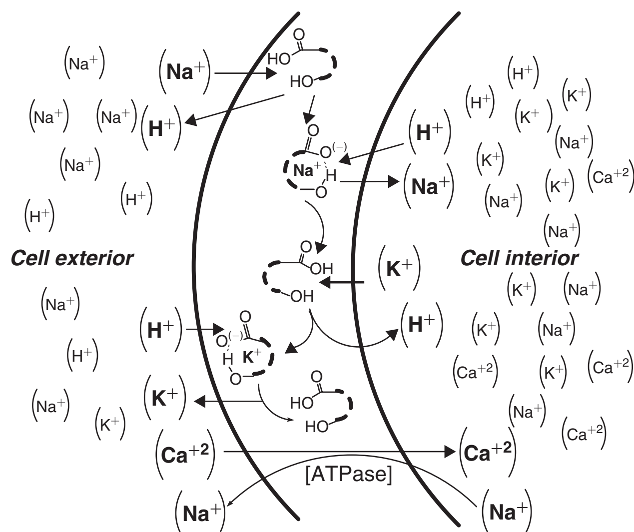


**FIGURE 83.2** Diagram of cation ion exchange diffusion across plasma membranes facilitated by monensin. Large arrows indicate major transport activity.

(Mubagwa *et al.*, 1997; Meral *et al.*, 2002). Monensin was reported to shorten the duration of the action potential and suppressed the pacemaker potential in cardiac tissue (Sutko *et al.*, 1977; Tsuchida and Otomo, 1990). These membrane current effects were related to transmembrane alterations in the gradients of $Na^+$ and $K^+$ions and to increased intracellular $Ca^{2+}$ following the increase in cytoplasmic $Na^+$ concentration, probably via a $Na^+/Ca^{2+}$ exchange mechanism. Lasalocid directly translocates $Ca^{2+}$ because it forms complexes with divalent cations with a range of complexing and transport capabilities including primary amines, e.g., catecholamines (Reed and Bokoch, 1982; Hanley and Slack, 1982). Monensin also causes release of catecholamines from cultured adrenal chromaffin cells and salinomycin causes a multifold augmentation of plasma catecholamines in animals. Catecholamines and toxic oxidation products have been implicated in myocardial necrosis through greater influx of $Ca^{2+}$ and formation of free radicals.

By special immunohistochemistry staining, monensin treatment was shown to increase $Na^+K^+$-ATPase and $Ca^{2+}$-ATPase and nitric oxide (NO) synthase activities in the heart of chickens (Calo *et al.*, 2002, 2003). NO, the product of NO synthases, is recognized as a regulator of calcium homeostasis. Alterations in the concentrations of $Ca^{2+}$ and other cations extracellularly as well as changes in their intracellular distribution have been associated with changes in subcellular organelles and cell damage (Dubardieu and Shier, 1992; Shier and Dubourdieu, 1992; Calo *et al.*, 2002, 2003; Sandercock and Mitchell, 2003, 2004).

Carboxylic ionophores directly affect the asexual and sexual developmental stages of eimerian coccidia by causing the normal transport of $Na^+$ and $K^+$ ions to fail (Smith and Strout, 1979; Smith and Galloway, 1983). In studies with free *Eimerian* sporozoites, monensin causes increased

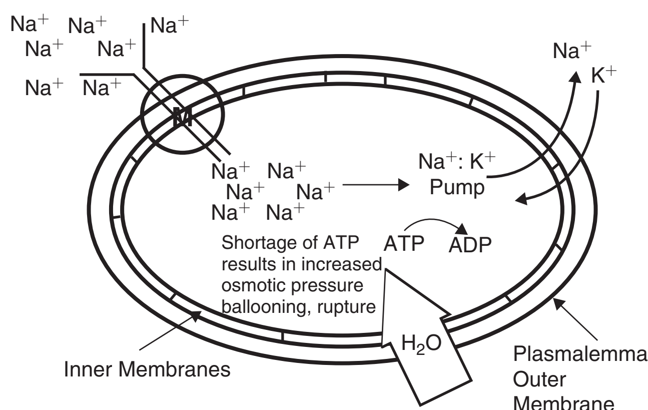


**FIGURE 83.3** Effect of ionophores on ion and water dynamics in the coccidia. M: monensin. From Thomas *et al.* (1985). Elanco Animal Health.

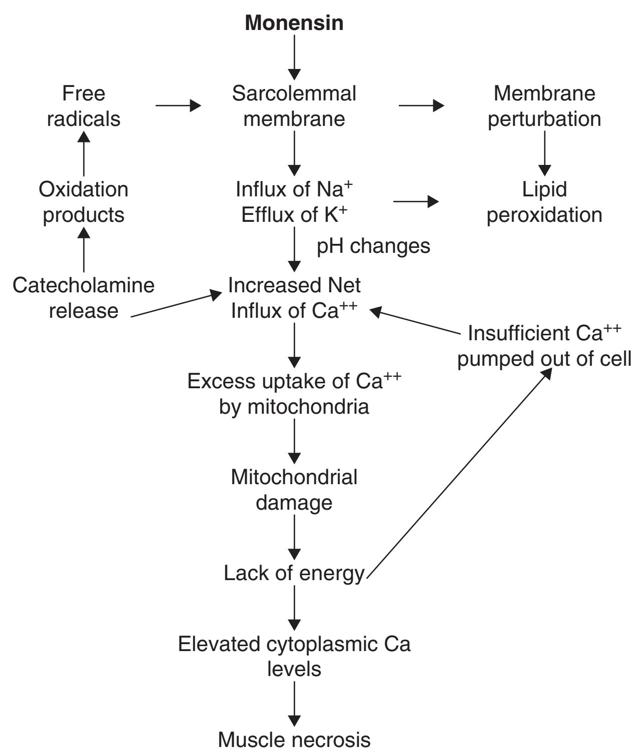


**FIGURE 83.4** Probable sequence of events induced by monensin in muscle cells. Adapted from Novilla and Folkerts (1986).

$Na^+$ levels in the parasite, increased activity of the sodium pump, decreased intra-sporozoite ATP, and stimulation of glycolysis (Smith *et al.*, 1981). Continuous exposure to monensin results in an increased osmotic gradient within the developing sporozoan parasite (Figure 83.3). Water follows and the organisms utilize energy to maintain intracellular homeostasis. When energy is exhausted the parasite swells with damage to organelles and death ensues (Smith and Strout, 1980; Smith *et al.*, 1981; Mehlhorn *et al.*, 1983). While most ionophores target free coccidial stages in the intestinal lumen (sporozoites and merozoites), the first intracellular generation (trophozoites) was most sensitive to lasalocid (Long and Jeffers, 1982; Ruff, 1982).

Similar events occur in susceptible bacteria. By reducing acetic and butyric acid producing (Gram-positive) bacteria and promoting growth of propionic acid producing (Gram-negative) bacteria, ionophores improve the efficiency of rumen fermentation resulting in a greater retention of feed energy in metabolizable nutrients (Richardson *et al.*, 1976; Bergen and Bates, 1984; Russel, 1987; Russel and Strobel, 1989). Monensin, lasalocid, and laidlomycin have been shown to alter the molar ratios of volatile fatty acids (VFAs) such that there is increased propionate and decreased acetate and butyrate without altering total VFA concentration.

# TOXICITY

## Pathogenesis

The exact mechanism of the toxicity induced by ionophores is not known. However, reasonable hypotheses have been generated based on their inherent ionophoric activity (Van Vleet *et al.*, 1983c, 1991; Novilla and Folkerts, 1986). All ionophores facilitate transmembrane ion fluxes and dissipation of ion gradients, which are exaggerated at toxic levels. Cells respond to the metabolic insult by expending energy to maintain homeostasis. When homeostatic mechanisms are exceeded, toxicity ensues from excessive influxes of $Na^+$ and $Ca^{2+}$ leading to degeneration and necrosis of cardiac and skeletal muscle cells (Figure 83.4). Although the ionophore mode of action is simple, four biochemical changes, including intracellular pH effects, calcium overloading, catecholamine release, and lipid peroxidation, probably occur during ionophore toxicoses.

In the diagram, monensin facilitates cation exchange diffusion as it intercalates with plasma membranes (Pressman, 1976; Reed, 1982; Riddell, 2002). The exaggerated pharmacologic activity at toxic levels disrupts not only osmotic gradients but the intracellular pH as well. Since drastic changes in acid base balance are incompatible with life, the pH shifts may be responsible for the peracute deaths observed with very high toxic levels of ionophores (Novilla and Folkerts, 1986). The monensin-induced entry of $Na^+$ is followed by entry of calcium, due to an ATPase-driven exchange mechanism at the cell membrane. A calcium ionophore like lasalocid or A23817 promotes $Ca^{2+}$ entry directly. Ionophores are known to trigger the release of $Ca^{2+}$ from intracellular stores further increasing $Ca^{2+}$ levels in the cytoplasm.

Two other mechanisms contribute to calcium overloading: exaggerated release of neurotransmitters like

catecholamines and increased peroxidation of lipids. Monensin, lasalocid, and salinomycin have been reported to release of catecholamines from adrenal chromaffin cells, transport catecholamines directly, or augment catecholamine plasma levels, respectively. The degradation products of catecholamines have been implicated in myocardial necrosis through the formation of free radicals and calcium influx (Singal *et al.*, 1981; Yates *et al.*, 1981; Reichenback and Benditt, 1982). Since ionophores are lipophilic and have detergent properties, dose-related perturbations of plasma membranes can promote increased lipid peroxidation. It is known that lipid peroxidation promotes membrane damage, and consequently, increased $Ca^{2+}$ influx.

Since they are not mutually exclusive, one or more of the above pathogenetic mechanisms may increase calcium concentrations in the cell. The rise in intracellular calcium can be buffered by calcium pumps in the plasma membrane, mitochondria, and sarcoplasmic reticulum. However, toxicity overwhelms this buffering mechanism and a vicious cycle ensues, resulting in calcium overloading. Elevated calcium levels then activate muscle proteases and phospholipases which initiate degradative processes (disassembly of myofilaments and membrane damage) in striated muscle and ultimately cell death (Van Vleet *et al.*, 1983c, 1991; Trump *et al.*, 1989; Sandercock and Mitchell, 2003, 2004).

## Occurrence

Generally, marketed ionophore products have been found to be safe and effective in the target species provided the approved dosage ranges. However, excessive ionophore feed concentrations have resulted in the ionophore toxic syndrome (Novilla, 1992). Toxic syndromes have occurred from the following: (1) feed-mixing errors or ingestion of premix concentrates with unsafe amounts of ionophores; (2) extra label use, either accidental or intentional, have resulted in adverse reactions in horses, dogs, rabbits, adult poultry, ostriches, camels, deer, water buffaloes, and humans; and; (3) drug incompatibilities with other compounds such as tiamulin, chloramphenicol, and macrolides such as triacetyloleandomycin have also resulted in toxicity.

Feed-mixing errors have caused most toxicity problems in animals for which ionophore use has been approved. Reports of toxicity in the target species have been reviewed (Potter *et al.*, 1984; Novilla and Folkerts, 1986; Dowling, 1992). Among the non-target species, horses appear to be the most susceptible and fish the most tolerant to high levels of ionophores. Except for a direct exposure to a monensin premix following a barn break-in, horse toxicity cases have resulted from feed contamination at the mill (Whitlock *et al.*, 1978; Ordidge *et al.*, 1979; Muylle *et al.*, 1981; Rollinson *et al.*, 1987; Matsuoka *et al.*, 1996; Bezerra *et al.*, 1999; Bila *et al.*, 2001; Peek *et al.*, 2004). Similarly, feed mill contamination of commercial dog food (Wilson, 1980; Hazlett *et al.*, 1992; Safran *et al.*, 1993), cat food (Van der Linde-Sipman *et al.*, 1999), and concentrate ration for ostriches (Baird *et al.*, 1997) have resulted in toxicoses.

In humans, there are two publications of intentional exposure to monensin. According to Kouyoumdjian and associates (2001), a 17-year-old Brazilian male admitted ingesting monensin premix (Rumensin®, exact amount unknown), probably to develop muscle. Instead he fell ill, was hospitalized, and died from acute rhabdomyolysis with renal failure. Although the amount of monensin ingested in this case was not estimated, in another case cited, two deaths among 6 people that consumed baked goods made with premix was attributed to monensin exposure of at least 10× the optimum daily dose fed to cattle. In another report from Brazil, a 16-year-old farm worker who ingested approximately 500 mg of monensin (5 g of Rumensin® 100 premix) "to become stronger" developed an early and severe rhabdomyolysis followed by acute renal failure, heart failure, and death (Caldeira *et al.*, 2001).

In target species, culprit feeds usually contained 5× the maximum approved ionophore use level in the total mix ration provided to the affected animals, with two exceptions. Toxic episodes have occurred from amending cattle and sheep rations with poultry litter (30% or above) containing maduramicin residues and incompatibilities with drugs concurrently administered via feed or drinking water. Poultry litter contained levels of maduramicin which proved to be toxic to cattle and sheep (Fourie *et al.*, 1991; Bastianello *et al.*, 1991; Shlosberg *et al.*, 1992). Various antibiotics, including tiamulin, chloramphenicol, and macrolides, and sulfa drugs, such as sulfachlorpyrazine, have been reported to potentiate ionophore toxicity (Frigg *et al.*, 1983; Weisman *et al.*, 1984; Dowling, 1992; Anadon and Reeve-Johnson, 1999). Among drug combinations that resulted in adverse effects in cattle (Basaraba *et al.*, 1999), chickens (Frigg *et al.*, 1983; Umemura *et al.*, 1995, Mazlum *et al.*, 1985), turkeys (Horrox, 1980; Weisman *et al.*, 1980, 1983; Dorn *et al.*, 1983; Broz and Frigg, 1987), and swine (Pott and Skov, 1981; Stanfield and Lamont, 1981; Wanner, 1984; Miller *et al.*, 1986), the most frequently reported drug interaction is with the pleuromotilin derivative, tiamulin. According to Meingassner *et al.* (1979), tiamulin interferes with the metabolic degradation of monensin in the liver, causing the ionophore to accumulate to toxic levels. The primary step in monensin metabolism is *o*-demethylation (Donoho, 1984) which is catalyzed by several P-450 enzymes (Ceppa *et al.*, 1997; Nebbia *et al.*, 1997). Witkamp *et al.* (1995, 1996) considered the formation of tiamulin metabolite inhibitory (MI) complexes with P-450 enzymes to be the basis of the toxic interaction, similar to that reported with macrolide antibiotics (Anadon and Reeve-Johnson, 1999). However, Szücs *et al.* (2004) reported no evidence of tiamulin MI complexes and that tiamulin directly inhibited CYP3A and two other enzymes, CYP1A2 and CYP2E1,

involved in monensin-*o*-demethylation. Whether similar alterations in ionophore metabolism occur with other drugs is not clear but is an active area of research.

The incidence of ionophore toxicity in all species appears to be low (Novilla and Folkerts, 1986). This is based on periodic reviews of product complaints submitted to the US FDA, as well as from a perusal of the literature. This is remarkable considering the widespread use of ionophore products. In the United States, more than 90% of broiler chickens and about 75% of cattle marketed yearly have consumed ionophores at least part of their lifetime. More information and reports of adverse reactions are available on monensin than for the other ionophores because of monensin's long-standing and widespread use in food animals. However, all the other ionophores can produce a similar toxic syndrome following overdosage, misuse, and drug interaction.

Over the years, several acute toxicity ($LD_{50}$) studies have been conducted in laboratory and domestic animals with monensin, lasalocid, salinomycin, and narasin. Due to a change in FDA regulations, there is limited or no $LD_{50}$ data for the other marketed ionophores (Oehme and Pickrell, 1997). Available data for most species are shown in Table 83.2.

The $LD_{50}$ of monensin varies from 214 mg/kg in broiler chickens to 1.4 mg/kg in horses, the most sensitive domestic animal species to ionophore toxicoses. In ionophore toxicoses, there is a progression of events from ingestion of a particular product. Dose and time factors influence the outcome. The greatest risk of intoxication is upon initial exposure, since animals will not consume highly contaminated feed. After an overdose, repeated daily intake of high levels of ionophores is not possible when anorexia occurs (Potter *et al.*, 1984; Matsuoka *et al.*, 1996).

Safety studies have shown that consumption by horses of complete feed containing the maximum approved use level of monensin for cattle is harmless. This is probably true for lasalocid or laidlomycin as well since there are no

**TABLE 83.2 Acute oral toxicity of ionophores in various animals ($LD_{50}$ + SE, mg/kg; [$LD_0$, mg/kg if available])[a]**

| Animal species | | Sex | Monensin[b] | Narasin[c] | Salinomycin[d] | Lasalocid[e] | Laidlomycin[f] |
|---|---|---|---|---|---|---|---|
| Chicken | (C[i]) | | 214<br>[100] | 67 | 40 | 71.5 | NR[h] |
| Turkey | (C) | | 253<br>[90] | ND[g] | NR | NR | NR |
| Cattle | | MF[j] | 26.4<br>[12.6] | ND | NR | <10>50<br>[10] | NR |
| Horse | | MF | 1.38 ± 0.2<br>[0.675] | 0.8 | NR | 21.5 | NR |
| Swine | | MF | 16.7 ± 3.6<br>[4] | 8.9 | NR | NR | NR |
| Dog | | M<br>F | [>20]<br>[>10] | [>10]<br>(MF) | NR | NR | NR |
| Rabbit | | MF | 41.7 ± 3.6<br>[25.2] | [>10.75] | NR | 40 | NR |
| Rat | | M<br>F | 40.1 ± 0.4<br>28.6 ± 3.8 | 22<br>24 | 48.9<br>47.6 | 122<br>(MF) | 63<br>(MF) |
| Mouse | | M<br>F | 70 ± 9<br>96 ± 12 | 15.8 ± 2.6<br>16.7 ± 2.1 | 57.4<br>(MF) | NR | NR |
| Sheep | | MF | 11.9 ± 1.2<br>[3] | ND | NR | NR | NR |
| Goat | | MF | 26.4 ± 4<br>[4] | ND | NR | NR | NR |
| Trout | | MF | >1000 | ND | NR | NR | NR |

[a] $LD_{50}$: amount of drug required to kill 50% of the group of animals; ($LD_0$: no deaths) within 7–14 days after a single oral dose. No data available for maduramicin and semduramicin.
[b] Updated from Todd *et al.* (1984) *J Anim Sci* **58**: 1512–17.
[c] From Novilla *et al.* (1994) *Vet Hum Toxicol* **36**: 318–23.
[d] Salinomycin sodium. Freedom of Information Summary. FDA NADA #D128686.
[e] From Galitzer *et al.* (1984) *Vet Hum Toxicol* **26**: 322–6; Lasalocid. Freedom of Information Summary. FDA NADA #96-298V.
[f] Laidlomycin propionate potassium. Freedom of Information Summary. FDA NADA #1410025.
[g] ND: not done.
[h] NR: no record.
[i] C: combined $LD_{50}$ values.
[j] M: male; F: female; MF: males and females represented.

confirmed reports of ionophore toxicoses in horses used in cattle feedlots. Results of acute toxicity studies with ionophores in domestic animals indicate that the horse is the most sensitive species; the chicken, least sensitive; and cattle, intermediate.

From studies in ponies given toxic doses of monensin, Amend *et al.* (1981) found that the death pattern from single, high toxic doses by gavage varied from peracute death in less than 24 h; acute death in 24–96 h; subacute death in 4–14 days; and chronic death after 14 days. The latter is not a chronic toxicity by definition, but delayed death secondary to congestive heart failure (CHF), a recognized consequence of ionophore toxicoses. Deaths from CHF may occur in some animals that survive the acute toxic episode depending upon the affected area in the heart and the cardiac reserve. In cattle, peracute deaths from monensin toxicity have not occurred. In fact, no deaths have been recorded earlier than 3 days even after high-level ($LD_{50}$) toxic exposure (Potter *et al.*, 1984).

Blood level studies suggest that horses, compared to cattle, are not able to clear monensin rapidly from the blood (Donoho, 1984). This was probably related to the oxidative efficiency of P-450 demethylating enzymes in the liver. Working with liver microsomes from horses, pigs, broiler chickens, rats, and cattle, Nebbia and associates (2001) found that horses had the lowest catalytic efficiency which may explain the greater susceptibility of horses to the ionophore-induced toxicosis, relative to the other species. However, not all levels of monensin are toxic to horses (Matsuoka, 1976; Matsuoka *et al.*, 1996). In a sub-chronic feeding study in horses, all 3 horses provided a complete ration containing 330-ppm monensin (or 300 g/ton, 10× the maximum cleared level of monensin in complete feeds for feedlot cattle) and one out of three horses given 121-ppm monensin died during the 1-month feeding period. However, 3 horses provided 33-ppm monensin have maintained typical levels of feed intake and suffered no ill-effects attributable to treatment (Table 83.3).

There are no confirmed field reports of ionophore toxicoses in horses used in cattle feedlots. After evaluating the effects of monensin in either supplements or blocks in pasture horses, Matsuoka and co-workers (1996) made the following conclusions that may apply to other marketed ionophores as well: (1) horses on pasture may consume, at initial exposure, enough supplement containing high levels of monensin to be toxic and/or lethal; (2) following an initial high-level monensin exposure, consumption of treated feed, supplement or block is negligible; and (3) horses can consume certain levels of monensin without suffering any ill effect indicative of toxicity.

**TABLE 83.3 Effect of feeding monensin in complete feed to horses for 28 days**

| Monensin level | Effect |
|---|---|
| 33 ppm (cattle use level) | Transient anorexia[a] |
| 121 ppm (broiler use level) | May be toxic |
| 330 ppm (10× cattle use level) | Lethal |

[a]Occurred in weeks 2 and 3 of the study.
From Matsuoka *et al.* (1996) *J Equine Vet Sci* **16**: 8–15.

## Clinical signs

Physical signs reported for ionophore toxicoses across several animal species are the following: anorexia, diarrhea, depression, hypoactivity/reluctance to move, dyspnea, leg weakness, ataxia, and recumbency. Most of these clinical signs occur in all animals studied but variations in their occurrence have been observed. For instance, diarrhea is commonly observed with monensin toxicity in cattle and poultry but has not been reported in either horses or dogs. The most consistent clinical sign in animals that have ingested toxic levels of ionophores is partial to complete anorexia (Novilla and Folkerts, 1986).

In chickens, anorexia is associated with diarrhea, drowsiness, extreme weakness, and sternal recumbency with legs extended posteriorly (Hanrahan *et al.*, 1981). In target animal safety and toxicity studies, depression of growth and feed conversion as well as higher mortality occur in broilers fed 5 times the recommended level of monensin for 8 weeks (Novilla, 2004). Broiler breeders exposed to high levels of ionophores manifest severe drops in egg production, anorexia, depression, paralysis, and death in sternal recumbency (Perelman *et al.*, 1993; Lin, 1995). Similar signs were reported in turkeys with toxicity induced by monensin, salinomycin, and narasin (Stuart, 1978; Beck and Harries, 1979; Salyi *et al.*, 1988; Ficken *et al.*, 1989). Mortality was variable but high death losses (up to 96.7%) were recorded with salinomycin (Halvorson *et al.*, 1982).

In cattle, clinical signs of monensin toxicosis include anorexia, diarrhea, ataxia, recumbency, depression, and non-responsiveness to visual stimuli (Potter *et al.*, 1984; Schweitzer *et al.*, 1984; Van Vleet *et al.*, 1983c). There is a definite correlation between onset of clinical signs and amount of monensin consumed. All intoxicated cattle manifest partial to complete anorexia. Severely intoxicated animals develop anorexia within 24 h. Animals exposed to lower levels may take up to 48 h to develop anorexia. Diarrhea usually is evident in 24–48 h in animals receiving high doses of monensin. Exposure to low doses may result in a delay of up to 5 days in the onset of diarrhea. Onset of depression follows a similar pattern, being observed by day 3 in animals receiving excessive monensin overdoses while being observed after 1 week in animals receiving less than excessive overdoses. Rapid breathing and ataxia may be present only in animals that have consumed extremely high levels. In cattle, death occurs without any manifestations of struggle.

Galitzer and co-workers (1982) found that lasalocid-intoxicated cattle manifested early signs of forced watery diarrhea, muscle tremors, and greater cardiac and respiratory rates followed by or concurrent with anorexia. Delayed signs of cardiac insufficiency were evident 7–10 days after administration of the toxic dose. The temporal occurrence of clinical signs (Table 83.4) and mortality from ionophore toxicity was clearly documented by Potter *et al.* (1984) in a multiple bolus dosing trial with monensin in cattle.

The onset and severity of toxic effects induced by monensin were dose-related. In the study, anorexia was the most consistent clinical sign of toxicity and no deaths occurred earlier than 4 days after exposure to high toxic doses of monensin. However, multiple bolus dosing does not simulate actual field conditions since repeated exposure to toxic levels is not possible when anorexia occurs. Further, the onset and severity of toxicity from bolus dosing is different than that via feed (Buck *et al.*, 1976)

Sheep and goats have clinical signs similar to cattle (anorexia, diarrhea, ataxia) but affected lambs frequently exhibit labored breathing, frothing at the mouth, and kicking at the abdomen (Confer *et al.*, 1983; Newsholme *et al.*, 1983; Bounque *et al.*, 1986; Egyed *et al.*, 1987; Agaoglu *et al.*, 2002). Dogs with ionophore toxicoses become weak and develop a rapid onset of paresis progressing to flaccid paralysis of the anterior and posterior limbs, tongue laxity, and dyspnea (Wilson, 1980; Safran *et al.*, 1993; Condon and Mckenzie, 2002; Segev *et al.*, 2004). In pigs, anorexia, diarrhea, lethargy, dyspnea, ataxia, knuckling at the fetlock, and myoglobinuria were present prior to lateral recumbency and death from ionophore toxicoses (Van Vleet *et al.*, 1983a, b; Miller *et al.*, 1986; Van Halderen *et al.*, 1993; Plumlee *et al.*, 1995).

As in cattle and other species, anorexia is the first and most consistent clinical sign finding (Table 83.5) in the horse with ionophore toxicoses.

Diarrhea has not been observed with either monensin or lasalocid but was reported with salinomycin and narasin (Matsuoka *et al.*, 1996; Whitlock *et al.*, 1978; Hanson *et al.*, 1981; Novilla and Folkerts, 1986; Rollinson *et al.*, 1987). Profuse sweating was also observed with monensin, salinomycin, narasin, and laidlomycin but not with lasalocid toxicity. Horses with leg weakness and ataxia generally progress to recumbency. Once recumbent, horses frequently attempt to rise with trashing of the limbs until death supervenes. This is in contrast to cattle that die without signs of struggling.

**TABLE 83.4 Clinical signs in beef cattle gavaged with multiple doses (7 days) of monensin**

| Monensin (mg/kg/day) | Day first observed | | | | | |
|---|---|---|---|---|---|---|
| | Anorexia | Diarrhea | Depression | Dyspnea | Ataxia | Death |
| 0 | –[a] | – | – | – | – | – |
| 1.5 | 2 | 5 | 7 | – | – | – |
| 2.3 | 1 | 4 | 6 | – | – | – |
| 3.8 | 1 | 3 | 4 | – | – | – |
| 7.6 | 1 | 2 | 4 | 5 | 5 | 5 |
| 15.5 | 1 | 2 | 3 | 5 | 4 | 4 |

[a] Not observed.

**TABLE 83.5 Clinical signs of ionophore toxicoses in the horse**

| Sign | Monensin | Lasalocid | Salinomycin | Narasin | Laidlomycin |
|---|---|---|---|---|---|
| Anorexia | X[a] | X | X | X | X |
| Diarrhea | –[b] | – | X | X | – |
| Depression | X | X | – | X | X |
| Dyspnea | X | – | X | X | X |
| Ataxia | X | X | X | X | X |
| Colic[c] | X | – | X | X | – |
| Leg weakness | X | X | X | X | X |
| Muscle tremors | X | – | – | X | X |
| Sweating | X | – | X | X | X |
| Recumbent | X | X | X | X | X |

[a] Observed.
[b] Not observed.
[c] Pain manifested by uneasiness, abnormal (wide) leg stance, pawing.

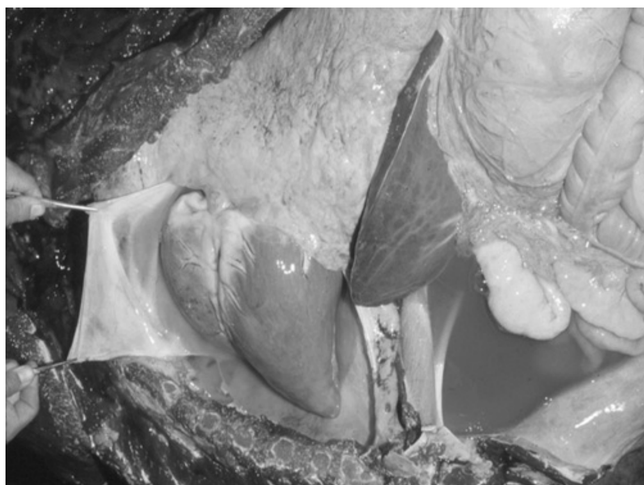

**FIGURE 83.5** Thoracic and abdominal viscera from a mare that died 7 days following a single gavage dose of 1.65 mg monensin/kg body weight. Note the fluid accumulation in the body cavities and the pale areas and epicardial hemorrhages on the heart. This figure is reproduced in color in the color plate section.

## Postmortem findings

Necropsy findings in animals with ionophore toxicoses include hemorrhages and pale areas in the heart, pale areas in some limb muscles, pulmonary edema, hydrothorax, ascites, and inflammation of the stomach and intestines (Nation *et al.*, 1982; Van Vleet *et al.*, 1983a, b, c; Galitzer *et al.*, 1986b; Salles *et al.*, 1994). Animals that die soon after exposure often will have no lesions, since they had not had time to develop or they may be masked by postmortem changes. Those with lethal doses surviving longer than a week may have hydropericardium, pulmonary edema, hydrothorax, ascites, reddish-mottled liver, and subcutaneous edema of the abdomen and limbs (Figures 83.5).

At necropsy, cutting the ventricular and thigh muscles transversely often shows the diffuse pallor (Figure 83.6) of myonecrosis induced by toxic levels of ionophores. Skeletal muscle lesions may be quite severe in sheep, pigs, and dogs, moderate in cattle and minimal to slight in horses. For this reason, myoglobinuria, evidenced by red urine, has been observed in pigs, sheep, and dogs but not in cattle or horses.

## Histopathologic findings

Target organs damaged by toxic doses of monensin and other ionophores were identified to include the heart and skeletal muscles in all species studied (Todd *et al.*, 1984; Novilla and Folkerts, 1986; Ficken *et al.*, 1989; Van Vleet *et al.*, 1991; Dowling 1992). In addition, neurotoxic effects have been reported for lasalocid (Shlosberg *et al.*, 1985; Safran *et al.*, 1993), narasin (Novilla *et al.*, 1994), and salinomycin (Van der Linde-Sipman *et al.*, 1999). The

**FIGURE 83.6** Diffuse pallor in cross sections of the heart from the same mare described in Figure 83.5 legend. This figure is reproduced in color in the color plate section.

development of muscle lesions varies among species. The heart is primarily affected in horses, skeletal muscle in pigs and dogs, and there is about equal tissue predilection in rats, chickens, and cattle. Morphologic effects include degeneration, necrosis, and repair of cardiac and skeletal muscle fibers with a variable inflammatory component and secondary lesions of CHF. Neuropathic changes occurred in peripheral nerves and the spinal cord. Focal swelling, fragmentation, loss of axons, and formation of digestion chambers filled with macrophages were observed in both sensory and motor nerves, and there was vacuolation with swelling, degeneration, and fragmentation of myelin sheaths and axons in the spinal cord.

The most important change is a toxic myopathy characterized by focal areas of degeneration, necrosis, and repair in cardiac and skeletal muscles with a variable inflammatory component (Van Vleet *et al.*, 1991; Novilla and Folkerts, 1986). Muscle lesions are similar to those described for compound A204, the first polyether antibiotic tested at Lilly Research Laboratories (Todd *et al.*, 1970). Doses and time

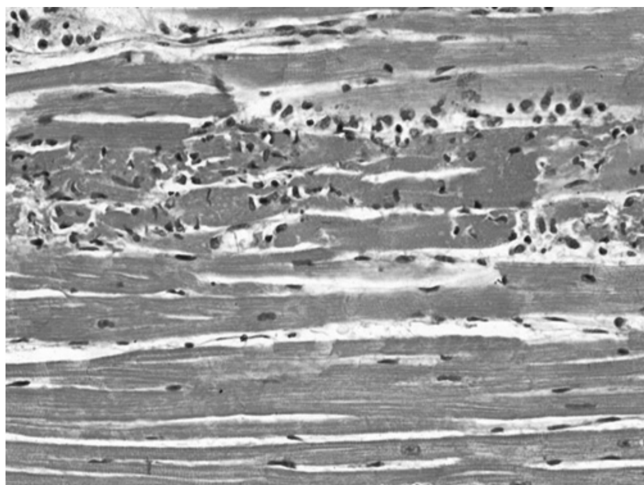

FIGURE 83.7 Early necrotic focus in the left ventricle of a gelding that died 20h following gavage with 2.5mg monensin/kg body weight. Necrotic muscle fibers have sparse infiltration of neutrophils and lymphocytes. H&E. Original magnification ×64. This figure is reproduced in color in the color plate section.

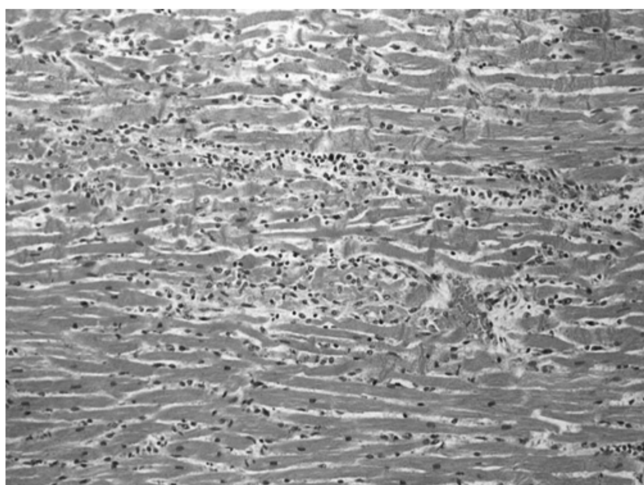

FIGURE 83.8 Larger foci of myofiber necrosis with contraction bands in the interventricular septum from a horse euthanized 72h following a single oral dose of 1.65mg monensin/kg body weight given by gavage. More cellular infiltrates with lymphocytes, macrophages, and few eosinophils are present. H&E. Original magnification ×10. This figure is reproduced in color in the color plate section.

factors influence the severity and distribution of lesions in ionophore toxicoses (Figures 83.7 and 83.8).

Generally, no significant lesions are seen by light microscopy in animals that die immediately, and animals that die after an acute course may have only a few scattered degenerated fibers in the heart and highly active muscles, such as the diaphragm. Lesions are most pronounced within 7–14 days following ingestion of a toxic dose and are accompanied by profound attempts at repair. Skeletal muscle fibers regenerate rapidly, and lesions are completely healed in about a month (Figure 83.9). Heart muscle fibers

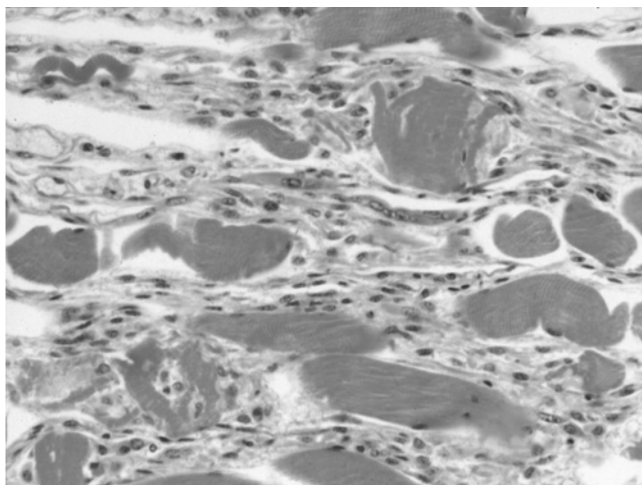

FIGURE 83.9 Skeletal muscle from a steer that died 6 days after a gavage dose of 39.8mg monensin/kg body weight. Notice the fragmented and regenerating fibers. H&E. Original magnification ×80. This figure is reproduced in color in the color plate section.

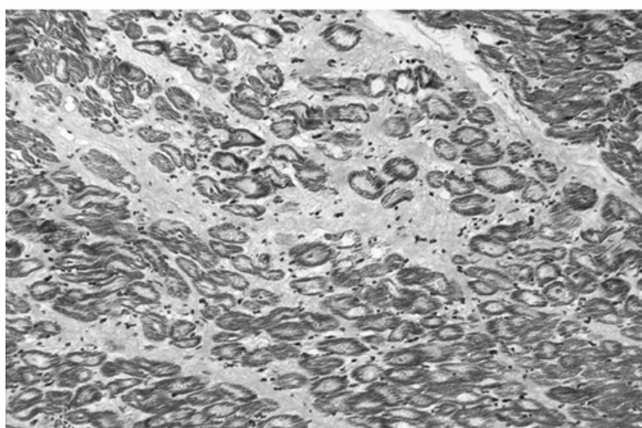

FIGURE 83.10 Hearts from animals that survived acute ionophore toxicity. Top: section of left ventricle with focal interstitial fibrosis from another steer, cohort of that described in Figure 83.9 legend, euthanized 28 days after a gavage dose of 39.8mg monensin/kg body weight. H&E. Original magnification ×20. Bottom: interstitial fibrosis in the left ventricle from a gelding that survived a gavage dose of 2mg monensin/kg body weight for 4 months. Masson's trichrome. Original magnification ×32. This figure is reproduced in color in the color plate section.

(Figure 83.10) do not regenerate, but repair takes place by replacement fibrosis. Secondary lesions of CHF, including heart dilatation and hydropericardium, lung edema, liver necrosis, and fluid accumulation in the thoracic and abdominal cavities, may be present, depending upon the severity of heart damage and the potential cardiac reserve.

Observations to date indicate that in horses, the heart suffers the greatest damage from monensin toxicoses with little or no involvement of skeletal muscles. This finding is in contrast to dogs and pigs in which the lesions are most pronounced in skeletal muscles. Chickens, cattle, and rodents have about equal predilection for cardiac and skeletal muscle lesions.

## Other laboratory findings

Mitochondrial swelling, myelin figures, and lipid vesicles were observed ultrastructurally in cardiac and skeletal muscles of ponies given monensin at 4 mg/kg and observed for 79 h (Mollenhauer *et al.*, 1981, 1984). Early degenerative changes of sarcoplasmic vascuolation from swollen mitochondria and accumulation of lipid were described in cattle and in pigs with monensin toxicosis (Van Vleet and Ferrans, 1983, 1984). Severely injured mitochondria had marked swelling, disrupted cristae, and dense matrical granules. Scattered myelin figures were present in muscle cells with numerous disrupted mitochondria. Subsequently necrosis occurred in degenerated myocytes and was characterized by dense fibers with intact sarcomeres or disrupted fibers with hypercontraction bands. Necrosis was followed rapidly by extensive macrophage infiltration with lysis of disrupted organelles and contractile material. Similar ultrastructural findings were observed in hearts and skeletal muscles of sheep given monensin (Confer *et al.*, 1983; Anderson *et al.*, 1984) and in skeletal muscles of broilers simultaneously given triacetyloleandomycin in the drinking water and monensin in the feed (Umemura *et al.*, 1984). Regeneration as evidenced by myoblast proliferation was observed as early as 4 days post-treatment in pigs and sheep.

Hematologic parameters are not significantly affected by ionophore toxicosis (Van Vleet *et al.*, 1983a, b, c; Condon and McKenzie, 2002, Segev *et al.*, 2004). Elevation of serum enzymes, notably creatine kinase, lactate dehydrogenase, and aspartate transaminase may indicate damage to cardiac and skeletal muscles (Wilson, 1980; Amend *et al.*, 1981; Van Vleet *et al.*, 1983b, c; Galitzer *et al.*, 1986a; Hall, 2001). Alkaline phosphatase, inorganic phosphorus, and total bilirubin levels are also higher, while serum levels of calcium and potassium are lower. The progressive hypokalemia and attendant cardiac conduction disturbances demonstrated in ponies were considered the life-threatening events in early acute monensin toxicosis (Amend *et al.*, 1981).

## Diagnosis and differential diagnosis

Since all ionophores in the market place are likely to produce a similar toxic syndrome in overdosage and misuse situations, six important criteria must be considered before a diagnosis of toxicity is given (Beck and Harries, 1979; Novilla and Folkerts, 1986). These include: (1) history of feed-related problem, usually affecting a group of animals; (2) ionophore laboratory assays; (3) clinical signs manifested during the toxicity episode; (4) gross postmortem lesions; (5) microscopic pathology; and (6) exclusion of nutritional, infectious, and other toxic factors.

History assumes great significance when the problem is connected to the introduction of newly formulated feed or supplement to the herd or flock. Since clinical signs and lesions are not pathognomonic, feed analysis for the amount and type of ionophore in the ration is necessary for diagnosis. With the availability of seven ionophores in the marketplace, the use of an efficient and highly selective laboratory assay is indicated. Newer methods have been developed to determine one or more ionophores in feeds and tissues (Hormazabal *et al.*, 2002; Bertini *et al.*, 2003; Ebel *et al.*, 2004). Several samples of the feed should be submitted for analysis, including residual material from the feeder, trough, auger, or feed bin. Feed assays may prove exposure to a particular product but significantly higher than the recommended levels must be found for a confirmatory diagnosis. Otherwise, concurrent use of an incompatible drug must be documented. In the absence of proof of a gross feed-mixing error, a wide list of differential diagnoses needs to be excluded in order to return a presumptive diagnosis.

Initially, ionophore toxicoses may be suspected when there is a history of a feed-related problem in a group of animals; clinical signs of anorexia, diarrhea, labored breathing, depression, locomotory disorder, recumbency, and death; lesions affecting heart and skeletal muscles; or CHF. The clinical signs and lesions induced by toxic levels of ionophores are not pathognomonic. However, a history of recent introduction of newly formulated feed or supplement to a flock or herd in which signs and lesions are present may cause one to suspect that acute intoxication has occurred. Dose and time factors influence the severity and distribution of lesions. Animals that die soon after exposure may not have muscle lesions discernible by light microscopy. Lesions are likely to be found in animals that survived longer than a week. The most active skeletal muscles may be involved when the heart is not affected or is only slightly affected. Since changes can be missed because of their focal distribution, more intense tissue sampling to include one section each of the atria, ventricles, and interventricular septum of the heart, the diaphragm, and muscles of the abdomen and thigh is desirable. Some animals with substantive heart damage from very high levels of monensin and other ionophores may later develop CHF.

Although a presumptive diagnosis of ionophore toxicosis can be made based on history, clinical signs, lesions, and considerations of differential diagnosis, specific assays are needed for confirmatory diagnosis. With seven ionophores currently in use, the need for confirmatory laboratory assays cannot be overemphasized. In monensin toxicosis, values greater than 5 times the recommended use level in the feed provided affected animals are usually confirmatory. Assays on stomach contents from peracute and acute cases of toxicity can prove exposure but values obtained have been low. Only minimal residues of monensin have been detected in target tissues of cattle and chickens given monensin (Donoho, 1984; Atef *et al.*, 1993). Further, blood levels of monensin are low or undetectable even in intoxicated animals, and accumulation in target tissues does not occur.

Ionophore toxicosis may be confused with acute infectious diseases, deficiencies, and other intoxications (Kingsbury, 1964; Hubert and Oehme, 1968; Beck and Harries, 1979; Van Vleet *et al.*, 1983a, b, c; Novilla and Folkerts, 1986; Dowling, 1992). In the differential diagnosis of monensin toxicosis, myopathic conditions should be considered first. In cattle, these would include: (1) ionophore toxicoses, (2) vitamin E and selenium deficiencies, (3) poisonous plant ingestion e.g., senna, coyotillo, white snakeroot, vetch, and (4) the common, yet puzzling, sudden death syndrome with myocardial necrosis. Ionophore toxicosis usually involves an accompanying history of feed supplementation or feed change and usually involves many animals. Clinical signs are anorexia, diarrhea, lethargy, ataxia, and the suggestion of damage to striated muscles. Vitamin E and selenium deficiency occur sporadically and produce prominent degeneration and necrosis with calcification of cardiac and skeletal muscles. Plant poisonings are usually localized to areas where the toxic plants are indigenous. For instance, coffee senna (*Cassia occidentalis*) poisoning occurs in the southeastern United States. It may cause anorexia, diarrhea, and the production of dark urine, but generally causes more pronounced lesions in skeletal muscles than in the heart. The coyotillo plant (*Karwinskia huntholdtiana*) in southwest Texas and Mexico produces limberleg in sheep and goats characterized by progressive weakness of the legs, muscular incoordination, recumbency, respiratory distress, and death. Lesions are observed both in cardiac and skeletal muscles as well as peripheral nerves and the liver. White snakeroot (*Eupatorium rugosum*), a plant indigenous to much of eastern Canada and the United States, causes "trembles" in goats, sheep, cattle, horse, and swine. Cardiac and skeletal muscle lesions may be present in animals that ingested this plant. However, with trembles there is constipation, blood in feces, an odor of acetone in the breath, and severe fatty degeneration in the liver and kidney that are not seen in ionophore toxicities. Hairy vetch (*Vicia villosa*) also produces myocardial necrosis but, unlike monensin toxicosis, it produces dermatitis, conjunctivitis, and abortion as well as lesions in the kidneys, adrenal glands, lymph nodes, and thyroid gland. The syndrome of sudden death with myocardial necrosis in cattle, especially calves, is common but sporadic in occurrence and is associated with lesions in cardiac but not skeletal muscle (Bradley *et al.*, 1981). Hence, clinical history and detailed pathologic studies will help distinguish among ionophore toxicosis, acute infectious diseases, deficiencies, and other intoxications.

From a clinical standpoint, respiratory diseases, particularly infectious bovine rhinotracheitis and the shipping fever complex, are initially considered in the differential diagnosis for cattle because of the respiratory difficulties that occur with ionophore toxicosis. At necropsy, however, pneumonic lesions are consistent with these diseases. Animals with acute bovine pulmonary edema and emphysema or fog fever, nitrogen dioxide intoxication, or rape, turnip, or kale poisoning also exhibit respiratory difficulties; but, in these conditions, gross lesions will include severe interstitial and interlobular emphysema or pneumonia or both rather than edema. The incoordination, stiff wobbly gait, and loss of visual reflexes may lead one to suspect polioencephalomalacia and thromboembolic meningoencephalitis but, in these conditions, histologic lesions present in the brain are confirmatory. Unequivocal central nervous system lesions have not been found in cases of monensin toxicity in any species. However, lesions in the spinal cord and peripheral nerves occurred with lasalocid toxicosis in chickens (Shlosberg *et al.*, 1985; Perelman *et al.*, 1993). Peripheral neuropathic changes have also been reported in cats exposed to salinomycin-contaminated feed (Van der Linde-Sipman *et al.*, 1999) and in dogs given narasin (Novilla *et al.*, 1994). Salt poisoning will cause nervous signs, paralysis, and diarrhea, but knowledge that insufficient amounts of water were available to the animals will also point to this problem. Eosinophilic meningoencephalitis is pathognomonic for salt poisoning in pigs, but this lesion does not occur in other species. Laboratory procedures used to confirm a diagnosis include assays of serum, cerebrospinal fluid, and brain tissue for sodium concentrations (Buck *et al.*, 1976). In cattle, urea toxicosis must be considered when sudden collapse, bloat, violent convulsions, terminal tetanic spasms, and high death losses occur within 10 min to 4 h from exposure to newly formulated feed. Deaths occurring earlier than 72 h have not been reported in cattle gavaged with high doses of monensin (Potter *et al.*, 1984).

In poultry, differential diagnoses should include nutritional (focal) myopathy, coffee senna toxicity, botulism, sodium chloride (salt) toxicity, mycotoxicosis by cyclopiazonic acid, and other myopathic mycotoxins, round heart disease, and in the turkey, the knockdown (downer) syndrome. Although no striated muscle lesions are produced in botulism and sodium chloride toxicity, clinical signs of limberneck and lesions of "water belly" may be confused

with ionophore toxicoses. On the other hand, birds affected with salt poisoning may have enlarged hearts or have enlarged pale kidneys from urate nephrosis (Swayne *et al.*, 1986). Monensin *per se* does not produce "barebacks" in broiler chickens, and downers among replacement birds may be suffering from viral arthritis. Therefore, this common reovirus infection must be excluded as a cause of the problem in chicken flocks. In commercial turkeys reared in confinement, focal myopathy has been attributed to deficiencies in vitamin E or selenium associated with rapid growth (Maronpot *et al.*, 1968; Wilson *et al.*, 1990). Nutritional myopathy may mimic skeletal muscle lesions induced by ionophore toxicoses; but unlike those of nutritional myopathy, ionophore-induced lesions are monophasic and polyfocal with little or no mineralization. Muscle lesions also occur in deep pectoral myopathy (Siller 1985), coffee senna toxicity (Simpson *et al.*, 1971), and toxicoses from the mold toxins, cyclopiazonic acid (Cullen *et al.*, 1988) and moniliformin (Engelhardt *et al.*, 1989). Their clinical presentation and presence of other lesions will help distinguish these conditions.

Round heart disease, also known as Roundheart–Edema–Ascites syndrome or spontaneous cardiomyopathy, occurs sporadically in turkeys and other fowl (Gough *et al.*, 1981; Czarnecki 1984; Julian *et al.*, 1992). Clinical signs indicate the presence of heart failure but the exact cause has not been determined. A genetic predisposition has been suggested because it is more common in certain lines of turkeys, particularly those having a rapid rate of early muscle development. Some of the predisposing factors include low levels of oxygen and high levels of carbon dioxide in the incubator, poor brooder house ventilation, and overcrowding during transport. Exposure to these factors may occur days or weeks prior to onset of illness. In contrast, monensin toxicity occurs as a sudden outbreak in a flock of birds accidentally provided toxic levels due to a feed-mixing error. Among birds that survive the acute toxic episode, a few, depending upon the extent and location of heart damage and the potential cardiac reserve, may subsequently develop dilatation of the heart and secondary signs of CHF.

Another condition that needs to be distinguished from monensin toxicity is the turkey knockdown syndrome. Turkey knockdown has been defined as any condition affecting the neuromuscular system to the extent that a bird is unable to stand or walk (Wages, 1993). A necrotizing myopathy, particularly of the rear limbs, has been reported in turkeys with the knockdown syndrome (Cardona *et al.*, 1992, 1993). Unfortunately, striated muscles have limited responses to injury (Van Vleet *et al.*, 1991) and lesions induced by any ionophore, including monensin are not pathognomonic (Novilla, 1992). Although monensin feed inclusion rates in knockdown cases are within or slightly above the maximum recommended level of 99 ppm (Wages and Ficken, 1988; Cardona *et al.*, 1992, 1993) monensin has been implicated as a causative factor because the clinical and pathologic findings are similar to those induced by monensin toxicity. However, unlike monensin toxicity, the turkey knockdown syndrome is characterized by: (1) low incidence and fatality rates; (2) acute onset of paresis/paralysis; (3) no anorexia, birds will eat if able to reach feeders; (4) no diarrhea; (5) affected birds are alert; (6) unable to vocalize; (7) presence of widespread skeletal muscle lesions that are subacute to chronic in character, generally no heart lesions; and (8) rapid recovery following change of feed. These observations were confirmed in an epidemiological case controlled study of knockdown cases in the Shenandoah Valley of Virginia that evaluated various factors, including management, environment, health, feed, water, pathology, and clinical pathology analytes (Evans *et al.*, 2000). Serum vitamin E levels were significantly lower in affected birds (Meldrum *et al.*, 2000). Feed and/or water intake alterations were highly correlated with the incidence of turkey knockdown and modification of practices affecting feed/water intake reduced the incidence of knockdown in one farm from 6 in 1999 to 0 in 2000.

In pigs, vitamin E and selenium deficiency, gossypol poisoning, and porcine stress syndrome (PSS) should be considered in the differential diagnosis since skeletal or cardiac lesions may be found in these conditions (Van Vleet *et al.*, 1984b; Van Vleet and Ferrans, 1984). In monensin toxicosis, the striated muscle lesions appear more frequently and are more severe in skeletal muscle than in cardiac muscle. Myoglobinuria may also be present. Widespread cardiac and skeletal muscle lesions, vascular damage, dietetic hepatosis, and gastric ulceration may be found in vitamin E and selenium deficiency. With PSS, cardiac, and skeletal muscle lesions may or may not be observed following a history of stress.

For horses, the exertional myopathies, such as equine rhabdomyolysis (Monday morning disease) and hyperkalemic periodic paralysis, plant poisoning from coffee senna and white snakeroot should be excluded along with blister beetle intoxication, colic, and laminitis (Whitlock *et al.*, 1978; Amend *et al.*, 1981; Novilla and Folkerts, 1986). Complete herd history, clinical examination, successful supportive treatment, and necropsy may help differentiate these conditions from ionophore toxicoses.

## TREATMENT

When ionophore toxicity is suspected, a feed change to a non-ionophore medicated ration must be made immediately and the affected group(s) of animals maintained on this ration until all diagnostic procedures are completed. At the present time, there is no known antidote or treatment for ionophore toxicity (Table 83.6). Although previous

**TABLE 83.6** Some biomarkers of ionophore toxicity

| | |
|---|---|
| • Elevated<br>– AST<br>– CK<br>– LDH<br>– Alkaline phosphatase<br>– BUN<br>– Bilirubin | • Decreased<br>– Calcium<br>– Potassium<br>• No change<br>– Sodium |

antidotal trials failed, the search continues (Mitema *et al.*, 1988). Nonetheless, it may be difficult to find a drug that can block up to four pathogenetic mechanisms, acting singly or in concert, that are involved in the toxicoses. Therapeutic interventions have been largely supportive. For horses, Amend *et al.* (1981) recommended supportive treatment with mineral oil, activated charcoal, and aggressive fluid and electrolyte replacement with potassium and phosphorus supplementation, including means to reduce cardiac work. However, supportive therapy may not be practical on a herd or flock basis.

Van Vleet and co-workers (1983a, 1987) obtained protection against the development of *clinical* monensin toxicity in pigs treated with selenium and vitamin E (selenium as selenite at 0.25 mg/kg and vitamin E as a-tocopherol acetate at 17 lU/kg) prior to a single oral dose of 50 mg monensin/kg ($LD_{50}$ 16.7 ± 3.57 mg/kg). A similar regimen in cattle administered toxic doses of monensin provided only partial protection (Van Vleet *et al.*, 1985) but clinical signs in pigs with tiamulin-induced salinomycin toxicity were ameliorated following administration of vitamin E. The protection against the toxicoses was theorized to be produced by stabilization of cellular membranes since selenium and vitamin E are known to prevent and control peroxidation-mediated cellular injury (Tappel, 1981; Van Vleet, 1986). Monensin, like all polyether carboxylic ionophores, is lipophilic and may produce dose-dependent membrane perturbations and increased lipid peroxidation, which could lead to degradative processes. Further studies are needed to determine whether vitamin E and selenium administration may have an important role in the prevention and treatment of ionophore toxicoses.

Until proper and effective therapy is available, prevention of ionophore toxicoses by (1) proper use from the implementation of good feed manufacturing and feeding practices at the feed mill and farm level; (2) avoidance of overdosing of feeds for approved species, which may carry over to feeds for non-target species; and (3) adherence to species restrictions will help prevent the adverse effects associated with this class of compounds.

## CONCLUSIONS

Seven ionophores – monensin, lasalocid, salinomycin, narasin, maduramicin, laidlomycin, and semduramicin – are marketed globally for use as anticoccidial drugs for poultry and/or growth promotants in ruminants. Off-label usage of ionophore products is known to occur since other uses continue to be investigated and applied in many countries. It is likely that basic and applied research on these versatile compounds could lead in the future to product line extensions to other target species and potential development of novel therapeutics for unmet needs in veterinary and human medicine.

Generally, these feed additives have been found to be safe and effective in target animal species, but toxic syndromes have resulted from overdosage, misuse, and drug interaction. Among the domestic species, horses are the most sensitive to ionophore toxicoses; poultry, the least sensitive; and cattle, intermediate. However, even for the horse, there is a threshold level of exposure below which no adverse effects are observed. Consumption of complete feed containing the maximum approved use levels of either monensin, lasalocid, or laidlomycin is harmless. Dose and time factors influence the severity and outcome of the toxic exposure. Results of controlled studies and confirmed field reports of toxicity indicate that the greatest risk of intoxication is upon initial exposure to ionophore-containing feed or supplement. Following sublethal exposure, consumption of culprit feed or supplement is negligible because of anorexia. Animals that die acutely after high levels of exposure often will have few or no lesions. Those that die later have profound striated (cardiac and/or skeletal) muscle lesions and changes secondary to CHF in some animals that survive the acute toxic episode.

Confirmatory diagnosis requires efficient laboratory assays to determine the identity and amounts of the ionophore involved and a thorough consideration of differential diagnosis. These cannot be overemphasized. There is no known antidote or specific treatment for ionophore toxicoses and treatment is largely supportive. Judicious use, avoidance of overdosing, and adherence to species recommendation will enhance livestock production and help prevent the occurrence of adverse effects associated with this class of compounds.

## REFERENCES

Adovelande JB, Schrevel J (1996) Carboxylic ionophores in malaria chemotherapy: the effects of monensin and nigericin on *Plasmodium falciparum in vitro* and *Plasmodium vinckelpetteri in vivo*. *Pharmacol Lett* **59**: 309–15.

Agaoglu ZT, Akgul Y, Keles I, Ugras S, Aksoy A, Cinar A (2002) Accidental salinomycin intoxication of Angora goats in Turkey. *Small Ruminant Res* **45**: 159–61.

Amend JF, Mallon FM, Wren WB, Ramos AS (1981) Equine monensin toxicosis: some experimental clinicopathologic observations. *Comp Cont Ed* **11**: S173–S183.

Anadon A, Reeve-Johnson L (1999) Macrolide antibiotics, drug interactions and microsomal enzymes: implications for veterinary medicine. *Res Vet Sci* **66**: 197–203.

Anderson TD, Van Alstine WG, Ficken MD, Miskimins DW, Carson TL, Osweiler GD (1984) Acute monensin toxicosis in sheep: light and electron microscopic changes. *Am J Vet Res* **45**: 1142–7.

Atef M, Ramadan A, Abo El-Sooud K (1993) Pharmacokinetic profile and tissue distribution of monensin in broiler chickens. *Brit Poultry Sci* **34**: 195–203.

Baird DJ, Caldow GL, Peek IS, Grant DA (1997) Monensin toxicity in a flock of ostriches. *Vet Rec* **140**: 624–6.

Basaraba RJ, Oehme FW, Vorhies MW, Stokka GL (1999) Toxicosis in cattle from concurrent feeding of monensin and dried distillers grains contaminated with macrolide antibiotics. *J Vet Diagn Invest* **11**: 79–86.

Bastianello SS, Fourie N, Prozesky L, Nel PW, Kellerman TS (1991) Cardiomyopathy of ruminants induced by the litter of poultry fed on ration containing the ionophore antibiotic maduramicin. II. Macropathology and histopathology. *Onderstepoort J Vet Res* **62**: 5–18.

Beck BE, Harries WN (1979) The diagnosis of monensin toxicity: a report on outbreaks in horses, cattle and chickens. *Proc Am Assn Vet Lab Diagn* **22**: 269–82.

Bergen WG, Bates DB (1984) Ionophores: their effect on production efficiency and mode of action. *J Anim Sci* **58**: 1465–83.

Bertini S, Feirrero S, Berny P (2003) A new improved high performance thin layer chromatography (HPTLC) method for the detection of ionophore antibiotics in feeds and animal tissues. *J Liq Chrom Rel Technol* **26**: 147–56.

Bezerra PS, Driemeier D, Loretti AP, Riet-Correa E, Kamphues J, De Barros CS (1999) Monensin toxicity in Brazilian horses. *Vet Hum Toxicol* **41**: 383–5.

Bila CG, Perreira CL, Gruys E (2001) Accidental monensin toxicosis in horses in Mozambique. *J S Afr Vet Assoc* **72**: 163–4.

Black B, McQuilken G (1980) A judgment against sudden death. *Calf News* **18**: 28.

Bradley R, Markson LM, Bailey J (1981) Sudden death and myocardial necrosis in cattle. *J Pathol* **135**: 19–38.

Bounque JG, Smart M, Wobeser C (1986) Monensin toxicity in lambs. *Can Vet J* **27**: 397–9.

Broz J, Frigg M (1987) Incompatibility between lasalocid and chloramphenicol in broiler chicks after long term administration. *Vet Res Comm* **11**: 159–72.

Buck WB, Osweiller GD, Van Odder CA (1976) *Clinical and Diagnostic Veterinaty Toxicology*, 2nd edn, Kendall/Hunt Publishing Co., Dubuque, IA.

Buxton D, Blewett DA, Trees AJ, McGolgan C, Finlayson J (1988) Further studies in the use of monensin in the control of experimental ovine toxoplasmosis. *J Comp Path* **98**: 225–35.

Caldeira C, Neves WS, Cury PM, Serrano P, Baptista MASF, Burdmann EA (2001) Rhabdomyolysis, acute renal failure, and death after monensin ingestion. *Am J Kidney Dis* **38**: 1108–12.

Calo M, Locascio P, Licata P, Richetti A, Zaccone G, Naccari F (2002) Effects of monensin on $Na^+$-ATPAse and $Ca^{2+}$ -ATPase activities in chick skeletal muscle and myocardium after subacute treatment. *Eur J Histochem* **46**: 309–15.

Calo M, Martini D, Locascio, Naccari F (2003) Effects of monensin on nitric oxide synthases in chick cardiac muscle. *J Vet Pharmacol Therap* **26**(Suppl. 1): 82–7.

Cardona CJ, Galey FD, Bickford AA, Charlton BR, Cooper G (1992) A syndrome in commercial turkeys in California and Oregon characterized by a rear-limb necrotizing myopathy. *Avian Dis* **36**: 1092–101.

Cardona CJ, Galey FD, Bickford AA, Charlton BR, Cooper GL (1993) Skeletal myopathy produced with experimental dosing of turkeys with monensin. *Avian Dis* **37**: 107–17.

Carpenter JW, Novilla MN (1992) Safety and physiologic effects of the anticoccidial drugs monensin and clasuril in sandhill cranes (*Grus canadensis*). *J Zoo Wild Med* **23**: 214–21.

Ceppa L, Nachtman C, Dacasto M, Carletti M, Nebbia C (1997) The effect of hepatic microsomal cytochrome P450 monooxygenases on monensin–sulfadimidine interaction in broilers. *J Vet Pharmacol Therap* **24**: 73–6.

Condon FP, McKenzie RA (2002) Fatal monensin toxicity in a dog after chewing a bovine intraruminal slow-release device. *Aust Vet Pract* **32**: 179–80.

Confer AW, Reavis DI, Panciera RI (1983) Light and electron microscopic changes in cardiac and skeletal muscle of sheep with experimental monensin toxicosis. *Vet Pathol* **20**: 590–602.

Coyle D, Walker A (2005) A study to determine the residues of monensin in edible tissues of growing cattle following treatment at 0.9 mg monensin/kg body weight for 28 days. Submitted to the European Medicines Agency CVMP by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Cruickshank JM (1992) The role of coronary perfusion pressure. *Eur Heart J* **13**(Suppl. D): 39–43.

Cullen JM, Wilson M, Hagler WM, Ort FJ, Cole RJ (1988) Histologic lesions in broiler chicks given cyclopiazonic acid orally. *Am J Vet Res* **49**: 728–31.

Czarnecki CM (1984) Cardiomyopathy in turkeys. *Comp Biochem Physiol* **77**: 591–8.

Davison KL (1984) Monensin absorption and metabolism in calves and chickens. *J Agric Food Chem* **32**: 1273–7.

De Guzman NT, Pressman BC (1974) The inotropic effect of the calcium ionophore X-537A in the anesthetized dog. *Circulation* **49**: 1072–7.

Donoho AL (1984) Biochemical studies on the fate of monensin in animals and in the environment. *J Anim Sci* **58**: 1528–39.

Donoho A, Manthey J, Occolowitz J, Zornes L (1978) Metabolism of monensin in the steer and rat. *J Agric Food Chem* **26**: 1090–5.

Dorn P, Weber R, Weikel J, Wessling E (1983) Intoxikation durch geichzeitge Verabreichung von Chloramphenicol und Monensin bei Puten. *Der Praktische Tierarzt* **64**: 240–3.

Dowling L (1992) Ionophore toxicity in chickens: a review of pathology and diagnosis. *Avian Pathol* **30**: 358–68.

DuBourdieu DJ, Shier WT (1992) Sodium- and calcium-dependent steps in the mechanism of neonatal rat cardiac myocyte killing by ionophores. II. The calcium carrying ionophore A23187. *Toxicol Appl Pharmacol* **116**: 47–56.

Duffield T, Bagg R, DesCoteaux L, Bouchard E, Brodeur M, DuTremblay D, Keefe G, LeBlanc S, Dick P (2002) Prepartum monensin for the reduction of energy associated disease in postpartum cows. *J Dairy Sci* **85**: 397–405.

Ebel JG, Wachs T, Henion JD (2004) Rapid forensic selected reaction monitoring liquid chromatography/mass spectrometry determination of ionophore antibiotics found at toxic levels in animal feeds. *J Assoc Off Anal Chem* **87**: 25–30.

Egyed MN, Perl S, Klopper U, Shlosberg A, Yakobson B, Nobel TA (1987) Monensin toxicosis in cattle and sheep with reference to its differential diagnosis. *Isr J Vet Med* **43**: 204–11.

Engelhardt JA, Carlton WW, Tuite JF (1989) Toxicity of *Fusarium moniliforme* var *subglutinans* for chicks, ducklings, and turkey poults. *Avian Dis* **33**: 357–60.

Evans RD, Edson RK, Watkins KL, Robertson JL, Meldrum JB, Novilla MN (2000) Turkey knockdown in successive flocks. *Avian Dis* **44**: 730–6.

Fajer-Avila EJ, Covarrubias MSM, Abad-Rosales S, Roque A, Mesa-Bojorquez P, Hernandez-Gonzalez C (2005) Effectiveness of oral Elancoban™ and Avimix-ST™ against *Nematopsis* (Apicomplexa: Porosporidae) gametocytes infecting the shrimp *Litopenaeus vannamei*. *Aquaculture* **244**: 11–8.

Feliciano L, Henning RJ (1999) Coronary artery blood flow: physiologic and pathophysiologic regulation. *Clin Cardiol* **22**: 775–86.

Ficken MD, Wages DP, Gonder E (1989) Monensin toxicity in turkey breeder hens. *Avian Dis* **33**: 186–90.

Food and Drug Administration, CFR Parts 556 and 558 (2004) Approval of supplemental NADA for the use of monensin Type A medicated articles to formulate Type and Type C medicated feeds for increased milk production efficiency in dairy cows. *Federal Register* **69**: 68783–4.

Fourie N, Bastianello SS, Prozesky L, Nel PW, Kellerman TS (1991) Cardiomyopathy of ruminants induced by the litter of poultry fed on ration containing the ionophore antibiotic maduramicin. I. epidemiology, clinical signs, and clinical pathology. *Onderstepoort J Vet Res* **58**: 291–6.

Frigg M, Broz J, Weber G (1983) Compatibility studies of ionophore anticoccidials with various antibiotics and chemotherapeutics in broiler chicks. *Archiv Fur Geflugelkunde* **47**: 213–20.

Galitzer SJ, Oehme FW (1984) A literature review on the toxicity of lasalocid, a polyether antibiotic. *Vet Hum Toxicol* **26**: 322–26.

Galitzer SJ, Bartley FF, Oehme FW (1982) Preliminary studies on lasalocid toxicosis in cattle. *Vet Hum Toxicol* **24**: 406–9.

Galitzer SJ, Kruckenburg SM, Kidd JR (1986a) Pathologic changes associated with experimental lasalocid and monensin toxicosis in cattle. *Am J Vet Res* **47**: 2624–6.

Galitzer SJ, Oehme FW, Bartley EE, Dayton AD (1986b) Lasalocid toxicity in cattle: acute clinicopathological changes. *J Anim Sci* **62**: 1308–16.

Gossett FO, Gibson WR, Koenig GR, Marroquin F, Young SS, Worth HM, Morton DM (1975) Dietary relay studies in dogs and rats to evaluate the safety of tissues from cattle fed Rumensin® (mycelial monensin sodium). Lilly Research Laboratories. Submitted to the European Medicines Agency CVMP by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Gough AW, Pinn S, Hulland TJ, Thomson RG, de la Iglesia F (1981) Spontaneous cardiomyopathy: histopathologic and ultrastructural alterations in turkey heart tissue. *Am J Vet Res* **42**: 1290–7.

Griffin T, Ryback ME, Recht L, Singh M, Raso V (1993) Potentiation of anti-tumor immunotoxins by liposomal monensin. *J Natl Cancer Inst* **85**: 292–8.

Gumila C, Ancelin ML, Delort AM, Jeminet G, Vial HJ (1997) Characterization of the potent in vitro and in vivo antimalarial activities of ionophore compounds. *Antimicrob Agents Chemothe* **41**: 523–9.

Hall JO (2001) Toxic feed constituents in the horse. *Vet Clin North Am Equine Pract* **17**: 1098–100.

Halvorson DA, Van Dijk C, Brown P (1982) lonophore toxicity in turkey breeders. *Avian Dis* **26**: 634–9.

Hamidinia SA, Shimelis OI, Tan B, Erdahl WL, Chapman CJ, Renkes GD, Taylor RW, Pfeiffer DR (2002) Monensin mediates a rapid and selective transport of $Pb^{2+}$ possible application of monensin for the treatment of $Pb^{2+}$ intoxication. *J Biol Chem* **277**: 3811–20.

Hammond AC, Carlson JR, Breeze RG (1980) Prevention of tryptophan-induced acute bovine pulmonary edema and emphysema. *Vet Rec* **107**: 322–5.

Hanley HG, Slack JD (1982) Pharmacology of lasalocid. *In Polyether Antibiotics* Westley JW (ed.). Marcel Dekker, New York, pp. 341–95.

Hanrahan LA, Corner DF, Naqi SA (1981) Monensin toxicosis in broiler chickens. *Vet Pathol* **18**: 665–71.

Hanson LJ, Eisenbeis HG, Givens SV (1981) Toxic effect of lasalocid in horses. *Am J Vet Res* **42**: 456–61.

Hazlett MJ, Houston DM, Maxie MG, Van Dreumel T, Ramsey J (1992) Monensin/roxarsone contaminated dog food associated with myodegeneration and renal medullary necrosis in dogs. *Can Vet J* **33**: 749.

Herberg R, Manthey J, Richardson L, Cooley C, Donoho A (1978) Excretion and tissue distribution of $^{14}C$ monensin in cattle. *J Agric Food Chem* **26**: 1087–90.

Heuer C, Schuken YH, Jonker, LJ, Wilkinson JID, Noorhuizen JPTM (2001) Effect of monensin on blood ketones bodies, incidence and recurrence of disease and fertility in dairy cows. *J Dairy Sci* **84**: 1085–97.

Holland DR (1978) Cardiovascular and respiratory effects of sodium monensin, IV and PO, in conscious dogs. Lilly Research Laboratories. Submitted to the European Medicines Agency CVMP by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Hormazabal V, Yndestad M, Ostensvik O (2002) Determination of amprolium, ethopabate, lasalocid, monensin, narasin, and salinomycin in feed by liquid chromoatography-mass spectrometry. *J Liq Chrom Rel Technol* **25**: 2655–63.

Horrox ME (1980) Monensin–tiamulin interaction risk to poultry. *Vet Rec* **106**: 178.

Hubert LC, Oehme FW (1968) *Plants Poisonous to Livestock*. 3rd edn, Kansas State University Printing Service, Manhattan, KS.

Jones TC, Overstreet RM, Lotz JM, Frelier FP (1994) *Paraphidioidina scolecoides* n. sp., a new aseptate gregarine from cultured Pacific white shrimp *Pennaeus vannamei Dis Aquat Org* **19**: 67–75.

Julian RJ, Mirsalimi SM, Bagley LG, Squires EJ (1992) Effect of hypoxia and diet on spontaneous cardiomyopathy (round-heart disease). *Avian Dis* **36**: 1043–7.

Kingsbury JM (1964). *Poisonous plants of the United States and Canada*. Prentice-Hall, Inc., Englewood Cliffs, NJ.

Kennington AS, Darby JM, Ehrenfried KM, Kiehl DE, Moran JW, Sweeney DJ (1995) [$^{14}$C]Monensin milk and tissue residues/metabolism in dairy cows. Submitted to the European Medicines Agency CVMP by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.{net}

Kouyoumdjian JA, Morita MD, Sato AK, Pissolatti AF (2001) Fatal rhabdomyolysis after acute sodium monensin (Rumensin) toxicity: case report. *Arq Neuropsiquiatr* **59**: 596–8.

Kyriakis SC (1989) The effect of monensin against swine dysentery. *Br Vet J* **143**: 373–7.

Kyriakis SC, Vlemmas JC, Mavromatis JC, Tsinas AC, Lekkas SG, Tsangaris TA (1993) The effect of monensin in the control of transmissible gastroenteritis (TGE) of pigs. *Swine Health Prod* **1**: 15–8.

Lin JA (1995) Mass salinomycin toxicity death due to concomitant use of tiamulin in a breeder flock. *Taiwan J Vet Med Anim Husb* **65**: 339–46.

Long P, Jeffers TK (1982) Studies on the stage of action of ionophorous antibiotics against Eimeria. *J Parasitol* **68**: 363–71.

Lowe BL, Ball GJ, Carruthers VR, Dobos RC, Lynch GA, Moate PJ, Poole PR, Valentine SC (1991) Monensin controlled-release intraruminal capsule for control of bloat in pastured dairy cows. *Aust Vet J* **68**: 17–20.

Maronpot RR, Bucci TJ, Stedham MA (1968) Focal degenerative myopathy in turkeys. *Avian Dis* **12**: 96–103.

Matsuoka T (1976) Evaluation of monensin toxicity in the horse. *J Am Vet Med Assoc* **169**: 1098–100.

Matsuoka T, Novilla MN, Thomson TD, Donoho AL (1996) Review of monensin toxicosis in horses. *J Equine Vet Sci* **16**: 8–15.

Mazlum Z, Pickles RW, Pradella G, Pagnani R (1985) Interaction between monensin, narasin, or salinomycin and the antibiotics erythromycin, chloramphenicol, or tylosin in broiler chicks. *La Clinica Veterinaria* **108**: 95–104.

Meingassner JG, Schmook FP, Czok R, Mieth H (1979) Enhancement of the anticoccidial activity of polyether antibiotics in chickens by tiamulin. *Poult Sci* **58**: 308–13.

Meldrum JB, Evans RD, Robertson JL, Watkins KL, Novilla MN (2000) Alterations in levels of various antioxidant factors in turkey knockdown syndrome. *Avian Dis* **44**: 891–5.

Mehlhorn H, Pooch H, Raether W (1983) The action of polyether ionophorous antibiotics (monensin, salinomycin, lasalocid) on

developmental stages of *Eimeria tenella* (Coccidia, Sprozoa) in vivo and in vitro: study by light and electron microscopy. *Z Parasitenkd* **69**: 457–71.

Meral I, Hsu W, Hembrough FB (2002) Digoxin- and monensin-induced changes of intracellular $Ca^{2+}$ concentration in guinea-pig ventricular myocyte. *J Vet Med A* **49**: 329–33.

Miller DJS, O'Connor JJ, Roberts NL (1986) Tiamulin/salinomycin interactions in pigs. *Vet Rec* **118**: 73–5.

Mitema ES, Sangiah S, Martin T (1988) Effects of some calcium modulators on monensin toxicity. *Vet Hum Toxicol* **30**: 409–13.

Mollenhauer HH, Rowe LD, Cysewski SJ, Witzel DA (1981) Ultrastructural observations in ponies after treatment with monensin. *Am J Vet Res* **42**: 35–40.

Mollenhauer HH, Rowe LD, Witzel DA (1984) Effect of monensin on the morphology of mitochondria in rodent and equine striated muscle. *Vet Hum Toxicol* **26**: 15–9.

Mollenhauer HH, Morre DJ, Rowe RD (1990) Alteration of intracellular traffic by monensin: mechanism, specificity and relationship to toxicity. *Biochim Biophys Acta* **1031**: 225–46.

Mubagwa K, Lin W, Sipido K, Bosteels S, Flameng W (1997) Monensin-induced reversal of positive force-frequency relationship in cardiac muscle: role of intracellular sodium in reas-dependent potentiation of contraction. *J Mol Cell Cardiol* **29**: 977–89.

Muylle E, Vandenhende C, Oyaert W, Thoonen H, Vlaeminck K (1981) Delayed monensin sodium toxicity in horses. *Equine Vet J* **13**: 107–8.

Nagaraja TG, Avery TB, Bartley EE, Roof SK, Dayton AD (1982) Effect of lasalocid, monensin and thiopeptin on lactic acidosis in cattle. *J Anim Sci* **54**: 649–58.

Nation PN, Crowe SP, Harries WN (1982) Clinical signs and pathology of accidental monensin poisoning in sheep. *Can Vet J* **23**: 323–6.

Nebbia C, Ceppa L, Dacasto M, Carletti M, Nachtmann C (1997) Evidence for CYP3A involvement in the hepatic microsomal metabolism of monensin. *J Vet Pharmacol Therap* **20**(Suppl. 1): 89–90.

Nebbia C, Ceppa L, Dacasto M, Nachtmann C, Carletti M (2001) Oxidative monensin metabolism and cytochrome P450 3A content and functions in liver microsomes from horses, pigs, broiler chicks, cattle and rats. *J Vet Pharmacol Therap* **24**: 399–403.

Newsholme SJ, Howerth EW, Bastianello SS, Prozesky L, Minne JA (1983). Fatal cardiomyopathy in feedlot sheep attributed to monensin toxicosis. *J South Afr Vet Assoc* **54**: 29–32.

Nocerini MR, Honeyfield DC, Carlson JR, Breeze RG (1985) Reduction in 3-methylindole production and prevention of acute bovine pulmonary edema and emphysema with lasalocid. *J Anim Sci* **60**: 232–8.

Novilla MN (1992) The veterinary importance of the toxic syndrome induced by ionophores. *Vet Hum Toxicol* **34**: 66–70.

Novilla MN (2004) Expert report on the safety file for monensin sodium. Submitted to the European Medicines Agency CVMP by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Novilla MN, Folkerts TM (1986) Ionophores: monensin, lasalocid, salinomycin, narasin. In *Current Veterinary Therapy-Food Animal Practice*, Howard JL (ed.). Academic Press, New York, pp. 359–63.

Novilla MN, Todd GC (1991) Cardiotoxicity of the ionophores rat. In *Monographs on Pathology of Laboratory Animals Cardiovascular and Musculoskeletal Systems*, Jones TC, Mohr U, Hunt RD (eds). Springer Verlag, Berlin.

Novilla MN, Owen NV, Todd GC (1994) The comparative toxicology of narasin in laboratory animals. *Vet Hum Toxicol* **36**: 318–23.

Oehme F, Pickrell J (1997) An analysis of the chronic oral toxicity of polyether ionophore antibiotics. *Vet Hum Toxicol* **41**: 251–7.

Ordidge RM, Schubert FK, Stoker JW (1979) Death of horses after accidental feeding of monensin. *Vet Rec* **104**: 375.

Osborne MW, Wenger JJ, Zanko MT (1977) The cardiovascular pharmacology of the antibioticionophore Ro2–2985 (X537A). *J Pharmacol Exp Ther* **200**: 195–205.

Parker RJ, Jones GW, Ellis KJ, Heater KM, Schroter KL, Tyler R, Holroyd RG (1986) Post-weaning coccidiosis in beef calves in the dry tropics: experimental control with continuous monensin supplementation via intraruminal devices and concurrent epidemiological observations. *Trop Anim Hlth Prod* **18**: 198–208.

Peek SF, Marques FD, Morgan J, Steiberg H, Zoromski DW, McGuirk S (2004) Atypical acute monensin toxicosis and delayed cardiomyopathy in Belgian draft horses. *J Vet Intern Med* **18**: 761–4.

Perelman B, Perak M, Smith B (1993) Effect of accidental feeding of lasalocid sodium in broiler breeder chickens. *Vet Rec* **132**: 271–3.

Pott JM, Skov B (1981) Monensin/tiamulin interactions in pigs. *Vet Rec* **109**: 545.

Potter EL, Van Duyn RL, Cooley CO (1984) Monensin toxicity in cattle. *J Anim Sci* **58**: 1499–511.

Plumlee KH, Johnson B, Galey FD (1995) Acute salinomycin toxicosis of pigs. *J Vet Diagn Invest* **7**: 419–20.

Pressman BC (1976) Biological applications of ionophores. *Annu Rev Biochem* **45**: 501–30.

Pressman BC, Fahim NI (1982) Pharmacology and toxicology of the monovalent carboxytic ionophores. *Annu Rev Pharmacol Toxicol* **22**: 465–90.

Pressman BC, Fahim NI (1983) Cardiovascular toxicity of ionophores used as feed additives. *Adv Exp Md Biol* **161**: 543–61.

Reed PW (1982) Biochemical and biological effects of carboxylic acid ionophores. In *Polyether Antibiotics*, Westley JW (ed.). Marcel Dekker, New York, pp. 185–302.

Reed PW, Bokoch GM (1982) Cardiovascular and renal effects of A23187 and monovalent polyether antibiotics. In *Polyether Antibiotics*, Westley JW (ed.). Marcel Dekker, New York, pp. 369–95.

Reichenback DD, Benditt EP (1982) Catecholamines and cardiomyopathy: the pathogenesis and potential importance of myofibrillar degeneration. *Human Pathol* **1**: 125–50.

Richardson LF, Raun AP, Potter EL, Cooley CO, Rathmacher RP (1976) Effect of monensin on rumen fermentation in vitro and in vivo. *J Anim Sci* **43**: 1501–8.

Riddell FG (2002) Structure, conformation, and mechanism in the membrane transport of alkali metal ions by ionophoric antibiotics. *Chirality* **14**: 121–5.

Rollinson J, Taylor FGR, Cheeney J (1987) Salinomycin poisoning in horses. *Vet Rec* **121**: 126–8.

Ruff MD (1982) Veterinary applications. In *Polyether Antibiotics*, Westley JW (ed.). Marcel Dekker, New York, pp. 303–67.

Russell JB (1987) A proposed mechanism of monensin action in inhibiting ruminal bacterial growth: effects on ion flux and protonmotive force. *J Anim Sci* **64**: 1519–25.

Russell JB, Strobel HJ (1989) Mini-review: effect of ionophores on ruminal fermentation. *Appl Environ Microbiol* **55**: 1–6.

Saini RK, Hester RK, Somani P, Pressman BC (1979) Characterization of the coronary vasodilator and hemodynamic actions of monensin, a carboxylic ionophore. *J Cardiovasc Pharmacol* **1**: 123–38.

Safran N, Aisenberg I, Bark H (1993) Paralytic syndrome attributed to laslocid residues in a commercial ration fed to dogs. *J Am Vet Med Assoc* **202**: 1273–5.

Salles MS, Lombardo de Barros CS, Barros SS (1994) Ionophore antibiotic (narasin) poisoning in rabbits. *Vet Hum Toxicol* **36**: 437–44.

Salyi G, Szabo E, Bago G, Banhidi G, Szilagyi M (1988) Narasin poisoning in turkeys. *Acta Vet Hung* **36**: 107–14.

Sandercock DA, Mitchell MA (2003) Myopathy in broiler chickens: a role for $Ca^{2+}$-activated phospholipase $A_2$? *Poult Sci* **82**: 1307–12.

Sandercock DA, Mitchell MA (2004) The role of sodium ions in the pathogenesis of skeletal muscle damage in broiler chickens. *Poultry Sci* **83**: 701–6.

Segev G, Baneth G, Levitin B, Shlosberg A, Aroch I (2004) Accidental poisoning of 17 dogs with lasalocid. *Vet Rec* **135**: 174–6.

Schweitzer D, Kimberling C, Spraker T, Sterner FE, McChesney AE (1984) Accidental monensin sodium intoxication in feedlot cattle. *J Am Vet Med Assoc* **184**: 1273–6.

Shier WT, DuBourdieu DJ (1992) Sodium- and calcium-dependent steps in the mechanism of neonatal rat cardiac myocyte killing by ionophores. *Toxicol Appl Pharmacol* **116**: 38–46.

Shaik MS, Ikediobi O, Turnage VD, McSween J, Kanikkannan N, Singh M (2001) Long-circulating monensin nanoparticles for the potentiation of immunotoxin and anticancer drugs. *J Pharm Pharmacol* **53**: 617–27.

Shlosberg A, Weisman Y, Klopper U, Perl S (1985) Neurotoxic action of lasalocid at high doses. *Vet Rec* **117**: 394.

Shlosberg A, Harmelin A, Perl S, Pano G, Davidson M, Orgad U, Kali U, Bor A, Van Ham M, Hoida G, Yakobson B, Avidar Y, Isaeli B-A, Bogin E (1992) Cardiomyopathy in cattle induced by residues of the coccidistat maduramycin in poultry litter given as a feedstuff. *Vet Res Commun* **16**: 45–58.

Siller WG (1985) Deep pectoral myopathy: a penalty of successful selection for muscle growth. *Poultry Sci* **64**: 1591–5.

Simpson CF, Damron BL, Harms RH (1971) Toxic myopathy of chicks fed *Cassia occidentalis* seeds. *Avian Dis* **15**: 284–90.

Singal PK, Yates JC, Beamish RE, Beamish NS (1981) Influence of reducing agents on adrenochrome-induced changes in the heart. *Arch Pathol Lab Med* **105**: 664–9.

Smith CK, Galloway RG (1983) Influence of monensin on cation influx and glycolysis of *Eimeria tenella* sporozoites in vitro. *J Parasitol* **69**: 666–70.

Smith II CK, Strout RG (1979) *Eimeria tenella*: accumulation and retention of anticoccidial ionophores by extracellular sporozoites. *Exp Parasitol* **48**: 325–30.

Smith II CK, Strout RG (1980) *Eimeria tenella*: effect of narasin, a polyether antibiotic on the ultrastructure of intracellular sporozoites. *Exp Parasitol* **50**:426–36.

Smith CK, Galloway RB, White SL (1981) Effect of ionophores on survival, penetration, and development of *Eimeria tenella* sporozoites *in vitro*. *J Parasitol* **67**: 511–6.

Stanfield DG, Lamont MN (1981) Monensin–tiamulin interactions in pigs. *Vet Rec* **109**: 545.

Stockdale PHG (1981) Effects of monensin on coccidiosis in ruminants. *Vet Med Small Anim Clin* November, 1575–8.

Stuart JC (1978) An outbreak of monensin poisoning in adult turkeys. *Vet Rec* **102**: 303–4.

Sutko JL, Besch Jr HR, Bailey JC, Zimmerman G, Watanabe AM (1977) Direct effects of the monovalent cation ionophores monensin and nigericin on myocardium. *J Pharmacol Exp Therap* **203**: 685–700.

Swayne DE, Shlosberg A, Davis RB (1986) Salt poisoning in turkey poults. *Avian Dis* **30**: 814–52.

Szűcs G, Tamasi V, Laczay P, Monostory K (2004) Biochemical background of toxic interaction between tiamulin and monensin. *Chemico-biological interactions* **147**: 151–61.

Taylor RW, Kauffman RF, Pfeifer DR (1982) Cation complexation and transport by carboxylic acid ionophores. In *Polyether Antibiotics*, Westley JW (ed.). Marcel Dekker, New York, pp. 103–84.

Tappel AL (1981) Vitamin E and selenium protection from to vitro lipid peroxidation. *Ann NY Acad Sci* **355**: 18–31.

Thomas EE, Smith CK, McGuffey RK, Quinn ME (1985) Monensin provides coccidiosis control: site and mode of action. Tech Talk Scientific Update from Elanco Animal Health, A Division of Eli Lilly and Company, Indianapolis, IN, USA.

Todd GC, Meyers DB, Pierce EC, Worth HM (1970) Acute reversible myopathy produced by compound A204. *Antimicrob Agents Chemother* 361–5.

Todd OC, Novilla MN, Howard LC (1984) Comparative toxicology of monensin sodium in laboratory animals. *J Anim Sci* **58**: 1512–17.

Trump BF, Berezesky IK, Smith MW, Phelps PC, Elliget KA (1989) The relationship between cellular ion deregulation and acute and chronic toxicity. *Toxicol Appl Pharmacol* **97**: 6–22.

Tsuchida K, Otomo S (1990) Electrophysiological effects of monensin, a sodium ionophore, on cardiac Purkinje fibers. *Eur J Pharmacol* **190**: 313–20.

Umemura T, Nakamura H, Goryo M, Itakura C (1984) Ultrastructural changes of monensin-oleandomycin myopathy in broiler chicks. *Avian Pathol* **13**: 743–51.

Umemura T, Nakamura H, Goryo M, Itakura C (1995) Ultrastructural changes of monensin oleandomycin myopathy in broiler chicks. *Avian Pathol* **13**: 743–51.

Van Halderen A, Bastianello SS, Fourie N, Zumpt IF (1993) An outbreak of narasin poisoning in swine. *J South Afr Vet Assoc* **64**: 43–6.

Van der Linde-Sipman JS, Van den Ingh TSGAM, Van Es JJ, Verhagen H, Kersten JGTM, Beynen AC, Plekkringa R (1999) Salinomycin-induced polyneuropathy in cats: morphologic and epidemiologic data. *Vet Pathol* **36**: 152–6.

Van Vleet JF (1986) Interactions of nutritional status and ionophore feed additives in animals. *Proceedings of 6th International Conference on Production Diseases in Farm Animals*, Belfast, Northern Ireland, pp. 268–76.

Van Vleet JF, Ferrans VJ (1983) Ultrastructural myocardial alterations in monensin toxicosis of cattle. *Am J Vet Res* **44**: 1629–36.

Van Vleet JF, Ferrans VJ (1984) Ultrastructural alterations in skeletal muscle of pigs with acute monensin toxicosis. *Vet Pathol* **114**: 461–71.

Van Vleet JF, Amstutz HE, Weirich WE, Rebar AH, Ferrans VJ (1983a) Acute monensin toxicosis in swine: effect of graded doses of monensin and protection of swine by pretreatment with selenium-vitamin E. *Am J Vet Res* **44**: 1460–8.

Van Vleet JF, Amstutz HE, Weirich WE, Rebar AH, Ferrans VJ (1983b) Clinical clinicopathological and pathologic alterations of acute monensin toxicosis in swine. *Am J Vet Res* **44**: 1469–75.

Van Vleet JF, Amstutz HE, Weirich WE, Rebar AH, Ferrans VJ (1983c) Clinical, clinicopathologic and pathologic alterations in acute monensin toxicosis in cattle. *Am J Vet Res* **44**: 2133–44.

Van Vleet JF, Runnels LJ, Cook JR, Scheidt AB (1987) Monensin toxicosis in swine: potentiation by tiamulin administration and ameliorative effect of treatment with selenium and/or vitamin E. *Am J Vet Res* **48**: 1520–3.

Van Vleet JF, Amstutz HE, Rebar AH (1985) Effect of pretreatment with selenium-vitamin E on monensin toxicosis in cattle. *Am J Vet Res* **46**: 2221–8.

Van Vleet JF, Ferrans VJ, Herman E (1991) Cardiovascular and skeletal muscle system. In *Handbook of Toxicologic Pathology*, Hascheck WM, Rousseaux CG (eds). Academic Press, San Diego, CA, pp. 539–624.

Wages DP (1993) Turkey knockdown is a multi-faceted puzzle. *Turkey World* **69**: 24–5.

Wages DP, Ficken MD (1988) Skeletal muscle lesions in turkeys associated with the feeding of monensin. *Avian Dis* **32**: 583–6.

Wanner M (1984) Unvertraglichkeit von Tiamulin und Salinomycin beim Schwein. Schweiz. *Arch Tierheilk* **126**: 521–6.

Weisman J, Herz A, Jegana J, Egyed M, Shlosberg A (1983) The effect of tiamulin administered by different routes and at different ages in turkeys receiving monensin in their feed. *Vet Res Comm* **6**: 189–98.

Weisman J, Shkap I, Egyed M, Shlosberg A (1984) Chloramphenicol induced monensin toxicity. *Refuah Vet* **41**: 3–6.

Weisman J, Shlosberg A, Egyed M (1980) Acute poisoning in turkeys caused by incompatibility of monensin and tiamulin. *Vet Res Comm* **4**: 231–5.

Whitlock RH, White NA, Rowland GN, Plue R (1978) Monensin toxicosis in horses; clinical manifestations. *Proc Am Assoc Equine Practnrs* **24**: 473–86.

Wilson JS (1980) Toxic myopathy in a dog associated with the presence of monensin in dry food. *Can Vet J* **21**: 30–1.

Wilson BW, Nieberg PS, Buhr RJ, Kelley BJ, Shultz FT (1990) Turkey muscle growth and focal myopathy. *Poult Sci* **69**: 1553–62.

Witkamp RP, Nijmeijer SM, Monshouwer M, Van Miert ASJPAM (1995) The antibiotic tiamulin is a potent inducer and inhibitor of cytochrome P450A via the formation of a stable metabolic intermediate complex. *Drug Metab Dispos* **23**: 542–7.

Witkamp RP, Nijmeijer SM, Van Miert ASJPAM (1996) Cytochrome P450 complex formation in rat liver by the antibiotic tiamulin. *Antimicrob Agents Chemother* **40**: 50–4.

Yates JC, Beamish RF, Dhalla NS (1981) Ventricular dysfunction and necrosis produced by adrenochrome metabolite of epinephrine: relation to pathogenesis of catecholamine cardiomyopathy. *Am Heart J* **102**: 210–21.

C H A P T E R

# 84

# Nonprotein nitrogen (urea) and hyperammonemia

*Patricia A. Talcott*

## INTRODUCTION AND BACKGROUND

Nonprotein nitrogen (NPN) intoxication is not an uncommon occurrence in ruminants. It has long been recognized that ruminal microbes of cattle and sheep can utilize NPN to synthesize proteins that can replace a portion of their total dietary protein requirements. Given that feeding NPN sources are typically cheaper than feeding expensive true protein, the practice of adding NPN to ruminant's diets is relatively commonplace. Feed grade urea ($CO[NH_2]_2$) is the cheapest, most effective, and most available nitrogen source. Other NPN compounds, which may be less readily available and more expensive, include ammonium acetate ($CH_3CO_2NH_4$), ammonium bicarbonate ($NH_4HCO_3$), ammonium carbamate ($NH_2CO_2NH_4$), ammonium lactate ($CH_3CHOHCO_2NH_4$), ammonium sulfate ($[NH_4]_2SO_4$), monoammonium phosphate ($NH_4H_2PO_4$), biuret ($NH_2CONHCONH_2$), dicyanodiamide ($NH_2C[NH]NHCN$), and diammonium phosphate ($[NH_4]_2HPO_4$). All these NPN sources consist of varying nitrogen content and protein equivalent; so addition of these products into the diet requires stringent nutritional dietary examination. Many of these supplements are solids and can be directly mixed into the mineral or grain component of the diet. Some, such as urea, can be added to molasses-based liquid products or into solid mineral blocks. Ammoniated beet pulp, citrus pulp, straw, silage, molasses, and rice hulls have also been used to supplement the protein content of a ruminant's diet. Ruminants can also be poisoned by gaining access to some fertilizers that can contain over 40% urea.

## TOXICITY

All mammalian species are potentially susceptible to NPN poisoning via the consumption of ammonium-containing feeds, if the dose is high enough. When urea is added to the ruminant's diet, it is rapidly broken down by bacterial urease in the rumen to ammonia. The ammonia is then utilized by the rumen bacteria, along with soluble carbohydrates, to synthesize amino acids and proteins (hence the name "nonprotein nitrogen"). Most of the excess ammonia produced is protonated to the ammonium ($NH_4$) ion and is trapped in the rumen. If this excess ammonia production continues, the rumen pH increases. When ammonia concentrations become too high too quickly, significant amounts get absorbed systemically. In the bloodstream, ammonia travels via the portal circulation to the liver where it is incorporated into the urea cycle and urea is then excreted in the urine via the kidneys or secreted into the saliva. Toxicities occur when this entire system becomes overwhelmed resulting in a clinical significant hyperammonemia. Excess ammonia has been shown to inhibit the citric acid cycle (Hatch, 1977), to cause a lactic acidosis, and to interfere with cerebral energy metabolism and the sodium–potassium ATPase pump. The systemic metabolic acidosis is thought to be related to a hyperkalemia, and it is this abnormality that can ultimately lead to cardiac arrest.

Factors that potentially can predispose ruminants to poisonings with these compounds include low energy diets, high rumen pH (e.g., rumen impaction), elevations

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in body temperature (enhances urease activity), dehydration, stress, concurrent disease, alterations in rumen microflora, and hepatic insufficiency. The lack of a readily available supply of soluble carbohydrates markedly affects the rumen bacteria's ability to utilize the ammonia. It is generally recommended to slowly introduce increasing levels of NPN in the diet over a period of several days to allow the rumen bacteria to adapt to the ammonia source. Most poisonings in ruminants occur as a result of inadequate implementation of an adaptation period, improper mixing of the supplement into the diet, and unlimited/unrestricted access to palatable liquid NPN supplements. NPN poisoning in monogastrics is not common.

Toxic and lethal doses are difficult to establish in ruminants due to all the predisposing factors that can enhance or decrease their sensitivity to these compounds. The oral lethal dose for urea in horses is 4.0 g/kg BW and the oral lethal dose for ammonia salts is 1.5 g/kg BW (Hintz *et al.*, 1970). A reported oral toxic dose for urea in nonacclimated cattle is 0.44 g/kg BW (Word *et al.*, 1960), whereas a lethal dose is in the 1.0–1.5 g/kg BW range (Osweiler *et al.*, 1976). Adapted cattle can tolerate much higher levels in their diets. Biuret is considered one of the safest of NPN compounds, and a reported lethal dose in cattle is 8.0 g/kg BW (Haliburton and Morgan, 1989). A single dose of 116 g of urea caused clinical signs of toxicity in cattle, whereas a dose of 57 g did not (Dinning *et al.*, 1948). Davis and Roberts (1959) reported that 0.3 g/kg BW urea was toxic to unacclimated cattle.

## CLINICAL SIGNS

The onset of clinical signs following introduction of a toxic NPN source into an animal's diet can be extremely quick; frequently within 30 min. A delay in the onset of clinical signs can be due to a wide variety of factors, including dose, method of introduction, and degree of adaptation. In general, most cases of NPN poisoning in ruminants involve some history of some type of "recent" feed change. Commonly reported clinical signs include uneasiness and ataxia, muscle tremors, excessive salivation, weakness, labored breathing, abdominal pain, and bloat. The progression of clinical signs is generally quite rapid and recumbency, convulsions and death typically ensue within 4 h. Hyperthermia, marked jugular pulse, cardiac arrhythmias, vomiting/regurgitation, and cyanosis have also been reported (Haliburton and Morgan, 1989). Rarely do animals survive once clinical signs start without some type of rapid treatment intervention.

## DIAGNOSTIC CRITERIA

The diagnosis of NPN intoxication rests mainly on a compatible history of exposure to a NPN source, compatible clinical signs with rapid progression to death, and clinical diagnostic laboratory data. Rarely do you find significant postmortem changes. Common findings at necropsy include generalized congestion of visceral organs, evidence of bloat, and mild to moderate pulmonary edema. Subepicardial and myocardial hemorrhages have also been reported. There are several diagnostic aids that can assist the veterinarian in confirming an NPN poisoning.

First, assessing the rumen pH either antemortem or shortly after death is a very useful tool. In animals succumbing to NPN intoxication, rumen pH is typically greater than 8–10. A markedly elevated rumen pH postmortem will gradually revert to normal over time due to continuous microbial activity postmortem. Other causes of excessive rumen pH can include rumen impactions or other problems leading to a "dead" rumen, or excessive salivary secretions.

Elevated ammonia concentrations in various biological specimens (e.g., serum, plasma, ocular fluid, and abomasal or rumen fluid) can be analytically confirmed by various methods. All samples should be collected and frozen immediately for delivery to the diagnostic laboratory, to prevent loss of the highly volatile ammonia. Samples collected postmortem should be retrieved shortly after death; decomposition of tissues can cause either increases or decreases in ammonia levels which can muddle the interpretation. Ammonia concentrations typically greater than 1.0 mg/dl in serum or plasma and greater than 80 mg/dl in rumen or abomasum contents are diagnostically significant.

Lastly, ammonia, nitrogen, or urea levels can be assessed in the "suspect" feed source. These analyses should be quantitative, and should assist the veterinarian in determining whether a mixing error was responsible for the clinical problem.

## TREATMENT

Rapid intervention is necessary in order for affected animals to survive. This is rarely possible except in experimental settings. Poisonings generally occur on a large scale (rarely a single animal problem) and in a setting (e.g. feedlots, pastures typically located in more rural settings) where it is nearly impossible to implement appropriate rapid therapies. Treatments are generally thought to be effective if initiated within 20 min after the onset of clinical signs, and prognosis is poor for recumbent animals.

Five percent acetic acid (i.e., vinegar) should be orally infused into the affected animal; 2–6 l in cattle and 0.5–1.0 l in sheep and goats (Lloyd, 1981). This should be immediately followed by a large volume (5–10 gallons) of cold water. The acetic acid will lower the rumen pH and shift the concentrations of ammonia to the ammonium ion, which will slow down the systemic absorption of $NH_3$. The cold water will lower the rumen temperatures and slow down the urease enzyme, responsible for the hydrolysis of urea to ammonia. Animals that respond to this treatment may relapse. If this should occur, the drench should be cut in half. Recumbent and convulsing animals respond poorly to this treatment. Other nonspecific, supportive therapies may include anticonvulsant therapy (e.g., phenobarbital, pentobarbital) and intravenous fluid therapy to correct fluid deficits and any existing elemental or electrolyte abnormalities.

Animals that do recover from the acute insult do not appear to suffer any recognizable long-term effects, though abortions have been reported to occur in pregnant animals.

## AMMONIA

NPN poisonings resulting in hyperammonemia should not be confused with ammonia poisoning. Synonyms include ammonia gas, anhydrous ammonia, and liquid ammonia. Livestock can be poisoned by ammonia, either through inhalation or ingestion, through its use as a fertilizer. Ammonia readily dissolves in water to form ammonium hydroxide. Anhydrous ammonia is colorless and highly irritating that causes corrosive injury to the mucous membranes of the eyes, lungs, and skin. Inhalation of ammonia can lead to severe bronchiolar and alveolar edema, and airway destruction that can result in respiratory failure and death. Dermal contact or oral exposures lead to corrosive skin lesions and corrosive damage to the mucosal lining of the oral cavity, esophagus and stomach.

## CONCLUSIONS

All mammalian species are susceptible to urea/ammonia poisoning. Though poisoning occurs with a greatest frequency in cattle by consumption of the contaminated feed. Diagnosis is based on clinical signs, and analysis of the body tissues/fluids and feed for urea and ammonia levels. Treatment includes oral administration of acetic acid (vinegar) and symptomatic and supportive therapies.

## REFERENCES

Davis GK, Roberts HF (1959) Urea toxicity in cattle. Gainesville, FL, *Agricultural Experiment Station, Bulletin 611*.

Dinning JS, Briggs HM, Gallup WD, Orr HW, Butler R (1948) Effect of orally administered urea on the ammonia and urea concentration in the blood of cattle and sheep, with observations on blood ammonia levels associated with symptoms of alkalosis. *Am J Physiol* **153**: 41–6.

Haliburton JC, Morgan SE (1989) Nonprotein nitrogen-induced ammonia toxicosis and ammoniated feed toxicity syndrome. *Vet Clin North Am Food Anim Pract* **5**(2): 237–49.

Hatch RC (1977) Veterinary toxicology. In *Veterinary Pharmacology and Therapeutics*, Jones LM, Booth LF (eds). The Iowa State University Press, Ames, IA, pp. 1253–9.

Hintz HF, Lowe JE, Clifford AJ, Visek WJ (1970) Ammonia intoxication resulting from urea poisoning by ponies. *J Am Vet Med Assoc* **157**: 963–6.

Lloyd WE (1981) Urea and other nonprotein nitrogen sources. In *Current Veterinary Therapy. Food Animal Practice*, Howard JL (ed.). W.B. Saunders, Philadelphia, PA, pp. 393–6.

Osweiler GD, Carson TL, Buck WB, Van Gelder GA (1976) Urea and nonprotein nitrogen. In *Clinical and Diagnostic Veterinary Toxicology*. Kendall/Hunt Publishing Company, Dubuque, IA, pp. 160–6.

Word JD, Martin LC, Williams DL, Williams EI, Panciera RJ, Nelson TE, Tillman AD (1960) Urea toxicity studies in the bovine. *J Anim Sci* **29**: 786–91.

CHAPTER

# 85

# Water quality and contaminants

*Michael P. Carlson and Steve Ensley*

## INTRODUCTION

Water is essential for all forms of life. The availability of adequate quantities and quality of water partially dictates the types of life and numbers of individuals that an environment can sustain, and may be a limiting factor for livestock production systems. Less water may be available for animal production and what is available may be of poorer quality as water supplies for human use become limited. Large-scale animal production practices and emphasis on animal performance may increase the demands on water delivery systems and increase concern about water quality. Water quality generally refers the suitability of water for some purpose. Parameters used to assess water quality vary depending upon its proposed use, and may include color, odor, taste, bacterial content, mineral content, salinity and the amounts of inorganic or organic compounds.

The intent for this chapter is to provide the reader with information about water to aid in the assessment of water quality for use by companion and production animals. Some of the more readily accessible water quality standards applicable to drinking water for animals are highlighted. Detailed discussions of the mechanisms by which those chemicals cause adverse effects may be found elsewhere in this book. Information is also provided about the daily amounts of water required by animals as an aide in the assessment of cases of possible exposure of animals to water-borne poisons. Lastly, the risk assessment of blue-green algae poisoning is discussed in this chapter.

## THE ASSESSMENT OF WATER QUALITY

Water quality may be assessed upon several occasions: to determine if the available water is suitable for a particular purpose, to determine if there has been a significant change in water quality or to determine if adverse health effects that are occurring may be attributable to water contamination. Numerous commercial, governmental and academic laboratories offer water quality analytical services. The assessment of the analytical results from such laboratories requires some sort of standards against which to compare the results. National, state or provincial, or local governments may regulate water quality and water quality standards may be established by any or all of those governmental bodies. The quality of drinking water intended for consumption by humans is almost always more extensively regulated than for any other water use.

Reports of results of water quality analyses often include water quality standard ranges against which to assess the reported results. They may include graphical comparisons of the results to the water quality standards, making identification of parameters that fall outside of the standard limits easily identifiable. Such reports often use water quality standards intended for human drinking water. Assessment of the results of water quality analyses must be done using standards that are applicable for the intended use of the water and which are in force for the venue at which the water is to be used. The suitability of water intended for use as drinking water by animals is better assessed using drinking water standards for animals instead of standards for humans. Otherwise, animal owners may take unnecessary and costly actions to mitigate what are erroneously believed to be unacceptably high risk of some sort of adverse health effect.

## WATER QUALITY STANDARDS FOR ANIMALS

Water quality standards for animals are not the same as nor are they enforced with the same authority as are those

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for humans. Water quality recommendations for animals have been made by the USEPA (1973) and the National Academy of Sciences (NAS, 1974) in USA, and in Canada in 1999 (Canadian Council of Ministers of the Environment, 2005a, b). Table 85.1 lists water quality recommendations for livestock taken from each of those sources for selected chemicals. The toxic effects cited in the NAS document are also listed in that table to give the reader some idea of the adverse health effects for the chemicals that were known at the time the document was written. These publications include recommendations for water use by animals other than livestock. Recommendations for other animals are

**TABLE 85.1 Water quality recommendations for selected chemicals for livestock**

| Chemical | Toxic effects* | Upper concentration limit (mg/l) | | |
|---|---|---|---|---|
| | | USEPA (1973) | NAS (1974) | Canadian (CCME, 2005a) |
| Arsenic | Mice @ 5 mg/l: non-toxic through three generations, tissue accumulation.<br>Guinea pigs @ 1 mg/l: increased thyroid colloid. | 0.2 | 0.2 | 0.025 (interim) |
| Cadmium | Mice @ 5, 10 mg/l: reduced longevity at lower dose, second generation did not survive at higher dose.<br>Rats @ 5 mg/l: reduced longevity.<br>Rabbits @ 0.1 mg/l: swollen kidney epithelium @ 6 months.<br>Dos @ 0−5 mg/l: tissue concentration proportional to intake. | 0.050 | 0.05 | 0.08 |
| Calcium | – | – | – | 1000 |
| Chloride | – | – | – | – |
| Chromium | Rats, rabbits @ 500 mg/l, 25 mg $Cr^{6+}$/l: first concentration is maximum non-toxic level based on growth, second concentration decreased water intake and tissue content 9 times that when $Cr^{3+}$ was substituted. | 1.0 | 1.0 | 0.050 ($Cr^{III}$, $Cr^{VI}$) (interim) |
| Copper | Steers @ 12 g $CuSO_4 \cdot 5H_2O$/day as drench: body weight decrease, fatal in 65 days.<br>Turkeys @ 625 mg/l: decreased feed and water intake, fatal. | 0.5 | 0.5 | 1.0 (cattle), 5.0 (swine, poultry), 0.5 (sheep) |
| Fluoride | Calves and cattle @ 100 mg/l, 11.8 mg/l: first concentration decreased feed intake, growth and Ca absorption, bones decalcified; second concentration mottled teeth.<br>Sheep @ 5–20 mg/l: severe mottled teeth, decreased wool production, decreased health.<br>Hogs @ 6–10 mg/l: severe mottling of teeth.<br>Mice @ 10 mg/l: no innate toxicity. | 2.0 | 2.0 | 2.0, 1.0 (if also in feed) |
| Hardness | – | – | – | – |
| Iron | Cattle @ 17 mg/l: pasture irrigation water produced scouring, decreased body weight and milk production. | – | – | – |
| Lead | Calves @ 100 mg/l: died after 4 months of drinking $Pb(NO_3)_2$.<br>Rats and mice @ 5, 25 mg/l: lower concentration reproduction ceased in second generation, higher concentration hypertension and higher death rates due to infections. | 0.1 | 0.1 | 0.1 |
| Mercury (inorganic) | Humans @ 30 μg/l, 75–30 mg/l: lower concentration readily absorbed into tissues, higher concentrations fatal. | 1.0 | 0.010 | 0.003 |
| Nitrate (as N)<br>(as $NO_3^-$) | Studies cited, too numerous to list here, involved concentrations from 90 to 4158 mg nitrate–nitrogen/l in sheep, pigs, cattle, chickens, turkeys and guinea pigs. | 23 ($NO_3^-$ + $NO_2^-$)<br>100 ($NO_3^-$ + $NO_2^-$) | 100 ($NO_3^-$ + $NO_2^-$) 440 | 100 ($NO_3^-$ + $NO_2^-$) |
| Nitrite (as N)<br>(as $NO_2^-$) | Studies cited, too numerous to list here, involved concentrations from 20 to 240 mg nitrite–nitrogen/l in pigs, cockerels, poults, chicks, laying hens, turkeys, rats and guinea pigs. | 3.0<br>10 | 10<br>33 | 10 |

**TABLE 85.1 (Continued)**

| | | Upper concentration limit (mg/l) | | |
|---|---|---|---|---|
| **Chemical** | **Toxic effects*** | **USEPA (1973)** | **NAS (1974)** | **Canadian (CCME, 2005a)** |
| Selenium | Rats and mice @ 2–3 mg $Se^{4+}$/l, 2–3 mg $Se^{6+}$/l: first concentrations decreased growth and caused early deaths in males; later concentrations caused no effects on growth or longevity, but tumorigenic, third generation failed to survive. | 0.05 | – | 0.05 |
| Sulfate | Cattle @ 100 mg/l, 3590 mg/l: first concentrations decreased water and feed intake, and creatinine excretion. Laying hens @ 2700 mg $Na_2SO_4$/l: reduced egg production. | – | – | 1000 |
| Total dissolved solids | – | – | – | 3000 |
| Zinc | Humans @ 5 mg/l: emetic. Rats @ 5 mg/l: increased mortality with 11 ppm Se in diet. Hens @ 2320 mg/l: decreased water consumption; egg production stopped after 3 days; body weight decreased. | 25 | 25.0 | 50 |
| Microorganisms | – | 5000 coliforms/dl (average of ⩾two samples/month), 20,000/dl (individual sample) | – | – |
| Fecal coliforms | – | 1000/dl (average of ⩾two samples/month), 4000/dl (individual sample) | – | – |

*Summary of effects reported in Table 11 of NAS (1974) at the listed animals and concentration or dosage.

listed in Table 85.2. Readers may find them to be more applicable than the livestock recommendations in certain circumstances.

## USEPA water quality criteria for animals

The USEPA published proposed water quality standards for irrigation, livestock, aquatic life, wildlife, public freshwater, marine aquatic life and recreational water in 1973. The criteria were formulated and published by the USEPA pursuant to the Federal Water Pollution Control Act Amendments of 1972 and the Water Quality Act of 1965. The report states: "Almost all of the criteria are taken from the recommendations of the National Academy of Science's report on Water Quality Criteria (1974) developed under contract to the Environmental Protection Agency." As far as we have been able to determine, those recommendations have not been revised since their publication.

## National Academy of Sciences recommended limits in drinking water for livestock and poultry

The NAS report published in 1974 summarizes what was known at the time of publication about effects of nutrient and toxic substances that were found in water consumed by domesticated animals. It also contains information about water requirements and the percentages of recommended intake of various substances provided by normal daily water consumption, and toxic concentrations for various species. The publication includes a table summarizing the effects of toxic concentrations of various chemicals in water for various domestic and laboratory animals. The information included in those tables is too extensive to reproduce in this chapter. Readers are urged to consult them as needed.

An *ad hoc* committee of the National Research Council's Committee on Animal Nutrition reviewed the scientific literature related to minerals and toxic substances in the

diets and water for animals. Its findings and recommendations were published as a second revision of mineral tolerances of animals (NAS, 2005). Drinking water standards cited in that revision still refer to the 1974 NRC publication (NAS, 1974).

## Canadian environmental quality guidelines

Canadian water quality standards for agricultural use, including livestock, were first published in a document titled *Canadian Water Quality Guidelines* in 1987 by the

**TABLE 85.2 Water quality recommendations for selected chemicals (upper concentration limit in mg/l) for aquatic water systems**

| | Freshwater aquatic life | | Freshwater wildlife | Marine aquatic life | |
|---|---|---|---|---|---|
| **Chemical** | **Canadian (CCME, 2005b)** | **USEPA (1973)** | **USEPA (1973)** | **Canadian (CCME, 2005b)** | **USEPA* (1973)** |
| Arsenic | 0.005 | – | – | 0.0125 (interim) | 0.01 × 96 h $LC_{50}$; 0.5 |
| Cadmium | 0.000017 (interim) | 0.03 in hard water,<br>0.004 in soft water | – | 0.00012 | 0.01 × 96 h $LC_{50}$;<br>0.01 mg/l |
| Calcium | – | – | – | – | – |
| Chloride | – | – | – | – | – |
| Chromium | $Cr^{III}$: 0.0089 (interim)<br>$Cr^{VI}$: 0.001 | 0.03 | – | $Cr^{III}$: 0.056 (interim)<br>$Cr^{VI}$: 0.0015 | 0.01 × 96 h $LC_{50}$;<br>0.1 mg/l |
| Copper | 0.002 @ [$CaCO_3$] = 0–120 mg/l<br>0.003 @ [$CaCO_3$] = 120–180 mg/l<br>0.004 @ [$CaCO_3$] = > 180 mg/l | 0.1 is 96 h $LC_{50}$ | – | – | 0.01 × 96 h $LC_{50}$;<br>0.5 mg/l |
| Fluoride | – | | | – | 0.1 × 96 h $LC_{50}$; 1.5 mg/l |
| Hardness | – | – | – | – | – |
| Iron | 0.300 | – | – | – | 0.3 |
| Lead | 0.001 @ [$CaCO_3$] = 0–60 mg/l<br>0.002 @ [$CaCO_3$] = 60–120 mg/l<br>0.004 @ [$CaCO_3$] = 120–180 mg/l<br>0.007 @ [$CaCO_3$] = > 180 mg/l | 0.03 | – | – | 0.02 × 96 h $LC_{50}$;<br>0.1 $LD_{50}$ |
| Mercury (inorganic) | Inorganic: 0.000026<br>Methylmercury: 0.000004 (interim)<br>May not protect against bioaccumulation | 0.0002 (total)<br>0.00005 (average)<br>0.05 mg/kg (total body burden) | 0.5 mg/kg in fish | Inorganic: 0.000016 (interim)<br>Methylmercury: no recommendation | 0.01 × 96 h $LC_{50}$;<br>0.1 mg/l |
| Nitrate (as N)<br>(as $NO_3^-$) | 13 (interim) | – | – | 16 (interim) | – |
| Nitrite (as N)<br>(as $NO_2^-$) | 0.06 | – | – | – | – |
| Total dissolved solids | – | – | – | – | – |
| Selenium | 0.001 | – | – | – | 0.01 × 96 h $LC_{50}$;<br>0.01 mg/l |
| Sulfate | – | – | – | – | – |
| Zinc | 0.030 | | | – | |
| Microorganisms | – | – | 2000/dl | – | – |
| Fecal coliforms | – | – | 2000/dl | – | – |

*Recommended concentration limits are expressed as the fraction of either lethal concentration-50 ($LC_{50}$) or lethal dose-50 ($LD_{50}$). The concentration listed thereafter is the maximum concentration considered acceptable.

Canadian Council of Resource and Environment Ministers (CCREM). That organization was later renamed the Canadian Council of Ministers of the Environment (CCME). In April 1996, CCME began work that consolidated national environmental quality guidelines for water, soil, sediment, tissue residues and air into one document, which was published as *Canadian Environmental Quality Guidelines* by CCME. The guidelines are continually reviewed and updated as deemed necessary.

## DRINKING WATER QUALITY STANDARDS FOR HUMANS

Drinking water quality standards for selected chemicals that are published by the USEPA (1973, 2004), Canada (Federal-Provincial-Terrestrial Committee on Drinking Water, 2004) and the World Health Organization (WHO, 2004) are listed in Table 85.3. They are included in this

**TABLE 85.3 Water quality recommendations for human drinking water: selected contaminants**

| Body establishing guidelines | Maximum acceptable concentration (mg/l) | | | Potential health effects from exposure above maximum acceptable concentration (USEPA, 1973) |
|---|---|---|---|---|
| **Chemical** | **USEPA (1973)** | **Canadian (Health Canada, 2004)** | **WHO (2004)** | |
| Arsenic | 0.010 | 0.025 | 0.01 (provisional) | Skin damage or problems with circulatory system; may have increased risk of cancer |
| Cadmium | 0.005 | 0.005 | 0.003 | Kidney damage |
| Calcium | – | – | – | – |
| Chloride | 250 (secondary) | ≤250 (esthetic objective) | No health concerns at usual concentrations | – |
| Chromium | 0.1 | 0.05 | 0.05 (provisional) | Allergic dermatitis |
| Copper | 1.3 | ≤1.0 (esthetic objective) | 2 | Short-term exposure: gastrointestinal distress<br>Long-term exposure: liver or kidney damage; in cases of Wilson's disease consult physician about maximum acceptable concentration |
| Fluoride | 4.0 | 1.5 (adjust to 0.8–1.0, optimal for control of dental caries) | 1.5 | Pain and tenderness of the bones; mottled teeth in children |
| Hardness | – | – | – | – |
| Iron | 0.3 (secondary) | ≤0.3 (esthetic objective) | No health concerns at usual concentrations | – |
| Lead | 0.015 | 0.010 | 0.01 | Infants and children: physical or mental development delays, slight attention span deficits and learning disabilities<br>Adults: kidney problems, hypertension |
| Mercury | 0.002 (inorganic) | 0.001 | 0.001 (total) | Kidney damage |
| Nitrate (as N) | 10 | | – | Infants <6-month old: blue-baby syndrome |
| (as $NO_3^-$) | – | 45 | 50 (short term) | |
| Nitrite (as N) | 1 | | – | Infants <6-month old: blue-baby syndrome |
| (as $NO_2^-$) | – | <3.2, where nitrite is measured in addition to nitrate | 3 (short term), 0.2 (long term, provisional) | |
| Selenium | 0.05 | 0.01 | 0.01 | Hair or fingernail loss; numbness in extremities circulatory problems |
| Sulfate | 250 (secondary) | ≤500 (esthetic objective) | No health concerns at usual concentrations | – |

(*Continued*)

TABLE 85.3 (Continued)

| Body establishing guidelines | Maximum acceptable concentration (mg/l) | | | Potential health effects from exposure above maximum acceptable concentration (USEPA, 1973) |
|---|---|---|---|---|
| **Chemical** | **USEPA (1973)** | **Canadian (Health Canada, 2004)** | **WHO (2004)** | |
| Total dissolved solids | – | – | No health concerns at usual concentrations | – |
| Zinc | 5 (secondary) | ≤5.0 (esthetic objective) | No health concerns at usual concentrations | – |
| **Microbes** | **USEPA** | **Canadian** | **WHO** | |
| Total coliforms | – | None in consecutive samples from same site; present in ≤10% of samples from distribution system in calendar month | | |
| Fecal coliforms | – | | | |
| *E. coli* | | None | | |

chapter to allow readers to compare standards for animals and humans. The adverse health effects listed in USEPA documents for each chemical are also listed in the table.

In the United States, legal limits called maximum contaminant levels (MCLs) have been established for about 90 contaminants. If contaminant concentrations in public water supplies and certain private supplies are found to be above applicable MCLs, then the water supplier must take action to bring the contaminant concentration down below the MCL. Meanwhile, an alternate source of water that meets the standards must be provided. In the United States, there are primary and secondary water standards for human drinking water (USEPA, 1973). Primary drinking water standards are legally enforceable and water providers cannot provide water containing contaminants higher than their respective MCLs. Secondary standards are non-enforceable guidelines for contaminants that may cause cosmetic effects, such as skin or tooth discoloration, or unacceptable esthetic effects, such as unacceptable taste of, odor from or discoloration of the water.

Primary standards are established considering the adverse health effect caused by the contaminant and the dose at which such effects occur. *A reference dose (RFD or RfD) is estimated based upon the amount of the contaminant to which a person may be exposed on a daily basis that is not anticipated to cause the adverse health effect over a person's lifetime*. Consequently, the standards may not reflect the risk of adverse health effects in animals, and care should be used applying them to water intended for consumption by animals. A complete listing of drinking water standards and health advisories applicable to humans is compiled as needed by the USEPA Office of Water. The latest available at the time this chapter was written was published in 2004 and may be accessed on the USEPA website (USEPA, 2004).

# WATER CONSUMPTION TABLES

The dose of a water-borne contaminate may have to be estimated in cases of suspected toxicoses and may be calculated if the contaminant's concentration in the water and the amount of water consumed by the victims are known. Contaminant concentration in the water may be determined by analysis, but water consumption by the victims is rarely known with certainty. Water consumption varies by species, weather condition, diet and state of health. All of those factors and their interactions make the determination or estimation of minimal water requirements difficult if not impossible. Additionally, daily water requirements are not necessarily met solely by drinking water consumption.

Use water consumption data to calculate doses of water-borne poisons, if such data are available. If not, use the data in Table 85.4, which was taken from the NAS publication (1974), to estimate water consumption. Those data should be modified for situations falling outside of the defining parameters listed in the title.

**TABLE 85.4 Anticipated water consumption of various adult animals of medium weight in temperate climates**

| Animal | Expected consumption (l/day) |
|---|---|
| Beef cattle | 26–66 |
| Dairy cattle | 38–110 |
| Horses | 30–45 |
| Swine | 11–19 |
| Sheep and goats | 4–15 |
| Chickens | 0.2–0.4 |
| Turkeys | 0.4–0.6 |

Data are from NAS (1974).

## RATIONALE FOR LIVESTOCK RECOMMENDATIONS

The rationales for the chemicals included in Table 85.1 livestock recommendations included in the USEPA Proposed Criteria for Water Quality Volume 1 (1973) are summarized below. Information listed was what was known at the time the recommendations were formulated and may not reflect current knowledge of the effects of the chemicals. Our addenda are so noted.

The EPA writes in its introduction to the document:

"Acceptable limits specified in the recommendations were derived by the application of scientific judgment to lethal dose or lethal concentration data in a manner that provides a margin of safety to test organisms. For those substances whose effects are more aptly described as undesirable such as impairing aquatic habitats, causing taste and odor problems in water supplies, or reducing the esthetic or recreational quality of a water body, limits which minimize these effects were established on the basis of field and laboratory investigations. Acceptable levels of toxic materials for which specific numerical maximum acceptable concentrations are not prescribed are determined by applying an application factor to locally derived $LC_{50}$ data. By basing criteria on effects on the most sensitive important species, a desirable degree of regional and local variation is introduced, allowing water quality standards to depend on local conditions. An 'important species' in the criteria is defined as an organism that is: (a) commercially or recreationally valuable; (b) is rare or endangered; (c) affects the well-being of some species within (a) and (b) or (d) is critical to the structure and function of the ecological system. A 'rare or endangered' species is any species so officially designated by the US Fish and Wildlife Service."

Toxicity of some of the common water toxicants is described here in brief.

### Arsenic

Toxicity of arsenic depends upon its chemical form, relating primarily to rate of excretion. Inorganic oxides are generally more toxic than are organic forms of arsenic. Acute toxicities for farm animals were given in Table 85.5. Arsenic acid fed to lactating cows at up to 1.25 mg/kg b.w. for 8 weeks, equivalent to an intake of 60 l of water containing 5.5 mg arsenic/l daily by a 500 kg animal was absorbed and rapidly excreted in the urine with no increase of arsenic content of milk. No toxicity was observed.

**TABLE 85.5 Acute toxicity of inorganic arsenic by species**

| Species | Toxicity (g/animal) |
|---|---|
| Poultry | 0.05–0.10 |
| Swine | 0.15–1.0 |
| Sheep and goats | 10.0–15.0 |
| Horses | 10.0–15.0 |
| Cattle | 15.0–30.0 |

Wadsworth 1952 as cited by USEPA (1973).

**TABLE 85.6 Effects of cadmium in various species**

| Species | Amount and source | Effect |
|---|---|---|
| Human | 15 mg/l in popsicles | Sickening |
| Male rats | 4.5 mg Cd/kg b.w. | Permanent sterility |
| Rats or mice | 5 mg/l in drinking water | Reduced longevity |
| Pregnant hamsters | 2 mg/kg b.w. of $CdSO_4$ by i.v. injection | Dose on day 8 of gestation caused fetal malformations |

Data are from USEPA (1973).

### Cadmium

Effects of cadmium in various species are listed in Table 85.6. A small fraction of cadmium is absorbed in ruminants, with most of what is absorbed going to the kidneys and liver. The cow is "found to be very efficient in keeping cadmium out of its milk." Most major animal products, including beef and milk, seem "quite well protected against cadmium accumulation."

### Chromium

Chromium is not readily absorbed by animals. Most of what is ingested is excreted in the feces. It does not appear to concentrate in mammalian tissue or its concentration to increase with age. It is concluded that up to 5 mg/l of chromium II or VI in drinking water should not be harmful to livestock. That level may be unnecessarily high and the 1.0 mg/l level is recommended to provide a "suitable margin of safety." Of course, recommended maximum level in drinking water for livestock is <0.1 ppm (Puls, 1994).

## Copper

Copper is an essential trace element so some is required in the diet to maintain good health. Swine appear to be more tolerant to copper with dietary concentrations of 250 mg/kg or higher improving live weight gains and feed efficiency. Copper does not appear to accumulate in tissues. Sheep are very susceptible to copper toxicosis. A diet containing 25 mg/kg fed to sheep is considered toxic. About 9 mg per animal per day is considered safe.

## Fluorine (Authors' addendum: fluorine and fluoride seem to be used synonymously)

Consumption of drinking water with 2.0 mg/l may produce some tooth mottling, but it is not excessive with respect to animal health or deposition in meat, milk or eggs. Chronic fluorosis of livestock has occurred with water content of 10–15 mg fluoride/l. Total ration content of 30–50 mg fluoride/l (sic) for dairy cattle is considered a safe upper limit. Transfer to milk occurs to a very small extent and to a greater degree in eggs. Fluoride at 1.0 mg/l in drinking water did not harm livestock.

## Hardness (Authors' addendum)

Hard water does not appear to have a deleterious effect on animals, but it often is reported as part of water quality analysis. Hardness is a measure of the calcium and magnesium ions present in the drinking water. Water with high total dissolved solids (TDS) or salinity may or may not be hard water. Hardness of water may be measured as grains/gallon or in ppm. One grain per gallon is equivalent to 17 mg calcium and magnesium per liter. Water is classified according to calcium and magnesium salt content as follows: soft water ranges from 0 to 60 ppm, hard water varies from 120 to 180 ppm and very hard water is >180 ppm.

## Iron

In small quantity, iron is essential to animal life, while in excessive quantity it is considered toxic. Due to its involvement in Fenton reaction, iron is known to produce excessive free radicals, thereby causing oxidative stress. Brain, lungs and liver are the major sites of iron toxicity. Maximum recommended levels of iron water for humans, livestock and poultry are <0.3, <0.4 and <0.4 ppm, respectively. Dietary iron concentrations of at least 4000 mg/kg were found to cause phosphorus deficiency and to be toxic to weanling pigs. Concentrations <3000 mg/kg apparently were not toxic. Elevated dietary iron may antagonize copper, manganese and sulfur in lactating dairy cattle. In poultry, iron level >200 ppm in diet is considered toxic (Puls, 1994).

## Lead

The toxicity of lead had not been clearly established quantitatively. Daily intake of 6–7 mg/kg b.w. had been suggested as a threshold dosage in cattle, but that is difficult to establish. A concentration of 0.05–0.1 mg/l in drinking water is considered safe for livestock. Drinking water containing 5 mg/l consumed by rats and mice over the course of their life times produced toxic effects. Death rates of older animals increased, especially in males. Death was not caused by overt lead toxicosis, but was due to an increased susceptibility to "spontaneous infections."

## Mercury

Mercury content in surface water was found to usually be <5 μg/l, but methylation of mercury in bottom sediments bordering mercury deposits resulted in the continuous presence of mercury in solution. The relative stability of methyl mercury and its high absorption from the gut contributes to its oral toxicity. It has been suggested that livestock blood and tissue lead content be kept below 0.1 and 0.5 mg/kg, respectively, to protect humans who might consume those tissues. The safe contamination level for fish consumed by humans has been set at 0.5 mg/kg by the USFDA. However, that did not take into consideration other possible sources of dietary mercury. "In views of these facts the limits prescribed herein are reduced by a factor of 10 to reduce the significance of levels from meat products in comparison with those of fish."

## Nitrate and nitrite

Nitrite is more toxic to livestock than is nitrate. Usually nitrite is produced by the reduction of nitrate to nitrite, which occurs in the rumen; in moistened feeds, such as freshly chopped green forage; or water containing sufficient organic matter to sustain microbes. Natural waters may contain "high levels" of nitrate, but they usually contain very low concentrations of nitrite. Nitrate in cattle feed did not seem to pose a hazard to humans consuming dairy products or meat produced from cattle eating the nitrate-containing feed. "Animals fed nitrate continuously develop some degree of adaptation (sic) to it." "Assuming maximum water consumption by dairy cattle of 3–4 times the dry matter intake, the concentration of nitrate to be tolerated in water should be about one-fourth of that tolerated in the feed. This would amount to about 300 mg/l of nitrate."

**TABLE 85.7 Effects of nitrate and nitrite administered to non-ruminants**

| Species | Concentration, dosage or dose | Observed effect(s) |
|---|---|---|
| Gilts from weaning through two farrowing seasons | 330 mg/l (nitrate) | No adverse effects |
| Growing pigs | 330 mg/l (nitrate) | No adverse effects |
| Chick and laying hens | Up to 300 mg/l (nitrite) | No effects on growth or production |
| | Up to 200 mg/l (nitrite) | No effects on growth or production |
| Turkey pullets | 200 mg/l (nitrite) | Decreased growth |
| Turkeys, laying hens and turkeys | 200 mg/l (nitrite) | Reduced liver vitamin A stores |
| | 50 mg/l (nitrite) | No adverse effects observed |

Data are from USEPA (1973).

Effects of nitrate and nitrite at various concentrations given to non-ruminants are summarized in Table 85.7. It appears that ingestion of water containing up to 300 mg/l nitrate or 100 mg/l nitrite was well tolerated by all classes of livestock and poultry studied under controlled experimental conditions.

Authors' addendum: Crowley and associates (1974) conducted a 35-month study in Wisconsin comparing reproductive efficiency and lactational performance for a 54 cow Holstein herd that consumed drinking water containing either 19 or 374 ppm nitrate. During the last 15 months of the study, cattle drinking the elevated nitrate-containing water had the highest services per conception and lowest first service conception rates. The average milk yield was not significantly different between the two groups but the total milk yield for the entire 35-month study was lower in the elevated water nitrate group. This small difference in milk yield in the elevated nitrate-containing drinking water was thought to be due to an increased dry period due to lower conception rates.

The authors have never encountered a case of acute nitrate or nitrite toxicoses due to the consumption of drinking water, unless the water had been hauled to the animals in a fertilizer tank. Such cases have always involved cattle and occur when the availability of drinking water is disrupted and drinking water must be hauled to the animals from someplace else. Such circumstances may occur when water freezes during stretches of very cold weather or the water pump fails and cannot be rapidly repaired. Deaths often occur relatively rapidly; owners often report finding dead victims within a few meters of the contaminated water tank. Ocular fluid collected from dead victims usually contains nitrate in excess of 100 μg nitrate/ml and nitrite in excess of 2 μg nitrite/ml. Ocular fluid collected from dead victims of forage nitrate toxicosis usually contains less nitrate than ocular fluid from dead victims of water nitrate toxicosis. If the fertilizer tank contained ammonium nitrate, then death may be the result of either excessive ammonia or nitrate exposure, and the determination of the exact cause of death is mostly academic. Under no circumstances do we recommend that a tank that has contained fertilizer be used to haul drinking water to animals, even if it has been "thoroughly washed out." Theoretically, a fertilizer tank may be cleaned sufficiently to haul drinking water, but not practically.

## Total dissolved solids

"Total dissolved solids" is defined in the NAS publication (1974) as the concentration of all dissolved constituents in water. "Salinity" is often used synonymously with "total dissolved solids," although that assumes that all of the dissolved solids are saline. Salinity is more than a measurement of the total amount of sodium and chloride present in water. Salinity also includes anions such as carbonates expressed as oxides, bromide and iodine expressed as chlorine, and cations such as calcium, magnesium, bicarbonate and sulfate but does not include organic matter.

The USEPA does not include recommendations for TDS, but the NAS provides criteria for classification of water based upon TDS content established for the US Geological Society. Criteria for two classification systems are listed in Table 85.8. Note the differences in the classification and the use of brine in both, but with different criteria for inclusion in that classification. It has been our experience that those classifications contribute little to the assessment of water quality for animals as we have never encountered an instance where those water classifications have been used to describe the TDS content. Nevertheless, they are included here. More useful information about the use of saline waters for livestock is provided under the section titled "A Guide to the Use of Saline Waters for Livestock" in the NAS publication, starting on p. 48 (1974):

> "Several factors need to be considered in assessing the suitability of saline water for livestock use, including kind, age and sex of the animals; pregnancy or lactation status; physical exertion; climatic conditions; diet and its moisture and mineral content; production expectations; water salt content; access to other water sources; and the adaptation to available water sources. Weight given to those factors is largely a matter of judgment, but TDS is the single most reliable factor that can be determined."

TABLE 85.8 Water classifications based upon total dissolved solid content

| Davis & DeWiest classification (1966) | | Robinove *et al.* classification (1958) | |
|---|---|---|---|
| Water classification | TDS content (mg/l) | Water classification | TDS content (mg/l) |
| Fresh water | <1000 | Slightly saline | 1000–3000 |
| Brackish water | 1000–10,000 | Moderately saline | 3000–10,000 |
| Salty water | 10,000–100,000 | Very saline | 10,000–35,000 |
| Brine | >100,000 | Brine | >35,000 |

Data are from NAS (1974).

TABLE 85.9 Guide to use of saline waters for livestock and poultry

| Water TDS content (mg/l) | Expected health or performance effects |
|---|---|
| <1000 | None expected for any livestock class. |
| 1000–2999 | None expected for any livestock class. May cause temporary diarrhea or watery droppings in animals unaccustomed to the water. |
| 3000–4999 | Should be none. May cause temporary diarrhea or be refused at first by animals unaccustomed to the water. Poor water for poultry, often causing watery droppings, increased mortality and decreased growth, especially in turkeys. |
| 5000–6999 | Reasonably safe for dairy and beef cattle, sheep, swine and horses. Avoid use of water approaching the higher limit for pregnant or lactating animals. Unacceptable for poultry; use for poultry will almost always be accompanied by some type of problem, especially near the upper limit, such as reduced growth or production. |
| 7000–10,000 | Unfit for poultry and probably swine. Considerable risk may exist for pregnant or lactating cows, horses, sheep and the young of those species. Considerable risk for animals subject to heavy heat stress or water loss. Generally use of such water should be avoided, although older ruminants, horses and even poultry and swine may subsist on it for long periods of low stress. |
| >10,000 | Unacceptable risks for any livestock class. |

Taken from NAS (1974).

The NAS did not recommend the use of highly saline water for livestock but acknowledged that circumstances may arise such that saline water may be all that is readily available. Table 85.9 lists the guidelines for use of saline water for livestock and poultry. If high-TDS water is to be used, the following points should be considered, too:

1 Alkalinities and nitrate should be considered whenever water contained >3000 mg TDS/l is to be used. Alkalinities of 2000 mg $CaCO_3$ detract from the suitability of water. Hydroxide is more harmful than carbonate, which is more harmful than bicarbonate.
2 If offered the choice between two water sources, one highly saline and one less saline, they will choose the less saline source.
3 Animals can tolerate high saline water for a few days if they are then given low saline water.
4 Water intake usually increases with soluble salt content, except in cases of extremely high saline content, which animals may refuse to drink.
5 Abrupt changes from a low to high saline water will likely cause more problems than a gradual change.
6 Decreased water intake is very likely accompanied by decreased feed intake. So, animals with high production expectations may not produce as well showing "deleterious effects from waters of lower salts content than animals on a maintenance regimen."
7 Highly saline water may furnish enough minerals to be considered in dietary mineral formulations. And salt content of the diet may contribute to the toxicity of saline water, particularly if salt is added to control feed intake. Generally, water should not normally be relied upon as a source on essential inorganic minerals.

## Selenium

At the time that the USEPA formulated its recommendations, no substantiated case of livestock poisoning by selenium in water had been found. However, selenium toxicosis was found in wildlife exposed to high-selenium-containing irrigation runoff water. Agricultural drainage water entering Kesterson Reservoir in California ranged from 0.140 to 1.40 mg/l (140 to 1400 ppb) during 1983–1985.

TABLE 85.10 Zinc toxicity in diets for various animals

| Dietary per water Zn content | Species and effect(s) |
|---|---|
| 1000 mg/kg in diet | Swine tolerated it; |
| ⩾2000 | Swine: toxic |
| 2320 mg/l in water | Chickens: reduced water consumption, egg production, body weight |
| >500 mg/kg in diet, as oxide | Ruminants: toxic |

Data are from USEPA (1973).

## Sulfate

No recommendation for sulfate was made by the USEPA probably because the association between sulfate exposure and polioencephalomalicia in ruminants had not yet been discovered. The Canadian recommendation is 1000 mg/l. Exposure to elevated sulfates in drinking water can result in a transient diarrhea. Animals usually acclimate to an elevated level of drinking water sulfate in 3–7 days and no longer exhibit diarrhea. Methemoglobin was increased 450% in cattle consuming drinking water containing sulfates at 3493 ppm.

## Zinc

Zinc is relatively non-toxic. The findings cited in the EPA document are summarized in Table 85.10. Increased zinc intake increased the zinc content of body tissue, but its accumulation was not great and tissue content fell rapidly after zinc intake was reduced.

# HEALTH EFFECTS OF CYANOTOXINS FROM WATER

The effects of cyanotoxins from water on humans are discussed by Kuiper-Goodman *et al*. (1999), and serve as the basis for risk assessment for exposure from drinking and recreational water by the World Health Organization (WHO, 2004). Relevant information from their chapter is summarized here. A more detailed discussion of cyanotoxins may be found elsewhere in this book (Chapter 58).

## Cyanotoxins

Toxic effects after exposure to cyanobacteria are evidenced by retrospective epidemiological studies of human disease, factual and anecdotal reports of disease in animals, and toxicological studies of cyanotoxins. Cyclic peptides, like microcystins, are specific liver poisons in mammals and of particular concern to human health. Acute toxicoses cause liver hemorrhage and death from liver failure. Chronic toxicoses may promote liver and other cancers.

Alkaloid neurotoxins, like anatoxins and saxitoxins/paralytic shellfish poison (PSP), have shown only acute toxicoses in mammals. Information about anatoxins is sparse. Some of the alkaloid toxins, especially PSP, accumulate in marine and freshwater biota, which may place animals and humans consuming such biota at risk of exposure after consuming them.

Cyanobacterial lipopolysaccharides (LPS) can elicit allergic and toxic responses in humans, although little is known about their acute or chronic effects. They should be considered whenever gastrointestinal (GI) and respiratory signs are reported or observed.

## Adverse health effects in humans

Drinking water has been associated with GI illness throughout history. Pathogenic bacteria and viruses are common causes of such illness, and evidence for GI illness caused by cyanotoxin exposure needs to take such etiologies into account. Cases of GI and hepatic illness that can be reliably attributed to cyanotoxins have all been coincidental with lysis of cyanobacterial blooms, either natural or artificial. Lysis releases cyanotoxins from the cells into the water.

Artificial lysis occurs after water treatment with copper sulfate to destroy blooms in water reservoirs, and poisonings subsequent to such treatment have been reported in the Unites States and Australia. Water filtration after the treatment can remove cell fragments, but not dissolved cyanotoxins.

Individuals at greater risk of cyanotoxicoses are the young, who drink a higher volume of water than adults in proportion to their body weights and subjects with liver disease or kidney damage. Chronic effects in humans are of great concern because of the potential of exposure to low concentrations of cyanotoxins in drinking water over the course of a lifetime. Results of animal experiments have indicated chronic exposure to microcystins may promote tumor growth.

Epidemiological studies of the incidence of human hepatocellular carcinoma in China have identified three risk factors: hepatitis B viral infection, exposure to aflatoxin $B_1$ in food and the source of drinking water. Cancer mortality is lower when the source of drinking water is from deep well than when it is from surface waters. Cyanobacteria are abundant in the same areas where the incidence of hepatic carcinoma is highest.

There have been repeated reports of adverse effects in swimmers exposed to cyanobacterial blooms, such as skin

irritation and GI symptoms. Deep blisters on skin under bathing suits have been reported, particularly after contact with the marine cyanobacterium *Lyngbya majuscule*, which is believed to occur when the organism becomes trapped against the skin under the bathing suit. Effects may be due to allergic reactions to cyanobacterial pigments or direct contact with the cyanotoxins. Individual sensitivity to cyanotoxins varies significantly.

Health effects in humans exposed to cyanobacteria in recreational waters include headache, nausea, vomiting, muscular pain, painful diarrhea, abdominal pain, blistering of the lips, sore throats, skin rashes, fever and eye or ear irritation, usually within 7 days of exposure. Severity of symptoms appears to be related to full-body immersion, the amount of water ingested and the duration of exposure. Severe pneumonia attributed to aspiration of a *Microcystis* toxin was reported to have occurred in two British army recruits after they swam in and underwent canoe training in water containing a dense bloom of *Microcystis* spp.

## Effects in animals

Reports of deaths due to cyanotoxicoses have been reported for cattle, sheep, dogs, horses, pigs, coots, ducks, skunks and mink exposed to water containing cyanobacteria. Animals consuming food in which cyanotoxins may accumulate, may also suffer from cyanotoxicoses. Secondary photosensitization may occur in animals exposed to hepatotoxic cyanotoxins. Post-mortem examination may reveal evidence of cyanobacteria in stomach content, or liver injury. However, in cases of neurotoxic cyanotoxins, no post-mortem lesions are usually found.

## Cyanotoxins in drinking water

Falconer *et al*. (1999) described how guideline values for cyanotoxins in water were derived. Information from that chapter that can assist in the assessment of risk from such exposure is summarized here. The World Health Organization has established a provisional guideline for human drinking water of 0.001 mg/l of microcystin-LR (total cell-bound and extracellular) (WHO, 2004). Canada has established a cyanobacterial toxin maximum acceptable concentration of 0.0015 mg/l (as microcystin-LR) (Health Canada, 1999). Australia has established a standard of 0.0013 mg/l of total microcystins as microcystin-LR toxicity equivalents (TE) (Australian Water Quality Guidelines, 2004). The USEPA has not yet established a standard. Potential carcinogenicity is the effect upon which those standards are based. Standards for drinking water for animals have not been established.

## Recreational water exposure

Routes of exposure to cyanotoxins for people and animals from recreational waters include direct contact with the skin (most important for sensitive areas such as the mouth, ears, eyes and throat, and the area covered by bathing suits worn by humans), swallowing and inhalation of water.

Absorption of cyanotoxins after swallowing, contact with nasal mucosa or inhalation is likely to be important during water-contact activities. Absorption through nasal and pharyngeal mucosa is more likely to occur when activities involve submersion of the head or inhalation of aerosols produced by water spray. Cumulative liver damage may occur by repeated microcystin intake during periods of daily water activities, especially if the activities take place in areas where scums are or have been present.

## Safe practices for drinking water

Draw drinking water from sources that do not harbor cyanobacteria, such as groundwater or surface water not supporting cyanobacterial growth.

Cyanobacterial-contaminated water may be used for drinking water if the cyanobacterial cells are removed without lysing them because most cyanotoxins are cell bound. Removal of released cyanotoxins from the water may be possible, but it may also be difficult and expensive. Risk of cyanotoxicosis should be considered high after algae-infested water is treated with copper sulfate. Critical control points for assessing the safety of drinking water are listed in Table 85.11.

All animals should be denied access to bodies of water containing visible discoloration that cannot be attributed to some other source other than algae or cyanobacteria. Areas in which scum has formed should be considered very high risk for acute cyanotoxicosis, regardless of the color of the scum. Identification of the microbial components of the scum may help with the risk assessment.

## Safe practices for recreational waters

Surveillance of recreational waters for algal blooms can be effective in minimizing or preventing exposure, but adequate surveillance of such waters for blooms may be difficult. Governmental agencies may not be able to survey all recreational waters within their jurisdiction adequately, so the burden of surveying recreational waters for the presence of blooms may rest with the users of the waters. Once blooms have been detected in public waters, provision of information to the public is usually the responsibility of public authorities.

Algal blooms are usually regarded as more of a nuisance than a toxic hazard. They are often associated with

**TABLE 85.11 Critical control points for assessing the safety of drinking water**

| Control point | Remarks |
|---|---|
| Water source | Groundwater less risky than surface waters. Water sources contaminated by surface runoff pose higher risks. |
| Presence of cyanobacteria; tendency of bloom formation | Bloom formation less likely during periods of high flow or deep vertical mixing, and are more likely during periods of low flow or stagnation. Historical evidence of algal blooms increases risk; blooms may be cyclic. Nutrient input, especially nitrogen and phosphorus, increases risk. Absence of cyanobacteria reduces risk. |
| Cell lysis | Most cyanotoxins are cell bound and may be removed with the cells. Lysis may occur naturally as the bloom dies, or artificially by treatment, pumping or transport. |
| Water treatment systems | Cyanotoxins may be removed by filtration through activated charcoal or by oxidation, but effectiveness of treatment should be monitored. |

Adapted from Table 5.1 in Falconer *et al.* (1999).

unpleasant odors and offensive appearances. They may be regarded as normal in areas where they occur frequently or regularly, and their toxic potential may be unknown or disregarded. The presence of any algal bloom, regardless of its color, location or time of occurrence, should be considered to pose a high risk of cyanotoxicosis and all animals should be denied access to such areas, whether it is in the water or on the shore. The detection of algal scum formation is problematic if routine monitoring is carried out at 1–2-week intervals. Scum formation may occur in the matter of hours to days. Daily inspection for scum formation after cyanobacteria are found to be present is recommended.

The availability of ELISA kits for the detection of microcystins makes it easier to monitor recreational waters for the presence of microcystins. Chorus and Cavalieri (2000) do not recommend risk assessment for cyanotoxicoses be based upon chemical analysis for specific cyanotoxins for two reasons: (1) only some of the substances causing health outcomes are known and can be detected and (2) epidemiological studies indicate some health effects are not due to known cyanotoxins. They recommend that a monitoring program focus surveillance efforts on sites that are likely to present a risk.

## Monitoring strategy for freshwater cyanobacteria

Kuiper-Goodman *et al*. (1999) recommend the following strategy for monitoring freshwater for cyanobacteria, consisting of the three steps listed below. Each will be briefly discussed, and serve a prerequisites for the following step:

1 Determine the carrying capacity of the water body for cyanobacteria.
2 Inspect the site to detect biomass developments.
3 Quantitatively assess the biomass as the basis for risk assessment when biomass developments occur.

### *Determine the carrying capacity of the ecosystem for cyanobacteria*

Algal and cyanobacterial growth requires nitrogen and phosphorus and the availability of those nutrients limits the numbers of algae and cyanobacteria that can grow in a body of water. Phosphorus is usually the limiting factor of the two and total phosphorus, not *ortho*-phosphorus, should be measured because algae and cyanobacteria can store sufficient phosphorus to support their growth. If total phosphorus is found to be $>0.02\,mg/l$, then Step 2 monitoring activities should commence. Inspect the water body area to determine if a source or sources of phosphorus or nitrogen input are present, like sewage, fertilization near the water body or erosion.

### *Inspect the site to detect biomass developments*

The frequency of monitoring should be no longer than every 2 weeks and should be concentrated in areas in which a biomass most likely will appear, i.e. leeward shorelines. Visual inspections should include the following activities, each serving as its successors prerequisite:

A Determine water transparency. If the lake bottom cannot be seen at 50 cm depth along the shoreline, or if the Secchi transparency is $<1\,m$, then go to Step B.
B Determine if greenish discolorations of the water are present along or near the shoreline, indicating the presence of algal or cyanobacterial masses. If so, go to Step C.
C Determine if greenish discolorations of the water are present on the surface anywhere on the body of water.

**TABLE 85.12 Guidelines for use of recreational waters**

| Situation | Health risk | Recommended action |
|---|---|---|
| Scum is present | High for acute poisoning and short-term health effects (skin irritation, GI illness). Humans are not likely to ingest scum mats or scum-contaminated water, but animals may. Animals may also be exposed when they groom themselves after contact with scum. Young animals or children playing with, in or around scum are at risk. | Prevent contact with scum; prohibit water-contact activities. |
| 100,000 cyanobacterial cells/ml; 50 μg chlorophyll a/l with dominance of cyanobacteria | Moderate to high for short-term adverse health effects, potential for chronic effects. | Watch for scums. Restrict water-contact activities; continue to monitor the situation, post on-site risk advisory signs. |
| 20,000 cyanobacterial cells/ml; 10 μg chlorophyll a/l with dominance of cyanobacteria | Low to moderate for short-term irritative or allergenic effects. | Post on-site risk advisory signs. |

Adapted from Falconer *et al.* (1999).

**TABLE 85.13 Expected microcystin content of water based upon the amount of microcystin-producing cyanobacteria present**

| Number of cyanobacterial cells | Expected microcystin concentration |
|---|---|
| 100,000 cyanobacterial cells/ml | Associated with about 20 μg/l of microcystins, if microcystin-producing cyanobacterial predominate. |
| 20,000 cyanobacterial cells/ml | Associated with 2–4 μg/l of microcystins, if microcystin-producing cyanobacterial predominate. |

Falconer *et al.* (1999).

***Quantitatively assess the biomass as the basis for risk assessment when biomass developments occur***

Either of two quantitative measurements may be performed: (i) microscopic examination of samples collected from the biomass to identify if cyanobacteria are present, and if so, what kind; (ii) quantitate the concentration of chlorophyll a present.

Guidelines for the use of recreational waters based upon those measurements are detailed by Falconer *et al.* (1999) and are summarized in Table 85.12. Falconer *et al.* (1999) do associate microcystin concentrations with numbers of cyanobacteria cells present in water, which may be valuable as an additional risk assessment variable if microcystin-producing cyanobacteria predominate in the biomass. The association is listed in Table 85.13.

Once algal blooms have occurred in a body of water, they should be considered to pose a high risk of cyanobacterial toxicosis until the microorganisms in them have been reliably identified. There are numerous publications on cyanobacteria that describe cyanobacterial blooms as being blue-green in color, but the absence of blue-green coloration in a bloom does not necessarily lessen its toxic hazard. The safest course of action is to deny access by animals to the bloom area.

The single most effective action that animal owners can take to protect their animals from cyanotoxicoses is to prevent their animals from making any contact with any scum or biomass in the water or on shorelines. It is not usually possible to determine if the scum or biomass is or is not cyanotoxigenic by visual inspection alone, so any biomass or scum should be considered potentially toxic.

Once an animal has been exposed to a bloom, it is advisable to monitor the animal closely for signs of cyanotoxicoses. Algal scum may be visible on its coat or around its mouth or muzzle and is a good indicator that dermal exposure to cyanobacteria may have occurred and is suggestive that algae may have been ingested. It is not usually possible to determine how much of the scum might have been ingested. It is expected that human swimmers involuntarily swallow 100–200 ml of water in one session, and water skiers and sailboard riders may ingest significantly more.

# REFERENCES

Canadian Council of Ministers of the Environment (2005a) Canadian water quality guidelines for the protection of agriculture water uses: summary table (updated October, 2005). In *Canadian*

*Environmental Quality Guidelines*, 1999. Canadian Council of Ministers of the Environment, Winnipeg, Man.

Canadian Council of Ministers of the Environment (2005b) Canadian water quality guidelines for the protection of aquatic life: summary table (updated October, 2005). In *Canadian Environmental Quality Guidelines*, 1999. Canadian Council of Ministers of the Environment, Winnipeg, Man.

Chorus I, Cavalieri M (2000) Cyanobacteria and algae. In *Monitoring Bathing Waters – A Practical Guide to the Design and Implementation of Assessments and Monitoring Programmes*, Bartram J, Rees G (eds), E&FN Spon, London.

Committee on Minerals and Toxic Substances in Diets and Water for Animals (2005) *Mineral Tolerance of Animals*, 2nd revised edition. The National Academies Press, Washington, DC, pp. 469–76.

Crowley JW, Jorgensen NA, Kahler LW, Satter LD, *et al*. (1974) *Effect of Nitrate in Drinking Water on Reproductive and Productive Efficiency of Dairy Cattle*. Wisconsin Water Resources Center, Madison, Wisconsin, WI.

Davis SN, DeWiest RJM (1966) *Hydrology*, John Wiley & Sons, New York.

Falconer I, Bartram H, Chorus I, *et al.* (1999) Safe levels and safe practices, Chapter 5. In *Toxic Cyanobacteria in Water: A Guide to Their Public Health Consequences, Monitoring and Management*, Chorus I, Bartram J (eds). E&FN Spon, London.

Federal-Provincial-Territorial Committee on Drinking Water (2004) Summary of guidelines for Canadian drinking water quality. In *Guidelines for Canadian Drinking Water Quality*, Health Canada, Ont., Canada (http://www.hc-sc.gc.ca/waterquality).

Health Canada (1999) Cyanobacterial Toxins – Microcystin-LR (http://www.hc-sc.gc.ca/ewh-semt/pubs/water-eau/doc_sup-appui/cyanobacterial_toxins/index_e.html).

Kuiper-Goodman T, Falconer I, Fitzgerald J (1999) Human health aspects, Chapter 4. In *Toxic Cyanobacteria in Water: A Guide to Their Public Health Consequences, Monitoring Management*, Chorus I, Bartram J (eds). E&FN Spon, London.

Microcystins, Fact Sheets (2004) Microoganisms toxic algae. In *Australian Water Quality Guidelines 2004*, pp. 238–42 (http://www.nhmrc.gov.au/publications/_files/awgfull.pdf).

Puls R (1994) *Mineral Levels in Animal Health: Diagnostic Date*, Second Edition, Sherpa International, Clearbrook, BC Canada.

Robinove JR, Langford RH, Brookhart JW (1958) *Saline-water resources of North Dakota*, USGS Water-Supply Paper 1428, Washington, DC.

Subcommittee on Nutrient and Toxic Elements in Water (1974) *Nutrients and Toxic Substances in Water for Livestock and Poultry*. National Academy of Sciences, Washington, DC.

USEPA (1973) *National Secondary Drinking Water Standards*. USEPA, Washington, DC (http://www.epa.gov/OGWDW/mcl.html#mcls).

USEPA (1973) *Proposed Criteria for Water Quality*, vol. 1. USEPA, Washington, DC.

USEPA (2004) 2004 *Edition of the Drinking Water Standards and Health Advisories*. USEPA, Washington, DC (http://www.epa. gov/waterscience/criteria/drinking/standards/dwstands.pdf).

USEPA *National Primary Drinking Water Standards* (2003). USEPA, Washington, DC (http://www.epa.gov/OGWDW/mcl.html# mcls).

*Water Quality Criteria 1972* (1974) National Academy of Sciences, National Academy of Engineering, US Government Printing Office, Washington, DC.

World Health Organization (2004) *Guidelines for Drinking-Water Quality*, 3rd edn. WHO, Geneva, Switzerland.

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# Part 17

# Diagnostic Toxicology

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CHAPTER

# 86

# Basic concepts of analytical toxicology

*Anant V. Jain and Beverly Arnold*

## INTRODUCTION

Veterinary analytical toxicology deals with the chemical analysis of ante-mortem, post-mortem, and environmental specimens to assist the veterinary practitioner in the diagnosis of poisoning. The chemical analytical data reported to the veterinarian is of utmost importance. The correct data will lead to correct diagnosis. The erroneous data may lead to disaster; the client may lose large number of animals. The erroneous data may also lead to legal ramifications. Thus, correctness of analytical data is of paramount importance for veterinary analytical toxicology. Chemical measurements are based on the principles of analytical chemistry. In practice, the analytical chemist–toxicologist uses analytical methods to get the final results. It should be pointed out that by nature analytical results are variable. The science of trace analysis (analysis at parts per million (ppm) or parts per billion (ppb)) is not as precise as most layman and many scientists view it to be (Rogers, 1986). Analytical methods once devised are, like life forms, subject to evolution. Natural selection as mediated by analytical chemists, ensures that only the fittest analytical methods survive. Therefore, an analytical method must be fit for purpose. It should be pointed out that most of the advancements and improvements in the analytical methods have been by making necessary changes and improvements in various steps used in an analytical method. The papers by Sawyer (1988) and Seiber (1988) clearly demonstrate this point for pesticide analysis. The important steps in chemical analytical measurements are (i) decomposition or extraction of the sample, (ii) clean up and separation of analyte from bulk impurities (sample matrix), (iii) pre-concentration of analyte, (iv) detection and measurement of the analyte, and (v) the generation of the report. The purpose of this chapter is to provide the analytical chemist/toxicologist, an overview of the brief history of early, and recent developments in chemical measurements, and above all the basis for scientifically sound chemical analytical measurements.

## HISTORY OF CHEMICAL ANALYSIS

### Early history of chemical analysis

Chemical analysis began on the 8th day (McCrone, 1987), Adam; recovering after cooperating with god, in creating Eve, felt first pangs of hunger. He went around and harvested different kinds of colorful berries, and set down for dinner with Eve. Eve rejected some berries due to foul smell (nose as detector). The bitter tasting ones were rejected next (taste as detector), and the delicious ones were consumed. Thus, first chemical detectors were nose, tongue, and eye; *the five senses were used as chemical detectors for a long time*.

The alchemists, in their futile attempts to make gold from base metals introduced the techniques of filtration, decantation, and distillation. Alchemist's zeal, however unsuccessful, leads to a reservoir of observations.

Observations followed observations, and when a large body of knowledge was available, it was named *chemistry*. Those observations, which dealt with the production of substances, were termed chemical synthesis, and those related to determining what the substances were called chemical analysis. The analysis could be carried out in solution as well as in dry state. The solution methods were termed "wet" methods, and were further divided into volumetric and gravimetric. These methods used sight (visual observation) as detection device. The dry methods also used sight as the detector.

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Archimedes, while running naked through the street, and shouting, *eureka, eureka* introduced the use of numerical properties for chemical analysis in the concept of specific gravity (density).

The volumetric and gravimetric methods were used for chemical analysis for a long time. These were the principle chemical analytical methods up to 19th century.

## Recent history of chemical analysis

In recent years, research in analytical chemistry has focused mainly on the development and application of physical and physicochemical analytical methods, instrumental methods of analysis. Instrumental chemical analysis began with Bunsen (Bunsen burner fame) in 1860, who introduced flame emissive spectrometry, and discovered metals rubidium and cesium. There are a large number of instruments available today for chemical analysis. The most important of these which have found routine use in veterinary analytical toxicology laboratories are (i) chromatographic techniques coupled with a large number of detectors, (ii) spectrometry and spectrophotometry, both for visible and ultraviolet range of the light spectrum, (iii) atomic absorption spectrophotometry, (iv) inductively coupled plasma spectrometry, and (v) mass spectrometry.

Chromatographic methods in general are the most common methods used at the determinative step for the organic compounds. Chromatography is a separation technique, which separates the impurities in the sample solution from analyte. According to International Union of Pure and Applied Chemistry (IUPAC), chromatography is a method used primarily for the separation of the components of a sample in which the components are *distributed* between *two phases*, one of which is stationary while the other moves. The stationary phase may be a *solid*, or a *liquid supported on a solid,* or a *gel.* The *stationary phase may be packed in a column, spread as a layer, or distributed as a film, etc.* The mobile phase may be gaseous or liquid. Thus there are two movements in chromatographic system, the mobile phase movement – which is usually at a constant rate; the other is the movement of the components of the sample. The movement of the component depends upon their relative distribution between the stationary phase and the mobile phase. This results in the separation of the components. This separation process coupled with the detection and measuring device completes the chromatographic system. Chromatography can be conducted in gas phase, known as gas chromatography (GC), liquid phase, known as liquid chromatography (LC), and on a glass or paper coated with solid adsorbent, known as thin layer chromatography (TLC). A variation of LC is known as high-pressure liquid chromatography (HPLC), since high pressure is used to force the solvent through the chromatographic column. Gas chromatographic equipment can be interfaced with large number of detectors such as, flame ionization (FID), electron capture (ECD), nitrogen phosphorus (NPD), flame photometric (FPD) (responds to phosphorus, and sulfur only), Hall electrolytic conductivity (HECD) (responds to halogens only), and mass spectrometer. HPLC can also be coupled with various detectors such as UV/VIS spectrophotometric detector, fluorescence detector, electrochemical detector, refractive index detector, and mass spectrometric detector. Chromatographic techniques coupled with a detector are represented by hyphenated abbreviations. For example a gas chromatograph equipped with FPD is known as GC–FPD. Sometimes this is also represented as GLC–FPD, due to the nature of the stationary phase of the chromatographic column used. Most of the time a liquid phase is coated on a solid support and packed in a glass column. Recently, silica columns of very small diameter are used in GC, and are known as capillary columns. In these columns, the liquid phase is usually bonded to the inner surface of the silica capillary.

Instrumental methods have surpassed the traditional gravimetric and volumetric analysis in speed and sensitivity.

# TRUENESS (ACCURACY) OF CHEMICAL ANALYTICAL RESULTS

With all the furious activity in the development of various analytical instruments, the question still remains, how good are your results. One can use the most expensive and sophisticated instrument on the market, but still have to prove that the analytical results so obtained are indeed accurate. As mentioned in the introduction, that the analytical results by nature are variable, it is more essential to prove the correctness of the results. Let me give a brief history why this is important.

After the Civil War (1861–1865), American agriculture has been on the march, and farmers have been trying to produce more and more per unit of land. Synthetic fertilizers are used extensively. Various state governments are trying to verify the claims made by the fertilizers manufacturers to protect the consumer (farmer). The problem was that different laboratories got widely different results for the same ingredient in the fertilizers. There were no uniform methods for fertilizer analysis. In order to control this chaos, Mr. J. T. Henderson, Agriculture Commissioner of Georgia, called a meeting of various interested parties. On May 15, 1884 the meeting took place at the Senate Chambers at the Capitol in Atlanta (Helrich, 1984).

Dr. Charles Shepard, a commercial chemist from Charleston, South Carolina, addressed the meeting and stressed the need for uniform and validated methods for

the analysis. He dramatized the effect of nonuniform methods of analysis was having on the monetary value of fertilizers. He told the convention "a 1% difference in the analysis of phosphoric acid made a difference of $50,000 in the annual production of a "good-sized factory". This presentation had a lasting effect on the attendees and was decided that the group again meet at AAAS, American Association for the Advancement for Science, in Philadelphia, on September 4–10, 1884. The Philadelphia meeting became the first meeting of "Association of Official Agricultural Chemists", then known as AOAC. Now this association is known as AOAC International. Over the past 122 years AOAC International has developed a process for validating and harmonizing analytical methods. Method validation is the key for determining the performance of analytical methods, and the reliability of the results obtained. AOAC International is committed to analytical excellence and worldwide confidence in analytical results using a defined method validation process.

## ANALYTICAL METHOD VALIDATION

There are several types of validation for analytical methods. AOAC International (Fredrick Ave, Gaithersburg, MD, USA) is the leading organization that conducts methods validation under its auspices. AOAC International conducts the following types of method validation:

1 Performance tested method (PTM)
2 Peer-verified method (PVM)
3 Official methods of analysis (OMA)

*AOAC Official methods* are referenced in the US Code of Federal Regulations (CFR) and are used worldwide by regulated industry, product testing laboratories, and academic institutions. AOAC International is an independent association devoted to promoting methods validation and quality measurements in analytical sciences. It provides full review and validation of approved standard methods of analysis, promotes uniformity, and reliability in statements of results, and develops and promotes criteria useful for laboratory accreditation and analyst certification.

### Performance tested methods

There are a large number of rapid test kits available for chemical and biological analysis. In order to verify the claims made by the kit manufacturer about the performance of test kits, AOAC International introduced the concept of PMT. The applicant (kit manufacturer) submits the performance data of the test kit along with the application fee. AOAC International appoints a panel of experts to review the performance data. If the review of performance data is satisfactory, an expert laboratory is selected to check the performance of the test kit. If the performance is as claimed the test kit is awarded the status of PMT.

### Peer-verified programs

PVM methods The AOAC® *Peer-Verified Methods*℠ Program is intended to provide a class of tested methods which have not been the subject of a full collaborative study. Through a less intensive process, the program provides a rapid entry point for methods, which are recognized by AOAC at a level of validation for methods not otherwise evaluated. The distinguishing aspect of an AOAC *Peer-Verified Methods*℠ is that its performance has been checked in at least one other independent laboratory. Eventually, it is expected that most PVMs will undergo full interlaboratory collaborative studies and obtain *Official Methods*℠ status. An explanation of the PMT, PVT, and OMA is available at www.aoac.org.

### Single laboratory validation

In order for a method to be validated as PTM, PVM, and finally as *Official method* it must first be validated in a single laboratory. In international trade and regulatory affairs, only validated methods are acceptable. The method need not to be validated under the auspices of AOAC International, but evidence is required that the method is suitable for the intended purpose such that similar results are obtainable from other competent laboratories. In order to gather this information, single laboratory validation (SLV) is essential. Various standard setting organizations such as International Standards Organization (ISO) and AOAC International (AOAC) have established standards for SLV of methods of analysis. AOAC has more than 120 years experience in collaboratively validating methods of analysis. These standards require that certain performance characteristics (attributes) of an analytical method must meet specified standards. Many analytical methods published in the scientific literature may not meet the criteria specified in the above standards. They usually lack the essential attributes required for the recognized validation process.

The performance characteristics are the specificity, the ability to distinguish the analyte from other substances; applicability – as the matrices and the concentration range, and reliability. Reliability is the most important characteristic of an analytical method and is expressed in terms of % recovery, repeatability, and reproducibility. Repeatability

and reproducibility are expressed in terms of relative standard deviation within laboratory and between laboratories, respectively ($RSD_r$, $RSD_R$). Intermediate precision, another term, which is important for SLV, is the intermediate repeatability relative standard deviation ($RSD_{ri}$). It is also very important that the analytical method is fit for its intended purpose. For example, a method intended for the determination of lead (Pb) in drinking water at ppm or ppb level is not fit for the analysis of lead ore (% composition level). If such a method is used for the determination of lead in an ore, the error, and the variability will be too large, and the analysis may not serve its purpose, i.e. whether to mine or not mine a certain location. Thus fitness for purpose is very important.

There are only few methods, which have been validated for use in veterinary analytical toxicology. These methods are listed in the *Official methods of AOAC International* (AOAC, 2000). An on-line version of OMA is available as the membership benefit for members of AOAC International. Thus, it is important that any method used in veterinary analytical toxicology is at least validated within the laboratory.

The American Association of Veterinary Laboratory Diagnosticians (AAVLD) is also an accrediting organization for veterinary diagnostic laboratories in USA. AAVLD has been emphasizing the use of validated methods in diagnostic laboratories. It should be pointed out that any method from the scientific literature does not become a validated method by simply rewriting it in ISO format. In order to be validated, the laboratory must prove that the method has acceptable performance characteristics of required attributes. If the laboratory hires a new analyst, the analyst should show that he (she) could get results consistent with the performance characteristics of the method. Many federal laboratories, such as the Food Safety and Inspection Service (FSIS), United States Department of Agriculture (USDA) (FSIS–USDA, 1995) require this.

## AN EXAMPLE OF AN SLV FOR THE GAS CHROMATOGRAPHIC DETERMINATION OF SELENIUM (Se) IN BLOOD AND LIVER

An illustrated example of the SLV for the determination of selenium in liver and whole blood is given here. In our laboratory there was a need for the determination of selenium (Se) in fresh liver and whole blood. The literature survey showed a large number of methods for the determination of Se using widely differing techniques such as fluorometry, AA, and GLC with electron capture detector (GLC–ECD). Due to the limitation in appropriate instrumentation, and conversation with another colleague, we decided on the GLC/ECD method. The method of Poole *et al*. (1977) as modified by Ross and Lund (1982) was selected. The method involves the wet digestion of the sample (0.1–1 g) in concentrated nitric acid with magnesium nitrate, the mixture is heated on a hot plate below 100°C till all the liquid evaporates, and after that the temperature is raised to remove the brown fumes of nitric acid. The residual nitric acid is removed by placing the sample in a muffle furnace at 500°C for 30 min. After cooling the sample, the ash is dissolved in concentrate hydrochloric acid for 10 min, at 100°C. To the solution, 10 ml of 20% urea is added and the mixture is allowed to cool; 4 ml of 1% solution of 1,2-diamino-4-nitrobenzene is added. The solution is transferred to a 50 ml stoppered polyethylene centrifuge tube quantitatively with the aid of 5 ml water. The contents of the tube are mixed, and 5 ml of toluene is added to the tube and vigorously shaken for 30 s, and phases are allowed to separate. About 2 ml layer of toluene is passed through a column of anhydrous sodium sulfate packed in a pasture pipette. The toluene layer is collected in pyrex screw cap tube, and 1 μl of the toluene (containing Se derivative) is injected in the gas chromatograph fitted with ECD. A Tracor (MT 222) gas chromatograph fitted with a 6 ft glass column with 1/4 in. internal diameter packed with 1.5% OV-17 and 0.95% OV-225 was used. The oven temperature was 200°C and detector temperature was 300°C. The carrier gas nitrogen flow was maintained at 60 ml/min. The Se retention time was about 3 min.

## CALIBRATION CURVE: HOW TO MEASURE A QUANTITY OF INTEREST

The modern chemical analytical instruments use physical properties for the measurement of analyte concentration. Thus, analytical instrument has to be optimized, and calibrated to detect and report the information about concentration of an unknown. The instrument is calibrated to test the response against a series of known standards of the analyte. The result of this experiment is a plot of response versus concentration, known as calibration curve. Calibration curves are functions of an instrument's response to a range of known concentration levels of reference material. In some cases the concentration level may be too low to be detected by the particular instrument in use, in other cases it may be too high and overload the detector. The optimal range of concentration level for detection is usually narrow. Ideally, this narrow range represents a linear response. The analytical chemist prefers to work in this linear range, since data analysis is comparatively easy in this range. The calibration standards from the reference material should be made with utmost care and

**TABLE 86.1** Se calibration curve data

| | Concentration | Response |
|---|---|---|
| | xi | yi |
| | 0 | 0 |
| | 0.1 | 2.1 |
| | 0.25 | 5.8 |
| | 0.5 | 10.9 |
| | 0.75 | 17 |
| $r$ | 0.99951739 | |
| | $b$ | $a$ |
| | 22.5308311 | −0.049865952 |

accuracy. The concentration of the analyte in the unknown sample is calculated by using a mathematical relationship between concentration and response, known as the regression equation for the calibration curve. The regression equation takes the form, response ($y$) = slope of the curve ($b$)+ $y$ intercept.

$$y = bx + a \quad \textbf{86.1}$$

We can determine the value of $b$ and $a$, from the calibration curve data, and the concentration of analyte in the sample, $x$, can be calculated according to Eqn. 86.2

$$x = \frac{\mathrm{y} - \mathrm{a}}{b} = \frac{Response - Intercept}{Slope} \quad \textbf{86.2}$$

The response is read from the instrument, and the slope and intercept are obtained from the calibration curve.

## GOODNESS OF LINEARITY OF CALIBRATION CURVE

The degree of linearity of the calibration curve is determined by the correlation coefficient, $r$, and as well as plotting the detector response on $y$-axis, and the concentration of the reference standards on the $x$-axis. The value of $r$, varies from −1.0 to + 1.0. A value of 1 indicates that there is positive linear relationship between the detector response and the standard concentration. The data for a typical calibration curve for Se is shown in Table 86.1. The data in Table 86.1 shows a linear relationship, and the calibration curve shows a straight line, shown in Figure 86.1.

The value of $r$, is calculated on Excel spreadsheet using the function, = correl(array $y$, array $x$). The values of the slope $b$, and intercept $a$, are calculated by using the function, = linest(array $y$, array $x$). In order to get both values ($b$ *and* $a$), place the data for concentration, $x$, *and* the response, $y$, columns as shown in Table 86.1. Run the "linest" function in a column just below marked, $b$. Hold the shift, and ctrl key and hit enter, both values will appear on the spreadsheet.

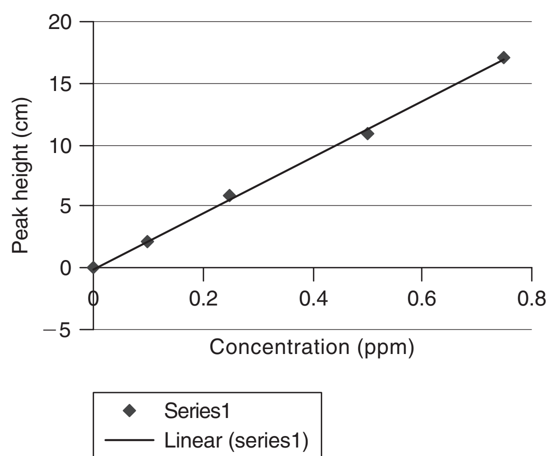


**FIGURE 86.1** Se calibration curve.

The correlation coefficient, slope, and the intercept can also be calculated with the aid of equations (3, 4, and 5) given below (Miller and Miller, 1992)

$$r = \frac{\sum_i \{(x_i - \bar{x})(y_i - \bar{y})\}}{\sqrt{\left\{\left[\sum_i (x_i - \bar{x})^2\right]\left[\sum_i (y_i - \bar{y})^2\right]\right\}}} \quad \textbf{86.3}$$

$$b = \frac{\sum_i \{(x_i - \bar{x})(y_i - \bar{y})\}}{\sum_i (x_i - \bar{x})^2} \quad \textbf{86.4}$$

$$a = \bar{y} - b\bar{x} \quad \textbf{86.5}$$

The regression equation for the calibration is as follows:

$$y = 22.53x + (-0.0499)$$

### Recovery and accuracy

The recovery and accuracy was determined with the use of standard reference bovine liver (1577a) from the National Institute of Standards and Technology (NIST), Gaithersburg, MD. The mean value of Se in the NIST standard 1577a was reported to be 0.71±0.07 ppm. Duplicate samples of NIST bovine liver sample were run on 10 different days. A new calibration curve was made each day with new Se standards (obtained from Perkin Elmer). The results of Se concentration in NIST bovine liver are shown in Table 86.2.

The mean recovery from NIST 1577a bovine liver is 0.6852 ppm. Thus, recovery % is:

% Recovery = (Laboratory mean recovery/NIST value) × 100

% Recovery = 0.6852/0.71 = 96.5%

The % recovery is well within the values recommended in AOAC guidelines (AOAC, 2003) that are shown in Table 86.3.

**TABLE 86.2 Se recovery data**

| Replicate | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 | Day 6 | Day 7 | Day 8 | Day 9 | Day 10 | Mean |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 0.754 | 0.678 | 0.614 | 0.517 | 0.696 | 0.769 | 0.662 | 0.690 | 0.560 | 0.740 | 0.668 |
| 2 | 0.804 | 0.753 | 0.709 | 0.663 | 0.691 | 0.772 | 0.663 | 0.619 | 0.560 | 0.790 | 0.7024 |
| Mean | | | | | | | | | | | 0.6852 |

**TABLE 86.3 Acceptable recovery values for various analyte concentrations**

| Concentration | Recovery limits (%) |
|---|---|
| 100% | 98–101 |
| 10% | 95–102 |
| 1% | 92–105 |
| 0.1% | 90–108 |
| 0.01% | 85–110 |
| 0.001% (10 ppm) | 80–115 |
| 0.0001% (1 ppm) | 75–120 |
| 1 ppb | 70–125 |

## Precision

The precision of an analytical method is represented by the standard deviation for the method for replicate measurements. There are two kinds of standard deviations that can be computed for the laboratory method. One is the standard deviation for within day run, i.e. the standard deviation for replicate samples that are analyzed on a given day, the other is the standard deviation for among days analysis. Since the routine samples are analyzed on days when the laboratory receives them, the standard deviation for "among days" applies for the routine samples. The manual calculations, for the calculations of this standard deviation, are complex. Fortunately, AOAC International has the software for calculating these standard deviations, and is available from Dr. Joanna Lynch (jl72@cornell.edu), Cornell University, Ithaca, NY. This software is developed for evaluating interlaboratory (collaborative) studies. However, this is also applicable for the determination of within day, and among days standard deviation. This software can also determine the % recovery for the method.

The within day standard deviation ($s_r$), also known as repeatability, is calculated, from the software as 0.0480 ppm, and the relative standard deviation, $RSD_r$, which is represented by the relationship

$$\%RSD_r = \frac{s_r}{\bar{x}} \times 100 = 7\% \qquad \textbf{86.6}$$

But our true standard deviation for repeatability is among days standard deviation, thus the true repeatability relative standard deviation is calculated by using $s_R$ (0.082) (from AOAC software). For replicate measurements made on different days within a laboratory, it is also known as intermediate precision. For clarity purpose, this relative standard deviation is represented as $\% RSD_{ri}$. *The true repeatability relative standard deviation* (among days) is:

$$\%RSD_{ri} = \frac{s_R}{\bar{x}} \times 100 = 11.97\% \qquad \textbf{86.7}$$

Although the recovery is within AOAC International guidelines, does it belong to the same population as the NIST bovine liver 1577a? The answer to this question is obvious. The 95% confidence limit for the NIST bovine liver is given in the certificate of analysis as $0.71 \pm 0.07$ ppm. The laboratory mean recovery of 0.69 ppm (0.6852 rounded to two decimal places) is within the confidence limit as provided by NIST certificate of analysis. Thus, it can be concluded that there is no significant difference between two means. At this point the only thing to be answered is how good the laboratory relative standard deviation ($RSD_{ri}$) is. This is determined by the use of HorRat values.

## HorRat values

For SLV work the HorRat value is given by the relationship,

$$HorRat_r = RSD_{ri} \ / \ PRSD_R \qquad \textbf{86.8}$$

where $RSD_{ri}$ is the relative standard deviation for among days analysis (Eqn. 86.8), and $PRSD_R$ is the predicted relative standard deviation for between laboratories analysis. How do we find $PRSD_R$? It can be predicted based on concentration of the analyte (Horwitz *et al.*, 1980; Horwitz, 1982). Based on the data from 100 years worth of interlaboratory method validation studies conducted under AOAC International auspices, Horwitz determined that relative standard deviation between laboratories and within laboratory is dependent on the analyte concentration. The relationship is expressed by the equation:

$$PRSD_R\% = 2C^{-0.15} \qquad \textbf{86.9}$$

where $C$, is expressed as the mass fraction. The above equation can be expressed in spreadsheet notation: $PRSD_R$ % =2 × $C$^ (−0.15). For example a concentration of 1 ppm is expressed as 1.000E−6 (1 μg/g). Thus for an analyte concentration of 1 ppm the calculated:

$$PSRD_R\% = 2 \times (10.00E{-}07)\text{^}(-0.15) = 16\% \quad \textbf{86.10}$$

Another observation of Horwitz that was confirmed by Thompson and Lowthian (1997) is that the precision of analytical methods at any given concentration does not improve with time, despite the advances in analytical technology. The mean value of selenium in NIST bovine liver is 0.71 ppm, thus, the predicted relative standard deviation for between laboratories is

$$PSRD_R\% = 2 \times (7.1E{-}7)\text{^}(0.15) = 16.7\%$$

Thus, the HorRat value for our method for the determination of selenium is:

$$HorRat = 11.97/16.7 = 0.71$$

The accepted HorRat values for single laboratory method validation are from 0.5 to 1.3. Thus, the relative standard deviation for our method is well within the accepted limit. The linearity of the calibration curve, the percent recovery, the relative standard deviation, and HorRat value clearly show that the method meets the validation criteria.

## Shewhart chart

The basic concept behind Shewhart's control chart is the distinction between two categories of variation. A process will either display "controlled variation" or it will display "uncontrolled variation." When a process displays controlled variation its behavior is indiscernible from what might be generated by a "random" or "chance" process. On the other hand, when a process displays uncontrolled variation, then something markedly different has occurred from what would have been expected from a random or chance process, and therefore an "assignable cause" can be attributed to that occurrence. Also, seeing its effect has been large enough to notice, it is surely worth the effort to try to identify this assignable cause.

Given this distinction, the control chart is a technique for detecting which type of variation is displayed by a given process. The objective is to give the user a guide for taking appropriate action – *to look for assignable causes* when the data display uncontrolled variation, and *to avoid looking for assignable causes* when the data display controlled variation.

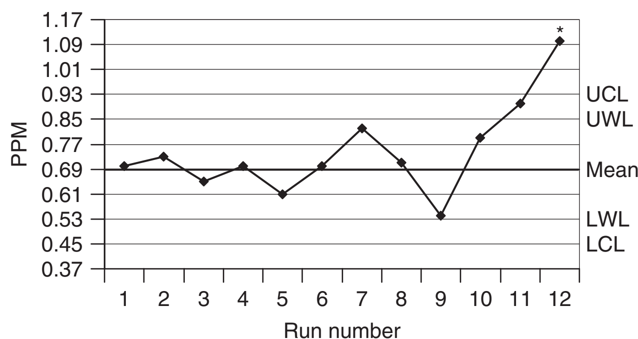


**FIGURE 86.2** Shewhart chart – Se assay.

Shewhart looked upon the control chart as the voice of the process – one can use the chart to understand how a process is behaving. Therefore, a process is said to be "in control" only when it has demonstrated, by means of a reasonable amount of data, that it is performing consistently and predictably. Moreover, from a practical perspective, such a process is, in essence, *performing as consistently as possible*. Therefore, any process that is not performing "as consistently as possible" may be said to be "out of control." Thus, the control chart is a procedure for characterizing the behavior of a process.

Figure 86.2 shows the Shewhart control chart for our GC method for selenium determination in NIST bovine liver 1577a. In normal distribution, two standard deviations of the mean consist of 95% of the values, and three standard deviations comprise 99% all the values, which can result from random variation in the process. Thus, for each run, if we plot the difference from the mean on $y$-axis, and number of run on $x$-axis, it provides us a pictorial view of the variation in the process. If our values fall within two standard deviations from the mean, the process is statistically under control. Thus, two standard deviations in positive or negative direction form the upper and lower warning limits. If the observation from the run is coming very close to the warning limit line that means the process is nearing its statistical control limit. If the values go beyond three standard deviations from the mean that means, the process is out of control, and that certain errors other than the random variation are entering the process. At this time, the analysis for routine samples should be stopped, and the reason for the error be investigated. When the problem is solved, only then the routine testing shall begin. In Figure 86.2, an asterisk marks the point at which the method is out of statistical control.

## EXAMPLE OF AN INTERLABORATORY (COLLABORATIVE) STUDY FOR THE VALIDATION OF SEMI QUANTITATIVE METHOD

Any method must be fit for the purpose. Nitrate causes poisoning in ruminants. The nitrate containing forages are usually responsible for this poisoning. The guidelines for feeding ruminant with nitrate containing forages are as follows (on dry matter basis) (Guthrie, 1986):

- Forages with nitrate concentrations less than 1000 ppm are considered safe under all conditions.
- Forages with 1000–5000 ppm nitrate should be safe to feed to *nonpregnant animals* under all conditions.
- Forages containing more than 2500-ppm nitrate should be limited to 50% of the total ration for *pregnant and lactating* animals. Erratic and low milk production is expected when nitrate concentration in forages exceeds 2500 ppm.
- Forages containing 5000–10,000 ppm nitrate may be fed safely to *nonpregnant and nonlactating* animals if limited to *50% of the total ration*. For *lactating and pregnant animals*, such forage should be limited to 25% of the total ration. Abortions and drop in milk production should be expected if such forage is greater than 25% of the total ration.
- With forages containing greater than 10,000-ppm nitrate, abortions, acute symptoms, and death should be expected. Thus, these forages should not be fed to ruminants.

Results of an intralaboratory study reported by Jain (1993) indicated that nitrate content of forage could be estimated using a test strip. Thus, a collaborative study was conducted to demonstrate the validity of the screening test for nitrate in forages.

Six forage samples were made for the collaborative study (Jain *et al*., 1999). The nitrate concentration in forage samples ranged from <1000 ppm nitrate to >10,000 ppm nitrate. Five samples were made from field cases submitted to the Athens Veterinary Diagnostic Laboratory. Two of them were mixture of forages. The sixth specimen was also a mixture of forages, and was provided by the Veterinary Diagnostic Center, University of Nebraska, Lincoln. The forage specimens were dried overnight at 60°C in a convection oven, ground using a Wiley Mill to pass through a 2-mm screen, and then thoroughly mixed in twin shell blender. Samples for each collaborator were prepared by placing 1.0 g of forage in a 50 ml plastic centrifuge tube labeled with a coded number. Five blind replicates of each of the six forage samples were prepared for each collaborator. The samples were labeled with numbers 1–30, and were sent to 20 different laboratories across USA.

Table 86.4 shows the results from various laboratories. The laboratory recorded the range number of a given sample. For example for sample number 21, which is in range number 1, all the laboratories, except laboratory U, identified the sample correctly in range number 1. The complete results were received from 19 laboratories. The statistical analysis of the data was conducted according McClure (1990). The published collaborative report does not show how the calculations for statistical parameters were

**TABLE 86.4 Response profiles of laboratory results for each range**

| | Range 1 samples | | | | | | | | | | Range 2 samples | | | | | Range 3 samples | | | | | Range 4 samples | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Laboratories | 2 | 11 | 12 | 13 | 16 | 4 | 6 | 10 | 21 | 27 | 8 | 14 | 22 | 28 | 30 | 15 | 17 | 20 | 23 | 29 | 3 | 5 | 7 | 9 | 25 | 1 | 18 | 19 | 24 | 26 |
| G | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 2 | 2 | 2 | *2 | 3 | 3 | 3 | 3 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | *3 | 4 | 4 |
| H | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 2 | 2 | 2 | 3 | 3 | 3 | 3 | 3 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| I[a] | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 2 | 2 | 2 | *2 | *2 | *2 | 3 | *4 | 4 | 4 | *3 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| J | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 2 | 2 | 2 | 3 | 3 | 3 | 3 | 3 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| K | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 2 | 2 | 2 | 3 | 3 | 3 | 3 | 3 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| L | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 2 | 2 | 2 | 3 | 3 | 3 | 3 | 3 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| M[a] | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | *3 | *3 | 2 | 2 | 2 | *4 | *4 | *4 | 3 | 3 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| N | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 2 | 2 | 2 | 3 | 3 | 3 | 3 | 3 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| O | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 2 | 2 | 2 | 3 | 3 | 3 | 3 | 3 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| Q | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 2 | 2 | 2 | 3 | 3 | 3 | 3 | 3 | *1 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| R | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 2 | 2 | 2 | 3 | 3 | 3 | 3 | 3 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| S | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 2 | 2 | 2 | 3 | 3 | 3 | 3 | 3 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| T | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 2 | 2 | 2 | 3 | 3 | 3 | 3 | *4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| U | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | *2 | 1 | 2 | 2 | 2 | 2 | 2 | 3 | 3 | 3 | 3 | 3 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| V | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 2 | 2 | 2 | 3 | 3 | 3 | 3 | 3 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| W | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 2 | 2 | 2 | 3 | 3 | 3 | *2 | *2 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| X | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 2 | 2 | *3 | 3 | 3 | 3 | 3 | 3 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| Y | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | *3 | 2 | 2 | 2 | 3 | 3 | 3 | 3 | 3 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| Z | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 2 | 2 | 2 | 3 | 3 | 3 | 3 | 3 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |

The * to the left of the number indicates that the range was incorrectly determined.

[a]Results of laboratories I and M were deleted from statistical analysis as outliers based on *chi-square* test and inspection, Mcclure (1990).

made, it only shows final results. The step-by-step calculations are shown in this chapter for the benefit of the reader.

## Outlier results

First, the results were tested for outlier values, if any, using *chi-square* statistics ($Q$). The null hypothesis is that labs do not have significantly different proportions of correct results. Table 86.5 shows the recast of the data in Table 86.4 for statistical purposes to reflect correct (1), and incorrect (0) results, and is used for computation of $Q$ *statistic*, by Eqn. 86.11:

$$Q = \frac{(L-1)\left(L\sum T_i^2 - \left(\sum T_i\right)^2\right)}{\left(L\sum S_j - \sum S_j^2\right)} \qquad \textbf{86.11}$$

where $L$ is the number of laboratories reporting the results, $T_i$ is the number of correct results by the laboratory ($i$) and $S_j$ is the total number of correct results for sample ($j$). Thus, referring to Table 86.5,

$$Q = \frac{(19-1)(19(16023) - (551)^2)}{19(551) - (10145)}$$

$$Q = \frac{18(836)}{324}$$

$$Q = 46.44$$

This calculated value of chi-square is greater than the critical value of 28.87 for 18 ($L$-$1$) degrees of freedom. Thus, the null hypothesis is disapproved, and there are significantly different proportions of correct results. By inspection, the laboratories I and M appear to be outliers. After rejecting the outliers the test was repeated, and the calculated value of statistic ($Q$) was found to be 15.775, which is less than the critical value of 26.29 for 16 degrees of freedom (note that the results of laboratories I and M were rejected).

## Sensitivity rate

The sensitivity rate ($p+$) is the probability that the method will classify a test sample in a "given range" provided that the sample is in that "given range." For example, samples 2, 4, 6, 10, 11, 12, 13, 16, 21, and 27 are range 1 samples; whereas, all other samples are in "another range." For statistical analysis, we are saying that all samples listed under range 1 in Table 86.4 are correctly identified if they are identified as 1 in the table. Any other number in table for range 1 is considered as misidentification, and is considered a negative value for range 1. The data for the calculations of sensitivity rate and its standard error for various ranges is shown in Table 86.6.

$$(p_+) = \frac{\sum a_i}{\sum m_i} \qquad \textbf{86.12}$$

where $\sum a_i$ is the total number of correct results for a given range, $\sum m_i$, is the total number of results for that range. Thus, the sensitivity for range 1,

$$(p_+) = \frac{169}{170} = 0.994$$

The standard error for sensitivity for each range can be calculated by the formula

$$s.e.(p_+) = \left\{\frac{\left(\sum a_i^2 - \left(\sum a_i\right)^2/L\right)}{\sum (\bar{m})^2 \; L(L-1)}\right\}^{1/2} \qquad \textbf{86.13}$$

Thus for range 1,

$$s.e.\ (p_+) = \sqrt{\frac{1681 - 1680.0588}{100 \times 17 \times 16}} = 0.006$$

This means that for range 1, if large numbers of observations were made, 6 out of 1000 observations will misidentify range 1, and 994 observations will correctly identify range 1.

The sensitivity rate and standard error for other ranges can be calculated similarly.

## Specificity rate ($p_-$)

The specificity ($p_-$) for the "given range" is the probability that the method will classify the test sample in "any other range" provided the test sample was in "another range." For range 1 all samples, which are not in range 1, are in "another range." The value of ($p_-$) can be calculated by Eqn. 86.12. For specificity rate calculations, $m$, *is* 20 since there are 20 samples in ranges 2, 3, and 4. Thus the total of all $m$ values is 20 × 17 (340, since there were 17 laboratories). The total of correct values for specificity rate is 339, since only laboratory Q has a range 2, 3, or 4 sample identified as range 1 sample. The data for calculations of specificity rate and its standard error is shown in Table 86.7.

Thus specificity rate for range 1 is, $(p_-) = \frac{339}{340} = 0.997$.

The standard error for specificity rate using Eqn. 86.13 is:

$$s.e.\ (p_-) = \sqrt{\frac{6741 - 6760.059}{400 \times 17 \times 16}} = 0.003$$

**TABLE 86.5 Recast of data in Table 86.4 to reflect correct (1) and incorrect (0) results**

| | Range 1 test samples | | | | | | | | | | Range 2 test samples | | | | | Range 3 test samples | | | | | Range 4 test samples | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Laboratories | 2 | 11 | 12 | 13 | 16 | 4 | 6 | 10 | 21 | 27 | 8 | 14 | 22 | 28 | 30 | 15 | 17 | 20 | 23 | 29 | 3 | 5 | 7 | 9 | 25 | 1 | 18 | 19 | 24 | 26 | Ti | $T_i^2$ |
| G | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 28 | 784 |
| H | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 30 | 900 |
| I | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 25 | 625 |
| J | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 30 | 900 |
| K | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 30 | 900 |
| L | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 30 | 900 |
| M | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 25 | 625 |
| N | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 30 | 900 |
| O | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 30 | 900 |
| Q | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 29 | 841 |
| R | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 30 | 900 |
| S | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 30 | 900 |
| T | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 29 | 841 |
| U | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 29 | 841 |
| V | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 30 | 900 |
| W | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 28 | 784 |
| X | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 29 | 841 |
| Y | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 29 | 841 |
| Z | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 30 | 900 |
| $S_j$ | 19 | 19 | 19 | 19 | 19 | 19 | 19 | 19 | 18 | 19 | 18 | 17 | 19 | 19 | 18 | 16 | 17 | 17 | 18 | 16 | 18 | 19 | 18 | 19 | 19 | 19 | 19 | 18 | 19 | 19 | | |
| $S_j^2$ | 361 | 361 | 361 | 361 | 361 | 361 | 361 | 361 | 324 | 361 | 324 | 289 | 361 | 361 | 324 | 256 | 289 | 289 | 324 | 256 | 324 | 361 | 324 | 361 | 361 | 361 | 361 | 324 | 361 | 361 | | |
| sum $S_j$ | 551 | | | | | | | | | | | | | | | | | | | | | | | | | | | | sum $Ti$ | | 551 | |
| sum $S_j^2$ | 10,145 | | | | | | | | | | | | | | | | | | | | | | | | | | | | sum $Ti^2$ | | | 16,023 |
| $(S_j)^2$ | 303,601 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |

**TABLE 86.6 Data for calculations of sensitivity rate and its standard error[a]**

| | Range 1 | | Range 2 | | Range 3 | | Range 4 | |
|---|---|---|---|---|---|---|---|---|
| Laboratories | $m_j$ | $a_i$ | $m_j$ | $a_i$ | $m_j$ | $a_i$ | $m_j$ | $a_i$ |
| G | 20 | 20 | 25 | 24 | 25 | 24 | 20 | 20 |
| G | 10 | 10 | 5 | 5 | 5 | 4 | 10 | 9 |
| H | 10 | 10 | 5 | 5 | 5 | 5 | 10 | 10 |
| J | 10 | 10 | 5 | 5 | 5 | 5 | 10 | 10 |
| K | 10 | 10 | 5 | 5 | 5 | 5 | 10 | 10 |
| L | 10 | 10 | 5 | 5 | 5 | 5 | 10 | 10 |
| N | 10 | 10 | 5 | 5 | 5 | 5 | 10 | 10 |
| O | 10 | 10 | 5 | 5 | 5 | 5 | 10 | 10 |
| Q | 10 | 9 | 5 | 5 | 5 | 5 | 10 | 9 |
| R | 10 | 10 | 5 | 5 | 5 | 5 | 10 | 10 |
| S | 10 | 10 | 5 | 5 | 5 | 5 | 10 | 10 |
| T | 10 | 10 | 5 | 5 | 5 | 5 | 10 | 10 |
| U | 10 | 10 | 5 | 5 | 5 | 5 | 10 | 10 |
| V | 10 | 10 | 5 | 5 | 5 | 5 | 10 | 10 |
| W | 10 | 10 | 5 | 5 | 5 | 5 | 10 | 10 |
| X | 10 | 10 | 5 | 4 | 5 | 3 | 10 | 10 |
| Y | 10 | 10 | 5 | 4 | 5 | 5 | 10 | 10 |
| Z | 10 | 10 | 5 | 5 | 5 | 5 | 10 | 10 |
| Total | 170 | 169 | 85 | 83 | 85 | 82 | 170 | 168 |
| $\Sigma a_i^2$ | | 1681 | | 407 | | 400 | | 1662 |
| $\Sigma(a_i)^2$ | | 28,561 | | 6889 | | 6724 | | 28,224 |
| $(\bar{m})^2$ | 100 | | 25 | | 25 | | 100 | |

[a]$m_j$ = number of "known" positive samples for a given range/lab.
[a]$a_i$ = number of positive results obtained among "known" positive samples for a given range/lab.

**TABLE 86.7 Data for specificity rate and its standard error[a]**

| | Range 1 | | Range 2 | | Range 3 | | Range 4 | |
|---|---|---|---|---|---|---|---|---|
| Laboratories | $m_j$ | $a_i$ | $m_j$ | $a_i$ | $m_j$ | $a_i$ | $m_j$ | $a_i$ |
| G | 20 | 20 | 25 | 24 | 25 | 24 | 20 | 20 |
| H | 20 | 20 | 25 | 25 | 25 | 25 | 20 | 20 |
| J | 20 | 20 | 25 | 25 | 25 | 25 | 20 | 20 |
| K | 20 | 20 | 25 | 25 | 25 | 25 | 20 | 20 |
| L | 20 | 20 | 25 | 25 | 25 | 25 | 20 | 20 |
| N | 20 | 20 | 25 | 25 | 25 | 25 | 20 | 20 |
| O | 20 | 20 | 25 | 25 | 25 | 25 | 20 | 20 |
| Q | 20 | 19 | 25 | 25 | 25 | 25 | 20 | 20 |
| R | 20 | 20 | 25 | 25 | 25 | 25 | 20 | 20 |
| S | 20 | 20 | 25 | 25 | 25 | 25 | 20 | 20 |
| T | 20 | 20 | 25 | 25 | 25 | 25 | 20 | 20 |
| U | 20 | 20 | 25 | 24 | 25 | 25 | 20 | 19 |
| V | 20 | 20 | 25 | 25 | 25 | 25 | 20 | 20 |
| W | 20 | 20 | 25 | 23 | 25 | 25 | 20 | 20 |
| X | 20 | 20 | 25 | 25 | 25 | 24 | 20 | 20 |
| Y | 20 | 20 | 25 | 25 | 25 | 24 | 20 | 20 |
| Z | 20 | 20 | 25 | 25 | 25 | 25 | 20 | 20 |
| Total | 340 | 339 | 425 | 421 | 425 | 422 | 340 | 339 |
| $\Sigma a_i^2$ | | 6761 | | 10,431 | | 10,478 | | 6761 |
| $\Sigma(a_i)^2$ | | 114,921 | | 177,241 | | 178,084 | | 114,921 |
| $(\bar{m})^2$ | 400 | | 625 | | 625 | | 400 | |

[a]$m_j$ = number of "known" positive samples for a given range/lab.
[a]$a_i$ = number of negative results obtained among "known" negative samples for a given range/lab.

**TABLE 86.8 Data for calculations of false positive rates and their standard error**

| | Range 1 | | | | Range 2 | | | | Range 3 | | | | Range 4 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Laboratories | $m_j$ | $m_j^2$ | $a_i$ | $a_im_j$ | $m_j$ | $m_j^2$ | $a_i$ | $a_im_j$ | $m_j$ | $m_j^2$ | $a_i$ | $a_im_j$ | $m_j$ | $m_j^2$ | $a_i$ | $a_im_j$ |
| G | 10 | 100 | 0 | 0 | 6 | 36 | 1 | 6 | 5 | 25 | 1 | 5 | 9 | 81 | 0 | 0 |
| H | 10 | 100 | 0 | 0 | 5 | 25 | 0 | 0 | 5 | 25 | 0 | 0 | 10 | 100 | 0 | 0 |
| J | 10 | 100 | 0 | 0 | 5 | 25 | 0 | 0 | 5 | 25 | 0 | 0 | 10 | 100 | 0 | 0 |
| K | 10 | 100 | 0 | 0 | 5 | 25 | 0 | 0 | 5 | 25 | 0 | 0 | 10 | 100 | 0 | 0 |
| L | 10 | 100 | 0 | 0 | 5 | 25 | 0 | 0 | 5 | 25 | 0 | 0 | 10 | 100 | 0 | 0 |
| N | 10 | 100 | 0 | 0 | 5 | 25 | 0 | 0 | 5 | 25 | 0 | 0 | 10 | 100 | 0 | 0 |
| O | 10 | 100 | 0 | 0 | 5 | 25 | 0 | 0 | 5 | 25 | 0 | 0 | 10 | 100 | 0 | 0 |
| P | 10 | 100 | 0 | 0 | 5 | 25 | 0 | 0 | 5 | 25 | 0 | 0 | 10 | 100 | 0 | 0 |
| Q | 11 | 121 | 1 | 11 | 5 | 25 | 0 | 0 | 5 | 25 | 0 | 0 | 10 | 100 | 0 | 0 |
| R | 10 | 100 | 0 | 0 | 5 | 25 | 0 | 0 | 5 | 25 | 0 | 0 | 9 | 81 | 0 | 0 |
| S | 10 | 100 | 0 | 0 | 5 | 25 | 0 | 0 | 5 | 25 | 0 | 0 | 10 | 100 | 0 | 0 |
| T | 10 | 100 | 0 | 0 | 5 | 25 | 0 | 0 | 4 | 16 | 0 | 0 | 10 | 100 | 0 | 0 |
| U | 9 | 81 | 0 | 0 | 6 | 36 | 1 | 6 | 5 | 25 | 0 | 0 | 11 | 121 | 1 | 11 |
| V | 10 | 100 | 0 | 0 | 5 | 25 | 0 | 0 | 5 | 25 | 0 | 0 | 10 | 100 | 0 | 0 |
| W | 10 | 100 | 0 | 0 | 7 | 49 | 2 | 14 | 3 | 9 | 0 | 0 | 10 | 100 | 0 | 0 |
| X | 10 | 100 | 0 | 0 | 4 | 16 | 0 | 0 | 5 | 25 | 0 | 0 | 10 | 100 | 0 | 0 |
| Y | 10 | 100 | 0 | 0 | 4 | 16 | 0 | 0 | 5 | 25 | 0 | 0 | 10 | 100 | 0 | 0 |
| Z | 10 | 100 | 0 | 0 | 5 | 25 | 0 | 0 | 5 | 25 | 0 | 0 | 10 | 100 | 0 | 0 |
| $\Sigma m_j$ | 170 | | | | 87 | | | | 82 | | | | 169 | | | |
| $\Sigma m_j^2$ | | 1702 | | | | 453 | | | | 400 | | | | 1683 | | |
| $\Sigma a_i$ | | | 1 | | | | 4 | | | | 1 | | | | 1 | |
| $\Sigma a_im_j$ | | | | 11 | | | | 26 | | | | 5 | | | | 169 |
| $\Sigma a_i^2$ | | | 1 | | | | 6 | | | | 1 | | | | 1 | |
| $(\bar{m})^2$ | 100 | | | | 26.19 | | | | 23.27 | | | | 98.52 | | | |

[a]$m_j$ = the number of positive readings for a given range among positive and negative test samples/laboratory.
[a]$a_i$ = the number of positive readings for a given range among known negative samples/laboratory.

**TABLE 86.9 Data for calculations for false negative rate and its standard error[a]**

| | Range 1 | | | | Range 2 | | | | Range 3 | | | | Range 4 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Laboratories | $m_j$ | $m_j^2$ | $a_i$ | $a_im_j$ | $m_j$ | $m_j^2$ | $a_i$ | $a_im_j$ | $m_j$ | $m_j^2$ | $a_i$ | $a_im_j$ | $m_j$ | $m_j^2$ | $a_i$ | $a_im_j$ |
| G | | | | | | | | | | | | | | | | |
| H | 20 | 400 | 0 | 0 | 24 | 576 | 0 | 0 | 26 | 676 | 1 | 26 | 21 | 441 | 1 | 21 |
| J | 20 | 400 | 0 | 0 | 25 | 625 | 0 | 0 | 25 | 625 | 0 | 0 | 20 | 400 | 0 | 0 |
| K | 20 | 400 | 0 | 0 | 25 | 625 | 0 | 0 | 25 | 625 | 0 | 0 | 20 | 400 | 0 | 0 |
| L | 20 | 400 | 0 | 0 | 25 | 625 | 0 | 0 | 25 | 625 | 0 | 0 | 20 | 400 | 0 | 0 |
| N | 20 | 400 | 0 | 0 | 25 | 625 | 0 | 0 | 25 | 625 | 0 | 0 | 20 | 400 | 0 | 0 |
| O | 20 | 400 | 0 | 0 | 25 | 625 | 0 | 0 | 25 | 625 | 0 | 0 | 20 | 400 | 0 | 0 |
| P | 20 | 400 | 0 | 0 | 25 | 625 | 0 | 0 | 25 | 625 | 0 | 0 | 20 | 400 | 0 | 0 |
| Q | 19 | 361 | 0 | 0 | 25 | 625 | 0 | 0 | 25 | 625 | 0 | 0 | 21 | 441 | 1 | 21 |
| R | 20 | 400 | 0 | 0 | 25 | 625 | 0 | 0 | 25 | 625 | 0 | 0 | 20 | 400 | 0 | 0 |
| S | 20 | 400 | 0 | 0 | 25 | 625 | 0 | 0 | 25 | 625 | 0 | 0 | 20 | 400 | 0 | 0 |
| T | 20 | 400 | 0 | 0 | 25 | 625 | 0 | 0 | 26 | 625 | 1 | 26 | 19 | 361 | 0 | 0 |
| U | 21 | 441 | 1 | 21 | 24 | 576 | 0 | 0 | 25 | 625 | 0 | 0 | 20 | 400 | 0 | 0 |
| V | 20 | 400 | 0 | 0 | 25 | 625 | 0 | 0 | 25 | 625 | 0 | 0 | 20 | 400 | 0 | 0 |
| W | 20 | 400 | 0 | 0 | 25 | 625 | 0 | 0 | 27 | 729 | 2 | 54 | 20 | 400 | 0 | 0 |
| X | 20 | 400 | 0 | 0 | 26 | 676 | 1 | 26 | 24 | 576 | 0 | 0 | 20 | 400 | 0 | 0 |
| Y | 20 | 400 | 0 | 0 | 26 | 676 | 1 | 26 | 25 | 625 | 0 | 0 | 20 | 400 | 0 | 0 |
| Z | 20 | 400 | 0 | 0 | 25 | 625 | 0 | 0 | 25 | 625 | 0 | 0 | 20 | 400 | 0 | 0 |
| $\Sigma m_j$ | 340 | | | | 425 | | | | 428 | | | | 341 | | | |
| $\Sigma m_j^2$ | | 6802 | | | | 10,629 | | | | 10,731 | | | | 6843 | | |
| $\Sigma a_i$ | | | 1 | | | | 2 | | | | 4 | | | | 2 | |
| $\Sigma a_im_j$ | | | | 21 | | | | 52 | | | | 106 | | | | 42 |
| $\Sigma a_i^2$ | | | 1 | | | | 2 | | | | 6 | | | | 2 |
| $(\overline{m})^2$ | 400 | | | | 625 | | | | | 633.9 | | | 402 | | | |

[a] $m_j$ = number of negative readings among both "known" positive and negative samples lab.
[a] $a_i$ = number of negative readings among "known" positive test samples/lab.

## False positive rate ($p_{f+}$)

The false positive rate is the probability that the test sample is in "another range" and has been classified in a "given range." The data for the calculations of false positive rate and its standard error is shown in Table 86.8. The equations for the calculations for false positive rate and its standard error are Eqns 86.14 and 86.15, respectively.

$$\text{False positive rate } (pf_1) = \frac{\sum a_i}{\sum m_j} \qquad \mathbf{86.14}$$

$$s.e.(pf_+) = \sqrt{\frac{\left(\sum a_i^2 - 2(pf_+)\sum a_i m_j + (p_{f+}^2)\sum m_j^2\right)}{L(L-1)(\overline{m})^2}} \qquad \mathbf{86.15}$$

Thus, the false positive rate ($p_{f+}$) for range 1 is = 1/170 = 0.0058823 rounded to 0.006

$$s.e.(p_{f+}) = \sqrt{\frac{1 - 2(0.0058823) \times 11 + 0.0000346}{17 \times 16 \times 100}} = 0.0058395$$

## False negative rate ($p_{f-}$)

The false negative rate is the probability that a test sample is in a "given range" and the sample has been classified in "another range." The data for the calculations of false negative rate and its standard error is shown in Table 86.9. Thus, the false negative rate ($p_{f-}$), and its standard error for various ranges can be calculated from the Eqns 86.14 and 86.15, respectively.

False negative rate ($p_{f-}$) for range 1 =1/340 = 0.0029411 = 0.003 and its standard error is

$$s.e.(p_{f+}) = \sqrt{\frac{1 - 2 \times (0.003) \times 21 + 0.0612}{17 \times 16 \times 400}} = 0.0029 = 0.003$$

**TABLE 86.10 Performance parameters for various ranges**

| Parameter | Range 1 | Range 2 | Range 3 | Range 4 |
|---|---|---|---|---|
| Sensitivity rate ($p_+$)[a] | 0.994 | 0.977 | 0.965 | 0.988 |
| Standard error of ($p_+$) | 0.006 | 0.016 | 0.026 | 0.008 |
| Specificity rate ($p_-$)[b] | 0.997 | 0.991 | 0.993 | 0.997 |
| Standard error of ($p_-$) | 0.003 | 0.005 | 0.004 | 0.003 |
| False Positive Rate ($p_{f+}$)[c] | 0.006 | 0.046 | 0.012 | 0.006 |
| Standard error of ($p_{f+}$) | 0.006 | 0.025 | 0.012 | 0.006 |
| False negative rate ($p_{f-}$)[d] | 0.003 | 0.005 | 0.007 | 0.006 |
| Standard error of ($p_{f-}$) | 0.003 | 0.003 | 0.006 | 0.004 |

[a] The sensitivity rate ($p_+$) is the probability that the method will classify a test sample in a "given range" provided the sample is in that "given range."
[b] The specificity rate ($p_-$) is the probability that the method will classify the test sample in "another range" provided the test sample is in "another range."
[c] The false positive rate ($pf+$) is the probability that the test sample is in "another range" and has been classified in a "given range."
[d] The false negative rate ($pf_-$) is the probability that a test sample is in given range, and the sample has been classified in "another range."

### Performance parameters for the method

Various performance parameters are shown in Table 86.10.

## LABORATORY QUALITY MANAGEMENT

The quality of a laboratory product depends on the quality system that is followed by the laboratory. Just what is quality and why is it so important? Quality system has a different meaning in different laboratories but they share the same key elements to ensure the quality and integrity of the product. A quality system has elements that are of quality assurance (QA) nature and others are of a quality control (QC) nature. The quality system is also referred as QA/QC. Some laboratories work under the standards of a certain organization such as ISO, AOAC, or AAVLD whereas some laboratories develop their own quality system customized to the needs of the laboratory (Tebbett, 2003).

### Quality assurance

ISO standard 9000:2000 defines QA as part of the quality management focused on providing the confidence that quality requirements will be fulfilled. All QA programs have similar elements: they are designed to ensure that (1) the sample is not contaminated, (2) is handled by personnel who are trained for that specific job and are using equipment that is properly calibrated and procedures that are validated, and (3) that everything is documented and that records can be accessed when needed (Tebbett, 2003). Good quality produces good results. It gives the laboratory an edge over competitors and makes the customers happy because they can rely on the integrity of the results produced. Poor quality wastes time, supplies, and money (Archer, 2005). The accuracy of the result is questionable which casts a shadow on everything that the laboratory produces.

### Quality control

Quality control refers to the steps taken during the analytical process to ensure that the process produces reliable measurements data that is fit for the purpose.

## CONCLUSIONS

"Prove it!" What would be your response to that challenge to the data generated by your investigation or services? Can you prove the accuracy of your data? Yes, you can prove the integrity and accuracy of your results if the validated analytical method was used in conjunction with reference materials traceable to recognized source, and you have on hand the validation data. Just saying that we use validated methods in our laboratory is not enough. The analysis of reference materials traceable to sources recognized by measurement bodies with client samples is essential to provide defensible data of such quality that is useful to the client. Even the validated methods must be repeated in the laboratory to confirm that the laboratory is able to get the performance parameters as reported in the validated method. The record keeping of the validation of performance parameters is part of the quality management plan. In order to ascertain that the method is performing under statistical control, a control sample should be run with each batch of samples. The value of the control sample gives the indication of the performance of the method on a given day. Analyzing control sample with each batch of samples is also part of the quality management program. Each laboratory must have a sound quality management (QA/QC) program.

## ACKNOWLEDGMENT

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## REFERENCES

AOAC (2000) *Official Methods of Analysis (OMA) of AOAC International*, 17th edn, Horwitz W (ed.). AOAC International, Frederick Avenue, Gaithersburg, MD, 20877.

AOAC (2003) *Guidelines for Single Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals*. AOAC International, Fredrick Avenue, Gaithersburg, MD, 20877.

Archer D (2005) *QA System Orientation*. NAHLN/AAVLD QA Committee Joint Symposium; Hershey, PA.

FSIS, USDA (1995) *Chemistry Quality Assurance Manual*. United States Department of Agriculture, Food Safety and Inspection Service, Quality Systems Branch.

Guthrie LD (1986). *Georgia Dairyfax*. The University of Georgia Cooperative Extension News Letter, July 1986, Athens, GA USA.

Helrich K (1984) *The Great Collaboration*. Association of Official Analytical Chemists (now known as AOAC International), Fredrick Ave, Gaithersburg, MD.

Horwitz W, Kamps, Boyer KW (1980) Quality assurance in the analysis of foods for trace constituents. *J Assoc Off Anal Chem* **63**: 1344–54.

Horwitz W (1982) Evaluation of analytical methods used for regulation of food and drugs. *Anal Chem* **54**: 67A–76A.

Horwitz W, Peeller JT, Albert R (1989) Precision parameters of standard methods of analysis for dairy products. *J Assoc Off Anal Chem* **72**: 784–806.

Jain AV (1993) Rapid test for the semiquantitative determination of nitrate in forages: intralaboratory study. *J AOAC Intl* **76** (5): 948–52.

Jain AV, Ross PF, Carlson MP (1999) Screening nitrate in forages with a test strip. Collaborative study. *J AOAC Int* **82** (1): 1–7.

McClure FD (1990) Design and analysis of qualitative collaborative studies: minimum collaborative program. *J Assoc Off Anal Chem* **71**: 953–60.

McCrone WC (1987) The evolution of chemical analysis. *Am Lab* **22**: 21–7.

Miller JC, Miller JN (1992) *Statistics for Analytical Chemistry*, 2nd edn, Ellis Horwood, New York.

Poole CV, *et al*. (1977) Determination of selenium in biological samples by gas chromatography with electron capture detection. *J Chrom* **136**: 73–83.

Rogers LB (1986) The inexact, imprecise science of trace analysis. *J Chem Edu* **63**: 3–6.

Ross PF, Lund SA (1982) *Proceedings of the 25th meeting of American Association of Veterinary Laboratory Diagnosticians*, 485–90.

Sawyer LD (1988) The development of analytical methods for pesticide residues. In *Pesticide Residues in Food: Technologies for Detection.* Office of Technology Assessment OTA-F-398, U.S. Government Printing Office, Washington, DC, pp. 112–22.

Seiber JN (1988) Conventional pesticide methods: can they be improved. In *Pesticide Residues in Food: Technologies for Detection.* Office of Technology Assessment OTA-F-398, pp 142-152, U.S. Government Printing Office, Washington, DC.

Tebbett I (2003) Laboratory QA/QC, Course Notes.

Thompson M, Lowthian PJ (1997) The Horwitz function revisited. *J. AOAC Int.* **80**: 459–62.

C H A P T E R

# 87

# Sample submission for toxicological analysis

*Anant V. Jain*

## INTRODUCTION

Is my patient poisoned? This question arises almost in every veterinary practice. An accurate diagnosis is the single-most important factor in dealing with animal poisons. The offending agent may be known or unknown. If the offending agent is known, specific treatment and prevention can be initiated. Often the toxic agent is unknown. In this instance, efforts are limited to supportive care and symptomatic therapeutic measures.

Chemical analysis is very important in establishing diagnosis of poisoning. Chemical analysis is usually done at the veterinary diagnostic laboratories, and there are many veterinary diagnostic laboratories in USA. A list of accredited and allied laboratories is available at www.aavld.org. Most of these laboratories are partially funded by respective states, thus, the services of veterinary diagnostic laboratories are available at a reasonable price to the client. Familiarity with the chemical analysis services provided by diagnostic laboratory in your area and its fee structure are extremely important in the effective use of the services provided by the laboratory.

The objective of this chapter is to provide with guidelines for proper samples submission to diagnostic laboratories. Each diagnostic laboratory has a submission form, which should be completed in full to get the most efficient service for chemical analysis. All of the diagnostic laboratories require complete history, clinical signs, and postmortem findings. This information greatly aids the laboratory personnel to decide on the best course for the chemical analysis.

## REQUIRED INFORMATION FOR SAMPLE SUBMISSION

### History

A detailed history of the circumstances associated with poisoning is the first step for a successful diagnosis. The investigation of poisoning case is as much detective work as is veterinary medicine, and chemical analysis. Three detectives are partners in this endeavor – veterinarian, client, and the analytical laboratory. The veterinarian must be ready to gather as many pieces of the puzzle together as possible. Fitgerald (2001) discusses in detail various elements of a good history. Osweiler *et al*. (1985) and Osweiler (1996) provides a checklist for a good history.

### Clinical signs

Clinical signs are very important in reaching the diagnosis of a poisoning. Clinical signs can narrow the search for toxic agents. Ask the client for sequence of clinical symptoms. Describe clinical signs in detail. It is not enough to say that the patient was observed with central nervous system (CNS) signs. It is essential to provide the complete sequence of events as to how the patient acted.

### Postmortem findings

If the animal has expired, it is imperative to perform a necropsy. It is better to perform the necropsy at site since

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it can provide very valuable information. If it is not possible to perform the necropsy at the site send the body to the diagnostic laboratory. A qualified pathologist will conduct the necropsy and collect the appropriate sample for histological as well as for toxicology testing. If it is poisoning then why worry about necropsy and histological examination? Simply test for toxicants and it should give us a diagnosis. Unfortunately, life is not that simple. There are thousands of chemicals that can cause poisoning in animals. *There is no simple test for all the poisons*. Some poisons produce extensive lesions, other only slight tissue alterations, and some produce on lesions at all. Based on this information the pathologist can provide assistance in the selection of toxicants to test for.

## Chemical analysis

Chemical evidence is often an indispensable aid in diagnosing toxicological problems. Used properly, chemical analysis can provide the most important evidence of poisoning. Analytical data should be used in conjunction with history, clinical signs, necropsy findings, and histological examination. The importance of clinical signs, postmortem findings, and chemical analysis has been described in detail in previous reports (Osweiler *et al*., 1985; Poppenga and Braselton, 1990; Galey, 2000, 2001, 2004; Puschner and Galey, 2001; Volmer and Meerdink, 2002).

# SAMPLE COLLECTION AND SUBMISSION FOR TOXICOLOGY ANALYSIS

A list of various types of samples which can be useful in the investigation of poisoning cases is given in Table 87.1.

Each sample should be stored in a separate container, and the container should be labeled with an indelible ink

**TABLE 87.1 Samples for toxicology testing**

| Sample | Amount | Tests for which sample can be used | Comments |
|---|---|---|---|
| *Antemortem Samples* | | | |
| Blood | 10–20 ml | Heavy metals, trace elements, anticoagulants, antifreeze, cholinesterase, cyanide, chlorinated hydrocarbon pesticides | Refrigerate sample. Use anticoagulant such as EDTA or heparin. Use *Royal blue tubes* with anticoagulant |
| Serum | 10–20 ml | Some metals like Cu, Zn, and nitrate/nitrite, ammonia, drugs (particularly sulfa drugs) | Separate clot from the serum. Refrigerate or freeze (if it will take long during shipping). Amount required for single test will be much less. Check with the testing laboratory. If Zn assay is desired use *Royal blue tubes* without any additive or anticoagulant |
| Urine | 50–100 ml | Alkaloids, some metals, antibiotics, cantharidin, drugs, sulfa drugs, oxalates, and paraquat or diquat | Freeze. Especially useful for paraquat or diquat |
| Feces | 250 g | Drugs, various poisons excreted mainly in bile | Freeze. Indicator of recent exposure |
| Biopsy specimen: liver | | Metals | Freeze |
| Milk | 250 ml | Antibiotics, chlorinated insecticides, polychlorinated biphenyls, | |
| Vomitus or gastric aspirate | All available | Various poisons. Heavy metals, anticoagulants, antifreeze, pesticides (all chlorinated hydrocarbons organophosphates, and carbamates) | Freeze. Indicator of recent exposure. In case of gastric aspirate make sure to submit first aspirate |
| Hair | 5–10 g | Pesticides, and some metals such as As and Se | Indicator of recent exposure. Chronic accumulation of metals |
| *Postmortem Samples** | | | |
| Brain | Half | Chlorinated pesticides, pyrethrins, cholinesterase, and metals like, Pb, Na, Hg | Brain should be separated by midline sagittal section. Collect frontal cortex for cholinesterase determination. Freeze |
| Fat | 250 g | Chlorinated pesticides and dioxins | |
| Kidney | 100 g or all available | Metals like As and Pb. Phenolic compounds, oxalates | Freeze |
| Liver | 100 g or all available | Chlorinated pesticides, alkaloids, anticoagulants, aflatoxin $M_1$, and metals | Freeze |

(*Continued*)

**TABLE 87.1 (Continued)**

| Sample | Amount | Tests for which sample can be used | Comments |
|---|---|---|---|
| Ocular fluid | All available | Nitrate/nitrite electrolytes | Freeze |
| Rumen content | 500 g | Various poisons. Heavy metals, pesticides (all chlorinated hydrocarbons organophosphates, and carbamates) | Freeze. Sample should be collected from several locations in the rumen. Rumen content is not a good sample for nitrate/nitrite evaluation |
| Stomach content | 500 g or all available | Various poisons. Heavy metals, anticoagulants, antifreeze, pesticides (all chlorinated hydrocarbons organophosphates, and carbamates) | Freeze |
| *Environmental samples* | | | |
| Baits | All available | Various poisons. Heavy metals, anticoagulants, antifreeze, pesticides (all chlorinated hydrocarbons organophosphates, and carbamates), anticoagulants | Any other information which is available about bait should be communicated to the testing laboratory |
| Feeds | 2 kg | Various poisons. Heavy metals, pesticides (all chlorinated hydrocarbons organophosphates, and carbamates), ionophores | Multiple representative samples should be taken from various places from the stock and mixed thoroughly for a composite sample or retained as individual samples to detect variability in the feed or both. Feed samples are required for ionophores screening |
| Forage (pasture) | 2–5 kg | Various poisons. Usually used for evaluation of nitrate/nitrite | Samples should be taken from multiple locations in pasture and mixed thoroughly for composite sample. Avoid contamination from soil. Refrigerate sample |
| Forage (hay) | 2–5 kg | Various poisons | Samples should be taken from multiple locations using a forage core sampler from baled hay or stacks and mixed thoroughly for composite sample |
| Forage (silage) | 2–5 kg | Various poisons | Freeze |
| Mushrooms | Whole 100 g | Identification cyclic peptide hepatotoxin | Keep cool and dry in paper bag |
| Plants | Entire plant | Identification | Press between sheets of paper |
| Soil | 1 kg | Various poisons | Samples should be taken from multiple locations using a soil core sampler and mixed thoroughly for composite sample |
| Water | 1 l | Nitrate/nitrite, pesticides, and metals | Use clean glass container. Refrigerate sample |

with animal and tissue identification. Place the sample container in a zip-lock bag, squeeze the air out of the bag, seal the zip-lock bag, and label the zip-lock bag also. *Do not add anything to the samples for chemical analysis: no antiseptic, no preservative, no fixative, unless otherwise noted (as for whole blood)?* These instructions apply to all the samples which are collected. Lorgue *et al*. (1996) provides a detailed account for sample collection and dispatch to the laboratory with particular reference to United Kingdom.

## Whole blood

Whole blood should be collected in royal *blue top* vacutainer tubes with anticoagulant. At least 10 ml of whole blood should be collected. If more than one test is required, collect 20 ml blood. Make sure the tube is properly sealed. Refrigerate the sample. *Do not freeze whole blood sample.*

## Serum

The blood for serum samples should be collected in *royal blue top* vacutainer tubes (plain) which contains *no additive or anticoagulants.* After the clot forms, usually less than 30 min, the serum should be separated promptly, and transferred to a clean plastic tube, and freeze the sample.

## Urine

Urine should be collected in a screw cap plastic bottle (4 oz.) and frozen.

## Liver biopsy specimen

Collect about 100–200 mg sample, and immediately freeze, some laboratories may not be able to provide results for

some metals due to small sample size. Contact the laboratory before proceeding with biopsy.

## Milk

Collect in a clean jar and refrigerate.

## Feces

Place feces in heavy duty aluminum foil, wrap it completely, place in zip-lock bag, and freeze.

## Vomitus or gastric aspirate

Place vomitus in a plastic bottle with screw cap and freeze. In case of gastric aspirate make sure to submit first aspirate.

## Hair

Hair samples should be collected using clean scissors or clippers. Do not contaminate with other materials such as blood on gloves. Place the hair sample in a zip-lock bag. Keep it at room temperature.

## Brain, liver, kidney, fat, and other tissue(s) with lesions

Collect appropriate amount of the particular sample (see Table 87.1), place the sample on a heavy duty aluminum foil, fold the corners of aluminum foil to seal the sample, and place the aluminum foil with the sample in the appropriate size zip-lock bag. Handle one specimen at a time and avoid cross contamination. Freeze the tissue samples and fat could be refrigerated. Special attention is needed for brain sample. Brain should be separated by midline sagittal section. Collect the frontal cortex for cholinesterase assay (Gupta, 2004), and freeze the sample until analysis. It should be pointed out that caudate nucleus should not be used for cholinesterase assay, since frontal cortex is better indicator of cholinesterase status (Gupta 2004).

## Ocular fluid

Collect ocular fluid in a small plastic tube and freeze.

## Ingesta (rumen content and stomach content)

Carefully examine the contents of rumen or stomach for extraneous material. Make sure that the extraneous material is sampled for submission. Collect appropriate amount of the particular sample (see Table 87.1), place the sample on a heavy duty aluminum foil, fold the corners of aluminum foil to seal the sample, and place the aluminum foil with the sample in the appropriate size zip-lock bag. Rumen contents should be collected from several locations in the rumen and mixed thoroughly. Rumen content is *not* a good sample for nitrate/nitrite evaluation.

## Baits

Collect all possible bait samples, wrap in heavy duty aluminum foil, and place in a zip-lock bag. Gather all possible information regarding the bait, and forward the information to the testing laboratory along with the sample.

## Feedstuffs

Feedstuffs should be collected from several different locations in bin, crib, or silo. Many mycotoxins, for example, are found in isolated pockets of the feed and therefore, could be missed if one sample is obtained. It is preferable to sample some feed from the troughs or bunks. If the ration is mixed on premises, it is advisable to collect at least one pound each of the various components in the total ration. Samples of dry ration can be submitted in plastic bags, and the bag labeled with the identification of the sample.

## Forage (pasture)

Samples should be taken from multiple locations in pasture and mixed thoroughly for composite sample. Avoid contamination from soil. Refrigerate the sample.

## Forage (hay)

Ideally, hay samples should be taken from multiple locations using a forage core sampler from baled hay or stacks and mixed thoroughly for composite sample. Additional attention should be paid when sampling hay for nitrate/nitrite assay. Nitrate is water soluble. When a bale of hay is exposed to rain, nitrate leaches from top of the bale and percolates through the bale. In this instance, it is preferable to sample hay in the area where water line has stopped. This area usually has very high nitrate concentration. Since this sample is wet, freeze it promptly. Make a notation on the history sheet if the animals were observed to eat hay from this area. In addition, the samples from other parts of the bale should also be submitted.

### Forage (silage)

Silage samples should be taken from multiple locations and frozen promptly.

### Mushrooms

Samples of mushrooms are collected for two purposes: identification of mushroom species and chemical identification of cyclic peptide hepatotoxin. For identification, collect a whole mushroom, and keep it cool and dry in a paper bag. For the presence of cyclic peptide hepatotoxin, collect about 100 g sample and freeze.

### Plants

Plant samples are collected for identification of the plant as well as the identification of plant toxins. It is desirable that the whole plant is collected intact, with roots, and any flowers or buds for identification. Plants can be pressed between sheets of newspaper and sent for identification. Plants for chemical analysis should be wrapped in aluminum foil, placed in a labeled plastic bag, and frozen.

### Soil

Samples of soil for chemical analysis should be taken from multiple locations using a soil core sampler and mixed thoroughly for composite sample.

### Water

Collect samples of water in clean glass containers.

### Samples for histological examination

In addition to samples for chemical analysis, samples of tissues fixed in formalin should also be submitted for histological examination. The importance of histological examination of tissue sample has already been discussed in an earlier section of this chapter.

### Samples for nutritional elemental analysis

Whole blood and serum are analyzed for this purpose. For elemental analysis, whole blood should be collected in royal blue top vacutainer tubes. For serum samples, the blood should be collected in royal blue top tubes (plain), which contains *no additive or anticoagulant*. After the clot forms, usually less than 30 min, the serum should be separated promptly and transferred to a clean plastic tube and frozen. The manuscript by Hancock *et al*. (1988) provides excellent information about the number of animals to be sampled for laboratory analysis for herd health management. It contains information tables for sample size required to estimate prevalence or attribute with a given confidence limit.

### Submission forms

All of the veterinary diagnostic laboratories have submission forms. Cases are not accepted in absence of submission forms. Complete the submission form as thoroughly as possible. Include all the information as discussed in earlier sections. Remember that there are three detectives in solving the poisoning problem – the client, the practitioner, and the analytical toxicologist at the diagnostic laboratory. It has been our experience that an accurate, fully completed submission form greatly helps laboratory colleagues to take the most appropriate analytical approach.

## SHIPPING REGULATIONS FOR DIAGNOSTIC SPECIMENS

Most of the countries have regulations for shipping diagnostic specimens. In USA the rules are published in the "Code of Federal Regulations" (CFR). One can access CFR on Internet. Go to google, type in code of federal regulations, and it takes you to the home page for regulations. In the search box, type diagnostic specimens, and it takes you to the regulations for transporting diagnostic specimens. Following is a summary of the current rules for transporting diagnostic specimens:

### Packing diagnostic specimens for transport: summary

- You as the shipper – not the transport company – are responsible for the shipment until the package reaches the consignee.
- The diagnostic specimens should be packaged in triple packing, consisting of primary, secondary, and the outer packing.
- *Primary packaging*: Primary receptacle(s) must be water tight, e.g. screw cap seal with Para film or adhesive tape or similar. Multiple primary receptacles must be wrapped individually to prevent breakage.
- *Secondary packaging*: Use enough absorbent material in the secondary container to absorb the entire contents

of all primary receptacles in case of leakage or damage. Secondary packaging must meet IATA packaging requirements for diagnostic specimens including 1.2 m (3.9 ft) drop test procedure.

- Secondary packaging must be watertight. Follow the packaging manufacturer or other authorized party's packing instructions included with the secondary packaging. Secondary packaging must be at least 100 mm (4 in.) in the smallest overall external dimension. Must be large enough for shipping documents, e.g. air waybill.
- *Outer packaging*: The outer packaging must not contain more than 4 l or 4 kg.
- Each package and the air waybill must be marked with the following exact wording:
  UN 3373 DIAGNOSTIC SPECIMEN
  PACKED IN COMPLIANCE WITH
  IATA PACKING INSTRUCTION 650
- An itemized list of contents must be enclosed between the secondary packaging and the outer packaging. Place in a sealed plastic bag to protect from moisture.
- A shipper's declaration for dangerous goods is *not* required.
- *Fines and penalties*: According to 49 CFR Sec. 171.1 (c) "Any person who knowingly violates a requirement of the Federal hazardous material transportation law. . . . Is liable for a civil penalty of not more than . . . $27,500 . . . and not less than $250 for each violation . . . and shall be fined under Title 18, United States code, or imprisoned for not more than 5 years, or both."

## Dispatch of samples

- Place one or two ice packs (as used for camping) in a polystyrene box. Place all frozen sample packets over ice packs. If whole blood is shipped together, make sure that some insulating material is between the frozen samples and blood. Place the polystyrene box in a rigid box.
- Write the address of the receiving laboratory legibly on the center of the outside box. On the upper left-hand corner write the sender's address.
- The submission form should be attached to the outside of the secondary packing and sealed in a plastic sleeve.
- The parcel should be clearly marked "*diagnostic specimen*" on the outside packing, and sent either by US parcel post, UPS, FedEx, or any other courier. Check with the courier for their special requirements.

# CONCLUSION

Great care should be taken during the collection and shipping diagnostic specimens for chemical analysis. Remember that laboratory results are as good as the submitted specimen. Follow transportation rules carefully, since it involves fine for violations. Diagnostic laboratories are not the enforcers of transportation rules. There was an incidence of a shipping foul up by a practitioner. The courier called the laboratory that they have a package for delivery to the laboratory. However, courier staff was not willing to handle the package, since it was shaking and making funny noises. The laboratory was asked to retrieve the package right away. The senior member of the laboratory went to pick up the package, opened the package, and found a live piglet in the container. Fortunately, the laboratory was informed the day before by the practitioner that he was sending one euthanized piglet for examination. Apparently, the piglet was not given enough euthanasia solution. This incidence happened about 10 or more years ago. Imagine, what would happen today, if a package like this arrived at the courier!

# REFERENCES


Fitgerald KT (2001) Taking a toxicological history. In *Small Animal Toxicology*, Peterson ME, Talcott PA (eds). W.B. Saunders Co, Philadelphia, PA, pp. 27–31.

Galey FD (2000) Diagnostic toxicology for food animal practitioner. *Toxicol Veter Clin North Am, Food Anim Pract* Osweiler GD, Galey FD guest editors, Smith RA consulting editor, W.B. Saunders Co., Philadelphia, PA, **16**(3) 409–21. .

Galey FD(2001) Approaches to diagnosis and initial treatment of the toxicology case. In S*mall Animal Toxicology*, Peterson ME, Talcott PA (eds). W.B. Saunders Co, Philadelphia, PA, pp. 99–113.

Galey FD (2004) Diagnostic toxicology. In *Clinical Veterinary Toxicology*, Plumlee KH (ed.). Mosby, St. Louis, MO, pp. 22–45.

Gupta RC (2004) Brain regional heterogeneity and toxicological mechanisms of organophosphates and carbamates. *Toxicol Mech Method* **14**: 103–43.

Hancock DD, Blodgett D, Gay CC (1988) The collection and submission of samples for laboratory testing. *Vet Clin North Am Food Anim Pract*, **4**(1): 33–59.

Lorgue G, Lechenet J, Riviere A (1996) Sampling for laboratory analysis and sending samples by Post. In *Clinical Veterinary Toxicology*, Chapman MJ (ed.). Blackwell Science, pp. 19–29.

Osweiler GD, Carson T, Buck WB, Van Gelder (1985) *Clinical and Diagnostic Veterinary Toxicology*, 3rd edn. Kendall/Hunt Publishing Co., Dubuque, IA, pp. 44–51.

Osweiler GD (1996) Toxicology. *The National Veterinary Medical Series for Independent Study.* Williams and Wilkins Publisher, Chapter 4, pp. 37–46.

Poppenga RH, Braselton WE (1990) Effective use of analytical laboratories for the diagnosis of toxicological problems in small animal practice. *Vet Clin North Am Small Anim Pract*, **20**(2): 293–306.

Puschner B, Galey FD (2001) Diagnosis and approach to poisoning in the horse. In *Toxicology, the Veterinary Clinics of North America, Equine Practice*, Galey FD (guest editor) Turner AS (consulting editor), pp. 399–409.

Volmer PA, Meerdink GL (2002) Diagnostic toxicology for the small animal practitioner. *Vet Clin North Am Small Anim Pract*, **32**(2): 357–65.

C H A P T E R

# 88

# Toxicoproteomics in diagnostic toxicology

*Christina R. Wilson and Stephen B. Hooser*

## INTRODUCTION

During the past decade, advances in genomics research have provided massive amounts of information resulting in the completion of entire genome sequences for multiple species. This plethora of genetic information, compiled in genome sequence databases, has been used to characterize changes in gene expression in response to external stimuli such as toxicants (toxicogenomics). Because genomic data reflects changes in gene expression at the mRNA level, DNA sequence databases can be queried to predict what proteins may be present in the cell. However, this approach is problematic because there is a poor correlation between mRNA levels and protein concentrations in cells (Gygi *et al.*, 1999a; Anderson and Seilhamer, 2005). Additionally, the biological activity of proteins is controlled by post-translational protein modifications or protein–protein interactions, both of which cannot be predicted by the genomic data. Therefore, characterizing the entire complement of proteins expressed by genes (proteome) in response to external stimuli serves as a better indicator of the response of an organism, tissue, or cell to toxic insult. This has given rise to the "proteomic" approach to toxicological evaluation.

Toxicoproteomics is broadly defined as the global identification and characterization of proteins expressed in complex biological systems in response to a toxin, toxicant or other adverse chemical or environmental stimulus. Toxicoproteomics can encompass global analysis of all of the proteins expressed, called "shotgun proteomics," or can entail analyzing a subset of proteins of interest, often termed "targeted proteomics." Irregardless of the approach, proteomics-based research has created an analytical challenge, requiring sophisticated, high-throughput analytical techniques and complex computer algorithms.

## ANALYTICAL TOOLS FOR PROTEOMICS ANALYSIS

### Multidimensional separation of proteins and peptides

#### *The proteome and sample complexity*

The initial step in proteome analysis typically involves the separation of intact proteins or peptides generated from proteolytic digestion of protein mixtures. Analysis of these heterogeneous components has proven to be an analytical challenge due to the large dynamic range of proteins and the microheterogeneity of protein expression in biological samples. For instance, serum albumin concentrations can range from 35 to 50 $\times 10^9$ pg/ml and proteins of lower abundance, such as interleukin 6, range in concentration from 0 to 5 pg/ml (Anderson and Anderson, 2002). Therefore, the dynamic range of proteins in serum can be as great as 10 orders of magnitude. The dynamic range problem is also complicated by protein heterogeneity due to polymorphisms, alternative mRNA splicing, or variations in their post-translational modifications. Currently, there are more than 100 different documented classes of post-translational modifications (O'Donovan *et al.*, 2001). When analyzing global proteolytic digests of proteins (i.e. at the peptide level), the sample complexity is compounded further. Proteolytic digests of the serum proteome, which

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could contain as much as 20,000 proteins, can potentially result in 200,000–600,000 peptides (Anderson and Anderson, 2002; Issaq *et al*., 2005). With this in mind, one can appreciate the analytical challenges encountered when conducting proteomics research in complex biological systems. Attempts to overcome these drawbacks have imposed improvements in sample preparation and separation methodologies, such as two-dimensional gel electrophoresis (2-DGE) and high-performance liquid chromatography (HPLC). Multidimensional levels of separation can be achieved when these techniques are combined affording increased resolution, sensitivity, and accuracy of detection.

### *Two-dimensional gel electrophoresis*

Since its inception in 1975 (O'Farrell, 1975), 2-DGE has become one of the most common platforms for separating and profiling complex protein mixtures. This technique is two-dimensional in that there are two levels of protein separation. The first dimension uses immobilized pH gradient-isoelectric focusing, which separates proteins based on differences in net charge (Görg *et al*., 2000). In the second dimension, proteins are resolved by electrophoresis and separated based on molecular weight. Visualization of resolved protein spots is achieved using universal stains such as Coomassie blue, silver, negative-reversible zinc, fluorescent, or radioisotope labeling dyes (Görg *et al*., 2000; Lopez *et al*., 2000; Ong and Pandey, 2001). If a particular subproteome is targeted for detection, more specific gel stains can be used. For example, detection of target proteins can be accomplished using stains containing antibodies for those proteins of interest or post-translationally modified proteins can be visualized using specialized stains for phosphorylated and glycosylated proteins (Görg *et al*., 2004; Vlahou and Fountoulakis, 2005).

After staining, the gel is digitized and protein concentrations quantitated using sophisticated 2-DGE image analysis software. Comparative analysis between control samples and samples of diagnostic interest can be accomplished using these image analyzers to superimpose the digitized data from multiple gel runs. However, due to the laborious nature of this procedure and the lack of gel reproducibility, comparative analysis is often difficult. In order to circumvent these limitations, fluorescent dyes, known as Cy dyes, have been developed permitting simultaneous analysis of two samples on one gel (Ünlü *et al*., 1997; Hamdan and Righetti, 2002). This technique, called two-dimensional differential in-gel electrophoresis (2-D DIGE), involves labeling each sample with one of two different Cy dye fluorophores, mixing the two samples together, and analyzing them using 2-DGE and fluorescent gel imaging.

While the gel staining techniques used to visualized and quantitate proteins may vary, in all instances protein identification is commonly accomplished using tandem mass spectrometry (MS/MS). Excised protein spots are digested into peptides using proteolytic enzymes and subjected, offline, to MS/MS analysis. The peptide mass fingerprints generated and peptide sequence data are then compared to theoretical peptide masses in protein or genome sequence databases using specialized bioinformatics algorithms.

Despite the fact that improvements in 2-DGE technology have enabled high resolution of separated proteins and enhanced protein identification, some intrinsic problems remain. Limited throughput capabilities, inability to measure low- and high-molecular mass proteins, inter-gel variability, and inefficient detection of basic and hydrophobic proteins are still inherent limitations of this proteomics platform. Irregardless of these limitations, 2-DGE is still a powerful tool for the separation of intact proteins and is the most widely used technique in comparative toxicoproteomic analyses.

### *Multidimensional high-performance liquid chromatography*

Attempts to improve protein separations have warranted the development of gel-free systems for large-scale analysis. Chromatographic strategies used to fractionate proteins and peptides have proven to be a successful alternative to 2-DGE. The chromatographic techniques commonly employed in proteomics research include affinity chromatography, capillary electrophoresis (CE), hydrophobic interaction chromatography, ion exchange chromatography (IEX), reversed-phase chromatography (RPC), and size exclusion chromatography (SEC) (Table 88.1) (Goheen and Gibbins, 2000; Levison, 2003; Goetz *et al*., 2004; Mahn and Asenjo, 2005; Mirzaei and Regnier, 2005; Babu *et al*., 2006; Mondal and Gupta, 2006). These modes of chromatography fractionate proteins and peptides based on their adsorption and desorption on stationary phase supports through mobile phase manipulation. On the protein level, they are commonly used to pre-fractionate samples in order to simplify complexity prior to analysis by 2-DGE or HPLC. Fractionation of proteins using these methods can also be accomplished online using high-throughput HPLC techniques. While this approach has proven to be a successful initial step in protein purification, HPLC fractionation of intact proteins is uncommon in proteomics.

**TABLE 88.1 Protein and peptide fractionation strategies**

| Chromatographic technique | Mode of fractionation |
|---|---|
| Affinity chromatography | Specific biomolecular interaction |
| Capillary electrophoresis | Net positive or negative charge |
| Hydrophobic interaction chromatography | Hydrophobicity |
| Ion exchange chromatography | Net positive or negative charge |
| Reversed-phase chromatography | Hydrophobicity |
| Size exclusion chromatography | Molecular weight |

HPLC is, however, the most widely used analytical tool for separating proteolytic digests of complex protein mixtures. In this approach, all of the proteins in the sample are digested into peptides using a proteolytic enzyme. After digestion, the peptides are separated using HPLC with ultraviolet (UV) detection. When interfaced with MS/MS instrumentation, the amino acid sequences of these peptides can be determined and their respective parent proteins identified using sophisticated bioinformatics tools.

Large-scale analysis of proteolytic digests can compromise the resolving power of HPLC when only one chromatographic fractionation technique is used (Davis and Giddings, 1985a, b). Therefore, orthogonal approaches using multidimensional chromatographic separations are preferred, affording greater peptide resolution and accuracy of detection. The most widely used high-throughput, separation technique is the coupling of IEX and RPC with MS instrumentation. Other examples of multidimensional HPLC/MS techniques can also include RPC/CE chromatography, affinity chromatography/RP chromatography, SEC/IEX/RPC, and SEC/RPC/CE (Issaq *et al*., 2005).

HPLC has also been used for quantitative analysis of protein expression. Relative quantitation of proteins by HPLC is based on the theory that the concentration of the parent protein can be determined by the relative peak areas of UV-detected peptides observed from that protein (Chelius and Bondarenko 2002). Another HPLC-based approach for relative protein quantitation involves the use of internal protein standards (Bondarenko *et al*., 2002). In this technique, biological samples are spiked with an internal standard protein prior to enzymatic digestion of the proteins into peptides. The chromatographic peaks observed are normalized to the peak area of the internal standard and the relative concentrations of the proteins are inferred. While these methods are less labor intensive and circumvent some of the limitations of current issues encountered with comparative proteomics, they are not widely used for relative protein quantitation.

Unlike 2-DGE/MS tools, multidimensional HPLC/MS is amenable to high-throughput analyses and has the ability to resolve peptide mixtures irregardless of molecular mass or hydrophobicity. This is important because resolving peptide mixtures by 2-DGE is impractical due to their narrow isoelectric points and molecular weight ranges and because 2-DGE tools cannot be directly interfaced with MS instrumentation. Even though 2-DGE is superior when separating intact proteins, methods based on pairing multidimensional HPLC and MS technology are becoming more refined and continue to advance proteomics research.

## Protein and peptide microarrays

Protein and peptide microarray technologies offer a complimentary approach to traditional separation methodologies and MS. This emerging technology incorporates the use of a variety of microarray platforms, in a high-throughput format, to probe protein function and determine protein abundance in complex biological samples.

Protein function microarrays use immobilized capture ligands, such as antibodies, antigens, enzymes, proteins, or peptides to screen for protein–protein, protein–enzyme, or protein–drug interactions (Kawahashi *et al*., 2003; Nielsen *et al*., 2003; Zhu *et al*., 2003; Cretich *et al*., 2006). Analytical microarrays, such as those used in surface-enhanced laser desorption (SELDI)/TOF MS, use retentate chromatography for protein profiling and detection (Merchant and Weinberger, 2000; Cretich *et al*., 2006). This technique is capable of on-chip sample fractionation utilizing various chromatographic surface chemistries and can probe for chemical properties in proteins such as phosphorylation, glycosylation, hydrophobicity, or anionic–cationic properties.

Both types of microarrays require spotting or immobilization of a capture ligand or chromatographic media onto a glass slide or other planar solid support, which can result in thousands of spots per slide (MacBeath and Schreiber, 2000; Kumble, 2003). After extensive washing of the array, the samples of interest are added to the microarray and the entire array is scanned to detect proteins or peptides that interact with the ligand or chromatographic media. Protein function can be probed using capture ligands, such as antibodies, antigens, enzymes, proteins, or peptides to screen for protein–protein, protein–enzyme, or protein–drug interactions (Kawahashi *et al*., 2003; Nielsen *et al*., 2003; Zhu *et al*., 2003). Analytical microarrays, such as those used in SELDI/TOF MS, use retentate chromatography for protein profiling and detection (Merchant and Weinberger, 2000; Cretich *et al*., 2006). This technique is capable of on-chip sample fractionation due to specific interactions of proteins or peptides with a variety of chromatographic surface chemistries. These surface chemistries can include affinity ligands such as lectins to probe for glycosylated proteins or other chromatographic media to select for other properties such as hydrophobicity, phosphorylation, or anionic–cationic characteristics.

Detection strategies for protein or peptide microarrays entail either label-free or labeled probe methods (Espina *et al*., 2004; Cretich *et al*., 2006). The label-free strategy is a direct detection method that includes MS, surface plasmon resonance, or atomic force microscopy. SELDI microarray chips commonly use MS-based detection of proteins or peptides. Labeled probe methods involve labeling proteins with a tag that allows them to be detected after binding to a particular ligand. The different labeled probes used include utilizing chromagens (similar to ELISA protocols), chemiluminescence, fluorescence, or radioactive decay-based detection techniques.

Even though microarray technology is relatively new, it is progressively becoming a versatile platform for its potential use in diagnostic toxicology. Although efforts to

standardize array analyses have been challenging, microarrays make it possible to simultaneously screen thousands of samples and profile large numbers of proteins from biological samples.

## Mass spectrometry

Recent developments in hybrid mass spectrometers have revolutionized the ability to analyze proteins and peptides, providing high-throughput automation combined with superior sensitivity and resolving power. In general, mass spectrometers have three components, an ion source, a mass analyzer, and a detector. The ion source is the component in which protein and peptide ions are produced; the mass analyzer separates or resolves these ions based on their mass-to-charge (m/z); and the detector detects the selected ions from the mass analyzer. One stage of mass analysis is commonly used to measure the molecular weights of intact proteins or peptides. However, in order to obtain peptide sequence information, hybrid mass spectrometers are employed. MS/MS, or tandem MS, detects intact peptide ions in the first mass analyzer. Once selected, the peptide ion enters the collision cell where it is fragmented into secondary ions by collision-induced dissociation (CID). These ions are then separated in the second mass analyzer, their m/z ratios detected, and MS/MS spectra are generated. Bioinformatics database tools use the MS/MS data to generate peptide amino acid sequence information and compare them with theoretical sequences in protein or genome databases to identify proteins.

In addition to being essential for protein identification, MS technology is being used for quantitative proteomic profiling. Through the use of stable isotope-coded mass tags, differential quantitation of changes in peptides from control and experimental samples is possible. Quantitation of changes in global protein expression involves proteolytic or chemical labeling of peptides with isotope-coded mass tags prior to separation by HPLC. These labeling reagents are chemically identical; however, one label contains light isotope atoms and the other heavy isotope atoms. Proteolytic labeling entails mass tagging peptides during proteolytic digestion. During proteolysis, enzymatic cleavage results in the incorporation of oxygen at the peptide carboxy-terminus. Exploiting this reaction, $^{18}O$ (heavy) and $^{16}O$ (light) isotopic oxygen labels can be used to differentially label two samples (Stewart *et al.*, 2001). In chemical labeling, which is the most frequently used isotope-coded mass tagging, all peptides are labeled after proteolytic digestion. Examples of these isotope-coded reagents and their labeling strategies are listed in Table 88.2. In isotope tagging, peptides from a control sample are labeled with the light isotope and the peptides from the treated sample are labeled with the heavy isotope. After labeling, both samples are mixed together and fractionated using HPLC. When subjected to MS analysis, the mass spectrometer can distinguish between the two isotope-labeled peptide samples because a predictable mass difference will be observed between the control and experimental peptides. MS-based quantitation is then achieved by calculating the difference between the ion intensities of the light-labeled control peptide and the heavy-labeled experimental peptide samples (Figure 88.1). From this data, differential displays of peptides that increase or decrease in response to a stimulus can be generated.

There are two major types of hybrid mass analyzers used in proteomics research, matrix-assisted laser desorption ionization/time-of-flight (MALDI-TOF/TOF) instruments and electrospray ionization/tandem MS instruments (ESI/MS/MS) (Karas and Hillenkamp, 1988; Fenn *et al.*, 1989; Hillenkamp *et al.*, 1991). The MS technology chosen primarily depends on the type of MS data that needs to be generated from toxicoproteomic experiments. For example, MALDI-TOF/TOF instruments are fast, robust mass analyzers, have a large dynamic range, and do not require labor-intensive sample preparation. MALDI-TOF/TOF instruments have high resolution, making them the instruments of choice when using MS for quantitative proteomic analyses. ESI/MS/MS instruments, including those containing hybrid combinations of quadrupole, 3-D ion trap, linear ion trap, TOF, and fourier transform-ion cyclotron resonance mass analyzers, have approximately four times less peak capacity than MALDI-TOF/TOF; however, they have higher-mass accuracy affording more accurate protein identification (Hopfgartner *et al.*, 2004; Hu *et al.*, 2005; O'Connor *et al.*, 2006; Yates *et al.*, 2006).

**TABLE 88.2 Isotope-coded reagents used for chemical labeling of peptides**

| Reagent | Labeling technique | Reference |
|---|---|---|
| ALICE | Thiol modification of cysteines | Qui *et al.* (2002) |
| AQUA | Synthetic internal standard peptide | Gerber *et al.* (2003) |
| ICAT | Iodoacetylation of cysteine | Gygi *et al.* (1999b) |
| iTRAQ™ | Modification of primary amines | Zieske (2006) |
| GIST | Acylation of primary amines | Ji *et al.* (2000) |
| MCAT | Guanidation of C-terminal lysine | Cagney and Emili (2002) |
| QUEST | Amidination of N-terminal lysine | Beardsley and Reilly (2003) |

Additionally, they are capable of analyzing low-molecular weight peptides and can be directly interfaced with HPLC instrumentation.

Innovative approaches to advance protein identification strategies have spurred the development of new MS technologies. Improvements in ion activation using electron capture dissociation or infrared multiphoton dissociation technologies have been shown to yield more extensive peptide sequence coverage when compared to CID, resulting in significant improvements in protein identification (Wysocki *et al.*, 2005; Bakhtiar and Guan, 2006). Developments in MALDI imaging mass spectrometry (MALDI-IMS) make it possible to simultaneously map peptides and proteins by direct MS analysis of thin tissue sections, providing a means to correlate and monitor changes in protein patterns associated with regions of the tissue that are histologically significant (Chaurand *et al.*, 2005). Additionally, technological advances in large biomolecule ionization and data analysis have enabled the development of miniaturized, portable mass spectrometers capable of direct analysis of complex biological samples (Laughlin *et al.*, 2005; Cooks *et al.*, 2006).

Due to the escalating interest in proteomics research and bioanalytical chemistry, there is an ever-increasing demand for mass spectrometers with improved mass resolution, mass accuracy, and automated data mining capabilities. Hence, development of next generation mass spectrometers is ongoing and constantly impacting proteomics research.

## Bioinformatics tools

As mentioned previously, protein identification is accomplished by using computer search algorithms that correlate MS and MS/MS data with predicted amino acid sequences contained in protein or genome sequence databases. Even though several types of MS and MS/MS search engines have been created, databases that are used more frequently include MASCOT, SEQUEST, Spectrum Mill, X!Tandem, and Protein Prospector (Eng *et al.*, 1994; Clauser *et al.*, 1995; Perkins *et al.* 1999; Robertson and Beavis, 2004; Kapp *et al.*, 2005). Recently, a searchable toxin protein database called Tox-Prot has been created that contains all known animal protein toxins (Jungo and Bairoch, 2005).

Even though the computer algorithms designed for each of these bioinformatics databases are slightly different, their general approach to protein identification is similar. Database search engines compare the experimental precursor m/z ions from each MS/MS scan with hypothetical peptide m/z values from the database. Hypothetical peptide masses from the database that correspond with the experimental mass values are then assigned probability scores. The proteins recognized with the highest scores are indicative of the best probable protein match to the experimental MS/MS data. Some bioinformatics tools assign *p*-values to the correlation scores, providing an additional means for evaluating credibility of protein matches.

Integrating MS technology with bioinformatics tools has become an indispensable tool in proteomics research.

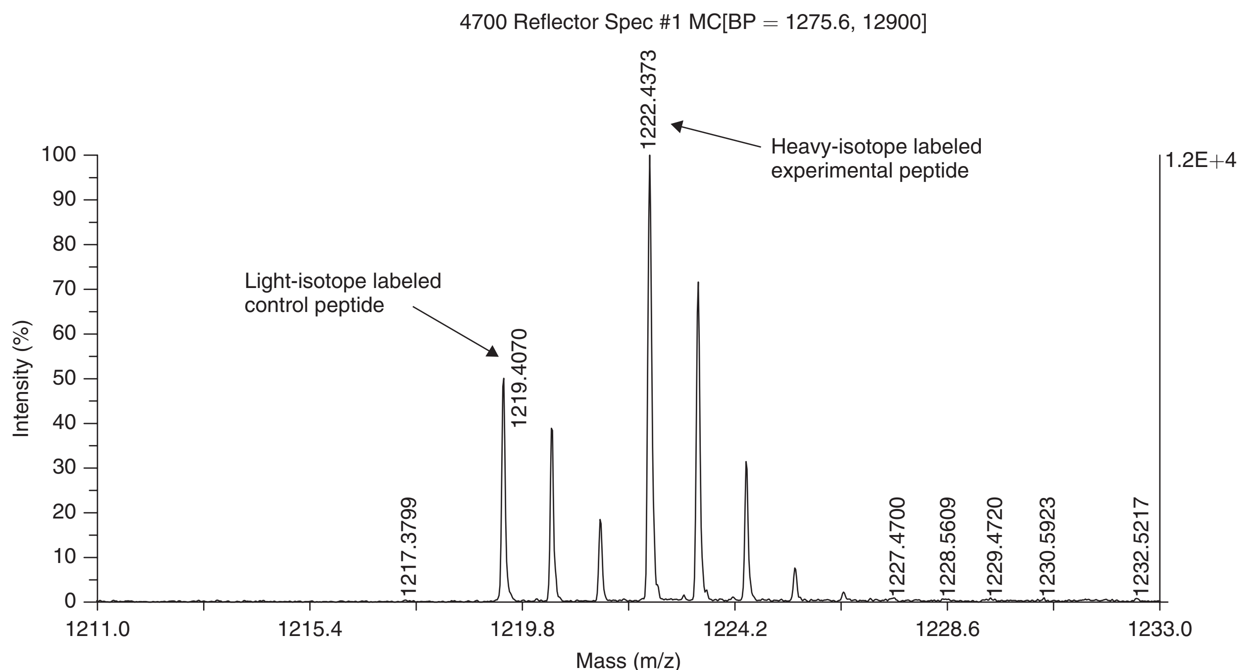


**FIGURE 88.1** MALDI-TOF MS spectrum of GIST isotopically labeled peptides from control and experimental serum samples. Comparison of the relative ion intensities between the two peptides indicate that the peptide present in the experimental sample (at 1222.43 m/z) was present at a higher concentration when compared to the control sample (at 1219.40 m/z).

However, due to the overwhelming amounts of MS and MS/MS data generated from typical proteomics experiments, creating bioinformatics tools that adequately identify and characterize the data has been a tremendous challenge. Credible protein identification is reliant on successful interpretation of MS and MS/MS data. Unfortunately, data interpretation is often complicated by ion suppression, atypical MS/MS peptide fragmentation patterns, ill-defined universal standards for evaluating credible database matches, and inability to define a single protein from one peptide spectrum (Kearney and Thibault, 2003). Additionally, variants of MS instrumentation are developing rapidly and the computer algorithms necessary to correlate the data from these next generation mass spectrometers with information contained in protein or genome sequence databases are lagging. Even though bioinformatics tools are in their early stages of development, integrating MS technology with bioinformatics tools will continue to be an indispensable tool in proteomics research.

## PROTEOMICS APPLICATIONS IN DIAGNOSTIC TOXICOLOGY

Toxicoproteomics embodies the integration of toxicology with current proteomic technology and signifies an innovative approach toward establishing a new knowledge base with regard to the recognition and characterization of cellular responses to toxic agents. The current objectives of toxicoproteomics research are to define molecular mechanisms of toxicity, screen for drug toxicities, and elucidate biomarkers or signature protein profiles in order to more accurately assess, predict, and diagnose toxicities (Kennedy, 2002; Guerreiro *et al*., 2003; Wetmore and Merrick, 2004). For decades, clinical laboratories have relied upon individual protein markers for assessing toxicity. However, some of these single biomarkers can be non-specific and reflect protein leakage from tissues as opposed to the direct effects of toxicants on the tissues alone (Plebani, 2005). Toxicities in biological systems are multifactorial and complex, warranting the identification of multiple biomarkers for accurately diagnosing and classifying toxicity. This makes proteomics research in toxicologic evaluation appealing because these technologies are capable of globally analyzing multiple protein systems. Hence, the potential to better define molecular signatures of toxicity for clinical and diagnostic toxicology is now possible. Several proteomic applications have been applied to gain a better understanding of target organ toxicities including mechanisms of action and biomarkers for a variety of toxicants.

Monitoring liver function is crucial in toxicologic evaluation. Hence, it is no surprise that several toxicoproteomic experiments have been conducted to gain a better understanding of proteins that change with hepatotoxicity. For example, toxicoproteomics has been used to identify metabolic pathways perturbed by drug-induced cirrhosis in rat models. In these studies, thioacetamide and ethanol have both been shown to down-regulate proteins involved in oxidative stress and fatty acid β-oxidation pathways during early phases of cirrhosis (Low *et al*., 2004; Venkatraman *et al*., 2004).

The need to identify early biomarkers of the effects of chemical pollutants and their underlying biochemical mechanisms are also essential in toxicologic evaluation. 2-DGE/MS analysis of aerosol inhalation of JP-8 jet fuel on kidney protein expression has revealed changes in proteins associated with nephrotoxicity, inferring a possible risk to humans exposed to JP-8 jet fuel vapors (Witzmann *et al*., 2000). In the environment, organisms are typically exposed to mixtures of chemical pollutants. The mechanisms of toxicity of these chemical mixtures often involve multiple biochemical pathways. Because this new technology can simultaneously screen for numerous proteins in a single test, proteomics will become a valuable asset for preventative and predictive toxicology and risk assessment.

## CONCLUSION

Toxicoproteomics is still in its infancy. Recognition of the diagnostic accuracy of proteomics data and standardization of methodologies remain a challenge to proteomics researchers. Despite these concerns, toxicoproteomics has the potential to better define molecular mechanisms of toxicity and elucidate biomarkers or signature protein profiles for clinical and diagnostic toxicology. This technology promises to establish new guidelines for preventative and predictive toxicity, ultimately revolutionizing and redefining our future understanding of toxicology.

## REFERENCES

Anderson NL, Anderson NG (2002) The human plasma proteome. *Mol Cell Proteomics* **1**: 311–26.

Anderson L, Seilhamer J (2005)A comparison of selected mRNA and protein abundances in human liver. *Electrophoresis* **18**(3–4): 533–7.

Babu S, Song EJ, Babar SME, Wi MH, Yoo YS (2006) Capillary electrophoresis at the omics level: towards systems biology. *Electrophoresis* **27**: 97–110.

Bakhtiar R, Guan Z (2006) Electron capture dissociation mass spectrometry in characterization of peptides and proteins. *Biotechnol Lett* **28**: 1047–59.

Beardsley RL, Reilly JP (2003) Quantitation using enhanced signal tags: a technique for comparative proteomics. *J Proteome Res* **2**: 15–21.

Bondarenko PV, Chelius D, Shaler TA (2002) Identification and relative quantitation of protein mixtures by enzymatic digestion followed by capillary reversed-phase liquid chromatography-tandem mass spectrometry. *Anal Chem* **74**: 4741–9.

Cagney G, Emili A (2002) *De novo* peptide sequencing and quantitative profiling of complex protein mixtures using mass-coded affinity tagging. *Nat Biotech* **20**: 163–70.

Chaurand P, Schwartz SA, Reyzer ML, Caprioli RM (2005) Imaging mass spectrometry: principles and potentials. *Toxicol Pathol* **33**: 92–101.

Chelius D, Bondarenko PV (2002) Quantitative profiling of proteins in complex mixtures using liquid chromatography and mass spectrometry. *J Proteome Res* **1**: 317–23.

Clauser KR, Hall SC, Smith DM, Webb JW, Andrews LE, Tran HM, Epstein LB, Burlingame AL (1995) Rapid mass spectrometric peptide sequencing and mass matching for characterization of human melanoma proteins isolated by two-dimensional PAGE. *Proc Natl Acad Sci USA* **92**: 5072–76.

Cooks RG, Ouyang Z, Takats Z, Wiseman JM (2006) Ambient mass spectrometry. *Science* **311**: 1566–70.

Cretich M, Damin F, Pirri G, Chiari M (2006) Protein and peptide arrays: recent trends and new directions. *Biomol Eng* **23**: 77–88.

Davis JM, Giddings JC (1985a) Statistical method for estimation of number of components from single complex chromatograms: theory, computer-based testing, and analysis of errors. *Anal Chem* **57**: 2168–77.

Davis JM, Giddings JC (1985b) Statistical method for estimation of number of components from single complex chromatograms: application to experimental chromatograms. *Anal Chem* **57**: 2178–82.

Eng JK, McCormack AL, Yates JR (1994) An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database. *J Am Soc Mass Spec* **5**: 976–89.

Espina V, Woodhouse EC, Wulfkuhle J, Asmussen HD, Petricoin III EF, Liotta LA (2004) Protein microarray detection strategies: focus on direct detection technologies. *J Immunol Method* **290**: 121–33.

FennJB, Mann M, Meng CK, Wong SF, Whitehouse CM (1989) Electrospray ionization for mass spectrometry of large biomolecules. *Science* **246**: 64–71.

Gerber SA, Rush J, Stemman O, Kirshner MW, Gygi SP (2003) Absolute quantification of proteins and phosphoproteins from cell lysates by tandem MS. *Proc Natl Acad Sci USA* **100**(12): 6940–5.

Goetz H, Kuschel M, Wulff T, Sauber C, Miller C, Fisher S, Woodward C (2004) Comparison of selected analytical techniques for protein sizing, quantitation and molecular weight determination. *J Biochem Biophys Method* **60**: 281–93.

Goheen SC, Gibbins BM (2000) Protein losses in ion-exchange chromatography and hydrophobic interaction high-performance liquid chromatography. *J Chrom A* **890**: 73–80.

Görg A, Weiss W, Dunn WJ (2004) Current two-dimensional electrophoresis technology for proteomics. *Proteomics* **4**: 3665–85.

Görg A, Obermaier C, Boguth G, Harder A, Scheibe B, Wildgruber R, Weiss W (2000) The current state of two-dimensional electrophoresis with immobilized pH gradients. *Electrophoresis* **21**: 1037–53.

Guerreiro N, Staedtler F, Grenet O, Kehren J, Chibout S (2003) Toxicogenomics in drug development. *Toxicol Pathol* **31**: 471–9.

Gygi SP, Rochon Y, Franza BR, Aebersold R (1999a) Correlation between protein and mRNA abundance in yeast. *Mol Cell Biol* **19**(3): 1720–30.

Gygi SP, Rist B, Gerber SA, Turecek F, Gelb MH, Aebersold R (1999b) Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nat Biotech* **17**: 994–9.

Hamdan M, Righetti PG (2002) Modern strategies for protein quantification in proteome analysis: advantages and limitations. *Mass Spec Rev* **21**: 287–302.

Hillenkamp F, Karas M, Beavis RC, Chait BT (1991) Matrix-assisted laser desorption ionization mass-spectrometry of biopolymers. *Anal Chem* **63**: 1139A–202A.

Hopfgartner G, Varesio E, Tschäppät V, Grivet C, Bourgogne E, Leuthold LA (2004) Triple quadrupole linear ion trap mass spectrometer for the analysis of small molecules and macromolecules. *J Mass Spectrom* **39**: 845–55.

Hu Q, Noll RJ, Li H, Makarov A, Hardman M, Cooks RG (2005) The orbitrap: a new mass spectrometer. *J Mass Spectrom* **40**: 430–3.

Issaq HJ, Chan KC, Janini GM, Conrads TP, Veenstra TD (2005) Multidimensional separation of peptides for effective proteomic analysis. *J Chrom B* **817**: 35–47.

Ji J, Chakraborty A, Geng M, Zhang X, Amini A, Bina M, Regnier F (2000) Strategy for qualitative and quantitative analysis in proteomics based on signature peptides. *J Chrom B* **745**: 197–210.

Jungo F, Bairoch A (2005) Tox-Prot, the Toxin Protein Annotation Program of the Swiss-Prot Protein Knowledgebase. *Toxicon* **45**: 293–301.

Kapp EA, Schütz F, Connolly LM, Chaker JA, Meza JE, Miller CA, Fenyo D, Eng JK, Adkins JN, Omenn GS, Simpson RJ (2005) An evaluation, comparison, and accurate benchmarking of several publicly available MS/MS search algorithms: sensitivity and specificity analysis. *Proteomics* **5**: 3475–90.

Karas M, Hillenkamp F (1988) Laser desorption ionization of proteins with molecular masses exceeding 10,000 daltons. *Anal Chem* **60**: 2299–301.

Kawahashi Y, Doi N, Takashima H, Tsuda C, Oishi Y, Oyama R, Yonezawa M, Miyamoto-Sato E, Yanagawa H (2003) *In vitro* protein microarrays for detecting protein–protein interactions: application of a new method for fluorescence labeling of proteins. *Proteomics* **3**: 1236–43.

Kearney P, Thibault P (2003) Bioinformatics meets proteomics – bridging the gap between mass spectrometry data analysis and cell biology. *J Bioinform Comp Biol* **1**(1): 183–200.

Kennedy S (2002) The role of proteomics in toxicology: identification of biomarkers of toxicity by protein expression analysis. *Biomarkers* **7**(4): 269–90.

Kumble KD (2003) Protein microarrays: new tools for pharmaceutical development. *Anal Bioanal Chem* **377**: 812–19.

Laughlin BC, Mulligan CC, Cooks RG (2005) Atmospheric pressure ionization in a miniature mass spectrometer. *Anal Chem* **77**: 2928–39.

Levison PR (2003) Large-scale ion exchange column chromatography of proteins comparison of different formats. *J Chrom B* **790**: 17–33.

Lopez MF, Berggren K, Chernokalskaya E, Lazarev A, Robinson M, Patton WF (2000) A comparison of silver stain and SYPRO ruby protein gel stain with respect to protein detection in two-dimensional gels and identification by peptide mass profiling. *Electrophoresis* **21**: 3673–83.

Low TY, Leow CK, Salto-Tellez M, Chung MCM (2004) A proteomic analysis of thioacetamide-induced hepatotoxicity and cirrhosis in rat livers. *Proteomics* **4**: 3960–74.

MacBeath G, Schreiber SL (2000) Printing proteins as microarrays for high-throughput function determination. *Science* **289**: 1760–3.

Mahn A, Asenjo JA (2005) Prediction of protein interaction in hydrophobic interaction chromatography. *Biotech Adv* **23**: 359–68.

Merchant M, Weinberger SR (2000) Recent advancements in surface-enhanced laser desorption/ionization-time of flight mass spectrometry. *Electrophoresis* **21**: 1164–7.

Mirzaei H, Regnier F (2005) Structure specific chromatographic selection in targeted proteomics. *J Chrom B* **817**: 23–34.

Mondal K, Gupta MN (2006) The affinity concept in bioseparation: evolving paradigms and expanding range of applications. *Biomed Eng* **23**: 59–76.

Nielsen UB, Cardone MH, Sinskey AJ, MacBeath G, Sorger K (2003) Profiling receptor tyrosine kinase activation by using ab microarrays. *Proc Natl Acad Sci USA* **100**(16): 9330–5.

O'Connor PB, Pittman JL, Thomson BA, Budnik BA, Cournoyer JC, Jebanathirajah J, Lin C, Moyer S, Zhao C (2006) A new hybrid electrospray Fourier transform mass spectrometer: design and performance characteristics. *Rapid Commun Mass Spectrom* **20**: 259–66.

O'Donovan C, Apweiler R, Bairoch A (2001) The human proteomics initiative (HPI). *Trend Biotech* **19**(5): 178–81.

O'Farrell PH (1975) High resolution two-dimensional electrophoresis of proteins. *J Biol Chem* **250**(10): 4007–21.

Ong S, Pandey A (2001) An evaluation of the use of two-dimensional gel electrophoresis in proteomics. *Biomed Eng* **18**: 195–205.

Perkins DN, Pappin DJ, Creasy DM, Cottrell JS (1999) Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis* **20**: 3551–67.

Plebani M (2005) Proteomics: the next revolution in laboratory medicine? *Clin Chim Acta* **357**: 113–22.

Qui Y, Sousa EA, Hewick RM, Wang JH (2002) Acid-labile isotope-coded extractants: a class of reagents for quantitative mass spectrometric analysis of complex protein mixtures. *Anal Chem* **74**: 4969–79.

Robertson C, Beavis RC (2004) Tandem: matching proteins with mass spectra. *Bioinformatics* **20**: 1466–7.

Stewart II, Thomson T, Figeys D (2001) $^{18}$O Labeling: a tool for proteomics. *Rapid Commun Mass Spectrom* **15**: 2456–65.

Ünlü M, Morgan ME, Minden JS (1997) Difference gel electrophoresis. A single gel method for detecting changes in protein extracts. *Electrophoresis* **18**(11): 2071–7.

Venkatraman A, Landar A, Davis AJ, Chamlee L, Sanderson T, Kim H, Page G, Pompilius M, Ballinger S, Darley-Usmar V (2004) Modification of the mitochondrial proteome in response to the stress of ethanol-dependent hepatotoxicity. *J Biol Chem* **279**(21): 22092–101.

Vlahou A, Fountoulakis M (2005) Proteomic approaches in the search for disease markers. *J Chrom B* **814**: 11–19.

Wetmore BA, Merrick BA (2004) Toxicoproteomics: proteomics applied to toxicology and pathology. *Toxicol Pathol* **32**: 619–24.

Witzmann FA, Bauer MD, Fieno AM, Grant RA, Keough TW, Lacey MP, Sun Y, Witten ML, Young RS (2000) Proteomic analysis of the renal effects of simulated occupational jet fuel exposure. *Electrophoresis* **21**: 976–84.

Wysocki VH, Resing KA, Zhang Q, Cheng G (2005) Mass spectrometry of peptides and proteins. *Methods* **35**: 211–22.

Yates JR, Cociorva D, Liao L, Zabrouskov V (2006) Performance of a linear ion trap-orbitrap hybrid for peptide analysis. *Anal Chem* **78**: 493–500.

Zieske LR (2006) A perspective on the use of iTRAQ™ reagent technology for protein complex and profiling studies. *J Exp Bot* **57**(7): 1501–8.

Zhu Q, Uttamchandani M, Li D, Lesaicherre ML, Yao SQ (2003) Enzymatic profiling system in a small-molecule microarray. *Org Lett* **5**(8): 1257–60.

CHAPTER

# 89

# Microscopic analysis of toxic substances in feeds and ingesta

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## INTRODUCTION

Feed microscopy is the scientific discipline and technology for the identification and evaluation of feed ingredients and formula feeds. Basically there are three essential components to a successful microscopic identification. These include an aid to the eye, a trained mind and a collection of reference standards. The feed microscopist uses both the stereomicroscope and the compound microscope to identify unknown particles by comparisons to known reference standards. The experience and training of the microscopist determine the efficacy and speed of the identification. A microscopic analysis can be applied to any type or size of particles but commonly observations ranges from the macroscopic level for the initial gross examinations to the stereoscopic level for centimeter- to millimeter-sized opaque to translucent particles. Translucent and transparent fine particles are examined with the compound microscope to confirm the particle identity at the cellular and sub-cellular levels. In addition, due to the ability of the human mind to sort and classify a wide range of different materials rapidly, the feed microscopist can accomplish a subjective analysis of a feed ingredient in just a few minutes that might take a chemist several hours to accomplish.

It is important to understand from the beginning that feed microscopy is subjective and visual in nature. It is not an objective technology that generates hard numbers. It is designed to identify the feed resources and their inherent quality visually but not to determine the quantity of the individual nutrients within those feed resources. Therein is the real essential value of feed microscopy to the feed manufacturer, quality assurance laboratory, veterinary toxicologist or anyone concerned about feed ingredient quality and its effects on the consuming animal. Feed microscopy is the entire other half of the quality assurance picture that is often discounted because it involves an opinion. It is, however, a powerful technology that plays an important role in the evaluation of any feed or toxicant analysis and an understanding of what microscopy can do needs to be introduced into every general veterinary and toxicology curriculum.

Microscopy is critical to the toxicological examination of feeds because it is the most rapid and practical way to define problems and focus in on potential solutions. Although feed microscopy has not generally been a part of veterinary school curricula, feed microscopists have been part of the diagnostic picture for many years. Veterinary diagnostic laboratories and poison control centers, often located in universities or in a state chemist's office, have utilized feed microscopists as part of their rapid response teams. Unfortunately the number of feed microscopists that are trained and available for veterinary diagnostic work today has declined dramatically to the point that integrating a feed microscopist into the initial toxicological examination is now very difficult. Because the microscopist can sort through more seemingly unrelated but potentially pertinent information about a feed than any other type of analyst and handle more complex situations in feeds than a chemist using standard methods, we need to re-insert a feed microscopist back into the modern veterinarian fast response diagnostic team.

For example, there are absolutely no rapid chemical assays to locate blister beetles or their fragments in alfalfa samples and the analysis of the cantharidin toxicant would take several hours. In contrast, a feed microscopist should be able to locate specific evidence of the beetles in a few

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minutes without any sample preparation. The knowledge of how and where to look for potential problems in feeds is also a critical part of defining a problem. Although it is more specific to look for the toxin itself, the low concentrations of toxins in feeds make them difficult to detect. It is often more efficacious to find an indicator of the toxin and take appropriate remedial action than spend the time in sample preparation and detailed confirmatory analysis.

## THE SEARCH FOR CLUES

Feed ingredients and finished feeds come in a bewildering array of sources, particle sizes, mixtures and forms. Added to that the other natural materials that are fed, or that animals consume, and the task of identifying toxicological problems appears totally impossible. Certainly that would be the case if we did not have an organized way of identifying and classifying all the potential ingredients in a feed. Basically the majority of feed ingredients and formula feeds are by-products from the food industry. Because they are materials processed out of foods, they are often wet and require drying to be safely stored prior to use. They may also be relatively dry but hygroscopic and difficult to store. They could also be dry but susceptible to oxidation or rancidity due to having been removed from their cellular compartmentalization in nature. For these and numerous other reasons, they are all potential problems for the toxicologist if they are not handled or stored properly. Nevertheless, the clues to identifying which ingredient(s) or feeds are or are not a problem in any situation is right in front of the toxicologist and available to be interpreted. The clues for feed quality are inherent in the visible appearance of each ingredient.

## BACKGROUND: PRINCIPLES OF FEED MICROSCOPY

Feed microscopy is founded on the most basic descriptive aspects of biology and the wide diversity of cellular structures found in natural organisms that are genetically controlled and that do not change from year to year. It is also based on the fact that many of these unique tissues, cellular traits and sub-cellular characteristics are not greatly affected by the moisture, heat, pressure and shear commonly used to manufacture formula feeds. Therefore, not only are these cellular characteristics inherently good identifiers of individual feed ingredients and mixtures, they are equally good for finished formula feed identifications because they maintain their characteristics after they have been intimately mixed, conditioned, pelleted, extruded, or otherwise manufactured into finished feeds. Consequently, feed microscopists can trust these characteristics to make accurate identifications of ingredients and confirm them to the cellular and sometimes the sub-cellular levels. If a micro-chemical or micro-biochemical test is applied to the sample, the feed microscopist can confirm ingredients to the molecular level and watch the reaction in real time in the cells. In addition, they can evaluate feed ingredients, finished formula feeds and, in special instances, the ingesta and fecal wastes from feeds and forages. The latter is a specialized part of the feed microscopy field but one that is particularly important for the toxicologist. In all these situations, the feed microscopist is very much like a medical examiner or a veterinarian toxicologist who uses the unique visual characteristics of organ or tissue morphology, red or white blood cell characteristics or sub-cellular tissue sections to diagnose diseases or other pertinent problems.

Feed microscopy is, in concept, a simple subjective science. It is sometimes further defined as being both art and science due to the need to have steady hands and to develop very good manipulative skills under the microscope. Therefore, if we build on the basic three essentials and add a few hand tools to the well-trained mind, the microscopes and the numerous reference standards mentioned above, we can produce a complete analytical system.

Feed microscopy, due to its conceptual simplicity, is a rapid and relatively accurate technology for identifying materials and problems for which no other direct tests or scientific instruments exist. It is also a relatively rapid way to identify animal problems and it can become an essential complement to objective laboratory analyses. For example, a feed microscopist can identify the residues in a feed bunker that had been rejected by cattle, the contents of dog vomit to identify a feed source or a problem, or perhaps locate evidence of mold growth or mites hidden in a pelleted feed. Subsequently the microscopist can, after locating evidence of a potential feed problem, advise the analytical or toxicology laboratory staff to select appropriate methods to confirm, negate or quantify the extent of the problem. Thus, the feed microscopist is very much like a diagnostician who examines an animal and, based on multiple observed criteria, concludes subjectively that certain definitive laboratory tests should be run to confirm the diagnosis and potential cure.

The feed microscopist, like the diagnostician and toxicologist, must be well versed in the significance of tiny observed differences from the expected norms. Toxicants are not visible at light microscope resolution and feed microscopists are not trained to evaluate tissue samples, organ morphology or animal behavior. However, the particles and structures associated with toxicity such as yew leaves, mold growth or moldy feed, a blister beetle wing fragment, caster bean seed coat fragments and many others can be detected and identified using common feed

microscopy methods. These are the keys to both the identification of potential feed problems and the selection of suitable chemical testing plus the evidence to correlate with the findings of the diagnostician.

Because there are often no specific tests for the small differences that must be detected to ascertain a particular problem, the feed microscopist uses all possible senses to evaluate the feeds. For example, although there is no single instrument that can identify an unknown sample as fish meal or ground corn without a lot of work, a feed microscopist can make those qualitative determinations in a matter of minutes. Thus, organoleptic observations, color of particles, breakage lines, presence of caramelized and charred particles and many other seemingly insignificant details may offer clues to the quality of a feedstuff or help to explain a suspected toxicant situation. An objective laboratory test would be necessary to confirm or deny the actual presence of the suspected toxicant.

Another very important facet of feed microscopy is to detect natural contaminants that are similar to the feed ingredients and particularly for adulterants that are deliberately selected to deceive and not be detected by standard methods. An example of a classical adulterant would be urea used in an ingredient for a monogastric animal ration. The ingredient could command a premium price due to the apparent superior protein content. It would not be detected by the feed manufacturer testing for crude protein. Unless non-protein nitrogen testing was routinely run to detect this type of protein spiking or if a microscopist spotted the urea particles and ordered a non-protein nitrogen test, this adulteration would escape detection.

It should have become obvious by this point that a feed microscopist familiar with the structural characteristics of plants, animals and microbial materials and trained in the proper use of the microscope or other aid to the eye can not only solve certain difficult to solve types of problems such as those of feed quality, but can often aid the chemist or other laboratory analyst to select appropriate tests to solve increasingly more complex problems that we often encounter today. Feed microscopy is particularly well suited for solving complex multifaceted contaminant, adulterant and toxicological problems and should be part of any rapid response team.

## THE APPLICATIONS OF FEED MICROSCOPY

Although the concepts of feed microscopy are quite simple, there are numerous ways to achieve accurate results. The more techniques and methods the microscopist masters, the easier it will be to handle the wide range of sample matrices and problem samples that will be encountered. Some techniques require essentially no sample preparation whereas others might require flotation, sieving or other separations to assist the analyst. There are no absolutes in feed microscopy as is the case with most subjective examinations. Therefore, it is essential that the microscopist have a full understanding of the available techniques and methods so that the most appropriate tests are applied and the most scientifically accurate information can be established from the available evidence in any sample. If the sample size is limited, then certain tests might have to be run before others so that the reagents of the first test will not confound the results of subsequent tests. Thus, the feed microscopist should be well versed in chemistry and biochemistry to be a key member of a rapid response team.

The trained mind of the feed microscopist may also be thought of as a computer with a massive hard drive and an extreme logic program to sort through the hundreds of facts and "what if" possibilities. That would be true also of any diagnostician dealing with complex situations. One way to understand and appreciate the capabilities of feed microscopy and some of the variables that should be considered in the sorting process to the most applicable methods and techniques for a particular sample is to consider what can be seen at various magnifications.

Feed microscopy is often defined by the level of magnification used. The initial examination would be analogous to a gross morphological description to establish the general direction of each later stage of an analysis. The stereomicroscope or dissecting microscope is the basic workhorse for most feed microscopists just as it is for the veterinary diagnostician or toxicologist. It would be used to aid the eye in a general review of the surfaces of pelleted feed, for example, although a lighted magnifier could be used as well. An examination of a feed ingredient or finished feed generally requires at least 7× magnification to see particles ground through a typical 2 mm hammermill screen. On site, field work can be done with a folding 6× linen tester or a battery operated 10× hand lens but more detailed laboratory work would require at least 7× to 10× magnification. The finer particles in the same hammermilled feed would require 30× to 75× magnification. The color, luster, particle shape, particle size, effects of processing, etc. would be visible at the magnification of the stereomicroscope. Seed coat particles, ground yew leaves, insects and insect fragments, mineral particles and many other particles associated with toxicants could also be examined and confirmed with the stereomicroscope. It is important to keep in mind the relative sizes of structures to be examined and the type of information one can obtain from each. For example, a macroscopic feed examination would involve those characteristics that can be defined by minimal magnification. The naked eye aided by a hand lens or magnifying glass provides sufficient magnification to determine the feed ingredient or formula feed color, shapes of particles and other very obvious

physical characteristics. A finished feed might be described as a densified, polished feed pellet, an extruded star-shaped petfood form, a floating fish particle, a granular crumble of a relatively soft pelleted feed for chickens or perhaps a mixture of silage, whole corn and a concentrated protein pellet for feeder cattle. All of these could be done with a hand lens and a needle probe. Similarly, a suspected feed ingredient or material that accidentally got into a feed might be described by its source. A description could be "weeds along a confinement area with potential toxic seeds," or a note that an on-farm feed mill was using roasted soybeans recovered from a sunken barge. Perhaps late harvested corn with extreme weather damage or restaurant cooking oil with floor sweepings was being used. Every piece of the puzzle needs to be collected and considered because when toxicants get into feeds the situation becomes a forensic analyses.

The higher magnifications of a stereomicroscope would be needed to complete the above examinations. The shift from macroscopic to stereoscopic would allow a more detailed picture of the weathered corn, any potential mold contamination of the recovered water-logged soybeans and the seed characteristics from the ingesta of a free roaming animal. The compound microscope plus some careful sample preparation would be needed to jump up to a cellular or histological analysis. The higher magnification would permit a visible examination of mold mycelia invading the seed structure, the cell types and sub-cellular calcium oxalate crystal inclusions of certain leaves and other definitive structures used for identifications. The compound microscope permits the unique cellular characteristics of all the ingredients to be used for the analysis.

## CLASSIFICATION OF FEED INGREDIENTS BY SOURCES

Feed microscopy can also be sorted, studied and examined by the ingredient sources. This allows parallel comparisons of similar structures which are related uniquely to each species. Each level of detail opens new opportunities for confirming an analysis.

*Processed feed grains, legumes and oilseeds* are whole seed sources that are minimally processed for feeds. Corn (maize) is one of the most common feed grains in the world. It is fed whole, ground or flaked. Grinding and steam flaking enhance the nutrient availability particularly of carbohydrates because corn is primarily a starchy energy source. The extent of starch gelatinization can be observed with polarized light. Grain sorghum and the several millets are smaller seeds that are fed in the same manner. Wheat, barley, rye, oats and rice are more typically food grains but lesser grades are used directly in feeds. The feed microscopist can identify ground particles from each of these sources by their inherent structural characteristics. For example, an examination of an unknown flat particle associated with intact starch could be compared to the modified leaf tissue that in grains is considered as chaff or hull. Wheat chaff is left in the field and is not in direct contact with starch in the seed. Therefore, logically the microscopist would search inwardly to the next flat layer under the hull which is pericarp. The outer pericarp, when ground, often remains attached to other layers of the original ovary wall plus the testa and aleurone layers and is called bran in commercial channels. The unknown would be compared to reference standards of the pericarp or bran for each cereal. If the initial observations that the particle was associated with starch was true, the particle would likely match the cell types and be confirmed as a bran particle of one of the cereals. Knowledge of the structure of all the seeds commonly used in feeds allows a logical comparative examination of any cereal or other feed ingredient.

An example of a confirmed minimal processed legume is cracked or whole fed soybeans. The soybean is the only major feed legume that is fed whole but it is usually roasted to minimize trypsin inhibitors. When whole soybeans are roasted, the seed coat splits transversely to the embryonic axis and remains on the seed. It is a unique characteristic associated with roasting. The extent of roasting could be measured by the residual urease activity. Other legumes such as lentils, lupine, dry beans and peas are found in feeds but not as mainline ingredients and they have no significant trypsin inhibitors that would require heat treatment. Each of these clues leads to a confirmed analysis.

Cottonseed is one of the few oilseeds fed whole as a fiber source in cattle. The seeds are very fuzzy and can be easily identified by the attached cotton fibers that rotate polarized light, have a dumbbell-shaped cross-section and exhibit a slow twist. In addition, the hull is a three-layered achene that is multicolored and unique to cottonseeds. Many of the characteristics exhibited by oilseeds are similarly so specific that tiny particles of the oilseeds can be identified even after they have been hammermilled and processed into finished feeds.

*Processed feed grain, legume and oilseed by-products* are food by-products on which the entire feed industry is based. A wide range of ingredients from the production of foods are available. These include corn bran, corn gluten feed, corn gluten, hominy feed, wheat middlings, wheat bran, barley hulls, rice hulls, oat hulls just to list a few. Legume and oilseed by-products include, soybean hulls, peanut meal and hulls, canola meal, linseed meal, sunflower meal and hulls, safflower meal and hulls, cottonseed meal and hulls, and many less common meals. Each ingredient has specific characteristics at each level of magnification to identify it with confirmation at the cellular level.

These structures can be found in the listed references of this chapter or created by careful dissection of authenticated reference standards.

*Processed by-products from other plants* include citrus pulp, bagasse from sugar cane and beet pulp from sugar beets, fruit pomaces, tomato pomace, potato meal, food gum meals and other food by-products. Almost everything that is not designated human food finds its way into feeds.

*Processed plant products from industrial sources* include distiller's grains and solubles from the power alcohol and distillery industries, fats and oils from off grade edible and detergent oil sources, Aspergillus meal from food and industrial enzyme production and many others. The oils are particularly problematic because they have no visible cellular structures and can get mixed accidentally with miscible industrial liquids and pesticides.

*Processed animal products and by-products* include whole animal meals such as chopped beef, fish meal or poultry, lamb or venison meat. Examples of meat meals include blended meat meal (beef and pork), fish meal from larger trimmed out or canned fish, and all manner of rendered meat products. Feed microscopy is currently the major firewall against BSE (Bovine Spongiform Encephalopathy) or mad cow disease tissues getting into cattle feed.

*Processed natural mineral products and metallic wastes* are the minerals in feed. On rare occasions, ground circuit boards and other electronic and industrial waste that contains metallic components get into feeds. Cadmium, arsenic, lead and other toxicants also find their way into feeds. These can be detected by the microscopist and suitable chemical testing initiated to determine the extent of the problems.

*Processed aquatic products and by-products* include fish meals, shellfish meal, oyster shells, shrimp meal, shrimp shell meal and shrimp head meal, squid meal and other specialized products. Of all the by-product meals, the aquatic ones are the most variable and potentially the most adulterated because they lack specific standard descriptions for trading. The feed microscopist is often quite challenged by these meals in feeds.

## CLASSIFICATION OF FINISHED FORMULA FEEDS BY PROCESSING

We can also classify finished feeds by the type of agglomeration and cooking processes used as an aid to understanding the effects of processing on the feed ingredients. Although the key identifiers will not change dramatically, it is important to understand that the extent of conditioning and moisture incorporation during processing will (1) affect the starch gelatinization and other binding reactions and (2) affect the methods used in the microscopic examination to reverse the hydrothermal bonds and permit easier examinations. Some of the common processes and applications are as follows.

*Pelleted and crumbled feeds* are used to maintain uniform nutritional distribution in feeds. The pelleting operation increases the bulk density and fixes the nutrients so that no segregation occurs during shipment and handling. The pellets are crumbled to make it easier for poultry and small animals to feed. Crumbling increases the surface areas for saliva or other digestive juices to be absorbed and to speed digestion. Crumbling also increases the available surface area for moisture absorption and in humid areas can lead to mold growth. Pelleted feeds have relatively friable structures due to minimum starch gelatinization. They can be readily hand ground in a mortar and pestle. The particles will usually separate adequately to conduct an analysis when aided by solvent flotation and sieving to separate fine particles and fat and ease the identification of fine particles.

*Expanded and extruded feeds* enhance nutrient availability and feeding efficacy primarily by increased gelatinization during conditioning and the ability of the extruder to process higher moisture feed mixtures. The extruder combined with the expander, particularly when the feed is reground or re-extruded for aquaculture feeds and some pet foods, reduces the sizes of the individual feed particles to the point that the feed microscopist can barely identify the sources of the ingredients. The extent of gelatinization is much greater for extruded matrices and they do not separate easily even after mortar and pestle grinding, flotation and sieving. They are a much more difficult matrix to separate and examine.

*Steam conditioned and rolled feeds* have enhanced cell disruption and nutrient availability. The process is used primarily for whole grain corn and sorghum that are to be fermented or reconstituted and fed with silage and other rations. Unless flaked grain has been mixed with other ingredients, no additional separations are needed prior to the microscopic examination.

*Canned pet foods* are specialty soft and wet products with unique attractants and flavors. They require thermal processing to maintain shelf life stability and palatability. They are particularly difficult to analyze but are commercially sterile and not prone to microbial growth or related toxicant problems.

## CLASSIFICATION OF FORAGES BY POINTS OF CONTACT OR POTENTIAL TOXICANTS

Lastly, we can classify feed resources by their natural habitats and their potential for toxicological problems. These

plants and toxicant situations are not commonly seen by the feed microscopist but a good broad knowledge of plant structures makes the feed microscopist particularly well suited to examine them. These include native forages and pasture plants that may be consumed due to animals roaming free on pasture and via casual contacts. Some cut and stored forages and silages that are fed to confined animals may be weathered and spoiled. Exotic and introduced plants may be consumed due to invasive growth in pastures or by accidental cuttings and unknowledgeable feedings. Finally, decorative house plants and some garden materials may be consumed by pets. All these type of forage samples are examined as they are taken. That is, no specific methods are used to reduce the particle size because the evidence will be compromised by additional processing. Instead the particles are separated by hand into appropriate classes to be examined. For example, a whole kernel corn, silage and concentrate pellet feed would be separated on a large tray by hand or with minimal sieving depending on the particle size classes involved. Hand separations are generally easiest, although time consuming. The corn and pelleted concentrate would be dried and each examined separately. The pellet would be handled like any other pelleted feed and analyzed for its component parts. The silage would also be dried and, if a toxicant was suspected, the examination would focus on the purity of the silage including weedy and herbaceous forages, evidence of wild fermentation, mold growth and the extent of uniformity and the presence of additives. Identifications would be based on collected forages from the farm or ranch or keyed to range and pasture plant references. Unknown forages could also be identified through herbarium collections.

## IDENTIFICATION OF INGESTA PARTICLES

The identification of fecal and ingesta samples is very similar to the routine examinations of feeds and forages. Sometimes particles can be isolated directly from the samples. Otherwise the samples require pre-analysis preparation for biological safety for the microscopist. A standard fume hood or air handling unit to isolate aromas and filter out dried dusty particles would also be needed for biological safety.

Digestion, even through a rumen, is similar to the feed manufacturing process and does not destroy the cellular characteristics used for identifications. Thus, the identification of yew or hemlock leaves, cockleburs, crotalaria and other toxic weed seeds and most other plant toxin sources is the same as for forage examinations. Reference standards of the seeds and plant tissues are the key to the identifications, although having some ingesta samples for comparisons is always helpful. Medical laboratory manuals also provide routine methods for the safe handling of fecal sample examinations that can be applied to feed microscopy.

## THE ROLE OF THE ATTENDING VETERINARIAN

Because few feed microscopists are available for on-site examinations, the attending veterinarian should have an understanding of feed microscopy and what it can provide for an investigation. The veterinarian should be aware of the sampling requirements for microscopy, the need to secure both the feed ingredients and the finished feeds from bagged storage, from feed bunkers, from augers and feed mixing, handling and feeding systems, and from any other on farm or confinement locations. Samples from baled hays or forages, silage bunkers, fence rows, pastures and other waste areas available to animals should be retained. In the case of pets and small animals, representative samples should include house plants and decorative plantings and garden areas around the house. Too often the feed microscopist is given only a sample of a suspect feed or ingredient which may or may not be related to the toxicant problem. A more encompassing view should be encouraged. Because sampling is one of the most critical aspects of any toxicant situation, samples representing the earliest possible point in time should be secured as quickly as possible and retained for microscopic examinations.

If an ingesta or fecal sample is available, it should be processed immediately or frozen (iced for short-time transport) at $-10$°C or below to limit microbial activity. The following is a simplified analytical scheme to handle these samples.

*Ingesta or fecal samples* should be separated into the portion for chemical testing and the microscopy aliquot. A 5 or 10 g wet weight aliquot can be rinsed and washed thoroughly through a 260 or 400 mesh stainless steel sieve to retain essentially all particulates but remove all the solubles (assuming no chemical or biochemical testing will be needed on that sample aliquot for extra-cellular constituents). The particulate residues should be transferred from the sieve to a hard surfaced but porous filter paper (standard pleated coffee filters work well) and the sample air dried. Do not heat the sample if any enzyme-based testing will be required. The dried particles, particularly the fibrous cellulosic particles, may be gently rolled in a mortar with the pestle to cause the fibrous particles to separate. Do not attempt to grind them. If the sample is too fatty to separate and the particles cannot be identified due to a

coating of fat, flotation on chloroform (observe the current laboratory safety procedures for the solvent) may be necessary as both a defatting step and a separation of mineral constituents and particulate fines. Defatting will take about three rinses using 2–3 times the volume of the fatty aliquot. When the chloroform has evaporated, the examination can proceed with standard feed microscopy methods comparing the unknown particles with reference standards and confirming the observations at the cellular level. Remember, most of the digestible nutrients will have been removed from the cellulosic cell matrix debris. That may change the appearance slightly but the cellulosic structures will not have changed, depending on passage time, and can be compared to reference standards of ingesta and feces if they are available. Animal tissues would have been digested and would not generally be available for examination.

## FEED MICROSCOPY TRAINING AND AVAILABLE LITERATURE

Although this chapter has briefly introduced feed microscopy and what it can do for toxicological examinations, some mention of where training is available, how one organizes a reference sample collection and sets up a laboratory may be of interest. Because feed microscopy is a "hands-on" practical technology, it is not easily learned from a book. It is instead taught as an intensive short course. An Internet search is currently the best way to locate feed microscopy short courses.

Feed microscopy draws on all the scientific disciplines and information exists but is widely scattered and often out of print and not readily available. The interpretive drawings of Winton and Winton (1932) still stands as one of the best reference sources ever published for food and feed structures. Vaughan (1970) updated the oilseeds with excellent interpretive drawings and some photographs. *The Manual of Microscopic Analysis of Feedstuffs* (Bates *et al.*, 1992) draws the most important technical information together into a single reference book specifically for feed microscopy but it is not a textbook or a training manual. Klein and Marquard (2005) put together an extensive *Atlas of Feed Microscopy* which provides many line drawings that had not been previously available in English. *The Aquaculture Feed Microscopy Manual* (Bates *et al.*, 1995) explored the ingredients commonly used in fish, shrimp and other confined aquatic organisms. *Introduction to Food-Borne Fungi* (Samson *et al.*, 1995) is one of the best references for detailed photos and interpretive drawings of the common molds attacking foods and feeds. What is most notable about the feed microscopy literature is the lack of a single treatise or detailed text for the feed microscopist that covers everything including the cellular keys for identification of forages and pasture plants and the identification of ingesta and fecal particles. Reference standard collections are critical to feed microscopy and ingesta identifications. Typically microscopists share samples or hard to find materials at professional meetings or via mailings. Sample collections of authentic materials are usually provided at feed microscopy short courses.

## THE FEED MICROSCOPY LABORATORY

Feed microscopists generally work alone, not in groups, and their laboratories are reflections of their work habits in whatever space is available. An ideal feed microscopy laboratory work station is centered in a "U" configuration with sinks, hoods, preparation areas, etc. located nearby but not in the immediate microscope area where fumes or solvents might damage the microscopes. Figure 89.1 shows a laboratory with shallow drawer cabinets full of reference standards supporting the work surfaces holding the microscopes. A boom stand stereomicroscopes is at the right side, a large research stereomicroscope is in the center equipped with video and still camera ports and a large frame compound microscope is seen on the left side. A microscope and a second compound microscope are just outside the picture borders along the legs of the "U." A balance, hand tools, reagents, reference materials and many other small items used in the analyses can be seen in the photo along with small cabinets for all types of reference standards.

## THE EQUIPMENT

The following equipment list details briefly the essential items plus many that are useful but may not be available in most laboratories. In all cases, purchase the best equipment that can be justified. Remember many extremely fine used microscopes are available for the starting microscopist:

- *Stereoscope*: Research grade, zoom microscope equipped with a brightfield/darkfield base and trinocular head. The newer models provide more options for digital photomicrography, videography, illumination and depth of field than the older, more simplified microscopes. Because the stereomicroscope is the heart and soul of the equipment used, it should be selected carefully to do all the types of examinations that are anticipated.
- *Compound microscope*: Large frame research model. Many excellent used Leitz, Zeiss and other brands from the

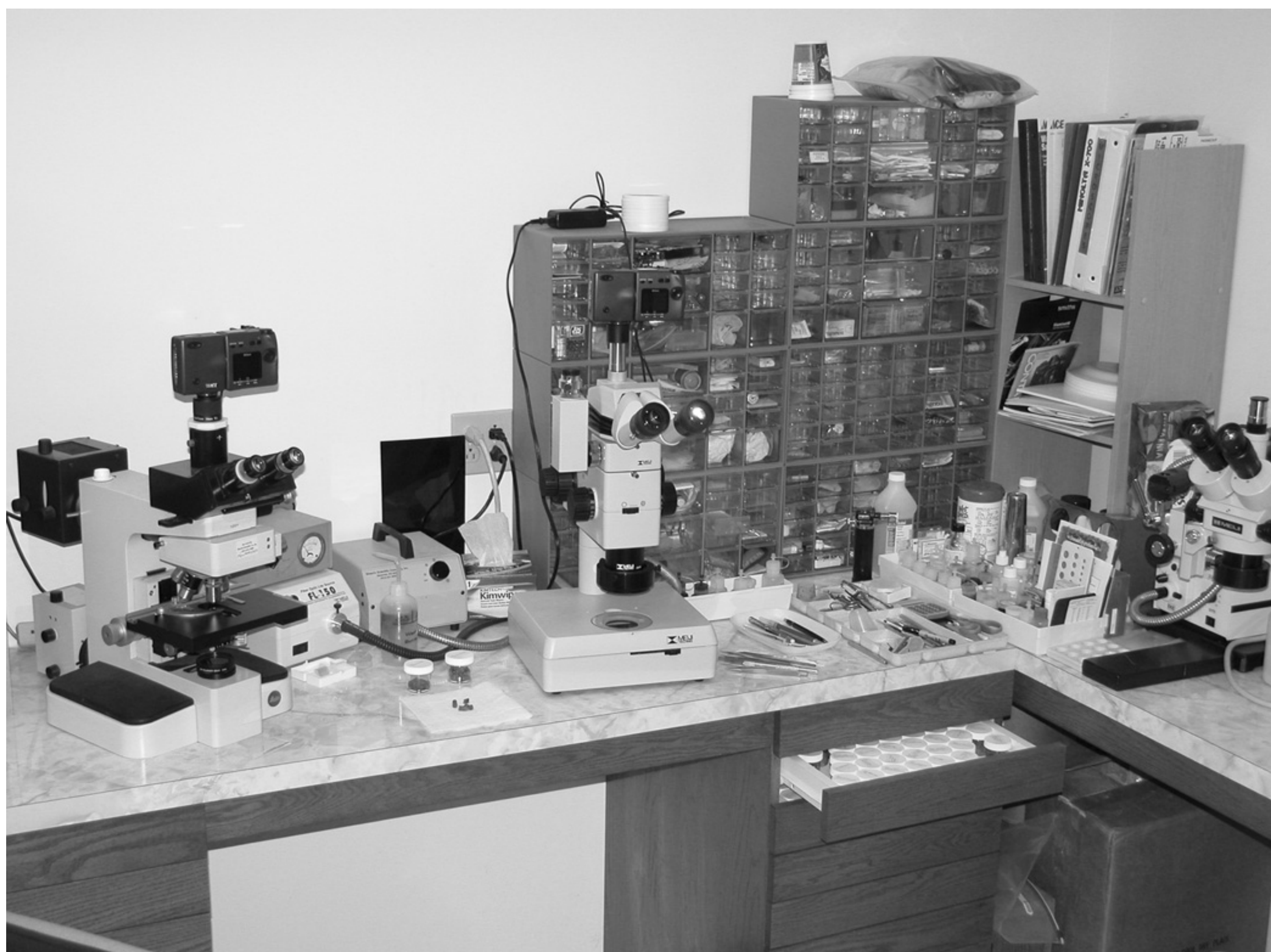

**FIGURE 89.1** An efficient "U"-shaped feed microscopy laboratory configuration.

mid-1980s are still available and serviceable. Most microscopy laboratories cannot justify purchasing the latest research models.

- *Dual compound microscopes*: Bridged for side-by-side comparisons of unknowns and authentic reference standards.
- *Documentation*: Digital cameras can be readily interfaced with microscopes to provide a digital record of observations, reference standards, side-by-side comparisons, etc.
- *Hand tools*: This should include tweezers, needles and probes for manipulating samples, small cutting instruments (scalpel, micro-scissors), glassware and sieves for flotation separations, measuring tools (manual or digital calipers, optical and stage micrometers and measuring cells for particulates). Many microscopists fabricate their own tools or adapt and modify tools from other disciplines. Dentistry picks and probes, tools for clay sculptors, model builders scissors, micro-soldering torches and many other small tools have been adopted for use under the microscope.
- *Reference standards set*: The collection of reference standards (authentics) is a critical aspect of feed microscopy. The reference collection is one of the central three requirements for microscopy and maintaining a collection is a career long endeavor. Every effort should be made to collect multiple samples of each feed ingredient to represent the variations from different parts of a country or different appearances due to heating, drying and other processing variations of manufactured by-products. The underlying cellular structures will remain essentially the same (except for agglomerated and co-mingled by-products) but the appearances may differ.

The reference collection should be preserved in glass or plastic containers that can be well sealed and maintained free of insects. Freeze potentially contaminated samples for 10–15 days before placing them in the collection. No pesticides or other chemicals should be used for preserving a reference collection because natural aromas are an inherent part of the ingredients.

## CONCLUSIONS AND FUTURE EXPECTATIONS

Feed microscopy offers a wide range of applications for the analysis of feed ingredients, finished feeds, ingesta and fecal samples. Although the classical applications of feed microscopy in the feed industry and state compliance laboratories have become relatively static due to a series of events and decisions ranging from load cell technology to least cost formulations to collective terms for labeling, the needs for microscopists in BSE screening and bio-security issues, toxicology and forensic applications have dramatically increased. Thus, this is a time of transition. This chapter illustrates just a fraction of the applications and opportunities for feed microscopists. The identification and evaluation of particulates from foods, feed ingredients, formula feeds, forages, weed seeds and all manner of other sources will continue to expand for many years if the current demand can be interpolated correctly into the future.

## REFERENCES

Bates LS, Barefield L, *et al*. (eds) (1992) *Manual of Microscopic Analysis of Feedstuffs*, 3rd edn. The American Association of Feed Microscopists (Now Div. of AOCS).

Bates LS, Akiyama DM, Shing LR (eds) (1995) *Aquaculture Feed Microscopy Manual*. American Soybean Association, Singapore.

Klein H, Marquard R (2005) Feed microscopy: atlas for the microscopic examination of feed containing vegetable and animal products. *Agrimedia Gmbh*, Bergen/Dumme.

Samson RA, Hoekstra ES, *et al*. (1995) *Introduction to Food-Borne Fungi*, 4th edn. Centraalbureau voor Schimmelcultures, Delft.

Vaughan JG (1970) *The Structure and Utilization of Oil Seeds*. Chapman and Hall, London.

Winton AL, Winton KB (1932) *The Structure and Composition of Foods*. John Wiley and Sons, New York.

CHAPTER

# 90

# Role of pathology in diagnosis

*Manu M. Sebastian*

## INTRODUCTION

All intoxications do not result in gross or histopathological lesions in tissues of exposed animals. Hence the pathological findings should be correlated and interpreted in terms of case history, clinical signs and chemical analysis of the body fluids or ingesta (e.g. no specific pathological lesions for *Clostridium botulinum* toxicity). The gross and histopathological examination of the tissues along with ancillary testing will help to attain a conclusive diagnosis and also rule out other etiologies. Many of the toxicants do leave some histopathological clues in organs like liver, kidney, gastrointestinal (GI) tract, skin, brain, etc. Appropriate samples should be collected for processing in order to attain a conclusive diagnosis. The tissue should be collected in 10% neutral buffered formalin for routine histopathological processing. In veterinary medicine, toxicological investigation of mortality in animals is conducted by postmortem examination. In such investigations pathologists play an important role by correlating and interpreting the case history, clinical signs and pathological findings with analytical findings of body fluids, gastric contents, etc. When field necropsies are conducted appropriate samples (sample collection and shipping have been mentioned in other chapters) should be submitted for analytical examination along with formalin-fixed samples for attaining a conclusive etiological diagnosis. In medico-legal cases the samples should be collected in the presence of qualified law officers and should be appropriately documented. The current chapter covers the pathological lesions observed in domestic animals which are exposed to common toxins/chemicals. This chapter is divided into nine sections which comprise of drugs, feed and food materials, gases, minerals and metals, mycotoxins, plants, pesticides, miscellaneous and zootoxins.

## DRUGS

Several drugs induce adverse reactions in domestic animals. Accidental ingestions of human prescription and over-the-counter medications have been reported in veterinary medicine literature. Antibiotics like penicillin lead to anaphylaxis and hemolytic anemia but there is a no definite microscopic finding. There are several cardiovascular medications like calcium channel blockers and angiotensin-converting enzyme inhibitors, beta blockers and diuretics, tranquilizers, antidepressants, anticonvulsants and muscle relaxants which have been reported to induce adverse reactions in domestic animals, but there are no specific histopathological changes which will aid in the diagnosis of these adverse reactions. Similarly illicit drugs or abused drugs like amphetamine, cocaine, opiates, marijuana, etc. have no specific histopathological lesions which will correlate with the accidental, intentional, or malicious exposure.

Felines are highly sensitive to toxicity for certain drugs as they are deficient in certain types of glucuronyl transferase. The common drugs to which cats are susceptible to toxicity include aspirin, acetaminophen, ibuprofen, preparations containing caffeine, theobromine and theophylline. Several drugs are reported to cause idiosyncratic reactions in different species of domestic animals and the specific pathogenesis is not known. This section covers the common drugs which are used in veterinary medicine and induce adverse reactions in domestic animals with histopathological changes.

### Aminoglycosides

The most important toxic effects manifested by aminoglycosides are nephrotoxicity, ototoxicity and neuromuscular

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blockage. Aminoglycosides accumulate in the proximal tubular epithelial cells where they are sequestered in the lysosomes and interact with ribosomes and mitochondria resulting in cellular damage (Kahn, 2005).

Gentamycin toxicity has been reported in dogs. Grossly the kidney appears pale. Histopathological changes include tubular epithelial necrosis with regeneration and mineralization. Predominantly the proximal tubules are affected. Lesions will progress from hyaline droplet degeneration with dilated lumen to necrosis of the renal tubular lining epithelium (Sprangler *et al*., 1980). Gentamicin toxicity has also been reported in snakes. In experimental studies conducted on gopher snakes, proximal tubules showed hydropic degeneration and this progressed to tubular necrosis 1 week after the gentamicin was discontinued. In the same experiment the snakes also developed visceral gout with urate crystals in multiple visceral organs (Montali *et al*., 1979).

Neomycin toxicosis has been reported in calves. Experimental exposure of neomycin in calves has reported both nephrotoxicity and ototoxicity (demonstrated clinically). The clinical pathological observations included granular casts in urine, proteinuria and low specific gravity of urine, azotemia, decreased creatinine clearance, polyuria and polydipsia. The histopathological findings included renal tubular epithelial degeneration and necrosis (Crowell *et al*., 1981).

Aminoglycosides can result in ototoxicity and cats are highly sensitive to ototoxicity. The important histopathological finding is the degeneration and loss of the hair cells and supporting sensory structures in the organ of Corti. The zootoxic potential is high for gentamycin (McCormick *et al*., 1985). Erythromycin ethylsuccinate has been reported to induce severe colitis in horses which is associated with major changes of the intestinal microflora. The subsequent growth of *Clostridium difficile* has been demonstrated as a potential etiological agent in antibiotic-induced acute colitis (Gustafsson *et al*., 1997).

## Antifungal agents

Amphotericin B is an antifungal agent and is a drug of choice for systemic mycosis. Toxicosis of this drug can happen at recommended dosage rates. Nephrotoxicity may be related to a decrease in intrarenal blood flow associated with arteriolar vasoconstriction, a subsequent decrease in glomerular filtration rate (GFR) and impaired tubular function. Dogs and cats are the common species affected with more susceptibility in cats. The common histopathological finding includes renal tubular necrosis, dilatation of the tubules and mineralization with mild lesions in the interstitium and glomeruli (Rubin *et al*., 1989). Ketaconazole, an antifungal agent, also has been reported to induce toxicity. The target organ is liver and toxicity is characterized by necrosis of hepatocytes (Ma *et al*., 2003).

Griseofulvin another antifungal agent is known to be teratogenic. Congenital malformations were reported in kittens of cats treated with griseofulvin which affected both brain and skeletal system. The changes in the brain consisted of exencephaly, malformed prosencephalon, caudal displacement and hydrocephalus. The changes in the skeletal system consisted of cranium iridium, spina bifida (C1 – C4 and sacral), abnormal atlantooccipital articulation, cleft palate, absence of maxillae and lack of tail vertebrae. Other pathological observations included cyclopia and anophthalmia with absence of optic nerves and rudimentary optic tracts atresia ani, atresia coli, lack of atrioventricular valves in the heart and absence of external nares and soft palate (Scott *et al*., 1975). Microphthalmia, brachygnathia superior and palatocheiliosis were reported in a foal born to a mare administered griseofulvin in early pregnancy (Schutte and Vanden Ingh, 1997).

## Antineoplastic drugs

Several antineoplastic drugs have been reported to be toxic in domestic animals. Experimental studies of methotrexate in cats have shown axonal degeneration and fibrin deposits in the walls of small vessels (Shibutani and Okeda, 1989).

Experimental studies of 5-fluorouracil and its masked compounds tegafur and carmofur in dogs have been reported to cause neurotoxicity (Okeda *et al*., 1988).

Vincristine, which is an alkylating agent, has been reported to produce peripheral neuropathy in dogs (Hamilton *et al*., 1991).

The common pathological manifestation of adverse reaction of cyclophosphamide therapy in dogs and cats is sterile hemorrhagic cystitis characterized by ulceration, edema and necrosis. Toxic effects of cyclophosphamide in the urinary bladder are by the action of acrolein, a metabolite of cyclophosphamide. Acrolein causes submucosal, edema, necrosis, hemorrhage and fibrosis of the urinary system epithelia (Dhaliwal and Kitchell, 1999; Charney *et al*., 2003). Another antineoplastic drug which has toxic effect is doxorubicin. Experimental studies have shown that doxorubicin can induce degeneration of the myocytes characterized by vacuoles.

Cisplatin is an antineoplastic drug which causes toxic changes in kidney, intestines and bone marrow. Cisplatin or its metabolites are eliminated through urine and hence cause renal toxicity. The exact mechanism of toxicity is not known and may involve the metabolites of cisplatin and not the platinum atom itself. The pathological findings associated with cisplatin treatment in dogs include mild renal tubular atrophy and tubular necrosis. In cats which had saline diuresis to avoid renal toxicity by cisplatin, the

medication resulted in pulmonary toxicity. The gross findings included severe hydrothorax, mediastinal and pulmonary edema. The microscopic observations associated with toxicity included thickening of alveolar septa and congestion with neutrophils, thrombi, mild necrosis and fibrin deposits. Microangiopathic changes were also observed in the alveolar capillaries (Choie *et al*., 1981; Knapp *et al*., 1987; Forrester *et al*., 1993).

## Chloramphenicol

Chloramphenicol is reported to be toxic in cats. The drug is metabolized by the liver and excreted as a glucuronide conjugate which accounts for the increased sensitivity in cats. In experimental studies toxic effects of chloramphenicol in cats include bone marrow suppression with marrow hypoplasia, maturation arrest of erythroid cells, inhibition of mitotic activity and vacuolation of lymphocytes, early myeloid and erythroid cells. Some cats had reduced marrow cellularity, or increased myeloid:erythroid ratio, or both of these. The hematological changes included decreased numbers of neutrophils, lymphocytes, reticulocytes and platelets. In dogs the toxic effects observed in experimental studies include suppression of erythropoiesis, decreased mitotic activity and reduced rate of granulocytopoiesis (Watson, 1977, 1980; Watson and Middleton, 1978).

## Fluoroquinolone

Quinolones have been reported to produce neurotoxicity and convulsions when administered in high dose. High-dose administration of quinolones for long time has reported embryonic loss and maternal toxicity. Joint is the target area for fluoroquinolone toxicity in dogs. Experimental studies in young dogs with difloxacin, a fluoroquinolone, show erosions or vesicles in the articular cartilage. The microscopic findings include necrosis of chondrocytes in the articular–epiphyseal cartilage complexes that was rapidly followed by disruption of extracellular matrix and formation of fissures (Burkhardt *et al*., 1992). The excessive use of quinolones in immature dogs may thus lead to lameness.

Enrofloxacin has been reported to be retinotoxic in cats resulting in blindness by acute and diffuse retinal degeneration. The histopathological observations include outer retinal degeneration, with diffuse loss of the outer nuclear and photoreceptor layers, hypertrophy and proliferation of the retinal pigment epithelium (Gelatt *et al*., 2001).

## Mebendazole and albendazole

Generally, the anthelmintic mebendazole is of low toxicity and has a wide margin of safety in animals. There have been reports of mebendazole toxicosis in a group of Doberman pinschers and dachshunds which were dosed at the recommended therapeutic level. The microscopic findings include centrilobular necrosis of the hepatocytes in the centrilobular areas with loss of hepatocytes, sinusoidal collapse and replacement by hemorrhage. Hepatocytes near portal areas had vacuolations with swollen nuclei. Occasional inflammatory cell infiltrates were present in the portal areas and central veins. The toxicity is considered to be due to an idiosyncratic drug reaction (Polzin *et al*., 1981).

There are reports of albendazole-induced bone marrow toxicity in dogs and cats. The toxicity appears to be dose related in dogs and idiosyncratic in cats. Clinical pathological findings include pancytopenia with severe bone marrow hypoplasia in dogs. In cats hypoplasia of erythroid and megakaryocytic series, but with a left-shifted granulocytic hyperplasia were observed (Stokol *et al*., 1997).

## Minoxidil

Minoxidil a vasoactive drug is used to stimulate hair growth in adult men and women and is available over-the-counter in United States. Pet owners may use the drug for alopecia in animals. Experimental studies have reported cardiovascular toxicity. Experimental studies in dogs indicate that minoxidil can induce pathological changes in the heart which include right atrial hemorrhage, right ventricular papillary necrosis, subendocardial necrosis and fibrinoid necrosis of the small arteries (Jett *et al*., 1988; Mesfin *et al*., 1995).

## Furazolidone

Furazolidone toxicity has been reported in goats. The gross observations included congestion in brain, liver and kidneys. The histopathological findings included degenerative changes in the renal tubules, hepatocyte necrosis and degeneration in the centrilobular areas. The changes in the brain included congestion of white matter in cerebellum and scattered vacuoles around nerve sheaths (Ali *et al*., 1984). The other species in which furazolidone toxicity is commonly reported is young turkey poults. The gross findings include ascitis, bilateral or right side dilatation of ventricles with round apex and congestion of the lung and liver. The histopathological findings include cardiomyopathy and secondary changes in the lung and liver due to cardiac changes (based on right or left-side failure) consisting of pulmonary edema and congestion, degeneration of hepatocytes, fibrosis and bile duct hyperplasia (Simpson *et al*., 1979; Good and Czarnecki, 1980).

## Non-steroidal anti-inflammatory agents and analgesics

NSAIDs are the most widely used analgesics in veterinary medicine, and all have some toxic potential. The NSAIDs and analgesics associated with toxicity in veterinary medicine include salicylates, ibuprofen, ketoprofen, carprofen, naproxen, indomethacin, etodolac, sulindac, oxyphenbutazone, phenyl butazone and piroxicam. The most common adverse effects are GI, renal, hepatic and coagulation disorders. The common species affected are dogs, cats and horses. Cats are more susceptible to salicylate toxicity due to the reduced hepatic glucuronyl transferase system.

NSAIDs cause toxicity of the GI tract, kidneys and liver. GI toxicity is attributed to the direct effect of the drug and inhibition of the cyclooxygenase isoenzyme. NSAIDs cause renal toxicity mainly by the inhibition of prostaglandin synthesis and renal blood flow resulting in renal papillary necrosis. The hepatic toxicity is mostly attributed to idiosyncratic reaction. Gross observations include GI irritation and possible ulceration with associated hemorrhage and possible perforation. Renal tubular or papillary necrosis is also reported in a limited number of cases in dog (Jones *et al.*, 1992).

The toxicity with carprofen has been reported in dogs and primarily affects the liver. Hyperbilirubinemia and high-serum activities of alanine aminotransferase (ALT), alkaline phosphatase and aspartate aminotransferase were the most important clinicopathological abnormalities. Gross findings may include icterus and yellowish

\discoloration of liver. The important histopathological findings are hepatocyte degeneration and necrosis. Hepatocellular necrosis is usually located in the centrilobular areas with mild lymphocytic–histiocytic periportal hepatitis; 13 of 21 reported cases of carprofen hepatocellular toxicosis occurred in the Labrador breed and the cause of breed predilection is not known (Figure 90.1). The toxicity of this drug is considered to be related to an idiosyncratic toxic reaction (MacPhail *et al.*, 1998). In another study carprofen appeared to be well tolerated by dogs after 2 months of administration (Raekallio *et al.*, 2006). Studies in dogs with Celecoxib, a cyclooxygenase-2 inhibitor, have shown that there is evidence for polymorphism in the canine metabolism of the cyclooxygenase-2 inhibitor which may account for some of the toxicity observed in dogs exposed to cyclooxygenase-2 inhibitor (Paulson *et al.*, 1999). Higher than approved dose of medication can result in toxicity. In a study conducted in dogs which received Deracoxib, a selective cyclooxygenase-2 inhibitor, at a dosage higher than that approved by the Food and Drug Adminstration (FDA) for the particular indication being treated, developed GI tract perforation leading to death. Multiple factors were associated with these GI tract injuries (Lascelles *et al.*, 2005).

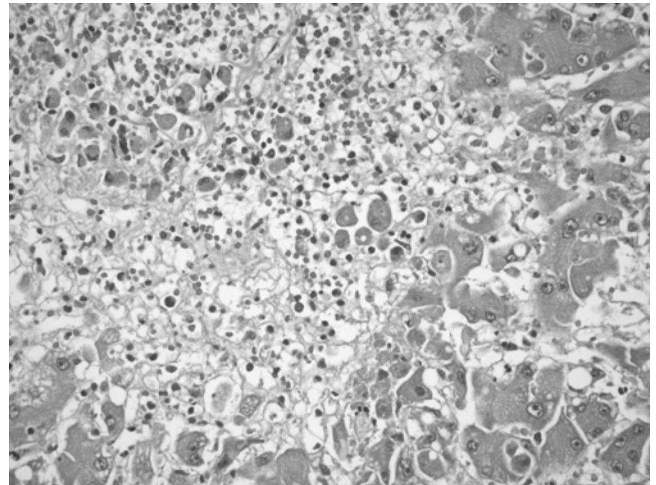

**FIGURE 90.1** NSAID-induced toxicity, liver, canine, hepatocyte necrosis and degeneration in the centrolobular areas with marked biliary stasis, H&E stain, 40×. This figure is reproduced in color in the color plate section.

In dogs other causes of GI tract ulceration include NSAIDs, hepatic disease, mast cell tumor, uremia, stress, and various systemic diseases like disseminated neoplasia, septicemia and disseminated intravascular coagulation.

Horses are susceptible to phenylbutazone toxicity especially when dehydrated and the target organ is kidney. The most common gross observation in experimental studies conducted in horse dosed with phenylbutazone and deprived of water was yellow green radial streak in the renal papillae. The microscopic observations include sloughing of the renal pelvic epithelium including the terminal lining of collective ducts with coagulation necrosis of the underlying papillary interstitium, mineralization of the calyx necrotic papillary interstitium containing nuclear debris and hemorrhage and dilatation of collecting ducts and cortical tubules in wedge-shaped segment of the kidney tissue above area of necrotic papillae. Other lesions observed in experimental studies of horses include ulceration and erosions of the glandular portion of the stomach, submucosal edema of the small intestine, erosions and ulcers of the large colon, ulceration of the gastric mucosa and tongue. Pathological findings in experimental studies of horses exposed to flunixin meglumine (amino nicotinic acid) were glandular mucosal erosion and ulcer of the stomach (Gunson and Soma, 1983; MacAllister *et al.*, 1993).

Recently report of vulture mortality was reported from the Indian subcontinent related to NSAID toxicity. Diclofenac is a NSAID widely available veterinary drug in the Indian subcontinent, used in domestic livestock. Vultures were exposed to the drug when they consumed carcasses of cattle that were treated with diclofenac shortly before death. Experimental studies of this drug in vultures showed marked nephrotoxicity. The gross

observations were primarily deposits of urate on the surface of internal organs related to renal failure. Histopathological findings were acute necrosis of the proximal renal tubular epithelium with minimal inflammatory response and deposits of urate crystals (Oaks *et al*., 2004; Meteyer *et al*., 2005).

## Analgesics

Aspirin, ibuprofen and acetaminophen are the important analgesics which are reported to cause toxicosis in veterinary medicine. Generally, toxicosis is a result of large overdoses, cats are predisposed to toxicosis, for acetaminophen and aspirin.

Cats are more susceptible to aspirin toxicosis than dogs due to saturation of elimination pathway. Anemia, with Heinz bodies and reduced RBC production, is an important clinicopathological finding in cats. Gastric irritation is the most common toxic effect of aspirin. The common pathological finding is gastric ulcers which lead to hematemesis. Toxic hepatitis is also reported rarely (Villar *et al*., 1998).

The common pathological finding with ibuprofen toxicosis is gastric ulcer and is observed in prolonged therapy in dogs. Perforated gastric ulcers are also reported with prolonged ibuprofen therapy. Centrilobular hepatocyte necrosis has been reported in dogs exposed to accidental ingestion of high doses of ibuprofen. Renal lesions including renal tubular necrosis and papillary necrosis have been reported in dogs.

## Acetaminophen

Acetaminophen is a widely used analgesic and antipyretic agent and toxicity has been reported in both dogs and cats. The toxic effects of acetaminophen are a result of increased production of a reactive intermediate, *N*-acetyl-*p*-benzoquinone imine (NAPQI). Another hypothesis cites oxidative stress as the mechanism of hepatocellular damage. NAPQI depletes the cell of glutathione, which is normally protective against oxidative stress. The stress to erythrocytes causes methemoglobinemia. Centrilobular necrosis of the hepatocytes is the most common histopathological finding associated with acetaminophen toxicity. When exposed to toxic levels it can lead to massive necrosis especially in cats. In studies conducted with acetaminophen centrilobular necrosis was observed in dogs, while cats had more diffuse hepatic necrosis. Heinz body formation is one of the most common clinical pathological manifestations of acetaminophen toxicity in cats (Villar *et al*., 1998; Taylor and Dhupa, 2000). Diagnosis is by correlating case history, clinical signs and pathological findings. Blood and serum levels of salicylate concentration are used in human medicine.

## Anticonvulsants

### *Phenobarbital*

Phenobarbital is one of the most commonly used anticonvulsant drugs in dogs. Hepatotoxicity and mortality have been reported infrequently as an adverse effect of phenobarbital medication. Increased serum liver-associated enzymes are reported in phenobarbital treated dogs both with and without clinical signs of liver disease. The common serum biochemical alterations associated with hepatotoxicity include marked increase in serum ALT, serum alkaline phosphatase (ALP), total bilirubin and prolonged sulfobromophthalein excretion. Neutropenia and thrombocytopenia are also reported in dogs treated with phenobarbital. The important histopathological findings in hepatotoxicity associated with phenobarbital therapy are inflammatory cell infiltration, single cell and piecemeal necrosis, fibrosis and vascular abnormalities characterized by markedly thickened vessel walls. In chronic cases macronodular or micronodular cirrhosis is observed (Bunch *et al*., 1982; Jacobs *et al*., 1998; Gaskill *et al*., 2005). Diagnosis is by correlating case history, clinical signs and pathological findings.

Diazepam is a benzodiazepine derivative and possesses anticonvulsant, sedative and skeletal muscle relaxant properties. Toxicity has been reported in cats and liver is the target organ. Elevated ALT and aspartate transaminase (AST) are the clinical pathological observations. The histopathological findings associated with toxicity include centrilobular hepatic necrosis, hyperplasia and proliferation of biliary ductules and suppurative intraductal inflammation. Toxicosis was considered to be an idiosyncratic reaction (Center *et al*., 1996).

### *Primapaste*

Primapaste is a digestive promotant given to new-born foals which contains an iron compound, ferrous fumarate along with probiotic bacteria. The histopathological lesion observed in toxicity associated with primapaste dosing includes massive hepatocellular loss with lobular collapse, with proliferation of bile ducts and postal tract fibrosis. Hepatic encephalopathy associated with liver damage will also be observed in these foals (Divers *et al*., 1983; Acland *et al*., 1984).

### *Albuterol*

Albuterol which is a beta 2-adrenergic agonist is reported to be cardiotoxic in dogs at very high dose (multiples $>$ or $=$ 19 times the clinical dose). The histopathological

findings observed in dogs experimentally exposed to such high clinical dose were mild degrees of left ventricle fibrosis in the papillary muscles. The cardiovascular effects observed were consistent with the known pharmacologic action of beta 2-adrenergic agonists (Petruska *et al.*, 1997).

### *Steroids*

Steroid administration has been reported to cause hepatopathy in dogs. In experimental studies conducted in dogs exposed to prednisolone acetate the light microscopical observations included progressive hepatocellular swelling and vacuolation. The ultrastructural changes included glycogen accumulation, peripheral displacement of organelles and prominent dilatation of bile canaliculi. The prominent clinical pathological changes include progressive increases in serum levels of alkaline phosphatase, gamma-glutamyltransferase and ALT (Rogers and Ruebner, 1977; Rutgers *et al.*, 1995). Monitoring the serum chemistry during therapy will give indication of the pathological changes in the liver.

### *Anabolic steroids*

Stanozolol is a synthetic anabolic steroid and has been used in both animal and human patients. Stanozolol has been reported to be toxic in cats and the target organ is the liver. The significant clinical finding is elevated serum ALT activity. Histopathological observations noted in biopsy specimens include hepatic lipidosis and cholestasis without evidence of hepatocellular necrosis (Harkin *et al.*, 2000).

### *Sulfonamides*

Sulfonamides can cause both acute and chronic toxic effects. The reactions are due to hypersensitivity and direct toxic effect. Anaphylactoid reactions have been reported with sulfonamides. Several adverse reactions have been reported after prolonged treatment including bone marrow depression, hepatitis, icterus, peripheral neuritis and myelin degeneration in the spinal cord and peripheral nerves, photosensitization, stomatitis, keratoconjunctivitis (Kahn, 2005).

The most important organ affected in toxicity is the kidney. Animals with renal toxicity show elevated levels of blood urea nitrogen (BUN) and creatinine. In some animals sulfonamide crystals will be observed in the renal pelvis as a gross finding and kidneys may be gritty in texture when cut. Histopathological observation is primarily renal tubular epithelial necrosis due to the direct action of the crystal and these crystals are visible with polarized light only if the kidney is processed to prevent the dissolution (specimens of kidney tissue should be fixed in a slightly acidified formalin solution or in absolute ethanol) especially with polarized light. The photosensitization associated with sulfonamide is a primary photosensitization due to the activation of the photodynamic substance by ultraviolet light in sunrays (Jones *et al.*, 1996). Rarely hepatic necrosis has been reported with trimethoprim sulfonamide administration in dogs and is considered to be idiosyncratic drug reaction (Twedt *et al.*, 1997).

### *Tetracyclines*

Tetracyclines have been reported to cause toxicity and kidneys are the primary organs affected. Renal tubular necrosis is the common pathological finding. Animals with renal toxicity will show oliguria, marked azotemia, moderate proteinuria, tubular casts in urinary sediment with inability to concentrate urine (Lairmore *et al.*, 1984; Vaala *et al.*, 1987). Intravenous administration of tetracyclines has been reported to cause fatalities and is not recommended in horses (Riond *et al.*, 1992).

### *Vitamins A and K*

The vitamins which are associated with toxicity in veterinary medicine are vitamins A, D and K. Vitamin A toxicity has been reported associated with consumption of improperly mixed feed or food material (liver) with very high concentration of vitamin A. The intoxication has been reported in felines and calves. Increased vitamin A induces premature growth plate closure in calves which is attributed to premature mineralization of columnar cartilage and subsequent endochondral ossification. The disease in cat is characterized by cervical ankylosis, forelimb lameness and in some animals cutaneous hyperesthesia. Pathological observations include marked exostosis on the dorsal and lateral aspects of the cervical vertebrae and sometimes in the occipital bone, fixation of joints of one or both forelimbs. In a cat which had serum vitamin A concentration 4 times normal levels, the pathological lesions observed were fusion of skull and the cervical and first few thoracic vertebrae, alteration of vertebral architecture by deposition of new bone with involvement of sternum and costal cartilages (Goldman, 1992; Palmer, 1993). Calves exposed to vitamin A/D3 in amounts greater than 10 times those recommended have been reported to result in Hyena disease which is an uncommon spontaneous disease of young dairy cattle that causes dwarfism characterized by short pelvic limbs. The pathological findings in these calves included focal to almost complete closure of physes in the humerus, tibia and femur (Woodard *et al.*, 1997). Excess vit A is reported to be teratogenic. Experimental studies in swine during pregnancy show high prevalence of cleft palate and lips.

### *Vitamin $K_3$ (menadione sodium sulfate)*

Toxicity is commonly reported in horse. In experimental studies the gross lesion observed was enlargement and paleness of the kidney. The microscopic lesions include

diffuse or multifocal tubular necrosis and dilation with proteinaceous and cellular debris from red blood cells and neutrophils and casts, diffuse interstitial edema and lymphocytic infiltration regeneration of the tubular epithelium. In animals which survived for 3 months renal tubules showed degeneration, necrosis and dilation. In chronic renal failure following vitamin $K_3$ administration, the kidneys were reduced in size, the capsule adherent and pale streaks of connective tissue may be grossly apparent. Microscopic findings include connective tissue proliferation with mononuclear cell infiltration, moderate hypercellularity of glomeruli with few sclerotic glomeruli. The tubules are dilated but the lining epithelium appears normal with mineral, cellular and proteinaceous casts may be seen in many tubules (Rebhun *et al*., 1984).

#### *Miscellaneous*

There are sporadic case reports of several compounds which are used as food materials or nutraceuticals but no specific pathological findings are associated with most of these cases.

Xylitol a sweetener used in sugar-free chewing gums has been reported to be toxic in dogs leading to severe hypoglycemia resulting in ataxia, seizures and collapse. Xylitol is a strong promoter of insulin release in dogs leading to hypoglycemia but has no or little effect in humans (Dunayer, 2004). Diagnosis is by correlating the history of exposure with clinical signs and clinical pathological findings. In a study conducted on dogs which had 5-hydroxytryptophan (over-the-counter dietary supplement for obesity, depression, etc.) toxicosis the gross finding in two dogs that died was congestion of multiple organs. The histopathological findings included pulmonary edema, diffuse congestion of liver and lung and acute renal tubular necrosis with hemorrhage (Gwaltney-Brant *et al*., 2000).

Halothane a halogenated hydrocarbon inhalant anesthetic agent has been reported to cause hepatic necrosis in human patients. Similar hepatic toxicity has been reported in a dog. The histopathological findings included centrilobular necrosis which extended between central veins and many of these necrotic areas replaced by erythrocytes, hemosiderophages, bilirubin and mixed inflammatory cells. A sharp line of demarcation between viable and necrotic hepatocyte which is a feature (other feature is centrilobular necrosis) of halothane-induced hepatopathy was also observed in this dog (Gaunt *et al*., 1984).

## FEED- AND FOOD-ASSOCIATED TOXICOSIS

The important feed-associated toxins are ammonia, urea, cottonseed meal, ionophores, sulfur (mentioned in metals) and nitrates. Many food materials which are consumed by humans are also shared with cats and dogs and few food materials do induce toxicity in domestic animals. The common food materials which induce some level of toxicity include macadamia nuts and raisin in dogs.

### Non-protein nitrogen (urea/ammonia)

Urea, biuret and ammonium salts serve as sources of non-protein nitrogen (NPN) for ruminants. Mostly ruminants are affected by excessive feeding and incorrect formulation. Pathogenesis is associated with inhibition of citric acid cycle resulting in reduced ATP. Animals show signs of tremors. No specific lesions are observed. Necropsy examination may reveal few changes, but an odor of ammonia is often noted in rumen contents and tissues. The pH of rumen contents is greater than 8. Animals bloat rapidly and carcasses seem to decompose more rapidly than normal (Haliburton and Morgan, 1989).

### Nitrite–nitrate

Toxicity is by consumption of fertilizers with nitrate, consumption of nitrate accumulator plants (accumulate excess nitrate), water from recently fertilized fields and manure pits and also during drought when plants accumulate nitrate. Ruminants are the common species affected but other species can also be affected. The rumen microorganisms reduce nitrate ($NO_3$) ion to nitrite ($NO_2$) ion which leads to methemoglobinemia. Fetuses and neonates are more susceptible than adults. During necropsy chocolate brown-colored blood is observed due to methemoglobin formation. Multifocal hemorrhages may be observed on the serosal surfaces. If fertilizers are consumed congestion may be observed in the GI mucosa. No specific histopathological findings are observed. Diagnosis is by clinical signs and history of exposure. Nitrate/nitrite analysis can be conducted on the blood, serum, forages, feed, water, rumen contents and ocular fluid (aqueous humor). In abortions aqueous humor of aborted fetuses may have increased nitrate concentrations (Kahn, 2005). Other causes of acute death should be ruled out.

### Gossypol

Swine is most common species affected by gossypol. The toxic component is gossypol in the glands of the cottonseeds which appear as tiny black spots. Most gossypol is removed during processing of the seeds. Gossypol has a cumulative effect. Poisoning usually appears abruptly, but signs tend to begin after the animals have been on cottonseed meal for 4 weeks to as much as a year.

The important clinical pathological observation is elevated liver enzymes. The primary organs affected are liver, heart and skeletal muscle. Gross lesions include widespread congestion and edema, large quantities of straw-colored fluid in the peritoneal, pericardial and thoracic cavities edema of lungs. Liver has prominent architecture and may be swollen, pale or congested. Ventricles are dilated. The most prominent histopathological lesion is massive hepatic necrosis and loss and these areas are replaced by erythrocytes leaving a thin zone of intact cells in the periphery. Skeletal and cardiac muscles show varying degrees of necrosis and degeneration. In chronic cases regenerative changes are observed. Pneumonia may also be observed in some cases (Jones *et al*., 1996).

Cottonseed can induce toxicity in dogs and in a report of accidental ingestion of cottonseed bedding by dogs which lead to mortality, the common histopathological lesions were multifocal myocardial degeneration and necrosis, severe pulmonary edema and chronic passive congestion of the lungs, heart, liver and kidneys (Uzal *et al*., 2005).

Diagnosis is based on case history, clinical signs and pathological findings. Differential diagnosis includes hepatosis dietetica, mulberry heart disease (vitamin E/selenium deficiency), coal tar poisoning (phenolics), selenium toxicity, monensin, lasalocid poisoning.

## Ionophores

Monensin, lasalocid, salinomycin, narasin and maduramicin are carboxylic ionophores commonly used as anticoccidial drugs for poultry and as growth promotants for ruminants. Horses are the most susceptible species for ionophore toxicity and other domestic animals are also susceptible. Clinical pathological findings include elevated serum creatine phosphokinase (CPK), lactate dehydrogenase (LDH) and AST. The gross lesions include multifocal pale areas and hemorrhage in the pericardium and epicardium. Microscopic findings include degeneration and necrosis of cardiac and skeletal myocytes with fibrosis in chronic cases (Figure 90.2). Degeneration of the renal tubular epithelium and myoglobinuria are also reported in some cases (Novilla, 1992). Histopathological findings in horses are mostly associated with heart, commonly in the ventricles. The changes in bovines are also common in the heart but pigs have lesion in the skeletal muscles and atrium. Experimental studies in pigs dosed with toxic level of monensin showed pathological changes in the left atrium and consisted of extensive necrosis with contraction bands. The study indicates the selective injury of monensin in pigs to atrial myocardium (Muylle *et al*., 1981; Van Vleet and Ferrans, 1984).

Salinomycin has been reported to induce toxicity to the nervous system in cats. Cats which were exposed to feed material containing salinomycin had acute onset of lameness and paralysis of the hind limbs and forelimbs. Microscopic findings include neuropathy, myelin degeneration of the sensory and motor nerves (van der Linde-Sipman *et al*., 1999). Diagnosis is by case history, chemical analysis of the feed and pathological findings.

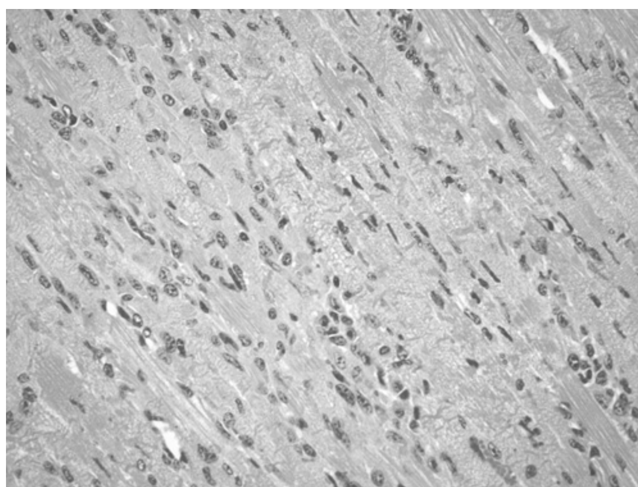

**FIGURE 90.2** Monensin toxicity, heart, bovine, cardiac myocytes show degeneration and some areas myocytes replaced and infiltrated by lymphocytes and histiocytes, H&E stain, 40×. This figure is reproduced in color in the color plate section. (Courtesy Dr. ME Hines, College of Veterinary Medicine, University of Georgia.)

## Raisin

Grape and raisin ingestion has been reported to cause renal toxicity in dogs. The specific mechanism is not known. Clinical pathological findings include hypercalcemia, hyperphosphatemia, elevated BUN and serum creatinine concentrations, all related to renal damage.

Gross lesions are absent and microscopic findings include degeneration and necrosis of proximal renal tubular epithelium with intact basement membrane, regeneration of the tubular epithelium and mineralization. The tubules contain granular and protein casts. In few animals fibrinous arteritis of the large colon was also observed (Eubig *et al*., 2005; Morrow *et al*., 2005).

## Macadamia nuts

Consumption of macadamia nuts has been associated with toxic clinical signs consisting of hind leg weakness, depression and tremors. No mortality has been reported. In experimental studies mild increase in serum triglycerides and serum alkaline phosphatase was detected. The lipase values increased sharply at 24 h and then returned to normal by 48 h after experimental dosing. No

histopathological findings are available in the literature as mortality has not been reported (Hansen *et al.*, 2000).

## GASES

Not much specific pathological findings are observed with exposure to toxic insult of gases. The common gases which induce toxicity in confinement animal housing are ammonia, hydrogen sulfide ($H_2S$), carbon monoxide and nitrogen dioxide. The toxicity of these gases rarely shows any histopathological changes. No gross or histopathological changes are associated with ammonia toxicity. Nitrogen dioxide is associated with silo fillers disease in humans, which is a disease seen in agriculture workers who are associated with silage production and results from the exposure to nitrogen dioxide produced during the fermentation of silage preparation.

### $H_2S$

$H_2S$ is associated with toxicity in pigs and leads to acute death. Liquid manure holding pits is the common source. Sulfide ions act as direct cytotoxic, bind to cytochrome oxidase within mitochondria and block electron transport. Animals dying as a result of $H_2S$ toxicosis are generally cyanotic and may exhibit pulmonary congestion and edema.

A report in cattle resulting in mortality as a result of exposure to $H_2S$ gas from manure pits showed no specific gross lesions. Microscopic findings include massive, diffuse cerebral cortical laminar necrosis and edema characterized by degenerative and necrotic neurons and vacuolations in subcortical white matter (Hooser *et al.*, 2000).

### Carbon monoxide

Exposure to carbon monoxide primarily occurs as a result of improper ventilation. This is common in farrowing houses and lambing sheds and leads to abortion as carbon monoxide cross placenta and cause fetal hypoxia. Common gross lesions in experimental studies in stillborn piglets born to carbon monoxide exposed sows were cherry red discoloration of the subcutaneous tissues, muscle, viscera and accumulation of a large volume of serosanguineous fluid in thoracic cavity. The histopathological findings included malacia of the gray and white matter infiltrated by gitter cell and reactive glial cells, glial cell and vascular proliferation, multifocal hemorrhage and vacuolation of the neuropil (Dominick and Carson, 1983). In many species of animals exposure to carbon monoxide results in acute death and blood has a bright cherry red color. The histopathological findings include degeneration of the neurons in the cerebral hemispheres indicative of hypoxia and ischemia.

## METALS AND MINERALS

Only the most common metals which induce toxicity in domestic animals are mentioned in this section and they include arsenic, copper, fluorine, iron, lead, mercury, molybdenum, selenium, sodium, sulfur and zinc.

### Arsenic

Arsenical compounds cause clinical symptoms related to the GI tract which may be acute or per acute. Herbicides, defoliants, insecticides and arsenic treated wood products are the common sources for toxicosis. Toxicosis is mostly reported in cattle and dogs but all species of domestic animals can be affected.

In acute toxicosis the gross lesions are mostly in the GI tract and consist of severe redness of the gastric/abomasal and intestinal mucosa. The intestinal lumen is dilated and filled with massive amount of fluid. The histopathological findings include marked congestion and hemorrhage in the mucosa of the abomasum and intestine with congestion in submucosa and necrosis of the mucosal epithelium. In some cases the necrosis of the GI tract can lead to perforations (Jones *et al.*, 1996). Arsenic toxicosis though rare is reported in horses also. The gross findings are similar to that mentioned above along with multifocal to coalescing, hemorrhagic ulcers of the mucosa of the cecum and large colon. Histopathological findings include mucosal necrosis and ulceration in the cecum and large colon, vascular thrombosis, necrosis of submucosal blood vessels and infiltration by mixed mononuclear inflammatory cells and neutrophils (Pace *et al.*, 1997).

In subacute cases pale swollen kidney and pale liver are observed and petichial hemorrhages can be observed in the serosal and mucosal surfaces. Renal damage affects all portions of the nephron leading to severe renal tubular necrosis. Histopathological lesions consist of dilation of the vessels, submucosal congestion and edema, necrosis of the intestinal epithelium, renal tubular necrosis and fatty degeneration of the hepatocytes. Nervous system is the target organ of organic arsenicals. The histopathological lesions associated with arsenilic acid are edema of the white matter in the brain and spinal cord with shrunken neurons in the medulla (Selby *et al.*, 1977; Jones *et al.*, 1996).

Diagnosis is by correlating the case history, pathological findings and analytical estimation of arsenic in liver, kidney and other tissues. Differential diagnosis of pathological lesions includes infectious causes and non-steroidal anti-inflammatory agent exposure.

## Copper

Acute copper toxicosis results from accidental ingestion or administration of copper-containing formulations like anthelmintics, foot baths used for livestock, pesticides include fungicides and algaecides at a toxic dose. Copper toxicosis is a problem observed frequently in the sheep. Sheep accumulate copper in the liver in proportion to intake, where there is slow build up of the copper and then there is sudden release which results in hemoglobinuria. Grossly the kidneys have a dark red or bluish black color and the liver will be pale and friable. Microscopic findings include coagulative necrosis of hepatocytes, fibrosis of the portal area and bile duct proliferation with mild infiltration of lymphocytes. The kidney tubules show degeneration of the epithelium. In some cases if the toxic exposure is acute abomasum or stomach has marked hemorrhage and edema (Maiorka *et al.*, 1998). In non-ruminants the lesions observed are pale livers with centrilobular necrosis and gastric ulcers. Diagnosis is by correlating liver copper values with pathological findings and case history.

Hepatic copper accumulation can occur secondary to primary cholestatic disease or as a primary storage disease. Primary copper storage disease is recognized in Bedlington terriers. Copper accumulation occurs secondary to a metabolic defect which inhibits appropriate biliary copper excretion and results in subsequent hepatocellular lysosomal copper accumulation. This defect has been identified as an autosomal recessive inherited trait in the Bedlington terrier. A defect in hepatic metallothionein which binds to copper within the hepatic lysosome is also considered as a pathogenesis. Other breeds reported to be affected by hereditary copper storage disease are West Highland white terrier, Skye terrier and Dalmatian. The animals have pale brown nodular liver. Histopathological findings include copper accumulation in the hepatocytes, starting in the centrilobular areas and extending to other regions resulting in hepatocellular necrosis, inflammation with copper-laden macrophages finally resulting in cirrhosis. Dogs of other breeds such as Doberman pinschers, Labrador retrievers and American and English cocker spaniels have been reported to have elevated copper concentrations in association with chronic hepatitis, but it has not been conclusively proven whether this copper accumulation is primary or secondary to chronic inflammation, fibrosis and cholestasis (Ludwig *et al.*, 1980; Thornburg, 2000).

## Fluoride

Fluoride toxicosis can be acute or chronic and chronic forms are observed commonly. Acute toxicosis is by exposure to insecticides/rodenticides and exposure to volcanic dust exposure. Chronic toxicosis is by exposure to mineral supplements, industrial effluents and fertilizers. Chronic toxicosis is commonly reported in herbivores grazing-contaminated pastures. Volcanic ash from volcanic eruptions has been reported to be a source of fluoride toxicosis in ruminants. Fluoride is metabolized via renal excretion and is preferentially deposited in bones and teeth.

Acute fluoride intoxication produces clinical signs and lesions of gastroenteritis and renal tubular necrosis. In a study conducted in sheep exposed to fluoroacetate, the microscopic findings in acute toxicosis included degeneration and necrosis of myocardial fibers. Subacutely and chronically intoxicated animals had the same findings but were more widespread and in various stages of development or resolution (Schultz *et al.*, 1982).

Chronic fluoride intoxication produces dental and/or skeletal lesions. The common pathological effects of fluoride toxicosis are on the teeth and bones. The common dental lesions include enamel hypoplasia, attrition of molar teeth and crown loss. Teeth have a typical mottling appearance. These lesions develop only if intoxication occurs when the teeth are in the developmental stages. The lesions associated with the skeleton system include kyphosis, hyperostosis and osteodystrophy. The lesions in the bones are observed mostly in the metacarpal, metatarsal and mandible. The marrow cavity becomes smaller in size and the periosteum is thickened. Microscopic findings include thickening of the bony trabeculae osteosclerosis, osteoporosis with periosteal hyperplasia and endosteal resorption. Fluoride toxicosis can also lead to renal injury. In a study conducted in sheep poisoned by sodium fluoride the pathological findings included necrosis of the proximal tubular epithelial cell, necrotizing rumenitis, reticulitis and abomasitis (Shupe, 1980; O'Hara *et al.*, 1982; Maylin *et al.*, 1987). The differential diagnosis includes exposure to heavy metals, ingestion of plants containing oxalates or tannins, mycotoxins, aminoglycosides and chlorinated hydrocarbons.

## Iron

The most commonly affected species is swine especially baby pigs although all other species can be affected. In piglets iron toxicosis results from when too much iron is injected or less often when too much is given orally. Any of the iron complexes and iron salts may be involved. In acute cases there is swelling at the injection site with respiratory distress. The gross lesion at the site includes edema with brownish black discoloration.

Hepatic changes are characterized microscopically by hepatocyte necrosis in the periportal areas. A rare form of less acute nature characterized by hard swelling develops at the injection site. This happens due to massive mobilization of calcium after injection and happens after some days of injection. Calcification may be observed in multiple parts of the body (Patterson and Allen, 1970; Kahn, 2005). Toxicity due to oral preparations leads to gastric ulceration and edema.

Foals given a nutritional preparation which contained an iron compound, ferrous fumarate, induced hepatopathy and histopathological lesions. The lesions are characterized by massive hepatocellular loss with lobular collapse, proliferation of bile ducts and portal tract fibrosis. Hepatic encephalopathy associated with liver damage was also observed in these foals (Divers *et al.*, 1983; Acland *et al.*, 1984). Diagnosis is based on case history, clinical signs and pathological findings.

## Lead

Lead toxicosis is by exposure to lead paint, lead-containing objects like toys foils, etc., plumbing material, tiles and linoleum. Lead toxicosis affects hemoglobin synthesis by inhibiting the enzymes delta-aminolevulinic acid synthetase and ferrochelatase resulting in anemia. Clinical pathological findings suggestive of lead toxicosis are basophilic stippling of erythrocytes and metarubricytosis with minimal polychromasia. Anemia may be microcytic hypochromic to normocytic normochromic (Prescott, 1983). Animals dying of acute lead poisoning have very few microscopic lesions other than mild degenerative neuronal necrosis in the cerebral cortex and mild renal epithelial degeneration.

In chronic cases also the lesions are not outstanding and in few cases the lead source may be found in the GI tract. Ruminants with chronic toxicosis have laminar cortical necrosis of the cerebrum which may appear as flattening and yellowing of the cortical gyri. Histopathological findings include degeneration and necrosis of the neurons in the cerebral laminar cortex with swelling of the cerebral and cerebellar capillary endothelium. Cerebral edema vascular congestion of the meningeal vessels and astrogliosis in the Purkinje and molecular cell layers have also been reported. The changes in the kidney are characterized by degeneration and necrosis of the renal tubular epithelium which may show regeneration. Precipitation of lead sulfide may be seen in the gingiva adjacent to the teeth as a blue discoloration "lead line" but is common in non-human primates and human. In dogs and cats rarely gross lesions are observed and sometimes the lead source may be found in the GI tract with GI hyperemia. Histopathological lesions include edema of the white matter of the brain and spinal cord, myelin degeneration within the cerebellum and cerebrum and spongiosis of the deep cerebral layers. Degenerative changes of the renal epithelial cells and hepatocytes have been reported in chronic lead poisoning in dogs. Intranuclear inclusions may be visible in the renal tubular epithelial cells, but their absence does not rule out lead toxicosis (Jones *et al.*, 1996).

In birds especially waterfowls, lead toxicosis has been reported and the lead shots may be found in the gizzard. The birds are generally emaciated with extensive muscle wasting with atrophy of the breast muscles. Pale streaks may be found in the myocardium which are areas of myocardial necrosis with fibrinoid necrosis of the vessels. Other histopathological lesions in the waterfowl include hepatocellular necrosis, renal tubular necrosis (with or without intranuclear inclusions) edema of the brain, myelin degeneration of the peripheral nerves and necrosis of the gizzard musculature (Locke and Thomas, 1996). Lead can cross the placental barrier and hence cause abortion, resorption and sterility.

Diagnosis is by analytical estimation and history of exposure. Liver and kidney are the preferred samples for analysis of lead. The differential diagnosis in large animals includes primarily rabies, viral encephalitis, polioencephalomalacia, thromboembolic meningoencephalitis, cyanide poisoning, hepatic encephalopathy. In small animals, rabies, distemper, other heavy metal exposure should be ruled out.

## Mercury

Fungicides, preservatives and fixatives are the main sources of mercury toxicosis. Often toxicosis is related to accidental ingestion of obsolete mercury products.

The lesions of mercury toxicosis are distributed in the GI tract, kidney and sometimes in the brain. Gross lesions include gastric ulcers, congestion of the intestinal mucosa, pale swollen kidney and pale liver. Histopathological findings include ulceration in stomach, necrosis of the intestinal mucosa in small intestine and colon, renal tubular degeneration and necrosis especially the proximal tubular epithelium and hepatocyte degeneration. In chronic exposure glomerulonephritis is observed due to antigen–antibody complex deposition. Degeneration of the Purkinje fibers and cardiac muscle fibers is reported in cattle and this may lead to fibros is and mineralization. Mercury compounds can cause fibrinoid degeneration of the media of the leptomeningeal arteries which can lead to cortical necrosis of cerebrum (Jones *et al.*, 1996).

Pathological changes associated with chronic mercury poisoning in cats are mostly confined to the nervous system and include degeneration of neurons and perivascular cuffing in the cerebrocortical gray matter. In the cerebellum the histopathological findings include focal spongiosis of the molecular layer, degeneration and loss

of Purkinje cells and focal atrophy of the granular layer. Other histopathological findings are demyelination in the fiber tracts of dorsal funiculus's in the lateral and ventral corticospinal tracts. Mercury can pass the placental barrier and cerebellar hypoplasia has been reported in kitten born to cats exposed to mercury before and during gestation (Charbonneau *et al.*, 1976; Gruber *et al.*, 1978). Inhalation of mercury vapor induces acute, corrosive bronchitis and interstitial pneumonia. Diagnosis and confirmation can be made by analytical estimation in the kidney. Differential diagnoses include toxicosis by lead, thallium, phenylarsonic feed additive and ethylene glycol toxicosis, encephalitis, polioencephalomalacia, hog cholera (pigs) and erysipelas (pigs).

## Molybdenum

Acute molybdenum toxicosis is mainly due to accidental exposure to oil and gas fields or motor oil. Chronic toxicity is associated with ingestion of green pasture plants grown on soils high in molybdenum. Cattle are highly sensitive for toxicosis. Molybdenum elimination occurs primarily through the renal system, with over 50% of excreted molybdenum found in the urine.

The molybdenum toxicosis induces copper deficiency and hence the symptoms and lesions of copper deficiency will be observed in molybdenum toxicity. This condition has also to be differentiated for a primary copper deficiency. The gross findings of molybdenum toxicity in feedlot cattle include swollen pale friable liver and swollen kidneys. Microscopic findings included hepatocellular degeneration progressing to massive periacinar necrosis and hemorrhage. Kidney showed hydropic degeneration with marked necrosis of the proximal and distal tubular epithelial cells. Experimental studies in sheep showed that molybdenum can induce hepatocellular damage probably due to a direct toxic effect of molybdenum on hepatocytes (Auza *et al.*, 1989; Jones *et al.*, 1996; Swan *et al.*, 1998). Diagnosis is by case history, clinical signs, pathological findings and analytical estimation in liver and kidney. Liver may show low concentration of copper.

## Selenium

Naturally occurring selenium toxicosis is reported mainly in pigs and birds. Excessive supplementation of selenium in rations and chronic exposure to environmental sources are the potential cause of selenium toxicosis. In birds selenium toxicosis is an important problem in selenium-contaminated western wetlands.

There are numerous reports of selenium toxicosis in pigs which is due to excess supplementation in the feed. Gross pathological lesions are mostly confined to the spinal cord and consist of focal, bilateral, depressed areas in the ventral horns of the cervical/lumbar/sacral segments of spinal cord. In a natural outbreak the histopathological lesions consisted of endothelial proliferation and glial cell reaction in the areas with gross lesions and also in some brain stem motor nuclei. Focal symmetrical poliomyelomalacia confined to the cervical and lumbar/sacral spinal cord and few brain stem motor nuclei were reproduced experimentally by feeding high levels of sodium selenite. Chronic lesions, such as roughness of the hair coat, coronitis and sloughing of the hooves, have been reported in cases which survive for longer periods (Harrison *et al.*, 1983; Wilson *et al.*, 1983; Summers *et al.*, 1995a).

In experimental studies conducted in mallard ducks exposed to different levels of selenium the gross lesions observed were bilaterally symmetrical alopecia of the scalp and dorsal cervical midline, broken or lost digital nails and necrosis of the tip of the beak (maxillary nail). Microscopic findings include single cell to full thickness necrosis of keratinocytes and multifocal parakeratosis in stratum corneum of digital and maxillary nails. Areas of alopecia showed mild necrosis. Some birds showed histopathological changes in the liver which consisted of mild-to-moderate generalized hepatopathy with single-cell necrosis, karyomegaly of hepatocytes, hyperplastic bile duct epithelium and/or iron accumulation in Kupffer cells (O'Toole and Raisbeck, 1997).

In acute poisoning heart is the target organ. The prominent pathological findings in experimental studies in sheep were degenerative and necrotic changes in heart (Blodgett and Bevill, 1987). The chronic form of selenium toxicosis is known as the alkali disease and is related to consumption of seleniferous plants over a period of weeks or months. This syndrome is observed in horses and other ruminants and is characterized by shedding of hair in the mane and tail, deep encircling grooves parallel to the coronary band and sometimes results in detachment of hoof from the sensitive laminae. Erosions of the long bones especially distal end of tibia and proximal end of metatarsus are also reported (Jones *et al.*, 1996). Diagnosis is by correlating clinical signs, liver and blood selenium levels and pathological findings.

## Sodium

Sodium toxicosis can occur directly by excess sodium in feed or water or indirectly by deprivation of water. Indirect toxicosis is the most common toxicity reported in ruminants and pigs which are highly sensitive to sodium toxicosis. Water deprivation caused by failure of automatic water system, overcrowding, unpalatable water or reduced temperature leading to freezing of water are the main causes for indirect toxicosis.

The prominent clinical pathological finding is hypernatremia. The gross pathological findings in sodium

intoxication are congestion and edema of the brain and meninges, with petechiae and ecchymosis within the gray and white matter of the cerebrum, cerebellum, brain stem and congestion of the abomasal mucosa. Histopathological changes in ruminants include varying degrees of vasculitis and edema in the meninges, gray and white matter with neuronal degeneration and necrosis. The vascular changes in the brain include endothelial cell swelling, fibrinoid necrosis and perivascular cuffing by neutrophils and eosinophils. Hemorrhages may be present in the brain. In swine the microscopic lesion includes cerebrocortical neuronal degeneration and necrosis, eosinophilic meningoencephalitis with perivascular cuffing by eosinophils. These eosinophils are later replaced by other mononuclear cells. Advanced cases show cavitation with malacia. Inflammation of the GI tract, hemorrhage and ulcers are also reported. Degeneration and edema of the skeletal muscles with anasarca, pulmonary edema and hydropericardium can occur in swine and ruminants (Trueman and Clague, 1978; Scarratt *et al*., 1985; Osweiler *et al*., 1995; Summers *et al*., 1995b). Pulmonary hemorrhage and edema has been reported in a dog that died of hypernatremia. The microscopic lesions in dog include diffuse vacuolation of the white matter of the brain with necrosis of the hepatocytes (Khanna *et al*., 1997).

Diagnosis is by correlating case history, clinical signs, pathological findings and analytical estimation of sodium in ocular fluid. Differential diagnoses should include thiamine deficiency, lead toxicity and sulfur toxicity.

## Sulfur

The important source for sulfur toxicity is high sulfur diet especially sulfur in water. The condition is seen more in summer when there is increased consumption of water with sulfur. Cattle is one of the common species in which toxicosis is reported. The studies conducted in cattle suggest that the level of thiamine is within normal range. Gross lesions include soft and flattened cerebral gyri. Hemorrhage may be seen in acute cases. Histopathological lesions include degeneration and necrosis of the neurons in the cerebral cortex with cortical glial and capillary endothelial hyperplasia. In acute cases there may be moderate to massive hemorrhage present in brain stem. The studies conducted in cattle suggest that the level of thiamine is within normal range (McAllister *et al*., 1993; Gould, 2000). Polioencephalomalacia has also been reported in cows associated with excess barley malt sprout intake because of its higher sulfur content (Kul *et al*., 2006).

Diagnosis is by correlating retrospective dietary analysis with pathological findings. Differential diagnoses should include thiamine deficiency, lead toxicity, water-deprivation/sodium ion toxicity, hypovitaminosis A and *Hemophilus meningoencephalitis*.

## Zinc

Zinc toxicosis has been reported in dogs as a result of ingestion of zinc-containing hardwares, pennies which contain zinc, drinking water, acidic food material from galvanized tanks and containers. Pancreas is the target organ in many species of animals which is exposed to toxic levels of zinc. Severe intravascular hemolysis is the most prominent clinical pathological finding associated with zinc toxicosis in dogs. The gross observations in dogs and cats exposed to zinc were splenomegaly, icterus, enlarged diffused red kidney with hemorrhage. Hepatomegaly may be observed. Liver showed vacuolar degeneration and necrosis of the hepatocytes. Pancreatic histopathological lesions include necrosis of the acinar cells, necrosis of the duct epithelium and interstitial fibrosis with mononuclear cell infiltrate. In pigs pancreatic epithelial cell necrosis, diffuse acinar atrophy and marked interstitial fibrosis are reported due to zinc toxicosis (Luttgen *et al*., 1990; Gabrielson *et al*., 1996).

In experimental studies conducted in sheep exposed to zinc, pancreatic lesions observed at day 7 included necrosis of the pancreatic duct epithelium, periductular inflammation and interlobular fat necrosis. These histopathological lesions were followed by edema, lobular cystic change, atrophy, fibrosis and a ductular hyperplasia. Other prominent lesions observed were necrosis of the hepatocytes and degeneration of the tubular lining epithelium in the kidney (Smith and Embling, 1993).

The histopathological changes observed in experimental studies in veal calves include atrophy and necrosis of pancreatic acinar tissue and multifocal fibrosis of pancreatic acini. The pathological changes in kidney included multifocal renal cortical fibrosis with necrosis in convoluted tubules and loops of Henle and intratubular mineralization. The histopathological observation in the liver was midzonal mineralization. Fibrosis of the adrenal zona glomerulosa was also observed in some animals (Graham *et al*., 1988).

Zinc toxicosis has been reported in foals also. The pathological changes observed in foals born near a zinc smelter were lameness-associated joint swellings that were attributable to severe generalized osteochondrosis. Separation and loss of hyaline cartilage were observed in the pastern and fetlock joints (Gunson *et al*., 1982). Diagnosis is by correlating case history, pathological findings and analytical estimation in liver, kidney and pancreas.

# MYCOTOXINS

Mycotoxins are fungal metabolites which cause pathological, physiological and or biochemical alterations in several species of animals, plants and microbes. The important

mycotoxins which cause natural toxicosis in pets are aflatoxins, penitrem A and roquefortine. Mycotoxicosis can occur as acute, subacute, chronic forms or subclinical conditions. Only mycotoxins which induce gross or microscopic lesions in domestic animals are described in detail in this section.

## Aflatoxins

Aflatoxins are produced by *Aspergillus flavus* and *A. parasiticus*. Aflatoxin B1 is the most toxic and accounts for most of the natural outbreaks. It also has carcinogenic properties. Among animals young turkeys and duckling are highly susceptible to aflatoxins. Young animals and males are more susceptible to the toxin. Natural disease outbreaks are commonly reported in cows, dogs and pigs but rarely in goat and sheep. Horses are rarely affected (Angsubhakorn *et al.*, 1981). Both acute (especially in dogs) and chronic aflatoxicoses are reported and chronic aflatoxicosis is the most common form reported. Prominent clinical pathological finding is elevated liver enzyme levels.

Acute aflatoxicosis due to consumption of feed material with high quantities lead to severe hepatic damage. Clinical pathological changes primarily show elevation of hepatic enzymes especially $\gamma$-glutamyl transpeptidase (transferase) (GGT). Gross pathological findings include citrus and yellowish discoloration of the liver. The common histopathological findings are necrosis and degeneration of the hepatocytes. Necrosis is primarily located in the periportal areas of liver in turkeys, chicken, ducklings, adult rat and cat. In rabbit the hepatic necrosis is observed in midzonal areas. Necrosis is observed in the centrilobular areas in pigs, cattle and dogs. Diffuse necrosis is observed in neonatal rats and trout. Edema and hemorrhage in the gall bladder are found in many cases of canine and porcine aflatoxicosis. Acute aflatoxicosis has also been reported to cause abortion in cows although no specific histopathological lesions were observed in the fetus (Liggett *et al.*, 1986; Jones *et al.*, 1996).

Chronic aflatoxicosis is the most common form reported. It may take weeks or months to develop the disease. Recently aflatoxicosis due to a commercial canine pet food has received national and global attention. The contamination of dog food by aflatoxin resulted in high mortality among dogs. The dogs affected were mostly from the northeastern states of United States and more than 25 countries throughout the world. Toxicosis resulted from contamination of corn used in formulating the feed. The primary lesions in dogs were mostly confined to the liver. The most important feature was hepatocytes degeneration and necrosis of varying degrees with proliferation of bile ductules at the periphery of the hepatic lobule within days of exposure (Figure 90.3). There was proliferation of fibrous connective tissue which leads to periportal fibrosis. There

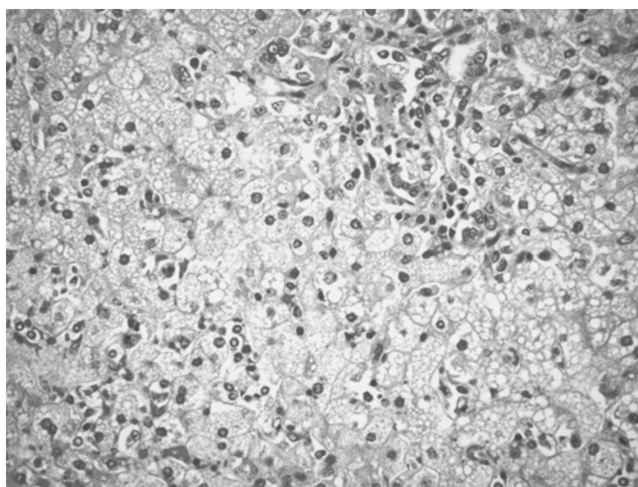

**FIGURE 90.3** Aflatoxicosis, liver, canine, hepatocellular vacuolation with biliary hyperplasia, H&E stain, 40×. This figure is reproduced in color in the color plate section. (Courtesy Dr. S Newman, College of Veterinary Medicine, University of Tennessee.)

was nodular regeneration of hepatocytes with marked variation in nuclear size and some hepatocytes showed megalocytosis (JAVMA, 2006; Newman *et al.*, 2006). Aflatoxin B1 has been reported to be teratogenic in laboratory animals. Diagnosis is by chemical analysis of feed material, correlating case history and pathological findings.

## Fumonisin

Fumonisins are mycotoxins produced by the fungus *Fusarium moniliforme* primarily in corn. The important toxin is fumonisin B1. The susceptible species are horses, ponies, donkeys, swine and rabbits. Sphingolipids which have important roles in membrane and lipoprotein structure and in cell regulation are formed by the turnover of complex sphingolipids and as intermediates of sphingolipid biosynthesis. Fumonisins inhibit ceramide synthase thereby blocking the biosynthesis of complex sphingolipids resulting in accumulation of sphinganine and sometimes sphingosine which act as cytotoxic agents or lead to cell proliferation affecting a wide variety of cellular systems. Alteration in sphingolipid concentrations and functions, especially in the vasculature, also is believed to contribute to the major signs and lesions of fumonisin toxicosis. In swine pulmonary edema induced by fumonisin is suggested to result from acute left-sided heart failure mediated by altered sphingolipid biosynthesis (Wang *et al.*, 1992; Merrill *et al.*, 2001).

In horse, brain and liver are the organs affected. The most common lesion is the liquefactive necrosis in the white matter which may occur as a result of low consumption of the toxin over a long time and the hepatitis which leads to secondary hepatoencephalopathy. Lesions of both types may be present within the same animal.

The clinical pathological findings in horses associated with liver lesion include elevated ALP, ALT, sorbitol dehydrogenase (SDH), GGT and total bilirubin and bile acids. The clinical pathological changes associated with lesions in the brain are high protein and leukocytic pleocytosis in the cerebrospinal fluid. The clinical pathological alterations in porcine toxicosis include increased ALT, ALP, GGT, total bilirubin, bile acids and cholesterol indicative of a hepatic damage.

Gross pathologic findings include liquefactive necrosis in the cerebral white matter (can be unilateral or bilateral) appearing as a cavity with hemorrhagic foci of various sizes in the brainstem. In some animals the liquefaction may extend into the cortex. Adjacent to these areas, the brain may have a greenish-yellow appearance. The gross lesions in the liver include swollen liver, yellowish brown discoloration and irregular pale foci or nodules. In pigs the gross lesions include heavy lung with widened interlobular septa, fluid in the airways and thoracic cavity. Histological findings in the horses with brain involvement are characteristic liquefactive necrosis in the cerebral white matter with hemorrhage predominantly around the cerebral vasculature. The microscopic observations in the liver lesion include centrilobular necrosis and fibrosis, bile stasis, bile duct proliferation, fatty degeneration of hepatocytes and portal fibrosis (Ross *et al*., 1993).

In pigs the histological observations include dilated lymphatics with fluid in the interlobar septa and subpleural areas and edema around vessels and bronchi. The liver lesions include multifocal areas of hepatocyte apoptosis, necrosis, hepatic cord disorganization and hepatocyte proliferation. In long-time experimental exposure studies fibrosis and hyperplasia of the hepatocytes cells with formation of nodules, right ventricular hypertrophy and esophageal plaques (hyperkeratosis and parakeratosis, formation of papillary down growths of the stratum basale of the distal esophageal mucosa) were the pathological observations. In experimental studies of prolonged exposure to low doses in pigs, the pathological observations consisted of proliferation of the connective tissue fibers, around the lymphatic vessels in subpleural and interlobular septa of the lungs, extending to the peribronchial and peribronchiolar areas. Although there was no clinical signs and significant performance impairment in pigs, it resulted in irreversible chronic changes (Osweiler *et al*., 1992; Casteel *et al*., 1993; Colvin *et al*., 1993; Haschek *et al*., 2001).

In experimental studies conducted in calves the microscopic findings were observed in liver and kidney and consisted of proliferation of hepatocytes and bile ductular cells with varying degrees of hepatocyte apoptosis, vacuole formation, karyomegaly, proliferation of proximal renal tubular cells and dilation of proximal renal tubules filled with cellular debris and proteins (Mathur *et al*., 2001).

In broilers the pathological findings include multifocal hepatic necrosis, thymic cortical atrophy, biliary hyperplasia and rickets (Ledoux *et al*., 1992). Experimental administration of fumonisin induces nephrotoxicity in rats, rabbits and sheep. Diagnosis is by history of ingestion, clinical signs, pathological observations and evaluation of the toxin in feed and corn samples by thin layer chromatography (TLC), high-performance liquid chromatography (HPLC) and enzyme-linked immunoabsorbent assay (ELISA).

## Citrinin and ochratoxin

Citrinin is a benzopyran metabolite produced by the toxic strains of *Penicillium* spp. and *Aspergillus* spp. Some hepatotoxic effects have been reported for citrinin but the lethal effects are largely due to the nephrotoxic effects. The specific mechanism of action in kidney is not known. Primary effect is on the kidney and leads to acute tubular necrosis. In pigs, rat and rabbit the proximal segments are affected. Experimental studies show that citrinin induces renal damage in turkeys and ducklings along with hepatic degeneration and lymphoid necrosis.

Ochratoxin is a mycotoxin produced by the toxic strains of *Penicillium* and *Aspergillus* spp. mostly related to improper storage conditions of grain, coffee, etc. Three different ochratoxins have been isolated and characterized (ochratoxins A, B and C). Of these ochratoxin A is the most commonly detected and most toxic among the three. The subchronic and chronic effects of this toxin are more of concern compared to the acute toxicity. Pigs are the most common species to be affected by ochratoxicosis. Clinical pathological changes include increased BUN values, reduced creatinine clearance and urine specific gravity. The gross pathological changes observed in experimental studies of pigs include enteritis, pale tan discoloration of liver, edema and hyperemia of the mesenteric and other lymph nodes. The microscopic findings are mostly confined to the kidney and GI tract which include necrosis of the renal tubular epithelium most frequently affecting the proximal convoluted tubules, dilatation of the tubules, necrotizing enteritis in multiple locations, fatty degeneration of liver and focal necrosis of lymphocytes in the germinal center of the lymph nodes (Szczech *et al*., 1973a; Zomborszky-Kovacs *et al*., 2002).

The gross pathological changes observed in experimental studies of dogs include severe hemorrhagic enteritis of the large intestine, tonsillitis with enlargement and hyperemia of the mesenteric and other lymph nodes. The microscopic findings are similar to that observed in the porcine studies and included necrosis and desquamation predominantly in the proximal convoluted tubules, eosinophilic granular casts in proximal and distal convoluted tubules necrotizing enteritis in multiple locations, fatty degeneration of liver and focal necrosis of lymphocytes in, germinal center of lymph nodes, spleen, tonsil, thymus, and lymphoid nodules in small and large intestine

and nictitating membrane and mild-to-moderate centrilobular necrosis and fatty change of liver (Szczech *et al.*, 1973b; Szczech, 1975).

Ochratoxicosis has been reported commonly in avian species. Experimental exposure of ochratoxin in chicken and turkey poults resulted in skeletal osteopenia with disturbed endochondral and intramembranous bone formation. Experimental studies in young broilers with ochratoxin A show renal edema and tubular necrosis (Wyatt *et al.*, 1975; Duff *et al.*, 1987). Chronic exposure of ochratoxin A is considered as an etiology in Balkan endemic nephropathy and urothelial tumors in human beings.

## Zearalenone

*Fusarium roseum* invade the grain and produce an estrogenic toxin known as zearalenone. Toxicity is commonly reported in swine and is mostly subacute. Anestrum or nymphomania is observed in swine based on the estrous cycle when exposed. Pseudopregnancy with reduced litter size due to fetal resorption or implantation failure may be observed. Gross findings include swelling of vulva, mammary gland hypertrophy, vaginal prolapse, atrophy of the ovaries and uterine hypertrophy. Microscopic observations include edema and hyperplasia of the uterus and proliferation of ducts in the mammary glands. Histopathological findings in experimental studies in pigs include squamous metaplasia in the uterus, uterine duct, cervix, vagina and mammary glands.

Abortions were reported in experimental studies but whether it occurs in field condition is not definitively proved. Abortions may occur at any stage of gestation but usually occur about 3 weeks following the initial consumption of the feed. No specific gross or microscopic findings are observed in the aborted fetuses. Diagnosis is by correlating history of exposure with clinical signs associated with hyperestrogenism and chemical analysis of the feed material (Kurtz *et al.*, 1969; Chang *et al.*, 1979; Stuart and Oehme, 1982). Differential diagnosis should include normal estrus and external genital injury.

## Ergot toxicosis

Commonly called the ergot, the *Claviceps* spp. mainly *Claviceps purpurea* and *Claviceps paspali* infect the developing grass and grain. The main action of the ergot alkaloid which is the important cause of toxicity is stimulation of the smooth muscles contraction, vasoconstriction and adrenergic blockage along with stimulation or inhibition of parts of the central nervous system (CNS).

The common lesion exhibited by ergotism is gangrene in the distal extremity. Grossly they appear as focal cracks on the hoofs and lead to multifocal areas of detachment of the hoof wall. Death has been reported in cattle related to ergotism by GI ulceration. Although inconclusive, cattle and swine may abort as a result of consumption of ergot alkaloid-containing feed material especially during the third trimester of pregnancy. Reproductive problems and neonatal mortality have been reported in horses by ergotism. Diagnosis is by examining the feed or grass for the presence of sclerotia or ergot bodies and analytical testing of the ergot alkaloids in feed material (Riet-Correa *et al.*, 1988; Coppock *et al.*, 1989; Cheeke, 1988). Differential diagnosis includes fescue toxicosis and hypothermia.

## Fescue toxicosis

Fescue toxicosis is caused by the consumption of fescue infested by the fungal endophyte *Neotyphodium coenophialum*. Ergoline and ergopeptine alkaloids are the major toxic principles responsible for this condition. Clinically the condition has four main manifestations which include the fescue foot, summer slump due to altered thermoregulatory mechanisms and hormonal imbalance resulting in reduced production and reproductive problems, fat necrosis in ruminants and equine reproductive problems.

The important clinical pathological finding is hypoprolactinemia in ruminants and hypoprolactinemia and reduced relaxin levels in late gestational mares (Ryan *et al.*, 2001). The gross observations of fescue foot include swelling of the pastern with necrosis and sloughing. Microscopic findings include perivascular edema and hemorrhage, thickening of the blood vessels and fibrosis. In advanced cases necrosis of the bone and septic arthritis are reported. Fat necrosis, is observed grossly as yellowish or chalky white nodules in the mesentery around the intestines. Microscopic finding includes necrosis of the adipocytes with occasional infiltration of macrophages (Strickland *et al.*, 1993; Blodgett, 2001).

In equine prolonged gestation is observed. Dystocia and retention of fetal membranes are commonly reported in pregnant mares. Those foals which are born to mares exposed to fescue toxicosis have high rate of perinatal mortality. The placental membranes show diffuse edema with increased connective tissue. Enlarged thyroid follicles are observed in foals from mares with fescue toxicosis and microscopically they are distended with colloid (Loch *et al.*, 1987; Putnam *et al.*, 1991).

## Sporodesmin

These are mycotoxins found on the spores of fungus *Pythomycus charatrum*. Toxicosis is reported commonly in sheep. The organs which are affected are skin and liver. The gross findings include swollen mottled liver with

icterus, enlarged gall bladder, edema and erythema in the areas exposed to sunlight particularly the ear, eyelid, face and lips. Histological observations in the liver include hepatocyte vacuolations, fibrosis, bile duct hyperplasia, necrotizing pericholangitis and bile stasis. The microscopic changes in the skin include ballooning degeneration of epidermal cells with edema in the epidermal–dermal junction, vesicles and pustule formation with thrombi in the vessels and perivascular inflammation (Hansen *et al*., 1994; Pinto *et al*., 2005).

## Other mycotoxins

Oosporein which is produced primarily by *Oospora colorans* and *Caetomium trilaterale* is observed in feed stuff, cereals and peanuts. It has been reported to be toxic in poultry resulting in nephrotoxicity, visceral and articular gout. The pathological observations in oosporein toxicity include necrosis of the tubular epithelial cells in the proximal tubules with basophilic casts, hyaline casts in the distal tubules with fibrosis and interstitial pyogranulomatous inflammation, urate deposits in various tissues and proventricular enlargement with mucosal necrosis (Pegram and Wyatt, 1981; Brown *et al*., 1987).

Lupinosis is a disease caused by the consumption of lupine plants infected by the fungus *Diaporthe toxica* which produce the toxin phomposin. This condition is totally different from lupine poisoning which is caused by the ingestion of lupines which contain the quinolizidine alkaloids. The disease is common in Australia where lupines are grown as a fodder crop and is a primary disease of sheep although other ruminants and equine can be affected. The primary target organ is the liver. Clinical pathological observation characterized by elevated liver enzymes indicative of hepatic injury is a prominent finding. The disease can be acute, subacute or chronic. In acute and subacute cases the gross findings include icterus and swelling of the liver with bright yellow discoloration and in chronic cases the liver is small and copper tan in color. The microscopic observations include apoptosis of the hepatocytes, karyomegaly and megalocytosis of hepatocytes, fatty degeneration, fibrosis and proliferation of Kupffer cell and bile ducts. Hepatoencephalopathy and myopathy of the skeletal muscles are also reported to be associated with lupinosis (Soler Rodriguez *et al*., 1991; Allen *et al*., 1992; Allen and Randell, 1993).

## Slaframine

Slaframine, also called the slobber factor, is an indolizidine alkaloid synthesized from the fungus *Rhizoctonia leguminicola* which causes the black patch disease in clover. This acts as a parasympathomimetic agent resulting in salivation, lacrimation, urination and defecation. Salivation is one of the first and prominent signs to develop and it continues as long as the toxin persists. Slaframine toxicosis is observed in several domestic animal species including horses, sheep, swine, cattle, cat and dogs. Pathological changes are not recorded in naturally occurring cases as mortality is rare. Diagnosis is by correlating the clinical signs and history of exposure to plants with black patch disease (Crump, 1973).

## Stachybotryotoxicosis

This is a mycotoxin produced by *Stachybotrys* spp. and affects several species of domestic animals. The toxic effect of this mycotoxin was first reported in horses from Eastern Europe. In horses the toxin primarily results in erosions and necrosis in the GI tract characterized by ulcerations in mouth, esophagus and stomach with hemorrhages in the intestinal tract, muscles and lymphoid necrosis. In a suspected field outbreak in sheep with secondary *Pasteruella* spp. septicemia the pathological findings included hemorrhages in the subcutaneous tissue, diaphragm, mediastinum, pericardium, membranes of the peritoneal and pleural cavity. The important histological observations were necrosis and atrophy of lymphoid organs with aplastic anemia (Schneider *et al*., 1979).

Trichothecens are a group of mycotoxins produced by different strains of *Fusarium* spp. of fungi. The toxins in this group which induce toxicity in domestic animals include T-2 toxin, DAS (diacetoxyscirpenol) vomitoxin (deoxynivalenol) and satratoxin (S*tachybotrys*). Natural outbreaks of these toxins causing toxicity are rare. The pathogenesis is related to their ability to inhibit protein synthesis producing a radiomimetic effect. The pathological findings of these toxins are mostly based on experimental studies. T-2 toxin and DAS are highly toxic causing necrosis of skin and in experimental studies these toxins cause necrosis of the lymphoid and myeloid tissues, tissues of the GI tract. Experimental studies in pigs exposed to T-2 toxin had gross lesions consisting of congestion and hemorrhage in lymph nodes, pancreas, GI mucosa, subendocardium, adrenal gland, and meninges with edema in gall bladder, lymph node and pancreas. Microscopic observations include hemorrhage in the organs with gross findings along with degeneration and necrosis of the lymphoid tissues, surface and crypt epithelium of the GI mucosa, mild multifocal necrosis of pancreatic acinar cells, myocytes of myocardium, bone marrow cells, cortical cells of adrenal, epithelial cells lining the tubules in the renal medulla and mild interstitial pneumonia. Vomitoxin ingestion results in vomiting especially in swine although other species are also susceptible (Smalley, 1973; Pang *et al*., 1986, 1987).

## Cyclopiazonic acid

Cyclopiaszonic acid was initially isolated from *Penicillium cyclopium* and later from several *Asperpgillus* spp. and *Penicillium* spp. Peanut is naturally contaminated with this mycotoxin. Chicken exposed to this mycotoxin show ulcerative changes in proventriculus with necrotic changes in liver and spleen. The prominent gross lesions observed in pigs exposed to cyclopiazonic acid included pale liver, gastric ulcers, mucosal hyperemia and hemorrhage throughout the small and large intestines. The histopathological observations included lesions in the GI tract, liver and kidney which were characterized by necrotizing gastroenteritis, hepatocellular necrosis, lobular fatty change in the hepatocytes of the peripheral lobules, necrosis of the renal tubular epithelium with focal suppurative tubulointerstitial nephritis (Lomax *et al.*, 1984).

## Tremorgenic mycotoxins

Tremorgenic mycotoxins (secondary fungal metabolites) contain an indole moiety, which produce tremors or seizures in animals consuming toxic amounts of contaminated foodstuffs. There are at least five groups of tremorgenic mycotoxins: the penitrem group, the paspalitrem group, the fumitremorgins group, the verruculogen group and the tryptoquivaline group. Penitrem and roquefortine are the most important tremorgenic mycotoxins associated with natural outbreaks in small animals. There are no specific microscopic findings in the nervous tissue or muscle tissue. Diagnosis is by analytical examination of the gastric contents, correlating the clinical nervous signs and case history of exposure. Differential diagnosis in small animals includes other causes of tremors like strychnine, organophosphate and carbamate insecticides pyrethroids, mushroom exposure, etc.

In large animals, the tremorgenic mycotoxins include lolitrem B in perennial rye grass, paspalitrems in Dallis grass and Bahai grass and unknown mycotoxin in Bermuda grass tremors. No specific lesions are seen at necropsy or on histopathology, secondary trauma is possible. Diagnosis is based on case history and clinical signs (McLeay *et al.*, 1999).

# PESTICIDES

Many pesticides, including rodenticides, act on the CNS and convulsion may be the only clinical sign observed. The common toxicants which induce acute convulsions in dogs are strychnine, insecticides, metaldehyde, zinc phosphide, methyl xanthines, drugs of abuse, bromethalin and the tremorgenic mycotoxins (roquefortine and penitrem A). Most of these compounds have very less or no specific pathological findings and hence gas chromatography–mass spectrometric analysis of the suspected bait material, stomach contents or vomitus should be conducted for conclusive diagnosis (Braselton and Johnson, 2003).

Insecticides like amitraz, anticholinesterase insecticides like organophosphates and carbamates, metaldehyde, organochlorine insecticides, pyrethrins and pyrethroids do not induce any specific gross or histopathological changes and hence they are not discussed in this section. Similarly avicides like 3 chloro-*p*-toluidine hydrochloride and 4-aminopyridine also do not induce any specific histopathological lesions.

## Anticoagulant rodenticides

Anticoagulant rodenticides are used extensively and accidental ingestion by domestic animals is very common. The most common first-generation coumarin anticoagulant is warfarin and the common second-generation anticoagulants include brodifacoum, bromadiolone and difenacoum.

All coumarins block the vitamin K 2,3-epoxide reductase enzyme. This prevents cycling between the inactive oxidized and the active reduced form of vitamin K in the liver. Reduced vitamin K is essential for the carboxylation and activation of the clotting factors II, VII, IX and X which leads to a coagulopathy due to depletion of the clotting factors.

Anticoagulant toxicity in small animals is diagnosed by the clinical pathological evaluation and includes an altered coagulation panel characterized by prolonged activated clotting time (ACT), one-stage prothrombin time (PT) and activated partial thromboplastin time (PTT). The common gross findings include hemorrhage in the abdominal cavity, thoracic cavity, subcutaneous tissue, subdural space, articular space, heart and lung and blood in GI tract. Histopathological findings include hemorrhages in the serosa of the intestine, meninges and GI tract mucosa. Coumarin-derived anticoagulants can cross the placenta and affect pups which can lead to death of the pups even without affecting the dam. Brodifacoum, a second-generation coumarin anticoagulant, was detected in the liver of pups which died as a result of *in utero* exposure (dam not affected) indicating that fetus is more susceptible to the toxicity than the dam. Differential diagnosis includes disseminated intravascular coagulation, hereditary clotting factor problems, liver diseases and autoimmune thrombocytopenia. Chemical analysis of the liver correlated case history, clinical pathological and necropsy findings will help to confirm diagnosis (DuVall *et al.*, 1989; Chua and Friedenberg, 1998; Sheafor and Couto, 1999; Munday and Thompson, 2003).

## Alpha-naphthyl thiourea

It is primarily used as a rodenticide and baits coating this are readily accepted by animals. The common domestic animals affected by the toxicity are cats, dogs and pigs. The toxicity is attributed to an increase in the permeability of capillaries which results in marked edema of the lungs. The common gross findings include hydrothorax and severe edema of the lungs. GI irritation can lead to congestion in the GI tract. Histopathological finding is predominantly diffused pulmonary edema. Diagnosis is by correlating the case history and pathological findings (Rutili *et al.*, 1982; Michel *et al.*, 1983).

## Bromethalin

Bromethalin is a non-anticoagulant rodenticide developed and released to combat the worldwide problem of rodent resistance to warfarin-like anticoagulant rodenticides. The mode of action is thought to be by uncoupling mitochondrial oxidative phosphorylation in the CNS and hence this compound acts as a neurotoxin in domestic animals. The common domestic animals affected are dogs and cats and it causes acute or chronic signs. Diagnosis is primarily by history of exposure, chemical detection and clinical signs (Dorman *et al.*, 1992; Dunayer, 2003).

The common gross lesions observed in experimental studies conducted in dogs include mild cerebral edema and pulmonary congestion. Microscopic findings included diffuse white matter spongiosis, mild microgliosis, optic nerve vacuolization, mild thickening of Bowman's capsule and occasional splenic megakaryocytes. Ultrastructural changes include occasional swollen axons, intramyelinic vacuolization and myelin splitting at the intraperiod line in the mid brainstem. Microscopic observations of experimental studies conducted in cats were similar to that of dogs and include edema in the white matter of brain with hypertrophied fibrous astrocytes and oligodendrocytes in the white matter of the cerebrum, cerebellum, brain stem, spinal cord and optic nerve. Bromethalin can be detected by gas chromatography in liver, kidney, fat and brain tissues. Diagnosis is primarily by history of exposure, chemical detection and clinical signs (Dorman *et al.*, 1990, 1992).

## Paraquat and diquat

Paraquat is one of the two widely used bipyridyl broad-spectrum herbicides (the other being diquat). The most common route of intoxication is ingestion. Among domestic animals toxic exposure has been reported in cattle, sheep, horses, pigs, poultry and dogs.

Type I pneumocytes are the target cells in acute and chronic poisoning. Paraquat gets actively concentrated in these cells, and causes an increase in both the consumption of oxygen and oxidation of NADPH via NADPH–cytochrome P450 reductase. The resultant reactive oxygen species generated are thought to play a key role in the acute lung injury.

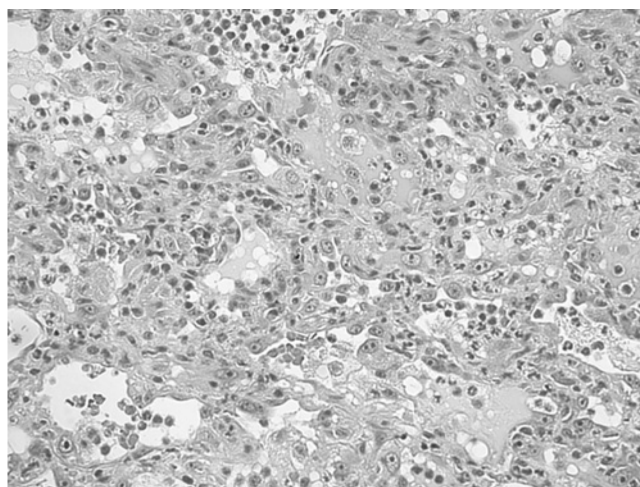

**FIGURE 90.4** Paraquat poisoning, lung, canine, diffuse interstitial pneumonia with type II pneumocyte hyperplasia, H&E stain, 40×. This figure is reproduced in color in the color plate section. (Courtesy Dr. ME Hines, College of Veterinary Medicine, University of Georgia.)

The common gross findings include congestion and hemorrhage of lungs with atlectasis and some areas may have bullous emphysema. In chronic cases the lungs may be shrunken with hemorrhage and edema. The histopathological findings include diffuse interstitial pneumonia with hemorrhage, hyaline membrane formation and type I pneumocyte loss in acute cases. In chronic cases microscopic observations include type II pneumocyte hyperplasia, intralveolar and interstitial fibrosis and emphysema (Figure 90.4). The lung fibrosis develops as soon as 5–10 days after exposure to paraquat. Kidney and liver may also be affected and the histopathological change includes renal tubular epithelial degeneration and hepatocyte degeneration mainly in the centrilobular areas. Diquat toxicity results in intracerebral hemorrhage and acute renal failure. Pulmonary fibrosis is not a common finding with diquat poisoning but studies in rat have shown that diquat may cause alveolar damage by the same mechanism as paraquat, although it is not actively taken up by the lung (Manabe and Ogata, 1987; Nagata *et al.*, 1992; Dungworth, 1993; Ali *et al.*, 1996). Diagnosis is by correlating history of exposure with pathological findings.

## Polychlorinated and polybrominated biphenyls

Polychlorinated biphenyls (PCBs) and polybrominated biphenyls (PBBs) are a class of organic compounds and are known to be causing classic skin lesions in human "chloracne". Isolated cases of poisoning have been reported in

animals and one of the most important cases was the contamination of cattle feed with PBB in Michigan. The only gross pathological change observed was hemorrhage in the serosa of duodenum, ileum and large intestine with hyperemia of the GI mucosa. Histopathological lesions include hemorrhage in the renal interstitial tissue, moderate fatty change without inflammation hemorrhage in the muscle layers and inflammation of the intestinal mucosa (Robens and Anthony, 1980).

Hexachlorophene is a polychlorinated bisphenol compound which has been reported to produce toxicosis in dogs and cats. Toxicosis is by exposure to preparations containing hexachlorophene like soaps and detergents. Prominent neurological signs observed in a litter of pups exposed to hexachlorophene consisted of muscle tremors, ataxia and muscle weakness. No specific gross findings are usually observed. The histopathological finding is characterized by diffuse vacuolation of the white matter and ultrastructurally observation is intramyelinic vacuolation. In another case study of toxicosis in cats the histopathological findings observed were proliferation of astrocytes and microglial cells in the cerebral and cerebellar white matter and corticospinal tracts with vacuolations (Thompson *et al.*, 1987; Poppenga *et al.*, 1990). Diagnosis is by analytical estimation of hexachlorophene in liver and kidney tissue and correlating clinical and pathological findings.

## Cholecalciferol/vitamin $D_3$

Vitamin $D_3$ toxicity in veterinary medicine is by overdosage of vitamin supplements or exposure to rodenticide. Dogs and cats are the common species affected but any species can be affected. The common clinical pathological finding is rapid increase in plasma phosphorus in acute cases and followed by an increase in plasma calcium levels. Common gross findings include hemorrhage in the GI tract and lungs. Histological findings include mineralization in multiple organs including the kidneys, lungs, myocardium, stomach and vessels. In kidneys mineralization of arteries as well as mineral deposition in the tubular basement membranes are observed. Diagnosis is by chemical evaluation of serum, plasma, bile, urine and kidney for PTH/25-hydroxycholecalceferol and correlating pathological findings (Gunther *et al.*, 1988; Fooshee and Forrester, 1990; Talcott *et al.*, 1991). Differential diagnosis includes ethylene glycol toxicity, hypercalcemia of malignancy, chronic renal failure and other causes of hypercalcemia.

## Zinc phosphide and aluminum phosphide

Toxicosis can happen by animals feeding directly on bait or by eating tissues of zinc phosphide-poisoned animals. All domestic animals can be affected and reports are common in dogs. Zinc phosphide and aluminum phosphide releases phosphine gas when in contact with acid contents of the stomach. Both phosphine and intact zinc phosphide are absorbed from the GI tract. The phosphine is believed to cause the majority of acute signs, while the intact phosphide may cause hepatic and renal damage later. These toxicants also induce CNS effects and the specific mechanism is not known.

Gross findings are not specific and include a characteristic acetylene odor of the stomach content, congestion of the gastric mucosa, pulmonary congestion with edema, subpleural hemorrhages, congestion of liver and kidney. Histopathological findings consist of fatty degeneration of hepatocytes in liver, renal tubular degeneration and necrosis in kidney and gastritis (Casteel and Bailey Jr, 1986). Diagnosis is by analysis of stomach contents/vomitus/liver and kidney for phosphine gas and the samples have to be submitted frozen in air-tight containers.

# PLANTS

This section on plants comprises those plants which produce pathological lesions associated with toxicity. Most of these plants cause toxicity as a result of accidental ingestion. This section is divided into subsections on plants affecting various organs, cardiovascular, nervous, GI and liver, skin, hemopoietic, respiratory, reproductive and urinary system.

## Cardiovascular system

This section covers plants which produce pathological lesions in the heart and blood vessels.

### *Summer pheasant's eye (Adonis aestivalis)*

Toxicity occurs by ingestion of contaminated grass or hay. Toxicity is attributed to cardiac glycoside strophanthidin. Gross findings include endocardial hemorrhage and gaseous distension of the GI tract. The microscopic lesions include multifocal necrosis and degeneration of myocytes infiltration of neutrophils with endocardial and epicardial hemorrhage (Woods *et al.*, 2004). Diagnosis is by correlating history of exposure with clinical signs, pathological findings and analysis of stomach content for glycosides.

### *Vitamin D containing plants*

The most common plants which contain calcitriol that accounts for pathogenesis are *Cestrum diurnum*, *Trisetum flavescens* and *Solanum malacoxylon*. The toxicity in *Cestrum*

is attributed to 1,25-dihydroxy-vitamin D-glycoside and that in *S. malacoxylon* to a molecule similar to or identical to 1,25-dihydroxy vitamin D: the plant (*C. diurnum*) is seen in warmer parts of the United States like Florida and is grown as an ornamental plant; *Solanum* spp. is seen in Brazil and Argentina; *Trisetum* spp. in Germany and Austria. The common species affected are cattle and horses. *S. malacoxylon* toxicity is commonly reported in sheep and cattle. Other animals are susceptible experimentally. Adult cows are more susceptible than calves.

The clinical pathological observation in the early stage is hypercalcemia and hyperphosphatemia and later the levels decrease resulting in normal calcium and phosphorus levels. The gross findings include white to tan gritty foci on the surface or inside of heart, great vessels, tendons, ligaments, kidneys, lungs, stomach and bones. In cattle the microscopic findings include cardiac mineralization commonly in the endocardium of left atrium, mineralization of the aorta primarily involving tunica intima and media. Mineralization is observed in several major and minor blood vessels. Other locations in which microscopic evidence of mineralization observed are the following: kidneys, alveolar septa of lung, bronchiolar epithelium, muscles of the intestine and stomach. In cattle osteoporosis, hyperplasia of thyroid C cells and atrophy of parathyroid are also reported in long-time exposure (Collins *et al.*, 1977). Hyperostosis may lead to bone thickening. A dark basophilic band (possibly mucopolysaccharides) separates new bone growth from pre-existing bone (Krook *et al.*, 1975; Durand *et al.*, 1999; Braun *et al.*, 2000). Diagnosis is by correlating history of exposure, pathological findings and estimation of 1,25-dihydroxy cholecalciferol levels in serum.

### *White snake root (Eupatorium rugosum)*

The common species affected by white snake root (*E. rugosum*) are cattle, horses, sheep and goats. The toxic component is tremetol and has been described as a fat soluble, high molecular weight alcohol. The toxic principle is a cumulative poison and repeated exposure to small amounts results in intoxication. The toxin is secreted in the milk more rapidly than by any other route which can result in relay toxicosis to offspring or humans.

The gross findings observed include pale linear streaks in the myocardium or skeletal muscles with or without hemorrhage, increased fluid in pericardial sac and ventral edema. In cattle the common histopathological findings include fatty degeneration of the liver, congestion and multifocal hemorrhage in the heart and GI tract, myocytes necrosis and degeneration of the skeletal muscles especially the shoulder muscles and congestion of the kidney.

In horses the toxin affects the heart. The histopathological findings include myocardial degeneration, necrosis and fibrosis, hepatic changes associated to cardiac changes including centrilobular hepatocyte degeneration with mild necrosis and fatty changes. Kidneys may have tubular degeneration with globin casts in the renal pelvis (Olson *et al.*, 1984; Jones *et al.*, 1996). Diagnosis is made by correlating clinical signs, history of exposure, pathological findings and chemical analysis of the toxin in liver and kidney. The differential diagnosis includes ionophore toxicity, selenium/vitamin E deficiency and oleander toxicity.

### *Coffee senna (Cassia occidentalis)*

Coffee senna, *C. occidentalis*, is a shrub which grows commonly in the southeastern United States. The common species affected are cattle, horse and sheep. The common clinical pathological observations are increase in CPK, serum glutamic oxaloacetic transaminase (SGOT) and LDH. Gross findings include diffuse or focal pale appearance of the skeletal and myocardial muscles. Microscopic observation includes degeneration and necrosis of the myocytes in the skeletal and cardiac myocytes. Lungs have edema and marked congestion. The liver will show secondary changes related to changes in the cardiac musculature consisting of hepatocyte necrosis and fatty degeneration in centrilobular areas. In many cases the kidney show renal tubular degeneration with protein casts (Martin *et al.*, 1981; Suliman *et al.*, 1982; Barth *et al.*, 1994; Jones *et al.*, 1996). Diagnosis is by correlating the history of exposure, pathological findings and appropriate clinical signs. Differential diagnosis includes toxicosis by ionophore, white snakeroot, gossypol and vitamin E/selenium deficiency.

### *Oleander: Nerium oleander*

It is an ornamental shrub seen commonly in southern and southwestern United States. The toxicity is attributed to the cardiac glycoside similar to digitoxin. Almost all species are susceptible. Pathological findings are not common, and rarely subendocardial and abomasal hemorrhages are observed. Pulmonary edema, myocardial degeneration and necrosis are the pathological findings which are rarely observed (Mahin *et al.*, 1984; Galey *et al.*, 1996). Diagnosis is by identification of oleander in the GI tract, analytical estimation of ingesta for oleandrin, history of consumption, appropriate clinical signs and pathological lesions.

### *Cyanogenic glycosides*

Cyanogenic glycosides are present in *Prunus* genus, grasses like *Sorghum*, Sudan and Johnson grass. Consumption may lead to mortality in domestic animals especially in ruminants. Cyanogenic glycosides yield hydrogen cyanide by hydrolysis. Rumen contains large amount of B glycosidase which hydrolyzes glycoside and hence they are more susceptible. Cyanide blocks the cellular respiration so that oxygen is not available for the electron transport in

the cytochrome system. There are no specific lesions as most cases are acute. Blood may have a cherry red appearance immediately after death. Diagnosis is by detecting cyanide content in the plant material from the GI tract, clinical signs and case history (Osweiler, 1996).

#### Water hemlock

Water hemlock (*Cicuta douglasii*) produces acute death and no pathological lesions are commonly observed in intoxication. Experimental studies in sheep have shown skeletal and myocardial degeneration when dosed orally with water hemlock (Panter *et al*., 1996).

#### Taxus

*Taxus* spp. contain an alkaloid toxine, which is a cardiotoxin. Horses and bovines are the most common species affected and it leads to sudden death. Gross lesion is absent except for hemorrhage in the endocardium of ventricles. Histopathological lesions in horses include occasionally multifocal areas of mild necrosis in the papillary muscle and ventricular musculature, congestion and interlobular edema in lungs. Diagnosis is by chemical analysis of the stomach content and correlating with clinical signs (Panter *et al*., 1993; Tiwary *et al*., 2005).

#### Black walnut

Toxicosis is observed in horses when exposed to shavings. Juglone, a toxic compound found in all parts of plants of the walnut tree family Jugans, is the toxic component. Most common clinical signs were moderate to severe laminitis (Obel grade 2 or 3), pitting edema of the distal portion of the limbs and rapid respiratory rate. Some animals may show signs of colic, anorexia and lethargy. Histopathological findings observed in experimental studies include necrosis of dermal tips of dorsal primary epidermal laminae. The laminae may also show proliferative epithelial response characterized by numerous mitotic figures and clusters of epithelial cells (Uhlinger, 1989; Galey *et al*., 1991).

### GI system

Many plants produce irritation of the oral cavity and also the GI tract. Irritation is caused by the oxalate crystals, their thorns which are sharp, and the histamine-releasing compounds in these plants. The common plants which produce these clinical signs are nettles (*Urtica* and *Laportea*), burdock (*Arctium lappa*), goatheads (*Tribulus terrestris*), wild barley (*Hordeum*) and *Cactus* spp. Some of these plants may penetrate the oral cavity or skin and lead to secondary bacterial infection and inflammation.

### Liver

#### Pyrrolizidine alkaloids

The common genus of plants which contain pyrrolizidine alkaloids are *Senecio*, *Crotalaria*, *Heliotropium*, *Echium*, *Tirchodesma* and *Amsinckia*. Liver is the target organ for toxicosis. Pyrrolizidine is activated in the liver by mixed function oxidases to the toxic alkaloid, pyrrole. Monocrotaline, the pyrrolizidine alkaloid in *Crotalaria* spp., has hepatotoxic, nephrotoxic, pneumotoxic and fetotoxic effects.

Clinical pathological changes include increased AST, SDH, ALP and GGT mostly in acute cases. Gross findings include swollen pale yellowish discolored liver or shrunken liver depending on the duration of exposure. The histopathological findings include periacinar necrosis and endothelial damage to the hepatic venules and small hepatic veins, widespread portal fibrosis, bile duct proliferation and megalocytosis. In cattle, chronic pyrrolizidine toxicosis produces pronounced hepatic bridging portal fibrosis which infiltrates along the sinusoids to dissect lobules and separate individual cells. Megalocytosis is by the continued nucleoprotein synthesis, coupled with mitotic inhibition. These megalocytic cells can range up to 20 times that of normal cells. The other compounds which cause megalocytosis are *Lantana camara*, *Lippia* spp., *Cycas* spp., aflatoxins and nitrosamines (Baker *et al*., 1991; Craig *et al*., 1991). In sheep, long-term consumption of pyrrolizidine-containing plants may lead to elevated levels of liver copper leading to copper toxicity. *Crotalaria* species induce pneumotoxicity. *Crotalaria* poisoning in horses is characterized by histopathological lesions in the lung which include diffuse alveolitis with fibrosis and hyaline membrane formation (Nobre *et al*., 1994).

#### Cocklebur (Xanthium spp.)

The glycoside carboxyatractyloside is the toxic principle of *Xanthium* spp. The seeds and the cotyledonous seedlings contain the toxin. Pigs and cattle are more commonly affected but the disease is also occasionally mentioned in sheep, and rarely in horses. Liver is the target organ. The most prominent histopathological finding in cocklebur poisoning is acute and severe centrilobular to massive necrosis of the liver. Ascitis may be seen related to liver injury. Chronic hepatitis is observed in animals which recover from toxic insult. Other pathological findings include gastroenteritis, with thickening of the mucosa, hepatic and cardiac hemorrhage. Renal injury leading to tubular degeneration is observed in some cases. The gross lesions in experimental studies of pigs include marked serofibrinous ascitis, edema of the gallbladder wall and lobular accentuation of the liver. Histopathological lesion is acute to subacute centrilobular hepatic necrosis (Stuart *et al*., 1981; Witte *et al*., 1990). The differential diagnosis of acute centrilobular hepatic

necrosis in ruminants includes blue-green algae, *Cestrum* spp., *Lantana camara*, etc.

#### *Lantana camara*

Toxicity is associated with triterpene acids lantadene A and lantadene B. Sheep and cattle are the common species affected. The major clinical effect of acute *Lantana camara* is photosensitization, the onset of which often takes place 1–2 days after consumption of a toxic dose. Subacute poisoning is more common. In severely affected cattle, lesions may appear at the muzzle, mouth and nostrils. Ulceration may be present in the cheeks, tongue and gums, while swelling, hardening, peeling of mucous membranes and deeper tissues occur in the nostrils. The common gross lesions in internal organs include yellowish discolored liver, icterus, general edema with gallbladder distention and gross changes in the skin. Histopathological lesions include megalocytosis, bile stasis in the canaliculi and focal hepatocyte necrosis. Skin lesions are characterized by ulceration and necrosis. Necrosis of renal tubular epithelium and myocardial injury are also observed in some cases. Hemorrhages are observed in some organs (Sharma *et al*., 1988). Diagnosis is by evidence of consumption, appropriate clinical signs and lesions.

#### *Cycads*

Cycad palms occur in tropical and subtropical climates. Cycasin is a glycoside that has been demonstrated to be responsible for acute hepatotoxic effects. Cattle, sheep, dogs, swine, horses, goats are susceptible. Two distinct syndromes, neurologic and hepatic–GI, have been described in ruminants. The prominent gross findings include ascitis and pale shrunken liver, in ruminants. Hemorrhage and GI mucosal necrosis is observed in dogs. Histopathological findings include hepatocyte necrosis and degeneration, centrilobular and extending to midzonal areas (dogs), hepatocellular megalocytosis, periacinar necrosis, fibrosis around central veins, cholestasis and bile duct hyperplasia in ruminants. The neurologic syndrome of cycad toxicosis in cattle is called Zamia staggers. The syndrome is characterized clinically by weight loss followed by lateral swaying of the hindquarters, with weakness, ataxia and proprioceptive defects in the rear limbs. Demyelination and axonal degeneration are present in the brain, spinal cord and dorsal-root ganglia. Surviving cattle remains ataxic and have hindquarter muscle atrophy (Hooper *et al*., 1974; Reams *et al*., 1993; Jones *et al*., 1996; Albretsen *et al*., 1998). Diagnosis is by evidence of consumption, appropriate clinical signs and lesions.

#### *Alsike and red clover*

These are members of the *Leguminosae* family. Toxicity is reported commonly in equines. Liver is the primary target organ and secondary photosensitization can occur. The specific toxin is not identified. The common significant clinical pathological finding is the elevated serum GGT and ALP levels and in some animals increased ALT and SDH. Gross findings include enlarged or shrunken liver. The prominent microscopic findings include bile duct proliferation with fibrosis. The fibrosis can be periportal, perilobular or centrilobular and is usually absent in the sinusoids. Chronic lesions will compress the hepatocytes leading to shrunken appearance of the liver. Mild-to-moderate inflammation and necrosis are also observed (Colon *et al*., 1996).

Diagnosis is by evidence of consumption, clinical signs of hepatic damage and pathological findings. The common differential diagnosis includes Theiler's disease and pyrrolizidine toxicity.

## Hemopoietic system

#### *Damaged/moldy sweet clover hay*

Sweet clover is grown throughout the central and northern United States and southern Canada. Toxicity is reported primarily in cattle. Formation of dicoumarol results in the dimerization and oxidation of the compound coumarin, a natural component of the plant; various fungi in the presence of moisture from coumarin to dicoumarol.

The toxic principle is dicoumarol which interferes with normal blood clotting by competitive inhibition of vitamin K 2,3-epoxide resulting in reduction of the concentrations of active forms of clotting factors II (prothrombin), VII, IX and X. The clinical pathological findings include significant changes in the clotting panel characterized by elevated PT, PPT and ACT.

Gross findings include multifocal hemorrhages (petechiae, ecchymosis and hematomas) in subcutaneous tissue, muscles and under the capsule of several organs. Histological findings include fatty change in liver, renal tubular degeneration, necrosis and hemorrhages (Jones *et al*., 1996; Puschner *et al*., 1998b).

## Reproductive system

The important plants which cause teratogenic effects or abortifacient potential are *Pinus* spp., *Veratrum* spp., *Lupine* spp., *Nicotiana* spp., *Conium maculataum* and hybrid sudan. Broomweed (*Gutierrezia* spp.) and Hoary alyssum (*Berteroa incana*) are reported to cause abortion in ruminants and equines, respectively. Carbon monoxide and nitrate do induce abortions in pigs and cattle, respectively, and are mentioned in other parts of this chapter.

#### *Pinus spp.*

Toxic principle is isocupressic acid which is a primary abortifacient compound. *Cupressus macrocarpa* and common

juniper (*Juniperus communis*) also contain high levels of isocupressic acid and induce abortion similar to *Pinus* spp. Toxin is present in green and dried needles and is water soluble. Cattle are the most susceptible species.

Abortion occurs mostly during the last trimester of pregnancy. Abortion usually occurs 2 days after pine needles have been fed but may occur up to 2 weeks after removed from source. Retained placenta is a common sequela of abortion. Live-born calves are weak and have poor viability. Parturition may be characterized by weak uterine contractions, incomplete dilation of the cervix and uterine hemorrhage. Metritis may occur. Gross findings are not specific. The observations in experimental studies conducted in pregnant cows include reduced number of trophoblastic giant cells in placentomes and more than normal number of necrotic luteal cells in corpora lutea (Jensen *et al.*, 1989; Stuart *et al.*, 1989; Gardner *et al.*, 1998). In experimental studies of cows exposed to pine needle tips both renal and neurological lesions were observed along with abortion, retention of placenta and endometritis. The renal lesions consisted of nephrosis characterized by tubular epithelial necrosis, proteinaceous and tubular casts in the tubular lumen, minimal inflammatory cell infiltration and congestion. The neurological lesions consisted of vacuolar changes in the neuropil of the basal ganglia, neuronal pyknosis and satelletosis in the cerebral cortex, diffuse perivascular and myelin edema which was prominent in the lumbar spinal cord. Focal skeletal myonecrosis of the large muscles (semimembranosus) was observed in animals which were paralyzed and recumbent. Suppurative endometritis limited to the mucosal surface was observed with no bacteria (Stegelmeier *et al.*, 1996). Diagnosis is made by evidence of consumption during gestation and clinical signs.

#### Hybrid sudan

Fetal malformations may occur when mares graze hybrid Sudan grass during the 20–50 days of gestation. Foals may be born with extreme flexion of the joints or ankylosis. Consumption of hybrid Sudan grass also may result in dystocia. Abortions are also reported associated with exposure (Prichard and Voss, 1967).

#### Lupines

Lupines are leguminous plants native to the western United States mostly in Rocky Mountain areas. Several quinolizidine alkaloids and piperidine alkaloids are isolated from *Lupine* spp. which are associated with the teratogenic action of these plants. Anagyrine is the most important teratogenic alkaloid which was experimentally been proved to induce teratogenic changes in cattle.

Ingestion of *Lupine* spp. results in a congenital syndrome called crooked calf syndrome which is characterized by arthrogryposis, scoliosis, torticollis and cleft palate. Some cases of abortions are also reported. Teratogenesis is observed when the plant is consumed by cows during 40–70 days of pregnancy. The crooked calf syndrome has been reproduced by the experimental feeding of the alkaloid, anagyrine the most teratogenic alkaloid of *Lupine* spp. Diagnosis is made by evidence of consumption during gestation, gross lesions and clinical signs (Panter *et al.*, 1998, 1999).

#### Veratrum spp.

The plant contains several alkaloids and the important teratogenic alkaloids include cyclopamine, veratrobasine, veratramine, cycloposine and jervine. Teratogenecity is reported commonly in sheep. The types of deformities are directly related to the stage of fetal development at the time of consumption. Teratogenic effect is manifested when the plant is consumed during 12–14 days of gestation. The most important deformity is the craniofacial effect. Consumption of the plant from days 0 to 10 may have no obvious effect. Days 12–30 of gestation are the most critical period and exposure during this period can lead to cleft palate and lips, syndactyly and brachygnathia. Consumption during days 12–14 of gestation results in cyclopia-type malformations which include hydrocephalus, displacement of nose and fusion of eyes. Experimental exposure during 30–36 days resulted in shortened metacarpals and metatarsals (Binns *et al.*, 1972; Keeler and Stuart, 1987). Diagnosis is made by correlating evidence of consumption, appropriate clinical signs and malformations.

#### Poison hemlock (Conium maculatum)

The toxic principle is a piperidine alkaloid. The most important toxic effect is teratogenecity. Common species affected is cattle, but can also affect pigs, sheep, goat and horse. The main teratogenic effects are arthrogrypotic skeletal malformations and cleft palate. This occurs when the plant is ingested by pregnant cows between days 40 and 100 of gestation. The skeletal lesions in cattle consist of torticollis, arthrogryposis, scoliosis and kyphosis. The cause of skeletal malformations and cleft palate is attributed to the lack of fetal movement during the critical stage of gestation. The maternal effect of poison hemlock is mortality and no significant pathological findings are observed (Keeler and Balls, 1978; Panter *et al.*, 1990, 1999). Differential diagnosis for teratogenic effects is *Lupine* species.

## Respiratory system

The most important toxicants which induce microscopic alterations in the respiratory system are ricin and the compounds which result in interstitial pneumonia.

### *Ricinus communis*

Ricin is the principle toxin and it is a phytotoxin (a lectin). All parts of the castor bean plant are toxic, the beans contain most ricin and need to be crushed or broken to release the toxic component. Ricin is 100 times more toxic parenterally than orally. The ricin molecule is composed of two glycoprotein chains A and B. One chain facilitates endocytosis, while the other chain, once in the cell, inhibits protein synthesis and causes cell death. Orally, ricin is readily absorbed from the stomach and intestine. Ricin is water soluble and not present in castor oil. Another phytotoxin in castor bean, ricinine, is reportedly goitrogenic. Common species affected are horses and dogs. Birds, cattle and pigs are also affected.

In dogs ingestion of castor bean causes severe GI clinical signs including vomiting with blood and diarrhea with blood and can lead to death (Albretsen *et al.*, 2000). Ingestion of castor bean/oral exposure of ricin results in catarrhal to hemorrhagic gastroenteritis. There may be petechial hemorrhages on the serosal surface of visceral organs and in subepicardium. Mesenteric lymph nodes are edematous. Hepatic and renal degenerative changes are reported. In birds it causes degeneration of hepatocytes and hemorrhage. In ducks there is ascending paralysis and the clinical signs appear similar to botulism (Dungworth, 1993).

Gross findings in inhalation experimental studies conducted in primates include edema of lungs; lungs do not collapse when the thoracic cavity is opened mottled red to purple discoloration. Microscopic findings include multifocal to coalescing areas of intra-alveolar fibrin, edema, and hemorrhage, acute alveolitis and necrosis of lower respiratory tract epithelium. Mild inflammation was also observed in the heart. Although it is suspected that ricin-induced pulmonary edema is due to increased pulmonary capillary endothelium permeability, the specific mechanism by which inhaled ricin crosses the respiratory epithelium to injure the vascular endothelium is not yet known (Wilhelmsen and Pitt, 1996). Diagnosis is based on the case history and pathological findings. Differential diagnosis includes other toxins which produce hemorrhages in the GI tract like arsenic and exposure to paraquat for pulmonary lesion.

### *Interstitial pneumonia*

Interstitial pneumonias in cattle have different names including acute bovine pulmonary emphysema and edema (ABPE), fog fever, atypical interstitial pneumonia (AIP) and cow asthma, the condition seems to occur predominantly in late summer or fall. The condition has an acute onset. Among the toxic etiologic causes, primary toxin is the amino acid L-tryptophan in lush pasture grasses, a compound which is converted to 3-methylindole by rumen microorganisms which is absorbed into the blood stream eventually reaching the lungs where the mixed function oxidase in the non-ciliated bronchiolar epithelial cells (Clara cells) convert them to a pneumotoxic compound which causes marked necrosis of the bronchiolar cells and pneumocytes. Other leading toxic causes of interstitial pneumonia are perilla mint and moldy sweet potatoes. Moldy sweet potato contaminated by pneumotoxic factor, 4-ipomeanol also produces the same clinical and pathological changes. Perilla mint, Stink weed, rape and kale (*Brassica* spp.) also produce similar clinical and pathological changes in the lung. The gross findings include enlarged pale lung with rubbery texture. Microscopic findings include alveolar and interstitial edema, emphysema with diffuse hyaline membrane formation in the alveolar lumen and necrosis of the alveolar epithelium. Those animals which survive for several days have proliferation of type II pneumocytes (Kerr and Linnabary, 1989; Dungworth, 1993). Diagnosis is by correlating history of exposure, clinical signs and pathological findings.

## Skin

This section consists of plants which produce skin lesions. The plants which produce primary photosensitization are St. Johnswort (*Hypericum perforatum*), buckwheat (*Fagopyrum esculentum*), spring parsley (*Cymopterus watsoni*), bishops weed (*Ammi majus*) and dutchmans breeches (*Thamnosma texana*), perennial rye grass (*Lolium perenne*) and Burr terfoil (*Medicago denticulata*). The other common compounds which produce photosensitization include phenothiazine, thiazides, some sulfonamides, tetracycline, acriflavins and methylene blue.

The toxic principle in St. Johnswort is hypericin, a fluorescent pigment. Susceptible species are mainly cattle, sheep, horses, goats, rabbits. Hypericin remains intact upon ingestion, is absorbed and passes through the liver and enter the general circulation and finally reach the skin leading to photosensitive reaction.

Toxicity in buckwheat is attributed to the naphthodianthrone derivative fagopyrin. All livestock with unpigmented skin are affected. The photodynamic agent in spring parsley, bishops weed and dutchmans breeches is psoralens. These plants also induce corneal edema and keratoconjunctivitis probably through the excretion of the compounds in the lacrimal secretion. The photodynamic substance in perennial rye grass is perloline.

Secondary hepatic photosensitization is due to hepatic injury and the plants include *Senecio*, *Crotalaria*, *Brassica* and *Heliotropium*. The other agents which can cause indirect photosensitization include blue green algae, neoplastic diseases, diseases which can induce diffuse, chronic and severe liver damage. The phylloerythrin which is the breakdown product of chlorophyll is removed by the liver and is excreted in the bile. When the liver or its ductal

system is injured leading to impairment of the excretory function, the phylloerythrin reaches the skin by the systemic circulation and cause photosensitization.

The common gross observations include edema and erythema, especially around the ears, face and eyelids leading to ulceration. In severe cases, inflammation may spread to unpigmented areas of the skin and vesicles may develop containing a yellowish fluid. Ruptured vesicles form a yellow or brown crusty scab. Erythema is followed by subdural edema and necrosis. Histopathological observations include dermatitis, necrosis and gangrene (Rowe, 1989; Jones *et al*., 1996). Diagnosis is made by identification of the plant material in the GI tract, evidence of consumption, appropriate clinical signs and pathological findings.

## Hairy vetch

Hairy vetch (*Vicia villosa*) is a legume that grows throughout United States and toxicity has been reported mostly from the midwestern states and California. The common species affected are cattle and horse. The gross pathological lesions include hemorrhage in heart, kidneys, omentum and intestinal mucosa. Dermatitis associated with hairy vetch is associated with pruritis. Lesions begin with alopecia of the udder, tail head, neck and later affect the face, trunk and the limbs leading to exudation, crusting and pruritis. The prominent histopathological findings include granulomatous inflammation predominantly in skin, liver, kidney and also heart, skeletal muscle, lymph node, intestine and urinary bladder. The inflammation is mostly around the vessels and consists of a mixture of lymphocytes, neutrophils, macrophages and eosinophils with and without multinucleate giant cells. In liver bile duct hyperplasia may also be observed (Anderson and Divers, 1983; Panciera *et al*., 1992; Woods *et al*., 1992). Diagnosis is made by correlating history of exposure, clinical signs and pathological findings.

## Urinary system

The most common plants which induce toxic pathological changes in the urinary system are Easter lily, Red maple, Oak, *Amaranthus*, *Rheum*, *Halogeton*, *Rumex* spp., *Solanum*, *Cestrum*, Brackenfern and Hybrid sudan grass.

### *Easter lilly*

Cats are the most common species affected. The aqueous extracts of leaves and flowers are proven to be nephrotoxic and pancreotoxic. The clinical pathological changes include an elevated creatinine and BUN with glucosuria, proteinuria with numerous tubular epithelial casts in urine sediment. The significant histopathological findings include acute necrosis of proximal convoluted tubules and degeneration of pancreatic acinar cells. The tubular epithelial cells show loss, degeneration and regeneration. Tubular lumen contains protein casts and cellular debris. Renal ultrastructurally changes include swollen mitochondria, megamitochondria, edema and lipidosis (Langston, 2002; Rumbeiha *et al*., 2004). Diagnosis is by correlating the clinical signs with histopathological examination and case history.

### *Oxalate-containing plants*

The common plants which contain toxic level of soluble oxalates are rhubarb (*Rheum rhaponticum*), halogeton (*Halogeton glomeratus*) and Grease wood (*Sacrobatus vermiculatus*), beets and dock (*Rumex* spp.) and lamb quarters (*Chenopodium* spp.).

The toxic principles are oxalic acid, sodium and potassium oxalates, which complex with calcium forming calcium oxalate and crystallize in the kidney when they are excreted. Sheep, cattle and swine are the common species affected. Hypocalcemia is observed due to formation of calcium oxalate. Increased BUN values are observed in chronic cases with nephritis. The gross lesions consist of radially arranged white streaks in the kidney and hemorrhages in the GI tract.

The common microscopic findings are hemorrhagic rumenitis in sheep associated with deposits of calcium oxalate in the walls of blood vessels, oxalate nephrosis characterized by irregular rhomboid crystals which are visible in sharply reduced or polarized light, protein casts in tubular lumen with varying degrees of renal tubular epithelial degeneration and necrosis (Dickie *et al*., 1978; Panciera *et al*., 1990).

In tropical and subtropical areas, certain grasses that are cultivated widely (genera of *Cenchrus*, *Panicum* and *Setaria*), accumulate large amounts of oxalate and have been associated with renal oxalate accumulation in cattle and sheep and with skeletal disease in the horse. Diagnosis is by evidence of consumption clinical signs and pathological findings.

### *Amaranthus spp.*

The toxic principles are unknown agents which cause perirenal edema and nephrosis. The most common species affected are cattle and swine. The significant clinical pathological findings include increased BUN, proteinuria, CPK and creatinine.

The most striking gross lesion is retroperitoneal edema in the perirenal connective tissue. Edema is often present in the ventral abdominal wall and per rectal area. Kidneys are normal or small in size and pale with subcapsular

petechiae. In calves free straw-colored fluid is observed in thoracic and peritoneal cavities. Histopathological findings include degeneration and necrosis of the tubular epithelium of the proximal tubules with interstitial edema, dilation of the renal tubules with protein casts and some may have oxalate crystals (Stuart *et al.*, 1975; Kerr and Kelch, 1998). Diagnosis is by evidence of consumption of the plant material with appropriate clinical signs and pathological findings.

### *Oak*

Oak (*Quercus* spp.) poisoning has been reported from many regions of the world, and more than 60 species of oak have been identified in North America. Tannins and their metabolites (digallic acid, gallic acid and pyrogallol) are responsible for the toxicity. High levels of toxic component are in young leaves and the shells of green acorns. Cattle and horses are the common species affected with reports more common in cattle.

Kidney is the target organ with pathological findings also in GI tract. Oak causes GI irritation which manifests in the early course of toxicosis, characterized by anorexia, constipation and colic. After a few days, constipation is followed by diarrhea, and fragments of acorns may be present within stools.

The gross findings include swollen pale kidney with petechial hemorrhage, perirenal edema, hemorrhage and ulcers in the GI tract (can extend from pharynx to the lower intestinal tract). The histopathological findings include degeneration and necrosis of tubular epithelium, regeneration of tubular epithelium, dilated tubules with erythrocytes, homogenous and granular casts and sloughed necrotic tubular epithelial cells and neutrophil infiltration. The renal interstitium has multifocal areas of hemorrhage and edema. GI lesions include hemorrhages in mesentery, stomach, small intestine and hemorrhage with ulcers in large intestine, especially in horses. Secondary lesions related to uremia and renal failure may also be observed in stomach and tongue. Oak toxicosis has high mortality due to renal failure (Anderson *et al.*, 1983; Plumlee *et al.*, 1998). Diagnosis is by evidence of consumption of the plant material with appropriate clinical signs and pathological findings.

### *Red maple (Acer rubrum)*

The most common species affected is equine. An unidentified toxin creates an acute hemolytic anemia associated with methemoglobinemia and/or Heinz body formation. Clinical pathological findings are hemolytic anemia and blood smears may show eccentrocytes, spherocytes, anisocytes and Heinz bodies. The gross findings include generalized icterus, splenomegaly, and enlarged liver with pale centrilobular areas, dark brown black discolored kidney, increased pleural and pericardial fluid, petechiae and ecchymosis in the serosal surfaces. The histopathological findings include hepatocyte degeneration in centrilobular areas, renal tubular epithelial degeneration with hemoglobin casts and erythrophagocytosis in spleen and liver. Pregnant mares may abort without showing any clinical signs (George *et al.*, 1982; Stair *et al.*, 1993). Diagnosis is by evidence of consumption of the plant material with appropriate clinical signs and pathological findings. Differential diagnosis includes nitrate toxicosis, equine infectious anemia and Babesiosis.

### *Onion (Allium)*

Dogs are the most common species affected. The toxic component of *Allium* spp. is *n*-propyl disulfide and its effects are on erythrocytes. The principal effects are related to hemolysis. This is believed to be secondary to oxidant-associated effects, and Heinz bodies may sometimes be evident. Discolored urine varying from port wine to almost black color is observed. Some animals may show icterus. Severe toxicoses may be lethal. No specific histopathological lesions are observed other than damage of the parenchymatous organs due to hemolytic anemia (Harvey and Rackear, 1985). Differential diagnosis includes zinc toxicosis, copper toxicosis, autoimmune hemolytic anemia, and hemolytic blood parasites and bacterial infections which cause hemolytic anemia. Diagnosis is by evidence of consumption with appropriate clinical signs and pathological findings.

### *Bracken fern (Pteridium aquilinum)*

Most common species affected are horse and cattle. Several toxic principles are recognized and poisoning in non-ruminants is due to thiaminase. In ruminants the toxins include a bone marrow toxin and the suspected carcinogen ptaquiloside which induces the bladder tumors in cattle. In horses it causes thiamine deficiency and leads to clinical signs of thiamine deficiency. The lesions in horses include enteritis, with some pericardial and epicardial hemorrhages. In sheep bracken fern poisoning is associated with progressive retinal degeneration and is called bright blindness. The condition is characterized by loss of rods and cons with reduction in width of the outer nuclear layer. The lesion is prominent in the tapetal region (Hirono *et al.*, 1993).

In cattle different clinical manifestations of the poisoning by bracken fern are reported. When large amounts of plants are consumed in a short period of time aplasia of the bone marrow develops and result in mortality. Thrombocytopenia, neutropenia and anemia develop and early myeloid cells are destroyed. Those animals consuming less quantity for longer period develop hematuria and is called enzootic hematuria. They also develop urinary

bladder tumors and squamous cell carcinoma in the GI tract. All these lead to death due to anemia and chronic wasting. The pathological findings include widespread petechial and ecchymotic hemorrhage in intestines, serosal surfaces of several internal organs, including urinary bladder, gall bladder, heart, subcutaneous tissue and muscles. Some animals develop abomasal ulcers. Hyperplasia and hemorrhage are observed in the urinary bladder and also ureter or renal pelvis. Chronic cystitis with hemangiomas develops which bleed resulting in hematuria. Adenocarcinomas, transitional cell carcinomas, squamous cell carcinomas, papillomas, fibromas and adenomas are reported in the urinary bladder, renal pelvis and ureters, related to exposure of bracken fern. Tumors in the GI tract are mostly squamous cell carcinomas located in pharynx, base of tongue, esophagus and rumen (Pamukcu *et al.*, 1976; Jones *et al.*, 1996; Gava *et al.*, 2002; Carvalho *et al.*, 2006). Diagnosis is by evidence of consumption of the plant material with appropriate clinical signs and pathological findings.

#### *Sorghum spp.*

In horses, especially females, a syndrome of "cystitis and ataxia" has been associated with the consumption of hybrid strain of *Sorghum* spp. Gross observations include edema and hemorrhage in the bladder mucosa with occasional ulceration of the mucosa. Histological findings include focal necrosis and ulceration of the urinary bladder mucosa, degeneration of axons and demyelination with gitter cells in cervical, thoracic, lumbar and sacral spinal cord segments. Fetal malformations associated with hybrid sudan is mentioned in the reproductive system section (Adams *et al.*, 1969; Morgan *et al.*, 1990).

## ZOOTOXINS

The toxic exposure from insects and reptiles is covered in this section. The clinical signs of exposure are very important in making conclusive diagnosis of most of these conditions and only few cases have gross or histopathological lesions suggestive of exposure to toxins. Most of the toxic exposures are severe and acute, with toxin entering the system resulting in mortality. The agents which are described in this section include hymenoptera, blister beetle, snakes, toad and spiders.

### Black blister beetle

Toxicosis is due to ingestion of blister beetle (*Epicauta* spp.) itself. Toxicity occurs primarily when beetles are present in alfalfa hay mostly in southwestern United States and occasionally in eastern and midwestern states of United States. The toxic principle is cantharidin, a bicyclic terpenoid, which is contained in the hemolymph, genitalia and other tissues of beetle. This is a very stable compound and remains toxic even in dead beetle. Ingested cantharidin causes irritation of oral and GI mucosa leading to colic. Horses are the most common species affected.

Clinical pathological findings include hemoconcentration, neutrophilic leukocytosis and hypocalcemia. Lesions are confined to GI tract, urinary tract and heart. Gross lesions are rare and may include ulceration and hyperemia in the entire GI tract and urinary bladder. Histopathological findings include ulceration and necrosis of the mucosal epithelium in the esophagus, stomach, urinary bladder and myocardial necrosis (Schoeb and Panciera, 1979; Helman and Edwards, 1997; Schmitz, 1989). Diagnosis is by clinical signs, pathological findings confirmed by analysis of urine, and gastric contents, or urine for cantharidin.

### Hymenoptera (bees, wasps) and ants

The toxins are not well characterized but may contain formic acid (in ants) which induces the pain. In acute cases the poison apparatus is observed in the wound by bees and certain wasps. Gross findings may include local edema and erythema. In dogs acute pruritic papular to facial nodular facial dermatitis are reported. Histopathological observations include eosinophilic folliculitis with collagen degeneration and mucinosis (Scott *et al.*, 2001). Fire ant bites in dogs cause erythema and pruritic swelling. Histopathology is characterized by vertically oriented linear band of full thickness necrosis of the dermis with edema and inflammatory cells predominated by eosinophils (Rakich *et al.*, 1993).

### Snakes

Most of the poisonous snakes in United States are of Crotalidae family which includes the rattlesnakes, water moccasins and copper heads. The most common snake of the Elapidae family seen in United States is the coral snake (*Micrurus euryxanthus*) which is distributed in southeast and southwestern United States. Crotalidae family produces venoms which are mostly necrolytic and hemolyzing. Hemorrhagins in crotalid venom are toxic to the vessels and hence cause hemorrhage and edema at the wound site, in addition to systemic hemorrhage and shock. Marked anemia is observed due to hemolysis and extravasation. Disseminated intravascular coagulation is observed in some cases. Coral snake venom is primarily neurotoxic and within a half hour neurotoxic effects may be noted. Coral snake venom causes local pain and swelling.

The rattlesnake bite in horses is mostly on or near the muzzle which results in head swelling, dyspnea and epistaxis. The most common complication is cardiac problems. The most common site of rattlesnake bite in dogs is the head. The most common finding is swelling at the bite area. The common initial clinical pathological changes include echinocytosis, thrombocytopenia, leukocytosis and prolonged ACT (Dickinson *et al*., 1996; Hackett *et al*., 2002). Generally edema and erythema along with fang marks (Crotalidae family) may be observed at the site of bite although it is difficult to identify due to thick hair coat in animals. These lesions may advance to necrotizing dermatitis with vasculitis. Major route of venom excretion is through the kidneys and hence kidney failure is observed in many cases of crotalid snake bites characterized by renal tubular necrosis with protein casts and hemorrhage and necrosis in the glomeruli.

### Spider

Spider bites are common in dogs. The most common spiders in United States are the brown recluse spider (*Loxosceles reclusa*), the black widow spider (*Latrodectus mactans*), the red widow (*Latrodectus bishopi*) and the common brown spider (*Loxosceles unicolor*). Brown recluse spider bite lesions are more common during the period of spring, fall and summer as they hibernate during winter. Brown recluse venom is cytotoxic and hemolytic. Several enzymes have been identified in the venom including lipases, hyaluronidase, alkaline phosphatase and sphingomyelinases. Sphingomyelinases are primarily responsible for both tissue necrosis and complement-dependent hemolysis. The pathogenesis is thought to involve intravascular coagulation locally at the site of the bite followed by necrosis and an influx of neutrophils. Endothelial damage and thrombosis of small capillaries have been demonstrated ultrastructurally as early as 3 h after envenomation.

Bites from brown recluse spiders are common at the face and forelegs and show considerable variation in clinical presentation. The early lesion is characterized by erythema, pruritis, pain, swelling and blister formation which may proceed to an indolent non-healing ulcer. In more severe cases, localized necrosis develops with a central area of deep blue to purple discoloration surrounded by a red rim of erythema. The lesion spreads in a gravity-dependent manner and eventually progresses to form a disfiguring dark black scar.

Grossly they appear as irregularly shaped, pale blue to blue-black mottled area of skin. Erythema with dermal and subcutaneous edema is also observed around the lesion. Histopathological findings include ulceration of the epidermis, necrosis and hemorrhage in the dermis (some times form cavitations) which may extend to the subcutaneous tissue with necrotizing vasculitis. Inflammatory cells are mixed and include neutrophils and macrophages and eosinophils may not be prominent. The vessels may contain fibrin thrombi, and the dermis may have collagen degeneration and edema (Gross *et al*., 1992). Differential diagnosis of the histopathological lesion includes other insect envenomation, snakebite, pythiosis, foreign body reactions or deep bacterial infections.

### Toads

The most common toad seen in United States is the giant tropical toad, *Bufo marinus* which produce toxins which include bufagins, bufotoxins, bufotenins and other compounds. Dogs and rarely cats are exposed to the toxin. Bufagin's and bufotoxin's action is described as digitalis-like, often resulting in ventricular fibrillation. The common clinical signs are related to cardiac and CNS. Profuse salivation, head shaking, pawing at mouth and wretching are the common initial signs. Other clinical signs include seizures, cardiac arrhythmia, dyspnea, vomiting and recumbency or collapse. No specific histopathological lesions are observed in exposure to these toxins. Toad poisoning is mostly reported from Florida and Texas (Roberts *et al*., 2000; Kahn, 2005).

## MISCELLANEOUS AGENTS

These agents include algae, citrus pulp, mushrooms, ethylene and propylene glycol.

### Algae

Toxic blooms of blue green algae in ponds and water reservoirs have been associated with acute, usually lethal toxicity in various species of domestic animals and humans. Algal blooms occur in the late summer or early fall due to the warm temperature. These toxic blooms of alga can be observed on top of water as a thick layer of blue, blue-green or green in color. Formation of the toxic bloom is promoted by fertilizer and animal waste runoff into these water reservoirs. Mostly ruminants are the affected species. Liver is the target organ in algal poisoning.

The toxins produced by blue green algae consist of primarily cyclic peptides (microcystins, nodularins) that target liver function and alkaloids (amatoxins) that target the nervous system and lipopolysaccharides that are potential irritants. The hepatic toxicity is commonly attributed to microcystin-LR which is produced by the blue-green alga *Microcystis aeruginosa*. The acute toxicity is attributed to cytoskeletal collapse due to aggregation of

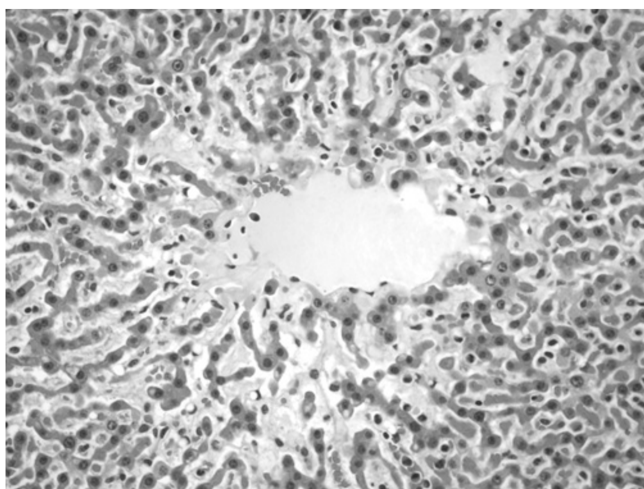

**FIGURE 90.5** Blue green algae poisoning, liver, bovine, centrilobular cord atrophy with dilated sinusoids and necrosis, H&E stain, 40×. This figure is reproduced in color in the color plate section. (Courtesy Dr. K Frazer, Philadelphia.)

actin filaments, microtubules and intermediate filaments within hepatocytes. The underlying pathogenesis involves rapid hyperphosphorylation of cytoskeletal proteins secondary to the potent inhibitory effects of this toxin on protein phosphatases 1 and 2A (Runnegar *et al.*, 1993; Guzman and Solter, 2002).

Grossly, liver is enlarged, hemorrhagic and friable and the gall bladder may have edema. Microscopic findings in acute toxicity are characterized by necrosis and degeneration of the hepatocytes primarily in the centrilobular areas and many of these hepatocytes are lost and replaced by erythrocytes (hemorrhage) (Figure 90.5). The coagulative necrosis and individualization of hepatocytes are a notable feature. Many cases have marked hemorrhage in the parenchyma (Frazier *et al.*, 1998; Puschner *et al.*, 1998a). Secondary (hepatogenous) photosensitization lesions also can develop due to the massive hepatic necrosis. Differential diagnoses for acute centrilobular hepatic necrosis in ruminants include aflatoxins, plant toxicants like *Cestrum* spp. and *Xanthium strumarium* spp.

## Citrus pulp

Citrus pulp has been used as cattle feed component and rarely do cause disease conditions. In a group of cattle fed with citrus pulp mortality was reported. The important gross findings included hemorrhages on the serosa of intestine and epicardium, lymphadenopathy, small intestinal hemorrhage and interstitial pneumonia. Histopathological findings included granulomatous inflammation with multinucleate giant cells and eosinophils in the heart, spleen, kidney, lymph nodes, liver, lung, pancreas and adrenal gland. The lesions and histopathological findings were similar to hairy vetch toxicosis and the pathogenesis is suspected to be due to a lectin in citrus pulp leading to a type IV hypersensitivity reaction (Saunders *et al.*, 2000).

Feeding of moldy citrus pulp which contained citrinin, a mycotoxin, has been reported to have resulted in an outbreak of pruritis, pyrexia and hemorrhagic syndrome in cows (Griffiths and Done, 1991).

## Ethylene glycol and propylene glycol

Intoxication with ethylene glycol is caused by consumption of antifreeze solution which contain up to 95% ethylene glycol. Mostly cats and dogs are the affected species but there are rare reports of intoxication in other species. The common clinical pathological findings in cats and dogs include neutrophilia, lymphopenia, azotemia, hyperphosphatemia, hypocalcemia, hyperglycemia and decreased whole blood bicarbonate. The common findings in urine analysis include proteinuria, glucosuria, hematuria, calcium oxalate and hippurate crystalluria, and the presence of renal epithelial cells, white blood cells and granular and cellular casts in the urine sediment (Thrall *et al.*, 1984).

Grossly the kidneys are firm with pale streaks at the corticomedullary junction. In some cases pulmonary edema and hyperemia of the gastric and intestinal mucosa are observed. The microscopic findings include marked renal interstitial fibrosis (in cases which survive for long), mild lymphocytic infiltration in the interstitium, atrophy, degeneration and necrosis and of the renal tubular epithelium with birefringent crystals, mineralization and glomerular atrophy in some cases. Microscopic demonstration of birefringent crystals in renal tubules using polarized light is pathognomonic for ethylene glycol toxicity in dogs and cats (Kelly, 1993; Jones *et al.*, 1996). In cats a hereditary disease called primary hyperoxaluria forms intraluminal birefringent oxalate crystals in the renal tubules (De Lorenzi *et al.*, 2005).

The antemortem diagnosis is by clinical pathological evaluation (increased anion and osmolal gaps, suggestive) colorimetric test kit, glycolic acid assay in serum and postmortem diagnosis is by pathological evaluation or chemical analysis of kidneys.

## Propylene glycol

Propylene glycol is a major component of soft-moist pet foods and is also used as a fungistat in pharmaceuticals, cosmetics and processed food. Experimental studies in cats have shown that propylene glycol induces Heinz body hemolytic anemia. This is relevant in cats with greater food intake like lactating queens and nursing kittens. The

histopathological findings in the cats dosed with propylene glycol included hepatic vacuolations in the periportal areas, intracellular aggregation of iron in liver and spleen (Christopher *et al.*, 1989). Mortality has been reported in a horse due to accidental iatrogenic oral administration of propylene glycol.

### Mushroom poisoning

Mushroom poisoning is commonly reported in small animals especially young dogs with few reports in horses. The most common mushroom is the *Amanita* spp. which contains amatoxins and is a thermostable toxin. Amatoxins are bicyclic octapeptides that are transported to the nucleus and inhibit RNA polymerase II, preventing DNA transcription, resulting in cessation of protein synthesis (Wieland, 1983; Tegzes and Puschner, 2002).

The common gross pathological findings include segmental congestion and hemorrhage in the mucosa of the small intestine and sometimes extending to the serosal surfaces serosa of distal ileum. Widespread necrosis and hemorrhage can be observed in the small intestine primarily in the posterior segments with necrosis of Peyer's patches in some cases. Hepatic necrosis is a prominent finding. There will be dissociation of hepatocytes, with degeneration of many with necrosis of random hepatocytes in all lobules (Figure 90.6). Edema and hemorrhage may be observed around central veins, with sparing of the portal tracts. The main lesion is nucleolar fragmentation in hepatocytes and enterocytes (Liggett and Weiss, 1989; Frazier *et al.*, 2000). Differential diagnosis includes other causes of hepatic necrosis like aflatoxicosis and exposure to other hepatotoxins. In large animals it should be differentiated from exposure to blue-green algae. Muscarinic/histaminic mushroom intoxications are reported in humans and are associated with no specific pathological lesions other than clinical signs associated with histamine release.

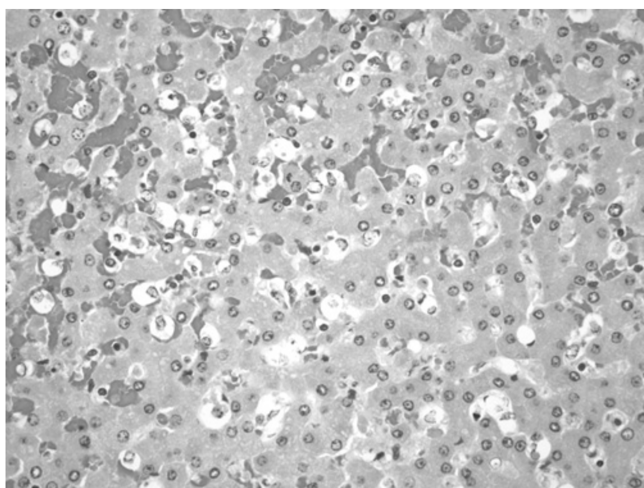

**FIGURE 90.6** Mushroom poisoning, liver, canine, hepatocyte dissociation and degeneration, H&E stain, 40×. This figure is reproduced in color in the color plate section. (Courtesy Dr. A Ligget, College of Veterinary Medicine, University of Georgia.)

## CONCLUSIONS

Domestic animals are exposed to a great number of toxins and most of these intoxications lead to marked alterations in biochemical profile of the body fluids, organs, tissues, cells and cellular organelles. These alterations are manifested as various pathological changes. Knowledge of the organs and systems affected in various intoxications will help clinicians to collect appropriate samples for both clinical pathological and histopathological investigations. With the advent of evidence-based medicine, the role of pathology in diagnosing toxic conditions is growing every day as pathological findings are the true reflections of structural and biochemical alterations of a toxic insult. Hence the clinical, gross and histopathological findings associated with toxicity are a great tool for etiological diagnosis of toxic exposure. The clinical and pathological findings related to experimental exposure of toxins in one species can be extrapolated in investigating natural cases of toxic exposure in multiple species (not in selective toxicity) of animals. Hence research in these areas will help clinicians to effectively use the data from these experiments to make a conclusive diagnosis and speed up therapeutic interventions especially in emergency medicine. Although we have several standardized *in vitro* testing methods for evaluation of toxic molecules, the histopathological changes associated with toxic exposure still remain the gold standard for assessing the toxic changes associated with their exposure. Though used routinely in preclinical toxicity evaluations of drug discovery, the new molecular pathology techniques will soon be used routinely for diagnosing clinical cases of toxic insults. Hence this branch of pathology plays an important role in diagnostic medicine. Finally, continued research in this area will increase the efficiency of clinical diagnosis for poisoning, and ultimately improve clinical practice.

## REFERENCES

Acland HM, Mann PC, Robertson JL, *et al.* (1984) Toxic hepatopathy in neonatal foals. *Vet Pathol* **21**(1): 3–9.

Adams LG, Dollahite JW, Romane WM, *et al.* (1969) Cystitis and ataxia associated with sorghum ingestion by horses. *J Am Vet Med Assoc* **155**(3): 518–24.

Albretsen JC, Khan SA, Richardson JA (1998) Cycad palm toxicosis in dogs: 60 cases (1987–1997). *J Am Vet Med Assoc* **213**(1): 99–101.

Albretsen JC, Gwaltney-Brant SM, Khan SA (2000) Evaluation of castor bean toxicosis in dogs: 98 cases. *J Am Anim Hosp Assoc* **36**: 229–33.

Ali BH, Hassan T, Wasfi IA, Mustafa AI (1984) Toxicity of furazolidone to Nubian goats. *Vet Hum Toxicol* **26**(3): 197–200.

Ali S, Jain SK, Abdulla M, *et al*. (1996) Paraquat induced DNA damage by reactive oxygen species. *Biochem Mol Biol Int* **39**(1): 63–7.

Allen JG, Randell AG (1993) The clinical biochemistry of experimentally produced lupinosis in the sheep. *Aust Vet J* **70**(8): 283–8.

Allen JG, Steele P, Masters HG, *et al*. (1992) A lupinosis-associated myopathy in sheep and the effectiveness of treatments to prevent it. *Aust Vet J* **69**(4): 75–81.

Anderson CA, Divers TJ (1983) Systemic granulomatous inflammation in a horse grazing hairy vetch. *J Am Vet Med Assoc* **183**(5): 569–70.

Anderson GA, Mount ME, Vrins AA, *et al*. (1983) Fatal acorn poisoning in a horse: pathologic findings and diagnostic considerations. *J Am Vet Med Assoc* **182**(10): 1105–10.

Angsubhakorn S, Poomvises P, Romruen K, *et al*. (1981) Aflatoxicosis in horses. *J Am Vet Med Assoc* **178**(3): 274–8.

Auza N, Braun JP, Benard P, *et al*. (1989) Hematological and plasma biochemical disturbances in experimental molybdenum toxicosis in sheep. *Vet Hum Toxicol* **31**(6): 535–7.

Baker DC, Pfister JA, Molyneux RJ, *et al*. (1991) Cynoglossum officinale toxicity in calves. *J Comp Pathol* **104**(4): 403–10.

Barth AT, Kommers GD, Salles MS, *et al*. (1994) Coffee Senna (*Senna occidentalis*) poisoning in cattle in Brazil. *Vet Hum Toxicol* **36**(6): 541–5.

Binns W, Keeler RF, Balls LD (1972) Congenital deformities in lambs, calves, and goats resulting from maternal ingestion of *Veratrum californicum*: hare lip, cleft palate, ataxia, and hypoplasia of metacarpal and metatarsal bones. *Clin Toxicol* **5**(2): 245–61.

Blodgett DJ (2001) Fescue toxicosis. *Vet Clin North Am Equine Pract* **17**(3): 567–77.

Blodgett DJ, Bevill RF (1987) Acute selenium toxicosis in sheep. *Vet Hum Toxicol* **29**(3): 233–6.

Braselton WE, Johnson M (2003) Thin layer chromatography convulsant screen extended by gas chromatography–mass spectrometry. *J Vet Diagn Invest* **15**(1): 42–55.

Braun U, Diener M, Camenzind D, *et al*. (2000) Enzootic calcinosis in goats caused by golden oat grass (*Trisetum flavescens*). *Vet Rec* **146**(6): 161–2.

Brown TP, Fletcher OJ, Osuna O, *et al*. (1987) Microscopic and ultrastructural renal pathology of oosporein-induced toxicosis in broiler chicks. *Avian Dis* **31**(4): 868–77.

Bunch SE, Castleman WL, Hornbuckle WE, *et al*. (1982) Hepatic cirrhosis associated with long-term anticonvulsant drug therapy in dogs. *J Am Vet Med Assoc* **181**(4): 357–62.

Burkhardt JE, Hill MA, Carlton WW (1992) Morphologic and biochemical changes in articular cartilages of immature beagle dogs dosed with difloxacin. *Toxicol Pathol* **20**(2): 246–52.

Carvalho T, Pinto C, Peleteiro MC (2006) Urinary bladder lesions in bovine enzootic haematuria. *J Comp Pathol* **134**(4): 336–46.

Casteel SW, Bailey Jr EM (1986) A review of zinc phosphide poisoning. *Vet Hum Toxicol* **28**(2): 151–4.

Casteel SW, Turk JR, Cowart RP, *et al*. (1993) Chronic toxicity of fumonisin in weanling pigs. *J Vet Diagn Invest* **5**(3): 413–7.

Center SA, Elston TH, Rowland PH, *et al*. (1996) Fulminant hepatic failure associated with oral administration of diazepam in 11 cats. *J Am Vet Med Assoc* **209**: 618–25.

Chang K, Kurtz HJ, Mirocha CJ (1979) Effects of the mycotoxin zearalenone on swine reproduction. *Am J Vet Res* **40**(9): 1260–7.

Charbonneau SM, Munro IC, Nera EA, *et al*. (1976) Chronic toxicity of methylmercury in the adult cat. *Interim Report Toxicol* **5**(3): 337–49.

Charney SC, Bergman PJ, Hohenhaus AE, *et al*. (2003) Risk factors for sterile hemorrhagic cystitis in dogs with lymphoma receiving cyclophosphamide with or without concurrent administration of furosemide: 216 cases (1990–1996). *J Am Vet Med Assoc* **222**(10): 1388–93.

Cheeke PR (1988) *Natural Toxicants in Feeds, Forages and Poisonous Plants*, 2nd edn. Interstate Publishers, Danville, IL.

Choie DD, Longnecker DS, Del Capmo AA (1981) Acute and chronic cisplatin nephropathy in rats. *Lab Invest* **44**(5): 397–402.

Christopher MM, Perman V, Eaton JW (1989) Contribution of propylene glycol-induced Heinz body formation to anemia in cats. *J Am Vet Med Assoc* **194**(8): 1045–56.

Chua JD, Friedenberg WR (1998) Superwarfarin poisoning. *Arch Intern Med* **158**: 1929–32.

Collins WT, Capen CC, Dobereiner J, *et al*. (1977) Ultrastructural evaluation of parathyroid glands and thyroid C cells of cattle fed *Solanum malacoxylon*. *Am J Pathol* **87**(3): 603–14.

Colon JL, Jackson CA, DelPeiro F (1996) Hepatic dysfunction and photodermatitis secondary to alsike clover poisoning. *Cont Ed Pract Vet Comp* **18**(9): 1022–6.

Colvin BM, Cooley AJ, Beaver RW (1993) Fumonisin toxicosis in swine: clinical and pathologic findings. *J Vet Diagn Invest* **5**(2): 232–341.

Coppock RW, Mostrom MS, Simon J, *et al*. (1989) Cutaneous ergotism in a herd of diary calves. *J Am Vet Med Assoc* **194**: 549–51.

Craig AM, Pearson EG, Meyer C, *et al*. (1991) Serum liver enzyme and histopathologic changes in calves with chronic and chronic-delayed *Senecio jacobaea* toxicosis. *.Am J Vet Res* **52**(12): 1969–78.

Crowell WA, Divers TJ, Byars TD, *et al*. (1981) Neomycin toxicosis in calves. *Am J Vet Res* **42**(1): 29–34.

Crump MH (1973) Slaframine (slobber factor) toxicosis. *J Am Vet Med Assoc* **163**(11): 1300–2.

De Lorenzi D, Bernardini M, Pumarola M (2005) Primary hyperoxaluria (L-glyceric aciduria) in a cat. *J Feline Med Surg* **7**(6): 357–61.

DuVall MD, Murphy MJ, Ray AC, *et al*. (1989) Case studies on second-generation anticoagulant rodenticide toxicities in nontarget species. *J Vet Diagn Invest* **1**(1): 66–8.

Dhaliwal RS, Kitchell BE (1999) Cyclophosphamide. *Compend Contin Educ Vet Pract* **21**: 1059–63.

Dickie CW, Hamann MH, Carroll WD, *et al*. (1978) Oxalate (*Rumex venosus*) poisoning in cattle. *J Am Vet Med Assoc* **173**(1): 73–4.

Dickinson CE, Traub-Dargatz JL, Dargatz DA, *et al*. (1996) Rattlesnake venom poisoning in horses: 32 cases (1973–1993). *J Am Vet Med Assoc* **208**(11): 1866–71.

Divers TJ, Warner A, Vaala WE, *et al*. (1983) Toxic hepatic failure in newborn foals. *J Am Vet Med Assoc* **183**(12): 1407–13.

Dominick MA, Carson TL (1983) Effects of carbon monoxide exposure on pregnant sows and their fetuses. *Am J Vet Res* **44**(1): 35–40.

Dorman DC, Simon J, Harlin KA, *et al*. (1990) Diagnosis of bromethalin toxicosis in the dog. *J Vet Diagn Invest* **2**(2): 123–8.

Dorman DC, Zachary JF, Buck WB (1992) Neuropathologic findings of bromethalin toxicosis in the cat. *Vet Pathol* **29**(2): 139–44.

Dunayer E (2003) Bromethalin: the other rodenticide. *Vet Med* **98**: 732–6.

Dunayer EK (2004) Hypoglycemia following canine ingestion of xylitol-containing gum. *Vet Hum Toxicol* **46**(2): 87–8.

Dungworth DL (1993) The respiratory system. In: *Pathology of Domestic Animals*, 4th edn, vol. 2, Jubb KVF, Kennedy PC, Palmer N (eds). Academic Press, San Diego, CA, pp. 603–6.

Durand R, Figueredo JM, Mendoza E (1999) Intoxication in cattle from *Cestrum diurnum*. *Vet Hum Toxicol* **41**(1): 26–7.

Duff SR, Burns RB, Dwivedi P (1987) Skeletal changes in broiler chicks and turkey poults fed diets containing ochratoxin A. *Res Vet Sci* **43**(3): 301–7.

Eubig PA, Brady MS, Gwaltney-Brant SM, *et al*. (2005) Acute renal failure in dogs after the ingestion of grapes or raisins: a retrospective evaluation of 43 dogs (1992–2002). *J Vet Intern Med* **19**(5): 663–74.

Fooshee SK, Forrester SD (1990) Hypercalcemia secondary to cholecalciferol rodenticide toxicosis in two dogs. *J Am Vet Med Assoc* **196**(8): 1265–8.

Forrester SD, Fallin EA, Saunders GK, *et al*. (1993) Prevention of cisplatin-induced nephrotoxicosis in dogs, using hypertonic saline solution as the vehicle of administration. *Am J Vet Res* **54**(12): 2175–8.

Frazier K, Colvin B, Styer E, *et al*. (1998) Microcystin toxicosis in cattle due to overgrowth of blue-green algae. *Vet Hum Toxicol* **40**: 23–4.

Frazier KS, Liggett AD, Hines, ME, *et al*. (2000) Mushroom toxicity in a horse with meningioamatosis. *Vet Hum Toxicol* **42**: 166–7.

Gabrielson KL, Remillard RL, Huso DL (1996) Zinc toxicity with pancreatic acinar necrosis in piglets receiving total parenteral nutrition. *Vet Pathol* **33**: 692–6.

Galey FD, Whiteley HE, Goetz TE, *et al*. (1991) Black walnut (*Juglans nigra*) toxicosis: a model for equine laminitis. *J Comp Pathol* **104**(3): 313–26.

Galey FD, Holstege DM, Plumlee KH, *et al*. (1996) Diagnosis of oleander poisoning in livestock. *J Vet Diagn Invest* **8**(3): 358–64.

Gardner DR, Panter KE, James LF, *et al*. (1998) Abortifacient effects of lodgepole pine (*Pinus contorta*) and common juniper (*Juniperus communis*) on cattle. *Vet Hum Toxicol* **40**(5): 260–3.

Gaskill CL, Miller LM, Mattoon JS, *et al*. (2005) Liver histopathology and liver and serum alanine aminotransferase and alkaline phosphatase activities in epileptic dogs receiving phenobarbital. *Vet Pathol* **42**(2): 147–60.

Gaunt PS, Meuten DJ, Pecquet-Goad ME (1984) Hepatic necrosis associated with use of halothane in a dog. *J Am Vet Med Assoc* **184**(4): 478–80.

Gava A, da Silva Neves D, Gava D, *et al*. (2002) Bracken fern (*Pteridium aquilinum*) poisoning in cattle in southern Brazil. *Vet Hum Toxicol* **44**(6): 362–5.

Gelatt KN, van der Woerdt A, Ketring KL A, *et al*. (2001) Enrofloxacin-associated retinal degeneration in cats. *Vet Ophthalmol* **4**(2): 99–106.

George LW, Divers TJ, Mahaffey EA, *et al*. (1982) Heinz body anemia and methemoglobinemia in ponies given red maple (*Acer rubrum* L.) leaves. *Vet Pathol* **19**(5): 521–33.

Goldman AL (1992) Hypervitaminosis A in a cat. *J Am Vet Med Assoc* **200**(12): 1970–2.

Good AL, Czarnecki CM (1980) The production of cardiomyopathy in turkey poults by the oral administration of furazolidone. *Avian Dis* **24**(4): 980–8.

Gould DH (2000) Update on sulfur-related polioencephalomalacia. *Vet Clin North Am Food Anim Pract* **16**(3): 481–96.

Graham TW, Holmberg CA, KeenCL, *et al*. (1988) A pathologic and toxicologic evaluation of veal calves fed large amounts of zinc. *Vet Pathol* **25**: 484–91.

Griffiths LB, Done SH (1991) Citrinin as a possible cause of the pruritus pyrexia, and hemorrhagic syndrome of cattle. *Vet Record* **129**: 113–7.

Gross TL, Ihrke PJ, Walder EJ (1992) *Veterinary Dermatopathology: A Macroscopic and Microscopic Evaluation of Canine and Feline Skin Disease*, Mosby, Philadelphia, PA.

Gruber TA, Costigan P, Wilkinson GT, *et al*. (1978) Chronic methylmercurialism in the cat. *Aust Vet J* **54**(4): 155–60.

Gunson DE, Soma LR (1983) Renal papillary necrosis in horses after phenylbutazone and water deprivation. *Vet Pathol* **20**(5): 603–10.

Gunson DE, Kowalczyk DF, Shoop CR, *et al*. (1982) Environmental zinc and cadmium pollution associated with generalized osteochondrosis, osteoporosis, and nephrocalcinosis in horses. *J Am Vet Med Assoc* **180**(3): 295–9.

Gunther R, Felice LJ, Nelson RK, *et al*. (1988) Toxicity of a vitamin D3 rodenticide to dogs. *J Am Vet Med Assoc* **193**(2): 211–4.

Gustafsson A, Baverud V, Gunnarsson A, *et al*. (1997) The association of erythromycin ethylsuccinate with acute colitis in horses in Sweden. *Equine Vet J* **29**(4): 314–8.

Guzman RE, Solter PF (2002) *Characterization of sublethal microcystin-LR exposure in mice*. *Vet Pathol* **39**: 17–26.

Gwaltney-Brant SM, Albretsen JC, Khan SA (2000) 5-Hydroxytryptophan toxicosis in dogs: 21 cases (1989–1999). *J Am Vet Med Assoc* **216**(12): 1937–40.

Hackett TB, Wingfield WE, Mazzaferro EM, *et al*. (2002) Clinical findings associated with prairie rattlesnake bites in dogs: 100 cases (1989–1998). *J Am Vet Med Assoc* **220**(11): 1675–80.

Haliburton JC, Morgan SE (1989) Nonprotein nitrogen-induced ammonia toxicosis and ammoniated feed toxicity syndrome. *Vet Clin North Am Food Anim Pract* **5**(2): 237–49.

Hamilton TA, Cook Jr JR, Braund KG, *et al*. (1991) Vincristine-induced peripheral neuropathy in a dog. *J Am Vet Med Assoc* **198**(4): 635–8.

Hansen DE, McCoy RD, Hedstrom OR, *et al*. (1994) Photosensitization associated with exposure to *Pithomyces chartarum* in lambs. *J Am Vet Med Assoc* **204**(10), 1668–71.

Hansen SR, Buck WB, Meerdink G, *et al*. (2000) Weakness, tremors, and depression associated with macadamia nuts in dogs. *Vet Hum Toxicol* 42(1): 18–21.

Harkin KR, Cowan LA, Andrews GA, *et al*. (2000). Hepatotoxicity of stanozolol in cats. *J Am Vet Med Assoc* **217**(5): 681–4.

Harrison LH, Colvin BM, Stuart BP, *et al*. (1983) Paralysis in swine due to focal symmetrical poliomalacia: possible selenium toxicosis. *Vet Pathol* **20**: 265–73.

Harvey JW, Rackear D (1985) Experimental onion-induced hemolytic anemia in dogs. *Vet Pathol* **22**(4): 387–92.

Haschek WM, Gumprecht LA, Smith G, *et al*. (2001) Fumonisin toxicosis in swine: an overview of porcine pulmonary edema and current perspectives. *Environ Health Perspect* **109**(Suppl. 2): 251–7.

Helman RG, Edwards WC (1997) Clinical features of blister beetle poisoning in equids: 70 cases (1983–1996). *J Am Vet Med Assoc* **211**(8): 1018–21.

Hirono I, Ito M, Yagyu S, *et al*. (1993) Reproduction of progressive retinal degeneration (bright blindness) in sheep by administration of ptaquiloside contained in bracken. *J Vet Med Sci* **55**(6): 979–83.

Hooper PT, Best SM, Campbell A (1974) Axonal dystrophy in the spinal cords of cattle consuming the cycad palm, Cycas media. *Aust Vet J* **50**: 146–9.

Hooser SB, Van Alstine W, Kiupel M, *et al*. (2000) Acute pit gas (hydrogen sulfide) poisoning in confinement cattle. *J Vet Diagn Invest* **12**(3): 272–5.

Jacobs G, Calvert C, Kaufman A (1998) Neutropenia and thrombocytopenia in three dogs treated with anticonvulsants. *J Am Vet Med Assoc* **212**(5): 681–4.

JAVMA News: Aflatoxin Contamination (2006) Contaminated food leads to death, illness in dozens of dogs. *J Am Vet Med Assoc* **228**(3): 334.

Jensen R, Pier AC, Kaltenbach CC, *et al*. (1989) Evaluation of histopathologic and physiologic changes in cows having premature births after consuming Ponderosa pine needles. *Am J Vet Res* **50**(2): 285–9.

Jett GK, Herman EH, Jones MF, *et al*. (1988) Influence of minoxidil on myocardial hemodynamics, regional blood flow, and morphology in beagle dogs. *Cardiovasc Drugs Ther* **1**(6): 687–94.

Jones RD, Baynes RE, Nimitz CT (1992) Nonsteroidal anti-inflammatory drug toxicosis in dogs and cats: 240 cases (1989–1990). *J Am Vet Med Assoc* **201**(3): 475–7.

Jones TC, Hunt RD, King NW (1996) *Veterinary Pathology*, 6th edn. Williams and Wilkins, Baltimore, MD.

Kahn CM (2005) *The Merck Veterinary Manual.* Merck, Whitehouse Station, NJ.

Keeler RF, Balls LD (1978) Teratogenic effects in cattle of *Conium maculatum* and conium alkaloids and analogs. *Clin Toxicol* **12**(1): 49–64.

Keeler RF, Stuart LD (1987) The nature of congenital limb defects induced in lambs by maternal ingestion of *Veratrum californicum*. *J Toxicol Clin Toxicol* **25**(4): 273–86.

Kelly WR (1993) The urinary system. In *Pathology of Domestic Animals*, Jubb KVT, Kennedy PC, Palmer N (eds), 4th edn, vol. 2, Academic Press Inc., San Diego, CA, pp. 491–494.

Kerr LA, Linnabary RD (1989) A review of interstitial pneumonia in cattle. *Vet Hum Toxicol* **31**(3): 247–54.

Kerr LA, Kelch WJ (1998) Pigweed (*Amaranthus retroflexus*) toxicosis in cattle. *Vet Hum Toxicol* **40**(4): 216–18.

Khanna C, Boermans HJ, Wilcock B (1997) Fatal hypernatremia in a dog from salt ingestion. *J Am Anim Hosp Assoc* **33**(2), 113–7.

Knapp DW, Richardson RC, DeNicola DB, *et al*. (1987) Cisplatin toxicity in cats. *J Vet Intern Med* 1(1), 29–35.

Krook L, Wasserman RH, McEntee K, *et al*. (1975) Cestrum diurnum poisoning in Florida cattle. *Cornell Vet* **65**(4): 557–75.

Kul O, Karahan S, Basalan M, *et al*. (2006) Polioencephalomalacia in cattle: a consequence of prolonged feeding barley malt sprouts. *J Vet Med A Physiol Pathol Clin Med* **53**(3): 123–8.

Kurtz HJ, Nairn ME, Nelson GH, *et al*. (1969) Histologic changes in the genital tracts of swine fed estrogenic mycotoxin. *Am J Vet Res* **30**(4): 551–6.

Lairmore MD, Alexander AF, Powers BE, *et al*. (1984) Oxytetracycline-associated nephrotoxicosis in feedlot calves. *J Am Vet Med Assoc* **185**(7): 793–5.

Langston CE (2002) Acute renal failure caused by lily ingestion in six cats. *J Am Vet Med Assoc* **220**(1): 49–52.

Lascelles BD, Blikslager AT, Fox SM, *et al*. (2005) Gastrointestinal tract perforation in dogs treated with a selective cyclooxygenase-2 inhibitor: 29 cases (2002–2003). *J Am Vet Med Assoc* **227**(7): 1112–7.

Ledoux DR, Brown TP, Weibking TS, *et al*. (1992) Fumonisin toxicity in broiler chicks. *J Vet Diagn Invest* **4**(3): 330–3.

Liggett AD, Weiss RW (1989) Liver necrosis caused by mushroom poisoning in dogs. *J Vet Diagn Invest* **1**: 267–9.

Liggett AD, Colvin BM, Beaver RW, *et al*. (1986) Canine aflatoxicosis: a continuing problem. *Vet Hum Toxicol* **28**(5): 428–30.

Lomax LG, Cole RJ, Dorner JW (1984) The toxicity of cyclopiazonic acid in weaned pigs. *Vet Pathol* **21**: 418–24.

Ludwig J, Owen C, Barham S, *et al*. (1980) The liver in the inherited copper disease of Bedlington terriers. *Lab Invest* **43**: 82–7.

Luttgen PJ, Whitney MS, Wolf AM, *et al*. (1990) Heinz body hemolytic anemia associated with high plasma zinc concentration in a dog. *J Am Vet Med Assoc* **197**(10): 1347–50.

Loch WE, Swantner LD, Anderson RR (1987) The effects of four levels of endophyte-infected fescue seed in the diet of pregnant pony mares. *J Reprod Fertil Suppl* **35**: 535–8.

Locke LN, Thomas NJ (1996). *Noninfectious Disease of the Wild Life*, Fairbrother A, Locke LN, Gerald GN (eds). Iowa State University Press, Ames, IA.

Ma YM, Ma ZQ, Gui CQ, *et al*. (2003) Hepatotoxicity and toxicokinetics of ketoconazole in rabbits. *Acta Pharmacol Sin* **24**(8): 778–82.

MacAllister CG, Morgan SJ, Borne AT, *et al*. (1993) Comparison of adverse effects of phenylbutazone, flunixin meglumine, and ketoprofen in horses. *J Am Vet Med Assoc* **202**(1): 71–7.

MacPhail CM, Lappin MR, Meyer DJ, *et al*. (1998) Hepatocellular toxicosis associated with administration of carprofen in 21 dogs. *J Am Vet Med Assoc* **212**(12): 1895–901.

Mahin L, Marzou A, Huart A (1984) A case report of Nerium oleander poisoning in cattle. *Vet Hum Toxicol* **26**(4): 303–4.

Maiorka PC, Massoco CO, de Almeida SD, *et al*. (1998) Copper toxicosis in sheep: a case report. *Vet Hum Toxicol* **40**(2): 99–100.

Manabe J, Ogata T (1987) Lung fibrosis induced by diquat after intratracheal administration. *Arch Toxicol* **60**(6): 427–31.

Martin BW, Terry MK, Bridges CH, *et al*. (1981) Toxicity of *Cassia occidentalis* in the horse. *Vet Hum Toxicol* **23**(6): 416–7.

Mathur S, Constable PD, Eppley RM, *et al*. (2001) Fumonisin B(1) is hepatotoxic and nephrotoxic in milk-fed calves. *Toxicol Sci* **60**(2): 385–96.

Maylin GA, Eckerlin RH, Krook L (1987) Fluoride intoxication in dairy calves. *Cornell Vet* **77**(1): 84–98.

McAllister MM, Gould DH, Raisbeck MF, *et al*. (1997) Evaluation of ruminal sulfide concentrations and seasonal outbreaks of polioencephalomalacia in beef cattle in a feedlot. *J Am Vet Med Assoc* **211**(10): 1275–9.

McCormick GC, Weinberg E, Szot RJS, *et al*. (1985) Comparative ototoxicity of netilmicin, gentamycin, and tobramycin in cats. *Toxicol Appl Pharmacol* **77**(3): 479–89.

McLeay LM, Smith BL, Munday-Finch SC (1999) Tremorgenic mycotoxins paxilline, penitrem and lolitrem B, the non-tremorgenic 31-epilolitrem B and electromyographic activity of the reticulum and rumen of sheep. *Res Vet Sci* **66**(2): 119–27.

Merrill Jr AH, Sullards MC, Wang E, *et al*. (2001) Sphingolipid metabolism: roles in signal transduction and disruption by fumonisins. *Environ Health Perspect* **109**(Suppl. 2): 283–9.

Montali RJ, Bush M, Smeller JM (1979) The pathology of nephrotoxicity of gentamycin in snakes. A model for reptilian gout. *Vet Pathol* **16**(1): 108–15.

Morgan SE, Johnson B, Brewer B, *et al*. (1990) Sorghum cystitis ataxia syndrome in horses. *Vet Hum Toxicol* **32**(6): 582.

Mesfin GM, Robinson FG, Higgins MJ, *et al*. (1995) The pharmacologic basis of the cardiovascular toxicity of minoxidil in the dog. *Toxicol Pathol* **23**(4): 498–506.

Meteyer CU, Rideout BA, Gilbert M, *et al*. (2005) Pathology and proposed pathophysiology of diclofenac poisoning in free-living and experimentally exposed oriental white-backed vultures (*Gyps bengalensis*). *J Wildl Dis* **41**(4): 707–16.

Michel RP, Hakim TS, Smith TT, *et al*. (1983) Quantitative morphology of permeability lung edema in dogs induced by alpha-naphthylthiourea. *Lab Invest* **49**(4): 412–19.

Morrow CM, Valli VE, Volmer PA, *et al*. (2005) Canine renal pathology associated with grape or raisin ingestion: 10 cases. *J Vet Diagn Invest* **17**(3): 223–31.

Munday JS, Thompson LJ (2003) Brodifacoum toxicosis in two neonatal puppies. *Vet Pathol* **40**(2): 216–19.

Muylle E, Vandenhende C, Oyaert W, *et al*. (1981) Delayed monensin sodium toxicity in horses. *Equine Vet J* **13**(2): 107–8.

Nagata T, Kono I, Masaoka T, *et al*. (1992) Acute toxicological studies on paraquat: pathological findings in beagle dogs following single subcutaneous injections. *Vet Hum Toxicol* **34**: 105–11.

Newman S, Smith J, Stenske K, *et al*. (2006) Aflatoxicosis in a dog. *Proceedings of the 34th Annual Southeastern Veterinary Pathology Conference*, Tifton, GA, May 20–21.

Nobre D, Dagli ML, Haraguchi M (1994) Crotalaria juncea intoxication in horses. *Vet Hum Toxicol* **36**(5): 445–8.

Novilla MN (1992) The veterinary importance of the toxic syndrome induced by ionophores. *Vet Hum Toxicol* **34**(1): 66–70.

Oaks JL, Gilbert M, Virani MZ, *et al*. (2004) Diclofenac residues as the cause of vulture population decline in Pakistan. *Nature* **427**(6975): 630–3.

O'Hara PJ, Fraser AJ, James MP (1982) Superphosphate poisoning of sheep: the role of fluoride. *NZ Vet J* **30**(12): 199–201.

Okeda R, Shibutani M, Matsuo T, *et al*. (1988) Subacute neurotoxicity of 5-fluorouracil and its derivative, carmofur, in cats. *Acta Pathol Jpn* 38(10): 1255–66.

Olson CT, Keller WC, Gerken DF, *et al*. (1984) Suspected tremetol poisoning in horses. *J Am Vet Med Assoc* **185**(9): 1001–3.

Osweiler GD, Ross PF, Wilson TM, *et al*. (1992) Characterization of an epizootic of pulmonary edema in swine associated with fumonisin in corn screenings. *J Vet Diagn Invest* **4**(1): 53–9.

Osweiler GD, Carr TF, Sanderson TL (1995) Water deprivation-sodium ion toxicosis in cattle. *J Vet Diagn Invest* **7**: 583–5.

Osweiler GD (1996) *Toxicology* .Williams and Wilkins, Philadelphia, PA.

O'Toole D, Raisbeck MF (1997) Experimentally induced selenosis of adult mallard ducks: clinical signs, lesions, and toxicology. *Vet Pathol* **34**(4): 330–40.

Pace LW, Turnquist SE, Casteel SW, *et al*. (1997) Acute arsenic toxicosis in five horses. *Vet Pathol* **34**(2): 160–4.

Palmer N (1993) Disease of bone. In Jubb KVF, Kennedy PC, Palmer N (eds), *Pathology of the Domestic Animals*, 4 edn. Academic Press, San Diego, CA.

Pamukcu AM, Price JM, Bryan GT (1976) Naturally occurring and bracken fern induced bovine urinary bladder tumors. *Vet Pathol* **13**: 110–22.

Panciera RJ, Martin T, Burrows GE, *et al*. (1990) Acute oxalate poisoning attributable to ingestion of curly dock (*Rumex crispus*) in sheep. *J Am Vet Med Assoc* **196**(12): 1981–4.

Panciera RJ, Mosier DA, Ritchey JW (1992) Hairy vetch (*Vicia villosa* Roth) poisoning in cattle: update and experimental induction of disease. *J Vet Diagn Invest* **4**(3): 318–25.

Pang VF, Adams JH, Beasley VR, *et al*. (1986) Myocardial and pancreatic lesions induced by T-2 toxin, a trichothecene mycotoxin, in swine. *Vet Pathol* **23**(3): 309–10.

Pang VF, Lorenzana RM, Beasley VR, *et al*. (1987) Experimental T-2 toxicosis in swine. III. Morphologic changes following intravascular administration of T-2 toxin. *Fundam Appl Toxicol* **8**(3): 298–309.

Panter KE, Bunch TD, Keeler RF, *et al*. (1990) Multiple congenital contractures (MCC) and cleft palate induced in goats by ingestion of piperidine alkaloid-containing plants: reduction in fetal movement as the probable cause. *J Toxicol Clin Toxicol* **28**(1): 69–83.

Panter KE, Molyneux RJ, Smart RA, *et al*. (1993) English yew poisoning in 43 cattle. *J Am Vet Med Assoc* **202**(9): 1476–7.

Panter KE, Baker DC, Kechele PO (1996) Water hemlock (*Cicuta douglasii*) toxicoses in sheep: pathologic description and prevention of lesions and death. *J Vet Diagn Invest* **8**(4): 474–80.

Panter KE, Gardner DR, Molyneux RJ (1998) Teratogenic and fetotoxic effects of two piperidine alkaloid-containing lupines (*L. formosus* and *L. arbustus*) in cows. *J Nat Toxins* **7**(2): 131–40.

Panter KE, James LF, Gardner DR (1999) Lupines, poison-hemlock and Nicotiana spp: toxicity and teratogenicity in livestock. *J Nat Toxins* **8**(1): 117–34.

Patterson DS, Allen WM (1970) Calcium mobilisation and calciphylaxis in piglets induced by iron injections. *Vet Rec* **86**(26): 791.

Paulson SK, Engel L, Reitz B, *et al*. (1999) Evidence for polymorphism in the canine metabolism of the cyclooxygenase 2 inhibitor, celecoxib. *Drug Metab Dispos* **27**(10): 1133–42.

Pegram RA, Wyatt RD (1981) Avian gout caused by oosporein, a mycotoxin produced by *Caetomium trilaterale*. *Poult Sci* **60**(11): 2429–40.

Petruska JM, Beattie JG, Stuart BO, *et al*. (1997) Cardiovascular effects after inhalation of large doses of albuterol dry powder in rats, monkeys, and dogs: a species comparison. *Fundam Appl Toxicol* **40**(1): 52–62.

Pinto C, Santos VM, Dinis J, Peleteiro MC, *et al*. (2005) Pithomycotoxicosis (facial eczema) in ruminants in the Azores, Portugal. *Vet Rec* **157**(25): 805–10.

Polzin DJ, Stowe CM, O'Leary TP, *et al*. (1981). Acute hepatic necrosis associated with the administration of mebendazole to dogs. *J Am Vet Med Assoc* **179**(10): 1013–6.

Poppenga RH, Trapp AL, Braselton WE, *et al*. (1990) Hexachlorophene toxicosis in a litter of Doberman pinschers. *J Vet Diagn Invest* **2**(2): 129–31.

Plumlee KH, Johnson B, Galey FD (1998) Comparison of disease in calves dosed orally with oak or commercial tannic acid. *J Vet Diagn Invest* **10**(3): 263–7.

Prescott CW (1983) Clinical findings in dogs and cats with lead poisoning. *Aust Vet J* **60**(9): 270–1.

Prichard JT, Voss JL (1967) Fetal ankylosis in horses associated with hybrid Sudan pasture. *J Am Vet Med Assoc* **150**(8): 871–3.

Puschner B, Galey FD, Johnson B, *et al*. (1998a) Blue-green algae toxicosis in cattle. *J Am Vet Med Assoc* **213**: 1605–7.

Puschner B, Galey FD, Holstege DM, *et al*. (1998b) Sweet clover poisoning in dairy cattle in California. *J Am Vet Med Assoc* **212**(6): 857–9.

Putnam MR, Bransby DI, Schumacher J, *et al*. (1991) Effects of the fungal endophyte *Acremonium coenophialum* in fescue on pregnant mares and foal viability. *Am J Vet Res* **52**(12): 2071–4.

Rowe LD (1989) Photosensitization problems in livestock. *Vet Clin North Am Food Anim Pract* **5**(2): 301–23.

Raekallio MR, Hielm-Bjorkman AK, Kejonen J, *et al*. (2006) Evaluation of adverse effects of long-term orally administered carprofen in dogs. *J Am Vet Med Assoc* **228**(6): 876–80.

Rakich PM, Latimer KS, Mispagel ME, *et al*. (1993) Clinical and histologic characterization of cutaneous reactions to stings of the imported fire ant (*Solenopsis invicta*) in dogs. *Vet Pathol* **30**(6): 555–9.

Riond JL, Riviere JE, Duckett WM, *et al*. (1992) Cardiovascular effects and fatalities associated with intravenous administration of doxycycline to horses and ponies. *Equine Vet J* **24**(1): 41–5.

Reams RY, Janovitz EB, Robinson FR, *et al*. (1993) Cycad (*Zamia puertoriquensis*) toxicosis in a group of dairy heifers in Puerto Rico. *J Vet Diagn Invest* **5**(3): 488–94.

Rebhun WC, Tennant BC, Dill SG, *et al*. (1984) Vitamin K3-induced renal toxicosis in the horse. *J Am Vet Med Assoc* **184**(10): 1237–9.

Riet-Correa F, Mendez MC, Schild AL, *et al*. (1988) Agalactia, reproductive problems and neonatal mortality in horses associated with ingestion of *Claviceps purpurea*. *Aust Vet J* **65**: 192–3.

Robens J, Anthony HD (1980) Polychlorinated biphenyl contamination of feeder cattle. *J Am Med Assoc* **177**: 613–5.

Roberts BK, Aronsohn MG, Moses BL, Burk RL, Toll J, Weeren FR (2000). Bufo marinus intoxication in dogs: 94 cases (1997–1998). *J Am Vet Med Assoc* **216**(12): 1941–1944.

Rogers WA, Ruebner BH (1997) A retrospective study of probable glucocorticoid-induced hepatopathy in dogs. *J Am Vet Med Assoc* **170**(6): 603–6.

Ross PF, Ledet AE, Owens DL, *et al*. (1993) Experimental equine leukoencephalomalacia, toxic hepatosis, and encephalopathy caused by corn naturally contaminated with fumonisins. *J Vet Diagn Invest* **5**(1): 69–74.

Rubin SI, Krawiec DR, Gelberg, *et al*. (1989) Nephrotoxicity of amphotericin B in dogs: a comparison of two methods of administration. *Can J Vet Res*. 53(1): 23–8.

Rumbeiha WK, Francis JA, Fitzgerald SD, *et al*. (2004) A comprehensive study of Easter lily poisoning in cats. *J Vet Diagn Invest* **16**(6): 527–41.

Runnegar MT, Kong S, Berndt N (1993) Protein phosphatase inhibition and in vivo hepatoxicity of microcystins. *Am J Physiol* **265**: 224–30.

Rutgers HC, Batt RM, Vaillant C, *et al*. (1995) Subcellular pathologic features of glucocorticoid-induced hepatopathy in dogs. *Am J Vet Res* **56**(7): 898–907.

Rutili G, Kvietys P, Martin D, *et al*. (1982) Increased pulmonary microvasuclar permeability induced by alpha-naphthylthiourea. *J Appl Physiol* **52**(5): 1316–23.

Ryan PL, Bennett-Wimbush K, Vaala WE, *et al*. (2001) Systemic relaxin in pregnant pony mares grazed on endophyte-infected fescue: effects of fluphenazine treatment. *Theriogenology* **56**(3): 471–83.

Saunders GK, Blodgett DJ, Hutchins TA, *et al*. (2000) Suspected citrus pulp toxicosis in dairy cattle. *J Vet Diagn Invest* **12**(3): 269–71.

Scarratt WK, Collins TJ, Sponenberg DP (1985) Water deprivation-sodium chloride intoxication in a group of feeder lambs. *J Am Vet Med Assoc* **186**(9): 977–8.

Schmitz DG (1989) Cantharidin toxicosis in horses. *J Vet Intern Med* **3**(4): 208–15.

Schoeb TR, Panciera RJ (1979) Pathology of blister beetle (Epicauta) poisoning in horses. *Vet Pathol* **16**(1): 18–31.

Schultz RA, Coetzer JA, Kellerman TS, *et al*. (1982) Observations on the clinical, cardiac and histopathological effects of fluoroacetate in sheep. *Onderstepoort J Vet Res* **49**(4): 237–45.

Schneider DJ, Marasas WF, Dale Kuys JC, *et al*. (1979) A field outbreak of suspected stachybotryotoxicosis in sheep. *J S Afr Vet Assoc* **50**(2): 73–81.

Schutte JG, vanden Ingh TS (1997) micropthalmia, brachygnathia superior and palatocheiloshisis in a foal associated with griseofulvin administration to the mare during early pregnancy. *Vet Q* **19**: 58.

Scott DW, Miller WH, Griffin CE (2001) *Muller and Kirks Small Animal Dermatology*. Saunders, Philadelphia, PA.

Scott FW, LaHunta A, Schultz RD, *et al*. (1975) Teratogenesis in cats associated with griseofulvin therapy. *Teratology* **11**(1):79–86.

Sharma OP, Makkar HP, Dawra RK (1988) A review of the noxious plant Lantana camara. *Toxicon* **26**(11): 975–87.

Sheafor SE, Couto CG (1999) Anticoagulant rodenticide toxicity in 21 dogs. *J Am Anim Hosp Assoc* **35**(1): 38–46.

Shibutani M, Okeda R (1989) Experimental study on subacute neurotoxicity of methotrexate in cats. *Acta Neuropathol (Berlin)* **78**(3): 291–300.

Shupe JL (1980) Clinicopathologic features of fluoride toxicosis in cattle. *J Anim Sci* 51(3): 746–58.

Simpson CF, Rollinghoff W, Preisig R, *et al*. (1979) Hepatitis, cardiomyopathy and hemodynamics in furazolidone-induced round heart disease of turkeys. *Can J Comp Med* **43**(4): 345–51.

Smalley EB (1973) T-2 toxin. *J Am Vet Med Assoc* **163**(11): 1278–81.

Smith BL, Embling PP (1993) Sequential changes in the development of the pancreatic lesion of zinc toxicosis in sheep. *Vet Pathol* **30**: 242–7.

Selby LA, Case AA, Osweiler GD (1977) Epidemiology and toxicology of arsenic poisoning in domestic animals. *Environ Health Perspect* **19**: 183–9.

Soler Rodriguez F, Miguez Santiyan MP, Pedrera Zamorano JD, *et al*. (1991) An outbreak of lupinosis in sheep. *Vet Hum Toxicol* **33**(5): 492–4.

Sprangler WL, Adelman RD, Conzelman GM, *et al*. (1980) Gentamicin nephrotoxicity in the dog: sequential light and electron microscopy. *Vet Pathol* **17**: 206–17.

Stair EL, Edwards WC, Burrows GE, *et al*. (1993) Suspected red maple (*Acer rubrum*) toxicosis with abortion in two Percheron mares. *Vet Hum Toxicol* **35**(3): 229–30.

Stegelmeier BL, Gardner DR, James LF, *et al*. (1996) The toxic and abortifacient effects of ponderosa pine. *Vet Pathol* **33**(1): 22–8.

Stokol T, Randolph JF, Nachbar S, *et al*. (1997) Development of bone marrow toxicosis after albendazole administration in a dog and cat. *J Am Vet Med Assoc* **210**(12): 1753–56.

Strickland JR, Oliver JW, Cross DL (1993) Fescue toxicosis and its impact on animal agriculture. *Vet Hum Toxicol* **35**(5): 454–64.

Stuart LD, Oehme FW (1982) Environmental factors in bovine and porcine abortion. *Vet Hum Toxicol* **24**(6): 435–41.

Stuart BP, Nicholson SS, Smith JB (1975) Perirenal edema and toxic nephrosis in cattle, associated with ingestion of pigweed. *J Am Vet Med Assoc* **167**(10): 949–50.

Stuart BP, Cole RJ, Gosser HS (1981) Cocklebur (*Xanthium strumarium*, L. var. *strumarium*) intoxication in swine: review and redefinition of the toxic principle. *Vet Pathol* **18**(3): 368–83.

Stuart LD, James LF, Panter KE, *et al*. (1989) Pine needle abortion in cattle: pathological observations. *Cornell Vet* **79**(1): 61–9.

Suliman HB, Wasfi IA, Adam SE (1982) The toxicity of *Cassia occidentalis* to goats. *Vet Hum Toxicol* **24**(5): 326–30.

Summers BA, Cummings JF, De Lahunta A (1995a) Selenium poisoning. In *Veterinary Neuropathology*, 1st edn. Mosby, St. Louis, MO, pp. 258–61.

Summers BA, Cummings JF, De Lahunta A (1995b) Salt poisoning. In *Veterinary Neuropathology*. Mosby, St. Louis, MO, pp. 254–5.

Swan DA, Creeper JH, White CL, *et al*. (1998) Molybdenum poisoning in feedlot cattle. *Aust Vet J* **76**(5): 345–9.

Szczech GM (1975) Ochratoxicosis in Beagle dogs. *Vet Pathol* **12**(1): 66–7.

Szczech GM, Carlton WW, Tuite J, *et al*. (1973a) Ochratoxin A toxicosis in swine. *Vet Pathol* **10**(4): 347–64.

Szczech GM, Carlton WW, Tuite J (1973b) Ochratoxicosis in Beagle dogs. II. Pathology. *Vet Pathol* **10**(3): 219–31.

Talcott PA, Mather GG, Kowitz EH (1991) Accidental ingestion of a cholecalciferol-containing rodent bait in a dog. *Vet Hum Toxicol* **33**(3): 252–6.

Taylor NS, Dhupa N (2000) Acetaminophen toxicity in cats and dogs. *The Compendium* **22**(2): 160–9.

Tegzes JH, Puschner B (2002) Toxic mushrooms. *Vet Clin North Am Small Anim Pract* **32**: 397–407.

Thompson JP, Senior DF, Pinson DM, Moriello KA (1987) Neurotoxicosis associated with the use of hexachlorophene in a cat. *J Am Vet Med Assoc* **190**(10): 1311–2.

Thornburg L (2000) A perspective on copper and liver disease in the dog. *J Vet Diagn Invest* **12**: 101–10.

Thrall MA, Grauer GF, Mero KN (1984) Clinicopathologic findings in dogs and cats with ethylene glycol intoxication. *J Am Vet Med Assoc* **184**(1): 37–41.

Tiwary AK, Puschner B, Kinde H, *et al*. (2005) Diagnosis of Taxus (yew) poisoning in a horse. *J Vet Diagn Invest* **17**(3): 252–5.

Trueman KF, Clague DC (1978) Sodium chloride poisoning in cattle. *Aust Vet J* **54**(2): 89–91.

Twedt DC, Diehl KJ, Lappin MR, *et al*. (1997) Association of hepatic necrosis with trimethoprim sulfonamide administration in 4 dogs. *J Vet Intern Med* **11**(1): 20–3.

Uhlinger C (1989) Black walnut toxicosis in ten horses. *J Am Vet Med Assoc* **195**(3): 343–4.

Uzal FA, Puschner B, Tahara JM, Nordhausen RW (2005) Gossypol toxicosis in a dog consequent to ingestion of cottonseed bedding. *J Vet Diagn Invest* **17**(6): 626–9.

Vaala WE, Ehnen SJ, Divers TJ (1987) Acute renal failure associated with administration of excessive amounts of tetracycline in a cow. *J Am Vet Med Assoc* **191**(12): 1601–3.

van der Linde-Sipman JS, van den Ingh TS, van nes JJ, *et al*. (1999) Salinomycin-induced polyneuropathy in cats: morphologic and epidemiologic data. *Vet Pathol* **36**(2): 152–6.

Van Vleet JF, Ferrans VJ (1984) Ultrastructural alterations in the atrial myocardium of pigs with acute monensin toxicosis. *Am J Pathol* **114**(3): 367–79.

Villar D, Buck WB, Gonzalez JM (1998) Ibuprofen, aspirin and acetaminophen toxicosis and treatment in dogs and cats. *Vet Hum Toxicol* **40**(3): 156–62.

Wang E, Ross PF, Wilson TM, Riley RT, *et al*. (1992) Increases in serum sphingosine and sphinganine and decreases in complex sphingolipids in ponies given feed containing fumonisins, mycotoxins produced by Fusarium moniliforme. *J Nutr* **122**(8): 1706–16.

Watson AD (1977) Chloramphenicol toxicity in dogs. *Res Vet Sci* **23**(1): 66–9.

Watson AD (1980) Further observations on chloramphenicol toxicosis in cats. *Am J Vet Res* **41**(2): 293–4.

Watson AD, Middleton DJ (1978) Chloramphenicol toxicosis in cats. *Am J Vet Res* **39**(7), 1199–203.

Wieland T (1983) The toxic peptides from Amanita mushrooms. *Int J Pept Protein Res* **22**: 257–76.

Wilhelmsen CL, Pitt MLM (1996) Lesions of acute inhaled lethal ricin intoxication in rhesus monkeys. *Vet Pathol* **33**: 296–302.

Wilson TM, Scholz RW, Drake TR (1983) Selenium toxicity and porcine focal symmetrical poliomyelomalacia: description of a field outbreak and experimental reproduction. *Can J Comp Med* **47**(4): 412–21.

Witte ST, Osweiler GD, Stahr HM, *et al*. (1990) Cocklebur toxicosis in cattle associated with the consumption of mature *Xanthium strumarium*. *J Vet Diagn Invest* **2**(4): 263–7.

Woodard JC, Donovan GA, Fisher LW (1997) Pathogenesis of vitamin (A and D)-induced premature growth-plate closure in calves. *Bone* **21**(2): 171–82.

Woods LW, Johnson B, Hietala SK, *et al*. (1992) Systemic granulomatous disease in a horse grazing pasture containing vetch (*Vicia* sp.). *J Vet Diagn Invest* **4**(3): 356–60.

Woods LM, Filigenzi MS, Booth MC, *et al*. (2004) Summer pheasant's eye (*Adonis aestivalis*) poisoning in three horses. *Vet Pathol* **41**: 215–20.

Wyatt RD, Hamilton PB, Huff WE (1975) Nephrotoxicity of dietary ochratoxin A in broiler chickens. *Appl Microbiol* **30**(1): 48–51.

Zomborszky-Kovacs M, Vetesi F, Horn P, *et al*. (2002) Effects of prolonged exposure to low-dose fumonisin B1 in pigs. *J Vet Med B Infect Dis Vet Public Health* **49**(4): 197–201.

# Part 18

# Therapeutic Measures

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CHAPTER

# 91

# Prevention and treatment of poisoning

*Camille DeClementi*

## INTRODUCTION

This chapter discusses prevention of poisoning, stabilization and care of the poisoned patient, decontamination techniques and antidotal therapies. Clinicians may find it beneficial to approach these patients no differently than any other patient. Using the same principles of history collection, physical examination and patient monitoring will help the clinician to concentrate on *treating the patient and not the poison*. Poisoning cases, like all cases, are often not predictable and oftentimes no antidote is available. A treatment plan should be developed based on the circumstances and progression of the individual case. Toxicant exposures often require immediate or urgent attention, so stabilization of the patient is the first priority. Once that has occurred, the clinician must decide if attempts at decontamination are needed. If an appropriate antidotal therapy is available and was not already given to assist with patient stabilization, it should then be administered.

In all instances, prevention of poisoning is preferable to treating a poisoning once it has occurred. Clients rely on their veterinarians to provide a great deal of information about their animal's well-being. Veterinarians are a great source of information on husbandry, disease prevention and safety. They are therefore the ideal source of information for clients on preventing poisoning as well.

## PREVENTION OF POISONING

Prevention of poisoning in household pets consists of controlling the animals' environment to decrease exposure to potentially dangerous substances. This requires animal caretakers to be diligent and knowledgeable of potential risks. While much of the advice offered to prevent poisoning will seem like common sense to many veterinarians, these guidelines are often unfamiliar to animal caretakers. Veterinarians are therefore encouraged to share this information with their clients.

Clients should be reminded to keep all veterinary and human medications, both prescription and over-the-counter (OTC), out of the reach of animals. Since some pets are able to climb onto high surfaces and open cabinets, medications are not adequately "out of reach" in those places. Owners should be instructed not to give their pets any medication, including their own, unless directed by their veterinarian. Clients may not realize that giving an OTC medication that they consider safe, like acetaminophen (APAP), could cause life-threatening illness in their pet. For example, treatment of alopecia in cats with topical minoxidil solution has led to pleural effusion, pulmonary edema and death (DeClementi *et al*., 2004). Clients should also store all other potentially hazardous products, including cleaning products, auto-care products, pesticides and insecticides, out of the reach of their animals. Garbage cans should be sealed with tamper-proof lids.

Animal caretakers should be urged to read all label information before using a product on an animal or in the animal's environment and to follow the label instructions exactly. Veterinarians should mention that it is often not safe to use a product on an animal species for which it is not intended. For example, using a concentrated permethrin flea product labeled for dogs could prove deadly if used on a cat (Richardson, 2000a).

Since many plants are poisonous, clients will want to be aware of the plants in an animal's environment, including those in outside areas. Additionally, they should be alert to any fluids leaking from vehicles and clean up leaks

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immediately. One effective method to remove liquid is to allow clay cat litter to soak it up. The litter should then be swept up or vacuumed and disposed of in a manner that will prevent animal exposure. If a rodenticide or other bait is necessary in the home or yard, the product should be placed in an area that is completely inaccessible to non-target animals. The bait should be removed as soon as it is no longer needed. An animal's enclosure should be routinely checked, and unfamiliar or questionable items removed. Companion animals should be supervised, when possible, if they are outdoors, and a securely gated, confined area should be provided when animals are left unattended.

The guidelines for keeping household pets safe from poisoning are very similar to those for children, especially toddlers. Some pets may even be more at risk than children because, unlike children, they are commonly left unattended. They are also likely to chew open some containers, including those considered child-safe. The ASPCA website has additional information regarding poison prevention (www.aspca.org).

Decreasing the risk of exposure to toxicants is also important in large animals. Caretakers should be urged to purchase hay and feed from reputable suppliers, to examine the feed for recognizable contamination and to verify cleanliness and high quality. Feed should be stored in a clean, insect-free area at the appropriate moisture level to prevent damage and lessen the risk of contamination or mycotoxin growth (Osweiler, 2001).

Learning to identify poisonous plants and their potential effects is critical for large animal clients. Once poisonous plants have been recognized in an area, many strategies can be employed to limit exposure. The most effective strategy is to remove animals from poisonous plant-infested areas either by herding or fencing off those areas. If this is impossible, attempts should be made to reduce the poisonous plant populations via mechanical (burning or pulling), chemical (herbicides), biological (such as using the larvae of the cinnabar moth to control tansy ragwort) or other control methods. Since treatment with herbicides may increase palatability or toxicity in some plants, animals should not be allowed access to herbicide-treated areas until all plant material is dead and removed. Once the plant populations are controlled, good grazing management can be used to maintain the area in a condition that limits regrowth of the plants (Cheeke, 1998).

If grazing in contaminated areas cannot be avoided and plant numbers cannot be reduced, other techniques can be used. The stocking rates can be adjusted so that the animals have ample non-toxic forage available relative to the amount of poisonous plants present. Or the client may opt to graze a species of livestock on the area that is less susceptible to poisoning by the plants present. Another successful management practice is to graze livestock in the area only when the plants are at their lowest toxicity or when the plants are unpalatable and will likely not be eaten (Cheeke, 1998). For instance, only the seeds and two-leaf seedling stages of cocklebur (*Xanthium*) are toxic. Once the plant reaches the four-leaf seedling stage, it no longer presents a risk (Burrows and Tyrl, 2001). Avoiding certain plants during the times when livestock are most susceptible may also prevent poisoning. Cattle farmers, for example, can use timing as described above to prevent their calves from developing crooked calf disease. This disease is caused by the teratogenic alkaloids of many species of lupine plants. The fetus is only susceptible between days 40 and 70 of gestation. If pregnant cows are kept from grazing the plants during this time frame, the calves will not develop the disease (Cheeke, 1998).

Caretakers should avoid placing hungry animals onto ranges infested with poisonous plants. This is especially true when animals are released after being transported. Given that animals naïve to an area are more likely to be poisoned by indigenous plants than native animals, feeding prior to setting them out may help decrease exposure (Cheeke, 1998). Large animal enclosures and barns should also be kept free of other potential toxicants, including insecticides, pesticides, petroleum products and medicated feeds. Following label instructions is just as important for large animals as household pets. Medicated feeds, medications and insecticides should be used only on the labeled species. If a pour-on amitraz product intended for cattle is instead used on horses, fatal ileus may result (Gwaltney-Brant, 2004).

## STABILIZATION AND MONITORING

Toxicant exposures often require immediate or urgent attention. Television and movies have led the public to believe that every toxicant has an antidote; consequently animal caretakers may expect their veterinarian to provide one. Unfortunately, this expectation is far from reality. There are very few antidotes and when they do exist, they may be cost prohibitive or difficult to obtain. Therefore, it is critical for the clinician to concentrate on *treating the patient and not the poison*. Poisoning cases should be managed following the same principles of triage and patient stabilization as other emergencies. Decontamination and antidote administration, if available, should follow initial stabilization.

A detailed history should be taken after the patient is stabilized but the following questions should be asked on presentation. To what toxicant was the animal exposed? What amount? When did the exposure occur? Has the patient shown any effects? Have any treatments been performed (e.g. dilution, emesis or bathing)? Have other animals also been exposed?

As in any emergency case, initial evaluation and stabilization of the patient should address the basic ABCs (*a*irway, *b*reathing, *b*leeding, *c*ardiovascular, *c*irculation and level of *c*onsciousness). The clinician must assure that the patient has an adequate airway and is not having difficulty breathing. If needed, an endotracheal tube should be placed or a tracheostomy performed to establish a patent airway. If the patient is dyspneic, 100% oxygen should be delivered via oxygen cage, mask or nasal cannula (Mathews, 2006). Oxygen supplementation is contraindicated in a paraquat exposure (Oehme and Mannala, 2006). Stress and handling should be minimized in a dyspneic patient.

The patient should be checked for bruising and for signs of active bleeding from the nose, mouth, anus and vulva or penis. The mucous membrane color and capillary refill time should be evaluated. Pulse rate, rhythm and strength should be assessed. Electrocardiogram (ECG) and blood pressure monitoring should also be performed. If needed, an intravenous (IV) catheter should be placed and fluid therapy initiated. Attempts should be made to control life-threatening arrhythmias (Mathews, 2006).

The patient's level of consciousness should be determined. If the patient is actively seizing, diazepam, given as an IV bolus at a dosage of 0.5–1 mg/kg, is often the initial drug used for dogs and cats. If this is not effective, other medications including phenobarbital, pentobarbital and propofol may be useful. Inhalant anesthesia may also be used to control the seizure activity (Mathews, 2006). The reader is referred to a formulary or reference on emergency seizure control for recommendations on doses and drug choices for the species being treated. Since some diagnostic tests, including that for ethylene glycol, may give false results once injectable medications are administered, the clinician should obtain blood samples prior to administering these medications. Body temperature should be checked and thermoregulation initiated if needed.

Once the patient has been stabilized a more thorough clinical evaluation can be completed including complete history, physical examination and appropriate diagnostic testing (Cantilena, 2001), and a treatment plan developed. The signalment and health history of the patient is important in developing a treatment plan. If the patient is a nursing or pregnant female, for example, precautions will need to be taken to prevent exposure to the fetus or young. Or if the patient is taking a highly protein-bound medication, it is more at risk from an overdose of another protein-bound drug. If there is no known exposure to a toxicant but poisoning is suspected based on presentation and clinical findings, the questions in Table 91.1 may be helpful to reveal a cause (Mathews, 2006).

Next, a complete treatment plan should be developed. It should include what method, if any will be used to prevent further toxin absorption and if an antidotal therapy will be used. The plan should address any clinical signs not addressed in the initial stabilization. It should list what additional findings may yet develop and a plan of action for each. The plan should define how often the patient will be monitored and what indices will be evaluated. And it should determine what diagnostic tests are appropriate for the situation. The treatment plan may need to be updated as the case progresses.

**TABLE 91.1 History gathering questions**

- When was the patient last normal?
- How long have the signs been present?
- Were there any initial signs that are no longer present?
- Was the onset of the signs gradual or sudden?
- Are there other pets in the area? Are they affected?
- What was the location of the animal in the last few hours prior to development of signs? Was the animal supervised?
- Is the animal indoors or outdoors mostly?
- If the animal is outdoors, is it confined or does it roam?
- To what areas of the home or garage does the animal have access?
- Have any new foods or treats been introduced to the animal's diet?
- Any access to sugar-free products?
- Has there been any recent access to garbage?
- Has the animal chewed or destroyed anything recently?
- Are there any medications in the house (human, veterinary, prescription, OTC)?
- Could the animal have been exposed to illicit drugs?
- Are there children or teenagers in the household?
- Have there been any recent visitors who may have dropped medication?
- Are there any rodenticides or other baits being used in the home or yard?
- Could the animal have ingested any plants (indoors or outdoors)?
- Have any medications, herbal products or insecticides been administered to this or any other animals in the household recently?
- Are there any mushrooms growing in the yard?
- Is there a compost pile in the yard?
- Have any yard treatments been applied recently?
- Are there any livestock in the animal's environment?
- Have any livestock been recently euthanized or buried on the property?

Diagnostic testing will be governed by clinical findings, history and in a known exposure, expected effects. The clinician should perform baseline testing and repeat as required throughout the course of the treatment. Appropriate testing may include a packed cell volume (PCV) and total solids (TS) to identify dehydration or blood loss in cases of anticoagulant exposure or agents that may cause gastrointestinal (GI) ulceration. A complete blood count (CBC) may be needed to check for inflammation or secondary infections in cases where GI ulceration and perforation are possible, such as in exposures to cationic detergents, corrosive materials or non-steroidal anti-inflammatory drug (NSAID) medications. Monitoring the CBC is also indicated in exposures that may cause bone marrow suppression such as estrogen overdoses.

A full chemistry panel is often appropriate. Blood glucose should be monitored closely with exposures to sulfonylurea hypoglycemic agents and in exposures to the sugar-free sweetener xylitol in dogs (Dunayer, 2004). Monitoring the renal values, blood urea nitrogen (BUN) and serum creatinine is important in many exposures to identify dehydration and renal alterations if the patient was exposed to potential renal toxicants such as lilies in cats (Volmer, 1999), grapes and raisins in dogs (Eubig *et al.*, 2005), NSAIDs and ethylene glycol. In these cases, urinalysis is also indicated. Electrolytes should be monitored in cases where IV fluid therapy is required and in cases where the toxicant may lead to electrolyte abnormalities. For example, hypokalemia is a common effect in dogs that puncture albuterol inhalers (Vite and Gfeller, 1994).

Monitoring of acid–base status is important in some cases such as exposures to aspirin and ethylene glycol. The cardiovascular system will need to be monitored via ECG and evaluating blood pressure in exposures to certain agents such as pseudoephedrine and methylxanthines in chocolate. Other general testing including pulse oximetry, radiographs and ultrasound may be required depending on the toxicant. Specific tests, such as an ethylene glycol test or serum iron panel, may also be suitable depending on the situation.

In addition to these physiological parameters, the patient's clinical signs, vitals, behavior and mentation should be monitored frequently for the duration of the treatment and abnormalities addressed. The frequency and degree of monitoring will depend on the situation. The patient's attitude should be noted regularly. Respiratory and cardiovascular status should also be checked at regular intervals, especially in cases where these systems may be affected by the toxicant. Capillary refill time and mucous membrane color can be used to assess peripheral perfusion. Body temperature should be monitored if the toxicant, clinical findings or treatments may cause abnormalities. Appetite, hydration status, bowel movements and urinary output should also be noted (Mathews, 2006).

# DECONTAMINATION

Once the patient has been stabilized, decontamination should be considered to prevent additional exposure to the toxicant. Although the basics of decontamination are similar amongst species, the specific method of decontamination that is chosen in each case must be guided by the exposure circumstances and the species exposed. For all decontamination methods, consider sedation or anesthesia if the procedure will be very stressful for the patient but only if the health of the patient will allow. The handler should wear appropriate protective clothing including gloves, mask and eye gear to prevent personal exposure. Most exposures to toxicants can be broken down into ocular, dermal and oral exposures. Following are methods of decontamination for each of these exposure types.

## Ocular exposure

Ocular exposures may cause effects ranging from mild irritation to corrosive injury and blindness depending on the substance, the concentration, the exposure time and the sensitivity of the patient. With any ocular exposure, the eyes should be flushed repeatedly with tepid tap water or saline solution for a minimum of 20–30 min (Rosendale, 2002). An eyedropper can be used for smaller patients like birds or reptiles. With a larger patient, fill a plastic cup and slowly pour the contents over the ocular area. Patients can be given a mild sedative prior to flushing if needed and if the health of the patient will allow. If not sedated, the patient should be allowed to rest at regular intervals during the flushing to minimize stress. Fluorescein staining should be performed after flushing and again at 12–24 h after exposure to check for corneal ulceration. Additional treatment with ophthalmic and systemic medications may be necessary.

## Dermal exposure

Dermal exposure to a large variety of substances, including petroleum products, pesticides and insecticides, corrosive or irritating materials and substances that are sticky (tar, asphalt, sap and glue) may occur. Removal of such substances may be less stressful for the patient and safer for the handler if sedation is used. Sedatives should only be used if the health of the patient will allow. If not sedated, the patient should be allowed to rest at regular intervals to minimize stress.

In birds, dermal substances can be removed by using a water bottle to spritz the bird lightly with room temperature water. This procedure should be done in a warm environment to prevent chilling. The bird should be misted until the feathers no longer smell or feel of the product. If misting alone does not remove the product, and a soap is needed, a liquid dishwashing detergent (e.g. Dawn™) may be diluted in the bottle and applied. After removal of the substance, the bird should be rinsed via misting with plain water until all soap is removed. In cases of heavy exposure, birds may be bathed with liquid dishwashing detergent and rinsed well. After misting or bathing, the bird should be wiped with a dry towel and kept in a warm environment away from drafts until completely dry. The procedure for reptiles is similar.

For dermal exposures in mammals, bathing in a mild liquid dishwashing detergent and warm water is recommended. Baths may be repeated in order to remove the toxicant completely (Rosendale, 2002). The animal should then be rinsed well with warm water and towel dried, then kept in a warm environment until completely dry. For smaller patients, like cats, that resent being sprayed with water, the bucket technique may be helpful. Fill a bucket with warm soapy water and, while supporting the hind legs, immerse the patient up to the neck. Remove the patient and continue washing. Use a fresh bucket with plain warm water to rinse well.

If the patient has a sticky substance on its fur, feathers or skin, do not use solvents to remove it since these may be irritating or corrosive to the patient. Instead, to remove sticky substances from mammals, remove as much of the substance as possible by trimming the fur. Then work a small amount of vegetable oil, mineral oil, mayonnaise or peanut butter through the rest of the substance until it breaks down into "gummy balls". Afterward, wash with liquid dishwashing detergent as described above (Rosendale, 2002). For birds, do not trim the feathers, just use vegetable oil, mineral oil, mayonnaise or peanut butter and then mist as described above.

## Oral exposure

When a patient is exposed to a potentially dangerous substance by ingestion, the clinician has many options for decontamination including dilution, induction of emesis, lavage, removal via endoscopy or gastrotomy, use of adsorbents, cathartics and administration of enemas. Oftentimes the best treatment plan will include more than one of these methods.

### *Dilution*

Dilution with a small amount of milk or water is recommended in cases where irritants or corrosive materials have been ingested. A suggested dose is 2–6 ml/kg (Mathews, 2006) which would be approximately only 1–2 teaspoons in an average-sized cat. It is important to use only a small amount of liquid for dilution. Using excessive amounts could lead to vomiting and re-exposure of the esophagus to the damaging material (Rosendale, 2002). For birds and reptiles, juicy fruits and vegetables can be fed to accomplish dilution. Dilution is not recommended in patients who are at an increased risk for aspiration, including those who are obtunded (Rosendale, 2002) or actively seizing. Additionally, milk, yogurt and cottage cheese have been useful as demulcents in cases of oral irritation following ingestion of plants containing insoluble calcium oxalate crystals (*Philodendron* species, for example) (Means, 2004b).

### *Emetics*

Emetics are usually most effective if used within 2–3 h post-ingestion (Rosendale, 2002) but in some instances emesis may be effective even after that time frame. For instance, if a timed-released medication was ingested or if the substance ingested could coalesce to form a bezoar in the stomach, emesis may be effective later than 3 h after the ingestion. Chocolate (Albretsen, 2004) and chewable medications may form bezoars. If the patient has not eaten in the previous 2 h, feeding a small moist meal before inducing vomiting can increase chances of an adequate emesis. Emetics generally empty 40–60% of the stomach contents (Beasley and Dorman, 1990).

Dogs, cats, ferrets and potbelly pigs are examples of domestic animals that are able to vomit safely. Emetics should not be used in rodents, rabbits, birds, horses and ruminants. Rodents are unable to vomit (Plumb, 2005) and rabbits have a thin-walled stomach putting them at risk for gastric rupture if they vomit (Donnelly, 2004). It is not safe to induce emesis in birds, horses or cattle.

Induction of emesis is contraindicated with ingestion of alkalis, acids or other corrosive agents. When one of these products is swallowed, the protective epithelial lining of the esophagus may be damaged. This damage can leave the muscular layer of the esophagus exposed and at risk for ulceration, perforation and scarring if vomiting does occur (Beasley and Dorman, 1990). Emesis is not recommended after ingestion of petroleum distillates due to the risk of aspiration. Pre-existing conditions of the patient that can cause vomiting to be hazardous, such as severe cardiac disease or seizure disorder, must also be taken into account by the clinician when deciding whether to induce emesis. In all instances the attending veterinarian must weigh the benefits of emesis against the risks. Emesis should not be attempted if the animal has already vomited or is exhibiting clinical signs such as coma, seizures or recumbency, which make emesis hazardous. Additionally, if the patient has ingested a central nervous system (CNS) stimulant and is already agitated, the additional stimulation of vomiting could elicit seizures (Rosendale, 2002).

### *Hydrogen peroxide*

Hydrogen peroxide, at a 3% concentration, is a useful emetic when given orally. It is an ideal emetic for household use because the 3% concentration is used in many households for cuts and scrapes. Additionally, it is inexpensive and easy to administer. Hydrogen peroxide is thought to induce emesis via gastric irritation (Peterson, 2006). The dosage is 1–2 ml/kg (Beasley and Dorman, 1990) generally not to exceed 50 ml for dogs and potbelly pigs and 10 ml for cats and ferrets (Peterson, 2006). The dose can be administered with a syringe or turkey baster (Rosendale, 2002) or can be mixed with a small amount of milk or ice cream to entice voluntary ingestion. Vomiting usually occurs within

10–15 min and the dose can be repeated once if emesis is not initially successful. Walking or other gentle movement may be beneficial in initiating emesis. More concentrated solutions of hydrogen peroxide, as found in hair bleaching agents or other products, should not be utilized to induce emesis as they can lead to protracted, severe vomiting and significant irritation of the GI mucosa (Rosendale, 2002).

### *Apomorphine hydrochloride*

Apomorphine hydrochloride is often used in the clinical setting to induce emesis in dogs. Use of apomorphine is considered controversial in cats since an effective and safe dose has not been established (Plumb, 2005). Nonetheless many veterinarians, including the author, have used apomorphine effectively in cats with limited adverse effects (Rosendale, 2002). Apomorphine hydrochloride is a synthetic opiate that stimulates the dopamine receptors in the chemoreceptor trigger zone to cause emesis. The dosage is 0.04 mg/kg intravenously (IV) or intramuscularly (IM). Emesis is expected rapidly following IV administration but may take 5 min with IM administration. Apomorphine can also be used conjunctivally by crushing and dissolving a portion of a tablet in a few drops of water. The solution is then administered into the conjunctival sac at a dose of 0.25 mg/kg (Plumb, 2005). This route may be preferred since the eye can be rinsed after emesis has occurred to prevent additional systemic absorption and decrease the likelihood of adverse events. At doses used to induce emesis, adverse effects may include CNS depression and protracted vomiting. CNS stimulation and respiratory and cardiac depression may be seen with excessive doses (Plumb, 2005). The opiate antagonist, naloxone, can be used to reverse the CNS and respiratory effects, but will not block the emetic effect (Rosendale, 2002).

### *Xylazine hydrochloride*

Xylazine hydrochloride is a potent $\alpha_2$-adrenergic agonist used in the veterinary clinic setting primarily as a sedative. It has also been used, with some success, as an emetic in cats (Beasley and Dorman, 1990). Xylazine does not produce predictable emesis in dogs (Plumb, 2005). The recommended emetic dose in cats is 0.44 mg/kg IM. This dose is lower than the dose used for sedation in cats. Emesis is expected within 5 min of administration. Possible adverse effects include CNS and respiratory depression, hypotension and bradycardia (Plumb, 2005). These effects, as well as the emetic effects (Beasley and Dorman, 1990), can be reversed by giving an $\alpha_2$-adrenergic antagonist; either yohimbine at a dosage of 0.5 mg/kg IV or atipamezole at a dosage of 50 mcg/kg IM (Plumb, 2005).

### *Other emetics*

Other emetics have been recommended by various sources, including table salt, liquid dishwashing liquid, syrup of ipecac and powdered mustard. However, these are not as effective as those mentioned, and salt and syrup of ipecac may cause significant adverse effects. Salt (sodium chloride) has been associated with hypernatremia and CNS dysfunction (Beasley and Dorman, 1990) and there are concerns that syrup of ipecac can be cardiotoxic (Rosendale, 2002). Human pediatricians no longer routinely recommend syrup of ipecac for home use. Further, the American Association of Poison Control Centers reported in 2001 that the use of ipecac in human exposures has fallen by >95% over a 15-year period (Shannon, 2003).

### *Lavage*

Lavage is sometimes used in cases where emesis is contraindicated, not possible or has been unsuccessful. If the patient is agitated, seizing or recumbent or has other health concerns, such as recent abdominal surgery, that increase the risks associated with induction of emesis, lavage is an option. Lavage should also be considered in species, like rabbits and rodents, which are unable to vomit safely. Lavage is unlikely to remove as much ingested toxicant as emesis (Beasley and Dorman, 1990) and is associated with significant potential risks (Rosendale, 2002). For these reasons, it should not be chosen haphazardly as a decontamination method over emesis. Lavage should also not be used to remove caustic substances or volatile hydrocarbons for the same reasons emesis is contraindicated in such cases (Rosendale, 2002).

Gastric lavage can be used in small mammals to remove recently ingested toxicants. If the patient is a species with cheek pouches, the cheek pouches should be emptied gently with a finger or swab prior to the lavage. General anesthesia must be performed when performing gastric lavage unless the patient is comatose. In all instances, a cuffed endotracheal tube should be in place to prevent aspiration. Body temperature water (5–10 ml/kg) should be instilled via a large bore gastric tube with a fenestrated end, inserted to a length equal to the distance from the nose to the xiphoid cartilage (Beasley and Dorman, 1990). The head of the patient should be kept lower than the chest throughout the procedure. Gravity should be used to instill the water by holding the tube higher than the patient and then drained by moving the tube lower than the patient. The flushing process should be repeated multiple times (15–40) until the lavage fluid runs clear. With each flush, approximately the same amount of fluid instilled should be removed. Occlude the free end of the tube before removal to prevent aspiration (Rosendale, 2002). The initial washings should be saved for toxicological testing if needed (Peterson, 2006).

When both gastric and intestinal decontamination is considered necessary, enterogastric lavage can be considered. The procedure involves first performing gastric lavage and leaving the lavage tube in place. A retrograde

high enema is then performed until the enema fluid passes through the lavage tube and runs clear (Peterson, 2006). Administration of a preanesthetic dose of atropine has been recommended prior to performing enterogastric lavage to relax the smooth muscles of the GI tract (Rosendale, 2002). This procedure is labor intensive and carries the same, although intensified, risks as lavage and should only be considered in cases where the benefits clearly outweigh the risks of the procedure.

In humans, hypernatremia following lavage with normal saline and hyponatremia following lavage with water have been reported. Additionally, one human study showed that as much as 25% of the fluid used for lavage passed into the small intestine. Thus there is a concern that lavage may actually propel a toxicant into the small intestine where the absorptive surface area is greater. These risks should be limited by allowing the entire amount of fluid to drain out after each flush and by using only gravity to instill the water. Other risks associated with gastric lavage include esophageal or stomach damage or perforation, hypothermia and the accidental placement of the tube in the trachea and the instillation of fluid into the lungs (Rosendale, 2002).

A lavage technique can also be used in birds to remove recently ingested toxicants from the crop. To prevent injury to patient and handlers, anxious and fractious birds should be anesthetized prior to the procedure. If the patient is under general anesthesia, an endotracheal tube should be placed to protect the airway (Richardson *et al.*, 2001). The patient should be held with the head up and the mouth held open. The bird's head and neck should be extended to minimize esophageal damage. An appropriately sized feeding tube (soft plastic or rubber) is then passed into the crop. The crop should be palpated to assure correct placement. Then 10–20 ml/kg of warm saline is infused into the crop, the crop is massaged gently and the liquid is aspirated. The first washing can be kept for toxicological evaluation if needed. The cycle is repeated three or four times (Echols, 2005).

### *Adsorbents*

Adsorbents may be utilized in addition to or instead of emesis or lavage to prevent further systemic absorption of a toxicant. These agents act by adsorbing to a chemical or toxicant in the upper GI tract and facilitating its excretion via the feces. The most commonly used adsorbent is activated charcoal. In the past, kaolin–pectin (Kaopectate®) had also been recommended as an adsorbent and demulcent in some instances. Kaolin is a form of clay (hydrated aluminum silicate) and pectin is a purified carbohydrate derived from fruits. However, Kaopectate®, and most generic kaolin–pectin combinations, now contain bismuth subsalicylate as the active ingredient instead of kaolin and pectin. Some commercial activated charcoal products also contain kaolin (Vet-A-Mix, Toxiban®). Another clay bentonite (colloidal hydrated silica) is recommended in some literature. In most instances, activated charcoal is a superior absorbent to the clays. Burnt toast had been recommended in the past but is no longer considered to be an effective adsorbent (Beasley and Dorman, 1990).

Activated charcoal is composed of large porous particles that are able to adsorb and therefore trap a wide range of organic compounds within the GI tract. It is created from materials such as coal, wood, rye starch and coconut shells through a process using steam and acid treatments. The surface binding area of these products is quite large, in the range of 900–1500 $m^2/g$ (Rosendale, 2002). Charcoal tablets and capsules, found in pharmacies and used to control flatulence and bloating, are not likely to be as effective as the commercially prepared products (Buck and Bratich, 1986) as the concentration of the charcoal is often low and may have a smaller binding area.

The recommended dose of activated charcoal for all species of animals is 1–3 g/kg (or 1–3 mg/g) body weight (Buck and Bratich, 1986). There are many products on the market, including both liquid and powder forms. If using the powder formulation in dogs and cats, the total calculated dose of activated charcoal is mixed with 50–200 ml of warm tap water to make a slurry (Rosendale, 2002). In horses and ruminants, each gram of activated charcoal powder is mixed with 3–5 ml of water (Plumb, 2005). In symptomatic small animal patients and large animals, the activated charcoal liquid or prepared slurry is administered via stomach tube (Bailey and Garland, 1992). Small animals receiving activated charcoal via stomach tube should be sedated and have a cuffed endotracheal tube in place to prevent aspiration. In small animal patients where no clinical effects are present, activated charcoal can be given orally with a large syringe or can be mixed with a small amount of canned food or chicken broth and offered to the patient (Rosendale, 2002). Some patients, especially dogs, will voluntarily ingest the mixture. Many birds will regurgitate a portion of the activated charcoal dose given and some dogs and cats will vomit after administration.

Repeated doses of activated charcoal may be indicated in some instances such as cases where toxicants undergo enterohepatic recirculation. The first step in this process involves the toxicant being carried to the liver by either the portal vein after absorption from the GI tract or via the systemic circulation. Once in the liver the toxicant then enters the bile and is excreted into the GI tract where it is again available for absorption. Many toxicants are known to undergo this type of recycling, including ibuprofen, marijuana and digoxin.

Another instance where multiple doses of activated charcoal are appropriate is ivermectin toxicosis. Ivermectin is a substrate for the P-glycoprotein pump that transports some drugs across cell membranes. This pump is found in various cells including intestinal epithelial cells and brain

capillary endothelial cells. In the intestine, ivermectin enters the enterocyte by absorption from the GI tract. However, once in the cell, the P-glycoprotein pump acts to move the ivermectin across the membrane and back into the GI lumen. This cycling allows the ivermectin molecules to have multiple opportunities to bind with the repeated doses of activated charcoal. Loperamide, diltiazem and doxyrubicin are some other substrates of P-glycoprotein (Mealey, 2006).

When repeated doses are indicated, half the original dose should be given at 4–8 h intervals, often for 2–3 days (Peterson, 2006). It is important to mention that with medications that are excreted in the bile, activated charcoal can be of benefit regardless of the route the medication was administered. Thus if a patient received an overdose of injectable ivermectin subcutaneously, activated charcoal will still be a very valuable decontamination option. The ivermectin molecules will be carried to the GI tract by the bile and then be subject to the P-glycoprotein pump within the enterocytes.

The use of activated charcoal does carry some risks and it does not bind all compounds equally. Some chemicals that are not effectively adsorbed include: ethanol, methanol, fertilizer, fluoride, petroleum distillates, most heavy metals, iodides, nitrates, nitrites, sodium chloride and chlorate. Activated charcoal should not be given to animals that have ingested caustic materials since it is unlikely to bind them, it can be additionally irritating to the mucosal surfaces and make visualization of oral and esophageal burns difficult (Buck and Bratich, 1986). If ethylene glycol testing will be performed, activated charcoal should be administered after blood is collected, since propylene glycol found in many formulations can cause a false positive on this test. Additionally, the timing of the activated charcoal administration should be taken into account when deciding on dosing of other oral medications since the charcoal can also bind them. In APAP overdose in humans, for example, it is recommended that *N*-acetylcysteine (NAC) not to be administered orally until 3 h after activated charcoal (Plumb, 2005).

Administration of activated charcoal carries a significant risk of aspiration. If the patient does aspirate the charcoal, the prognosis is poor. Hence proper placement of the stomach tube and a protected airway is a must in symptomatic patients. The patient may also experience constipation and black bowel movements making it difficult to determine if melena is present. If the activated charcoal sits within the GI tract for a significant period of time, it may release the compound it has adsorbed, leading to systemic absorption and recurrence of the clinical signs. It is for this reason that activated charcoal is frequently administered with a cathartic. In fact, many commercially available preparations do contain a cathartic such as sorbitol.

Another possible adverse effect of activated charcoal administration is the development of hypernatremia. In humans, hypernatremia has been reported primarily in children when multiple doses of a charcoal–sorbitol mixture were administered. The hypernatremia is attributed to a water shift from the intracellular and extracellular spaces into the GI tract as a result of the osmotic pull of the sorbitol cathartic (Allerton and Strom, 1991). The ASPCA Animal Poison Control Center (APCC) has also received reports of elevated serum sodium following activated charcoal administration in dogs. Hypernatremia seems to be more often reported in small dogs receiving multiple doses of activated charcoal, but it has also been reported in large dogs and in cases receiving only a single dose. Furthermore, unlike the human reports, hypernatremia has also been noted in cases where no cathartic was present in the charcoal product given (APCC, unpublished data). Perhaps one of the other components of the product is also osmotically active. In these cases, the APCC has found that administration of a warm water enema is very effective at lowering the serum sodium and easing the resultant CNS effects.

### *Cathartics*

Cathartics enhance elimination of substances, including activated charcoal, by promoting their movement through the GI tract. Since activated charcoal only binds to toxicants by weak chemical forces, without cathartics the bound toxicant can eventually be released and reabsorbed (Rosendale, 2002). When used with activated charcoal, the cathartic should be given immediately following or mixed with the charcoal. Cathartics are contraindicated if the animal has diarrhea, is dehydrated, if ileus is present, or if intestinal obstruction or perforation is possible (Peterson, 2006).

There are bulk, osmotic and lubricant cathartics. The most commonly used bulk cathartic is psyllium hydrophilic mucilloid (e.g. Metamucil®). Psyllium is found in the ripe seed coatings of *Plantago* species. It absorbs water and swells in the intestine increasing bulk to induce peristalsis and decrease GI transit time. The dose in dogs is 1–2 tablespoons, and the dose in cats is 1–4 teaspoons (Plumb, 2005). Psyllium can also be used in birds by mixing ½ teaspoon with 60 ml of baby food to form a gruel, then administering by a dosing syringe or eyedropper (Richardson *et al.*, 2001).

Another bulking cathartic that can be used in dogs and cats is unspiced canned pumpkin. Dilute peanut butter, fruit or vegetables can also be used as bulking cathartic agents in birds and reptiles. Timothy hay can be utilized in rabbits. Bulking cathartics are also used to assist the passage of physical agents through the GI tract (Beasley and Dorman, 1990). Examples include small pieces of plastic, coins, button batteries and sand in horses (Plumb, 2005).

Osmotic cathartics have limited absorption from the GI tract so that they are able to pull electrolyte-free water

into the GI tract, thereby increasing the fluid volume and stimulating motility to hasten expulsion in the feces. There are saline and saccharide osmotic cathartics. Sorbitol is the most commonly used saccharide osmotic cathartic; it is the cathartic of choice and is frequently combined with activated charcoal in commercially prepared charcoal products. The dose is 1–2 ml/kg of a 70% solution (Peterson, 2006). Sorbitol can be utilized in mammals, birds and reptiles. The saline cathartics include sodium sulfate (Glauber's salts) and magnesium sulfate (Epsom salts). The recommended dose is 250 mg/kg. The use of magnesium sulfate has led to hypermagnesemia in some cases which presents as depression of the CNS and cardiovascular systems (Rosendale, 2002). Saline cathartics should not be used in patients with renal insufficiency or in birds or reptiles.

Of the lubricant cathartics, mineral oil is the most often used. Heavy mineral oil is preferred to light mineral oil since it is thought to carry less of a chance of systemic absorption and aspiration following oral administration. Mineral oil lubricates fecal material and the intestinal mucosa easing elimination and reduces absorption of water from the GI tract, increasing fecal bulk and cutting transit time (Plumb, 2005). Mineral oil is not recommended as a cathartic following activated charcoal administration as the mineral oil may render the charcoal less adsorptive (Buck and Bratich, 1986; Galey, 1992). Mineral oil has been recommended to bind lipid-soluble toxicants including metaldehyde, nitrate and cantharidin (Stair and Plumlee, 2004; Plumb, 2005) and is used to treat impaction in horses (Buck and Bratich, 1986). The recommended dose in horses is 2–4 l per 500-kg body weight (Brown and Bertone, 2001). Possible adverse effects include diarrhea and aspiration of the oil leading to lipid pneumonitis. Ensuring proper placement of the stomach tube and slow administration lessen the risk of aspiration (Plumb, 2005).

As mentioned above, hypernatremia attributed to sorbitol administration has been reported in humans. Because all cathartics alter the water balance in the GI tract, electrolyte abnormalities, especially hypernatremia, are a potential risk to their use. A patient's hydration status should be monitored frequently and fluids administered, IV or via an enema, as needed.

### *Enemas*

Enemas are also appropriate when elimination of toxicants from the lower GI tract is desired (Beasley and Dorman, 1990). Many extended-release or controlled-release medications are absorbed from the entire GI tract, including significant absorption from the colon (Buckley *et al.*, 1995). An enema is used to move those medications quickly through the colon and lessen additional systemic effects. The general technique is to use plain warm water or warm soapy water. Commercial phosphate enema solutions should be avoided due to the risk of electrolyte and acid–base disturbances (Beasley and Dorman, 1990). Enemas are not recommended for birds since they already have a rapid GI transit time. In reptiles, enemas may be useful since ingested materials often lag for prolonged periods in the colon.

In some cases, *endoscopy* or *gastrotomy* may be indicated to prevent further clinical effects. Endoscopy can be used to remove small objects such as pennies, lead paint chips and small batteries. Pennies, minted after 1982, contain a significant amount of zinc which can be released by the acidic environment of the stomach. Since zinc toxicosis can cause hemolytic anemia, it is prudent to remove the coin to prevent release and systemic absorption of the zinc (Richardson *et al.*, 2002). Coins can be removed via gastrotomy if endoscopy is not available. Gastrotomy is also used to prevent obstruction from expanding foreign objects. Uncooked yeast dough can rise in the warm environment of the stomach and produce an obstructive mass. In addition, as the dough rises, ethanol is released which can be absorbed from the stomach and lead to alcohol poisoning (Means, 2003). Expandable isocyanate-containing glues (i.e. Gorilla Glue™) can swell once ingested to fill the entire volume of the stomach. A gastrotomy is needed in these cases to remove the mass (Horstman *et al.*, 2003).

## ANTIDOTAL THERAPIES

If an antidote to the toxicant a patient has been exposed to exists, the appropriate time to administer it will depend on the situation. In some instances, the antidote will be crucial in stabilizing the patient. For example, atropine should be used immediately in cases of organophosphate (OP) toxicosis in which the patient has life-threatening bradycardia and bronchial secretions (Gwaltney-Brant, 2002). On the other hand, in some cases, administration of the antidote is best done later. This is true for vitamin $K_1$ being used in anticoagulant rodenticide exposures. In a recent exposure, decontamination first would be the most appropriate course of action. In an anticoagulant patient that is symptomatic, supportive measures like a blood transfusion should be started first since the vitamin $K_1$ will not be immediately lifesaving (Merola, 2002).

Unfortunately few antidotes exist. There is little economic incentive for pharmaceutical companies to seek approval for antidotal medications with only a small projected market (Post and Keller, 1999). Additionally, organizing clinical trials for antidotal medications is uniquely problematic. The US Food and Drug Administration (FDA) has offered incentives to develop antidotal therapies through the Orphan Drug Act. As a result, at least one antidotal therapy was released for veterinary

patients: fomepizole was approved for use in treating ethylene glycol intoxication (Cantilena, 2001). In situations where an antidote does exist, its use may be limited by its expense or availability.

Antidotes are generally divided into three groups, based on the mechanism by which they are protective. The groups are chemical antidotes, pharmacological antidotes and functional antidotes. Chemical antidotes act directly on the toxicant. They may decrease the toxicity of the agent or increase its excretion. Pralidoxime chloride, for example, does both; it binds to OP insecticide molecules making them unable to bind to their target and the pralidoxime–insecticide complex is then readily excreted (Mowry *et al.*, 1994).

Pharmacological antidotes antagonize the poison at the target site. Flumazenil, for example, has a high affinity for benzodiazepine receptors, thus it competes with benzodiazepines to reverse their depressive effects (Gwaltney-Brant, 2002). Functional antidotes act to lessen the symptoms of the poisoning. They have no interaction with the toxicant. An example of a functional antidote is the use of methocarbamol to control fasciculations and tremors associated with tremorgenic mycotoxins (Schell, 2000). Below are some examples of antidotal therapies available for use in veterinary medicine.

## Antidotes for amitraz: atipamezole and yohimbine

Amitraz, a synthetic formamidine pesticide, is used topically to control ticks, mites and lice on cattle, pigs and dogs, as well as to treat demodectic mange in dogs (Grossman, 1993). It is also available in a collar form (Preventic®) for tick control in dogs. The acaracide action of amitraz is not well understood, but it may have effects on the CNS of susceptible organisms. It also exhibits significant $\alpha$-2 adrenergic agonist activity (Plumb, 2005). This activity is believed to be responsible for the clinical signs associated with amitraz toxicosis including ataxia, CNS depression, hypotension, hyperglycemia, mydriasis, hypothermia, GI stasis and bradycardia (Grossman, 1993; Gwaltney-Brant, 2004). In dogs, toxicosis can occasionally result from exposure to a topical product but is more commonly due to ingestion of an amitraz-containing collar (Grossman, 1993; Gwaltney-Brant, 2004).

### *Atipamezole*

Atipamezole (Antisedan®) is an $\alpha$-2 adrenergic antagonist labeled for use as a reversal agent for the sedative medetomidine. It has been used successfully in the treatment of amitraz toxicosis to reverse CNS depression, bradycardia, GI stasis and hyperglycemia (Grossman, 1993; Gwaltney-Brant, 2004). The recommended dose in dogs is 50 mcg/kg IM (Plumb, 2005). The drug has a good margin of safety; dogs injected with 10 times the therapeutic dose did not have significant effects. Potential adverse effects of atipamezole administration include vomiting, diarrhea, hypersalivation, a brief period of excitation or apprehensiveness and tremors (Plumb, 2005). In cases where an amitraz collar was ingested and has not been retrieved from the GI tract, the atipamezole dose may need to be repeated each time signs recur until the collar is passed through the GI tract.

### *Yohimbine*

Yohimbine is another $\alpha$-2 adrenergic antagonist that has been used to counter the toxic effects of amitraz. The recommended dose in dogs is 0.1 mg/kg IV. Yohimbine has a short half-life (1.5–2 h in dogs), consequently the dose may need to be repeated. Potential adverse effects include temporary apprehension or CNS excitation, tremors, hypersalivation, elevated respiratory rate and hyperemic mucous membranes. Careful dosing is recommended, because tremors and seizures have been reported in dogs receiving five times the therapeutic dose (Plumb, 2005).

## Antidotes for anticholinesterase agents: atropine and pralidoxime

### *Atropine*

Atropine is used to counter the muscarinic effects of anticholinesterase (AChE) agents. Acetylcholine (ACh) is a neurotransmitter that transmits impulses at cholinergic nerve synapses and neuromuscular junctions. The enzyme AChE is responsible for the catabolism of ACh. AChE agents, which include the carbamate and OP insecticides, produce their effects by binding with AChE to disrupt the break down of ACh. The ACh accumulates within the synapse leading to over-stimulation of the end organ (Meerdink, 2004). There are two basic cholinergic receptor types: muscarinic and nicotinic. The muscarinic are the postganglionic parasympathetic receptors in smooth muscle, the heart and endocrine glands. Over-stimulation of these receptors leads to a complex of signs, often described as SLUDDE, which includes *s*alivation, *l*acrimation, *u*rinary incontinence, increased peristalsis and *d*iarrhea, increased bronchial secretions and *d*yspnea, miosis, nausea, *e*mesis and abdominal discomfort. Excessive stimulation of the nicotinic receptors, which are found in the skeletal muscles and autonomic ganglia, leads to tremors, and possibly seizures, potentially followed by muscle fatigue, weakness and paralysis (Meerdink, 1989).

Atropine competes with the accumulated Ach in the synapse to block only the muscarinic effects. Atropine does not block the nicotinic effects. The dosage used to counter AChE agents is: dogs and cats 0.2–2 mg/kg; cattle

0.5 mg/kg; horses 0.22 mg/kg. In all of those species, ¼ of the dose is given IV and the remainder intramuscularly (IM) or subcutaneously (SC) (Plumb, 2005). In some cases, administration of atropine may need to be repeated but great care should be exercised to prevent over-atropinization. Auscultation should be performed to monitor the patient for bradycardia and continued bronchial secretions, since these are the most life-threatening of the muscarinic signs. Additional atropine should only be given if these signs are present. The patient will not die from miosis or hypersalivation. Horses are quite susceptible to ileus caused by atropine administration, and a total dose of no more than 65 mg is recommended for a horse of average weight (Meerdink, 2004).

Atropine is not an effective antidote for other types of insecticides including pyrethroids. Because some of the signs of pyrethroid exposure, including tremors, seizures and hypersalivation, are also present with AChE agents, some veterinarians may erroneously administer atropine in these cases. Atropine is not only ineffective for pyrethroid toxicosis but in fact contraindicated, because it could exacerbate the CNS stimulatory effects and tachycardia that may be present in these cases. When presented with a suspected case of AChE exposure, the clinician can use a test dose of atropine to assist in making a preliminary diagnosis. The patient should be given the preanesthetic dose of atropine (0.02 mg/kg) IV. If this dose is able to produce typical anticholinergic signs such as mydriasis and tachycardia, then the patient has likely NOT been poisoned by an AChE agent (Fikes, 1990). If the patient truly had been poisoned by an AChE agent, the dose of atropine required to produce those effects would be at least 10 times higher (0.2 mg/kg).

### *Pralidoxime chloride*

Pralidoxime chloride (2-PAM) is used with atropine in the treatment of OP poisoning to relieve nicotinic signs such as tremors and muscle weakness (Fikes, 1990). 2-PAM reactivates the acetylcholinesterase enzyme (AChE) that has been inactivated by the OP. Normally acetylcholine (ACh) binds to the enzyme at the anionic binding site. OPs and carbamates bind nearby on the esteric site thus physically blocking the anionic site from ACh and inactivating the enzyme. 2-PAM is able to squeeze in via nucleophilic attack and bind to the anionic binding site. It then attaches to the OP forming a pralidoxime–OP complex. This complex detaches from the enzyme reactivating it and is then excreted in the urine (Fikes, 1990; Mowry *et al.*, 1994; Plumb, 2005). Please see Figure 91.1.

Administration of 2-PAM is most effective if given within 24 h of exposure (Plumb, 2005). If the OP remains attached to the AChE much longer, aging of the bond may occur so that it can no longer be broken by 2-PAM (Meerdink, 1989; Mowry *et al.*, 1994). There are instances when later administration is warranted. For example, in large OP exposures, pralidoxime may still be of some benefit if given within 36–48 h (Plumb, 2005). And 2-PAM may still be indicated even later if clinical signs have been present for an extended period of time (Fikes, 1992). Please see Table 91.2 for dosing information.

Treatment with 2-PAM should continue until the animal is asymptomatic. If no improvement is seen after 24–36 h following initiation of treatment, 2-PAM should be discontinued (Fikes, 1990). In acute feline chlorpyrifos toxicosis, cats with persistent tremors can be maintained on 1–2 times daily treatment for up to 4 weeks (Fikes, 1992). The typical presentation in these cats differs from the classical signs expected from other OPs. The onset may be delayed 1–5 days and the cats have neurological signs, including tremors, especially of the muscles of the back, neck and top of head, ataxia and seizures in addition to non-specific depression and anorexia which can persist 2–4 weeks (Fikes, 1992).

As indicated in Table 91.2, when given IV, administration of 2-PAM should be slow. Rapid IV administration can cause tachycardia, muscle rigidity, transient neuromuscular blockade and laryngospasm (Plumb, 2005). At therapeutic doses, 2-PAM is generally safe and has no significant adverse effects (Plumb, 2005). However, careful dosing is recommended. At high doses, 2-PAM may exhibit AChE activity including muscle weakness, ataxia, vomiting, hyperventilation, seizures, respiratory arrest and death. The LD50 in dogs is 190 mg/kg (Plumb, 2005).

Patients receiving 2-PAM should be monitored for hypersensitivity reactions (Plumb, 2005). 2-PAM is generally not recommended for carbamate toxicosis because AChE inhibition due to carbamates is rapidly reversible (Plumb, 2005), and 2-PAM has less affinity for carbamates than OPs (Meerdink, 2004). In addition, there is evidence that 2-PAM can reduce the protective effects of atropine in the treatment of one carbamate, carbaryl (Fikes, 1990). Since the drug is excreted by the kidneys, patients with underlying renal impairment should receive a decreased dose and be monitored closely for signs of toxicity (Plumb, 2005).

## Cyproheptadine HCl

Cyproheptadine HCl has been successful in treating serotonin syndrome in dogs and people (Gwaltney-Brant, 2002). Cyproheptadine is an antihistamine that is most commonly utilized in veterinary practice as an appetite stimulant for cats. It also is a potent serotonin antagonist (Plumb, 2005).

Serotonin is a neurotransmitter in the CNS. It also acts to promote platelet aggregation and as a stimulant on the smooth muscle of the respiratory, GI and cardiovascular systems. The term serotonin syndrome is used to describe

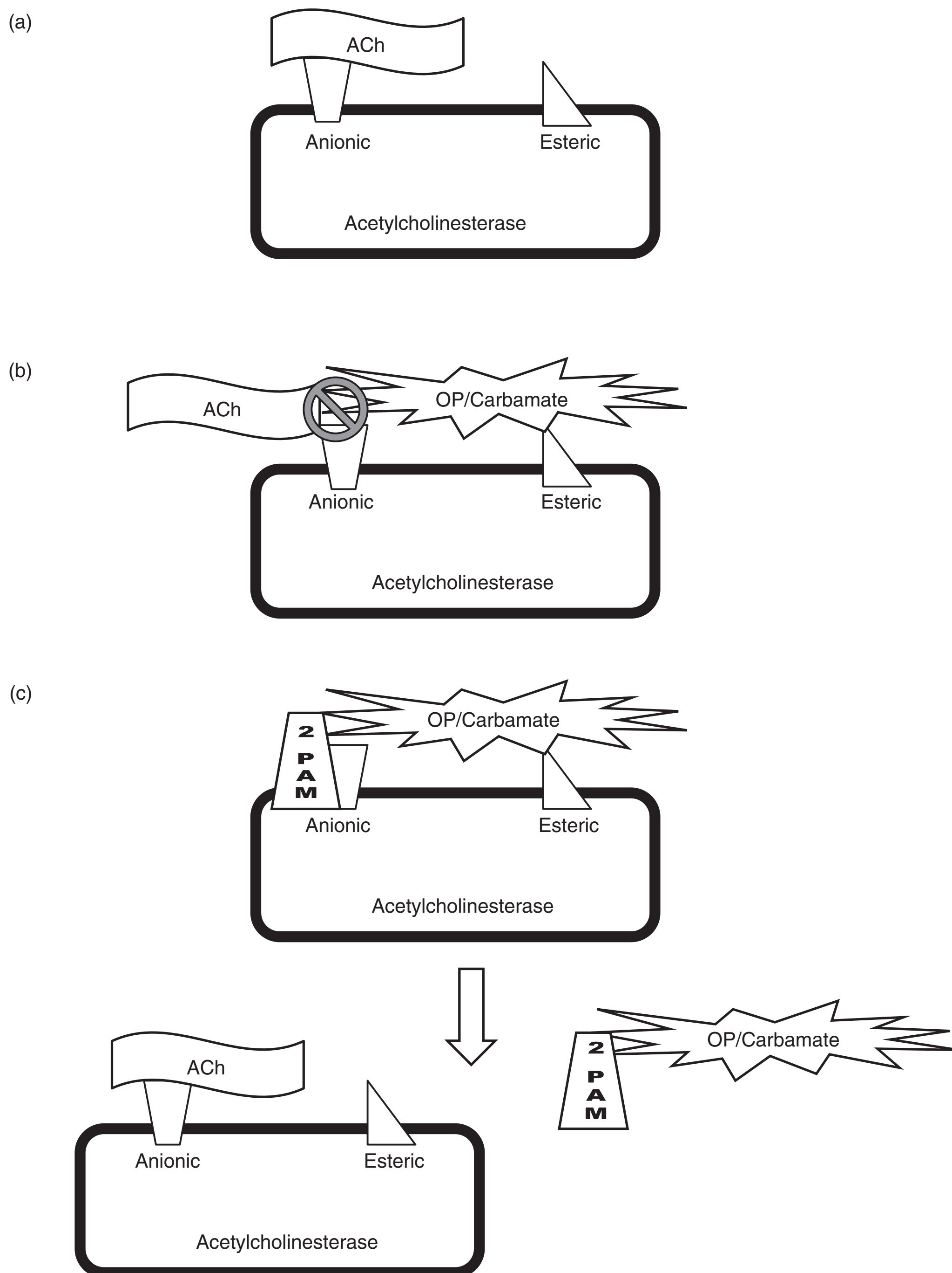


**FIGURE 91.1** Acetylcholinesterase binding and 2-PAM (Mowry et al., 1994). (a) ACh binds normally to the anionic site; (b) ACh cannot bind to the anionic site because the OP is blocking it; (c). 2-PAM slips in to bind to the site, forms 2-PAM-OP complex and detaches to reactivate the enzyme.

the characteristic signs which develop from excessive serotonin including autonomic, neuromuscular, behavioral and cognitive abnormalities (Gwaltney-Brant *et al.*, 2000). Excess serotonin may result from use or accidental overdose of medications that increase brain serotonin levels. These medications include selective serotonin reuptake inhibitors (SSRIs), like venlafaxine (Effexor®), paroxetine HCl (Paxil®) and fluoxetine HCl (Prozac®), as well as 5-hydroxytryptophan which is a serotonin precursor sold OTC as a dietary supplement. See Table 91.3 for a list of medications that carry a high potential of increasing brain serotonin levels (Gwaltney-Brant, 2002).

Dogs with serotonin syndrome typically have hyperthermia, central nervous abnormalities including tremors, seizures, ataxia, excitation or depression and hyperesthesia, and GI effects of vomiting, diarrhea and abdominal discomfort (Gwaltney-Brant, 2002). Death is possible if the signs are not controlled quickly. When used as a serotonin antagonist, the recommended dose of cyproheptadine in dogs is 1.1 mg/kg up to every 8 h until signs do

**TABLE 91.2 Pralidoxime dosing in OP treatment**

| Species | Dosing instructions |
|---|---|
| Dogs and cats | Pralidoxime works best when combined with atropine<br>Pralidoxime at 20 mg/kg, 2–3 times a day<br>Initial dose may be given either IM or slow IV<br>Subsequent doses may be given IM or SQ |
| Cattle | 25–50 mg/kg as a 20% solution IV over 6 min or as a maximum of 100 mg/kg/day as a CRI IV infusion |
| Horses | 20 mg/kg (may require up to 35 mg/kg) slow IV and repeat every 4–6 h |

**TABLE 91.3 Drugs with high serotonergic potential**

| | |
|---|---|
| Amitriptyline (e.g. Elavil®) | Lithium |
| Amphetamines (e.g. Adderall®) | Meperidine (e.g. Demerol®) |
| Clomipramine (e.g. Clomicalm®) | Moclobemide |
| Dexfenfluramine | Paroxetine (e.g. Paxil®) |
| Fenfluramine (e.g. Ponderal®) | Phenelzine |
| Fluoxetine (e.g. Prozac®) | Selegiline (e.g. Anipryl®) |
| Hydroxytryptophan | Sertraline (e.g. Zoloft®) |
| Imipramine (Tofranil®) | Tranylcypromine |
| Isocarboxazid | Tryptophan |
| | Venlafaxine (e.g. Effexor®) |

not recur (Gwaltney-Brant, 2006). The dose can be given orally if the patient is alert, not vomiting and activated charcoal was not given within 2 h. In those instances, the dose can be crushed, mixed with a small amount of saline and given per rectum. The drug should be discontinued after the first 2 doses if little or no improvement is noted (Gwaltney-Brant *et al.*, 2000). A similar dose of cyproheptadine has also been of some benefit in controlling vocalization and disorientation in some cases of baclofen toxicosis (Wismer, 2004).

## Digoxin immune Fab

Digoxin immune Fab fragments (e.g. Digibind® from Glaxo SmithKline) are a promising treatment for life-threatening digoxin toxicosis. The Fab fragments are used as a specific antidote for digoxin since they inactivate the drug by directly binding to it. The fragments are produced by first immunizing sheep with digoxin–human albumin complexes. In response, the sheep produce antibodies that are collected, purified and cleaved with papain into Fab fragments and Fc portions (Kittleson and Kienle, 1998). Digoxin immune Fab fragments are quite expensive, so their use may be cost prohibitive and they may be difficult to obtain. A local human hospital pharmacy may be willing to sell the product to the veterinary clinic if needed.

**TABLE 91.4 Plants containing cardiac glycosides**

| Plant species | Common name |
|---|---|
| *Acokanthera oblongifolia* | |
| *Adonis microcarpa* | Pheasant's eye |
| *Asclepias physocarpa* | Balloon cotton bush |
| *Byrophyllum tubiflorum* | Mother of millions |
| *Calotropis procera* | King's crown |
| *Carissa laxiflora* | |
| *Cerbera manghas* | Sea mango |
| *Convallaria majalis* | Lily of the valley |
| *Crytostegia grandioflora* | Rubber vine |
| *Helleboros* | |
| *Nerium oleander* | Oleander |
| *Thevetia neriifolia* | Yellow oleander |

Digoxin and other digitalis glycosides are thought to cause their effects by inhibition of the sodium–potassium ATPase pump (Na/K ATPase) through competition with potassium for binding sites (Kittleson and Kienle, 1998). Fab fragments can actually remove a digoxin molecule that is bound to the ATPase since their affinity for digoxin is much stronger than the affinity of digoxin for the Na/K ATPase target (Gwaltney-Brant, 2002). Fab fragments may also be used as an effective antidote for digitoxin toxicosis since they bind to it, though with less affinity than for digoxin. Moreover, sufficient cross reactivity exists with cardiac glycosides derived from *Bufo* toads and a wide variety of plants; consequently, Fab fragments may be effective at controlling toxicoses from these agents as well (Clark *et al.*, 1991; Gwaltney-Brant, 2002). Please see Table 91.4 for a list of plants containing cardiac glycosides that Fab fragments may be effective against.

The antidotal use of Fab fragments is indicated for treatment of potentially life-threatening cardiac glycoside toxicoses. Fab fragments should be considered when the patient has developed cardiac arrhythmias that are unresponsive to other antiarrhythmic therapy (Gwaltney-Brant, 2002). Ideally, the appropriate dose of Fab fragments is determined by using the patient's serum digoxin level. In some cases, the laboratory at a local human hospital may be willing to perform serum levels for the veterinarian. If a serum level can be obtained, the following formula is used to calculate the number of vials (Gwaltney-Brant, 2002). This formula assumes each vial will bind 0.5 mg of digoxin:

$$\text{Dose (number of vials)} = \text{Serum digoxin concentration (ng/ml)} \times \text{Body weight (kg)}/100$$

In cases where the cost of the calculated number of vials is prohibitive, if digoxin levels cannot be measured, or if the Fab fragments are being used to treat another cardiac

glycoside, treatment can be initiated with 1–2 vials and the patient observed for improvement. The reconstituted Fab fragments are administered over approximately 30 min. If the clinician feels the patient's clinical signs are immediately life threatening, the product can instead be given as a bolus. Fab fragments act quickly and dramatically. The patient's cardiac status is expected to begin improving within 20–90 min of administration and complete resolution of the clinical effects is usually seen within 4 h (Gwaltney-Brant, 2002). If significant improvement does not occur, additional vials may need to be given.

While monitoring the patient, it is important to note that the total serum digoxin concentration will be markedly increased with most commercial assays after Fab fragment administration. This occurs because previously tissue-bound digoxin binds to the Fab fragments, and the Fab–digoxin complexes then move into the blood to be excreted by the kidneys (Ward *et al.*, 1999). Since these patients have a compromised cardiovascular system as a result of the toxicosis, it is likely that renal perfusion is also compromised. These animals should be monitored carefully because decreased renal clearance of Fab–digoxin complexes may allow for dissociation of the toxin and recurrence of signs (Gwaltney-Brant, 2002).

The patient should also be monitored for hypokalemia. Elevated serum potassium is expected with cardiac glycoside toxicosis due to interference with the Na/K ATPase pumps. After administration of the Fab fragments, potassium moves quickly back into the cells leading to a significant drop in serum potassium levels. Serum potassium levels should be checked frequently within the first few hours and hypokalemia treated if needed (Gwaltney-Brant, 2002). The patient should also be monitored for anaphylaxis, fever and hypersensitivity reactions (Ward *et al.*, 1999).

## Antidotes for ethylene glycol: ethanol and fomepizole

### *Ethanol*

Ethanol can be used as an antidote for ethylene glycol poisoning. Ethylene glycol is found in many products at many concentrations. Most animal intoxications occur from ingestion of antifreeze since these products may have concentrations of ethylene glycol exceeding 90%. With intoxication, the GI, central nervous, cardiopulmonary and renal systems can be affected. Death is usually due to acute renal failure. The ethylene glycol itself does not cause the life-threatening clinical findings. It must be converted, primarily in the liver, to its more toxic metabolites including glycolic, glycoxalic and oxalic acids (Dalefield, 2004). The first step in the metabolism is conversion of the parent ethylene glycol molecule to glycoaldehyde via the enzyme alcohol dehydrogenase (ADH). Ethanol is also metabolized by ADH. It acts to block this conversion by competing for the enzyme, allowing time for ethylene glycol to be excreted unchanged in the urine (Mathews, 2006). Early intervention is very important to prevent the conversion to toxic metabolites. If a toxic dose was ingested and treatment is not initiated within a few hours of exposure, the patient's prognosis is grave.

**TABLE 91.5 Ethanol dosing in ethylene glycol treatment**

| | Intermittent dosing | CRI |
|---|---|---|
| Dogs | • Start with a 20% solution<br>• Give 5.5 ml/kg IV q4 h for 5 treatments<br>• Then give 5.5 ml/kg IV q6 h for 4 additional treatments | • Start with a 5% solution<br>• Give at CRI rate of 5.5 ml/kg/h until ethylene glycol test is negative |
| Cats | • Start with a 20% solution<br>• Give 5 ml/kg IV q6 h for 5 treatments<br>• Then give 5 ml/kg IV q8 h for 4 additional treatments | • Start with a 5% solution<br>• Give at CRI rate of 5 ml/kg/h |

The dosing recommendations for ethanol vary among authors. Intermittent dosing is possible but a constant rate infusion (CRI) is preferred to avoid high blood concentrations of the ethanol which can acerbate the potential clinical effects (Mathews, 2006). Please see Table 91.5 for examples of intermittent and CRI dosing recommendations for dogs and cats (Plumb, 2005). The ethylene glycol test by PRN Pharmaceutical Inc. can be used in dogs to determine when it is safe to discontinue ethanol treatment. The test measures serum concentrations $>50$ mg/dl. Since ethanol interrupts the metabolism of ethylene glycol, ethanol treatment should be continued until the test is negative indicating the parent ethylene glycol has been eliminated via the kidneys (Mathews, 2006). The test is not sensitive enough to be used in cats since they are significantly more sensitive to ethylene glycol intoxication than dogs and could develop life-threatening effects at serum concentrations $<50$ mg/dl. Treatment duration in cats should be based on clinical signs and laboratory findings; treatment may need to continue for more than 18 h (Mathews, 2006).

Ethanol is easy to obtain and is inexpensive. However, significant adverse effects will likely develop at the high doses required for treatment of ethylene glycol toxicosis including severe respiratory and CNS depression and metabolic acidosis. Since the signs of ethylene glycol intoxication mirror those effects, gauging patient progress can be challenging (Plumb, 2005).

### *Fomepizole*

Fomepizole, also called 4-methylpyrazole (4MP), is marketed as Antizol-Vet® by Jazz Pharmaceuticals as an antidote for ethylene glycol toxicosis in dogs. Recent studies

have found that 4MP can also be used in cats but must be used at higher doses to be effective (Connally *et al.*, 2002). 4MP is a synthetic ADH inhibitor that works similar to ethanol to prevent the conversion of ethylene glycol to its toxic metabolites. However, while ethanol acts by competing for the enzyme, 4MP forms a complex with ADH and its coenzyme (Connally *et al.*, 1996). Fomepizole is significantly more expensive than ethanol but has a therapeutic advantage because it causes fewer serious side effects (Gaddy, 2001). Dose-related CNS depression is the only adverse effect expected and tends to be mild if it develops. Cats are more at risk, because they require higher doses. Since 4MP has few serious risks, treatment is recommended in cases where exposure to ethylene glycol is suspected but not confirmed (Gwaltney-Brant, 2002).

4MP is excreted in the urine and accumulates over time depending on the dosage amount and frequency of administration. Since the drug accumulates, lower doses are given following the initial dose (Gaddy, 2001). In dogs, an initial dose of 20 mg/kg of the 5% reconstituted solution is given IV. At 12 and 24 h after the first dose, additional doses of 15 mg/kg are given. Finally, at 36 h after the first dose, a dose of 5 mg/kg is given. In cases where the patient has not fully recovered following the above doses or if an ethylene glycol test is positive, additional dosing of 3 mg/kg every 12 h is recommended (Gwaltney-Brant, 2002). This should continue until a negative ethylene glycol test indicates that the parent ethylene glycol has been eliminated via the kidneys.

The dosing schedule in cats is an initial dose of 125 mg/kg IV, then subsequent doses at 12, 24 and 36 h with 31.25 mg/kg (Plumb, 2005). Treatment in cats with either 4MP or ethanol must begin within 3 h post-ingestion of ethylene glycol or prognosis is grave. In dogs, fomepizole is most effective if given within 3–6 h of ethylene glycol ingestion; however some benefit was seen as late as 36 h post-ingestion. A late dose may prevent additional injury to the kidneys (Gwaltney-Brant, 2002). An ethylene glycol test can be used to determine if any parent ethylene glycol is still present.

## Flumazenil

Flumazenil, 1,4-imidazobenzodiazepine, is a benzodiazepine antagonist (Plumb, 2005). It is derived from the antibiotic anthramycin (Gwaltney-Brant, 2002) and blocks benzodiazepines by competition for the benzodiazepine receptor in the CNS (Plumb, 2005). Flumazenil has a higher affinity for the receptor than that of the benzodiazepines; as a result, it displaces receptor-bound benzodiazepines to reverse their depressive effects (Gwaltney-Brant, 2002).

Flumazenil has been used as a successful therapy for benzodiazepine toxicosis in dogs and cats (Wismer, 2002; Plumb, 2005). Please see Table 91.6 for a partial list of benzodiazepines that flumazenil may antagonize. It is also used to improve neurological signs in dogs with severe hepatic encephalopathy (Plumb, 2005). Flumazenil has been used in humans, with some success, to reverse ethanol and Tegretol-induced CNS depression. Consequently, it is reasonable to assume flumazenil also could be effective in small animal patients in such cases (Gwaltney-Brant, 2002).

**TABLE 91.6 Benzodiazepines**

| Generic name | Trade name |
|---|---|
| Alprazolam | Xanax® |
| Brotizolam | |
| Chlordiazepoxide | Librium® |
| Clobazam | |
| Clonazepam | Klonopin® |
| Clorazepate | |
| Diazepam | Valium® |
| Estazolam | |
| Flunitrazepam | Rohypnol® |
| Flurazepam | Dalmane® |
| Halazepam | |
| Lormetazepam | |
| Lorazepam | Ativan® |
| Medazepam | |
| Midazolam | Versed® |
| Nitrazepam | |
| Oxazepam | |
| Prazepam | |
| Quazepam | |
| Temazepam | |
| Triazolam | Halcion® |
| Zolazepam | |

The recommended flumazenil dose in dogs and cats is 0.01–0.02 mg/kg administered by rapid IV injection (Plumb, 2005). The medication rapidly crosses the blood–brain barrier, and a swift reversal of benzodiazepine-induced sedation is expected within 1–2 min (Gwaltney-Brant, 2002). The dose may need to be repeated multiple times, since flumazenil's half-life is shorter than most of the benzodiazepines. The half-life of flumazenil is only 1 h in humans (Plumb, 2005), but the half-life of diazepam in the dog is 6 h (Gwaltney-Brant, 2002). The patient should be monitored carefully for recurrence of signs and additional doses of flumazenil given as needed (Gwaltney-Brant, 2002). Repeat doses should not be given in asymptomatic patients. Flumazenil may act as a benzodiazepine agonist if administered at high doses despite its antagonist action at therapeutic doses (Gwaltney-Brant, 2002).

Flumazenil is a costly medication and carries the risk of significant adverse effects. It should be reserved for cases where the patient has life-threatening benzodiazepine induced clinical signs (Gwaltney-Brant, 2002). The drug should be given through a patent IV catheter, because extravascular leakage can cause extensive local tissue irritation and necrosis (Gwaltney-Brant, 2002). In humans,

vomiting, cutaneous vasodilation, vertigo, ataxia and blurred vision have been reported following flumazenil administration (Plumb, 2005). Seizures and death are also rarely reported in humans after treatment with flumazenil (ASHSP, 2003).

Flumazenil may lower the seizure threshold and is contraindicated in cases when seizures are anticipated. It also may increase intracranial pressure and should not be used in patients with head trauma (Gwaltney-Brant, 2002). Additionally, flumazenil is contraindicated in patients with life-threatening tricyclic antidepressant toxicosis and should be used only after careful consideration in cases of multiple drug overdosage (Plumb, 2005). Most human deaths and seizures associated with flumazenil followed use in cases of tricyclic antidepressant toxicosis (ASHSP, 2003) and mixed overdoses (Plumb, 2005).

## Methocarbamol

The skeletal muscle relaxant methocarbamol has proved to be very useful in the management of severe muscle fasciculations, tremors and seizures associated with a variety of toxicologic agents (Gwaltney-Brant, 2002). The exact mechanism by which methocarbamol works is unknown but it is thought to act centrally to block nerve impulses in the brain stem, spinal cord and subcortical levels of the brain ( Gwaltney-Brant, 2002; Plumb, 2005).

Methocarbamol has been used successfully in veterinary medicine to treat the following types of toxicoses: permethrin in cats (Richardson, 2000a), metaldehyde (Richardson *et al.*, 2003), strychnine (Gwaltney-Brant, 2002; Plumb, 2005) and tremorgenic mycotoxins (Schell, 2000). It is able to reduce the severity of tremors without causing the severe CNS depression often encountered when using a barbiturate medication (Gwaltney-Brant, 2002).

The dosage for dogs and cats is 55–220 mg/kg administered slowly IV at a rate of no more than 2 ml/min (Schell, 2000). Methocarbamol administration should be repeated if the signs recur; however, the total dose should not exceed 330 mg/kg in a 24-h period to avoid CNS and respiratory depression (Gwaltney-Brant, 2002; Richardson *et al.*, 2003). The dosage for horses is 15–25 mg/kg by slow infusion (Plumb, 2005). The drug should not be given SQ and extravasation of methocarbamol should be avoided as the solution can be irritating (Plumb, 2005).

In dogs and cats, adverse effects may include sedation, salivation, emesis, lethargy, weakness and ataxia. Slow administration of the drug can help to avoid salivation and emesis. Sedation and ataxia can be seen in horses following methocarbamol administration (Plumb, 2005). This medication is contraindicated in animals intended for food purposes and in patients hypersensitive to it (Plumb, 2005). The manufacturer lists known or suspected renal insufficiency as a contraindication to injectable methocarbamol therapy since the injectable product contains polyethylene glycol 300, which has been found to increase acidosis and urea retention in renal-impaired humans (Plumb, 2005). For this reason, veterinary patients treated with injectable methocarbamol should receive IV fluid support and have kidney values monitored if renal impairment is suspected or is a possible outcome of their clinical signs. For example, patients with prolonged tremors or seizures can release muscular myoglobin. The myoglobin is excreted by the kidneys and can lead to renal damage (Volmer, 2004).

## Naloxone HCl

Naloxone HCl (Narcan®) is an injectable opiate antagonist used to reverse the effects of opiate medications. It is able to reverse opioid agonist/antagonists such as butorphanol as well. This drug is also being investigated for treatment of other conditions like septic, hypovolemic and cardiogenic shock (Plumb, 2005).

Naloxone is a pure opiate antagonist, and it has no analgesic activity or other agonist effects (Plumb, 2005; Volmer, 2006). Although the exact mechanism for its activity is not fully understood, it is thought the drug acts as a competitive antagonist by binding to multiple opioid receptor sites. The drug apparently has its highest affinity for the $\mu$ receptor which makes it an ideal antagonist since most of the clinically useful opioids work by binding to the $\mu$ receptor (Plumb, 2005; Volmer, 2006).

Naloxone reverses most of the effects of opioids including analgesia and respiratory and CNS depression (Plumb, 2005). It does not reverse the emetic actions of apomorphine in dogs (Volmer, 2006). At high doses, naloxone increases dopamine levels and acts as a $\gamma$-aminobutyric acid (GABA) antagonist (Plumb, 2005).

Because orally administered naloxone is only minimally absorbed and the drug is destroyed rapidly in the digestive tract, an injectable formulation is preferred (Plumb, 2005). The onset of action is normally within 1–2 min when given IV and within 5 min when given IM (Plumb, 2005). The duration of action is usually 45–90 min but may be as long as 3 h (Plumb, 2005). Since the duration of action of naloxone may be shorter than the medication being reversed, additional doses may be required (Plumb, 2005).

The recommended dosage for dogs is 0.04 mg/kg IV, IM or SC. For cats, 0.02–0.04 mg/kg IV and for horses, 0.01–0.02 mg/kg IV (Plumb, 2005). In patients that have been intentionally given an opioid to treat a painful condition, abrupt reversal can lead to tachycardia, vasoconstriction and hypertension. In these cases, the unwanted CNS and respiratory depression can be reversed without reversing the analgesia. To achieve this, dilute 0.04–0.1 mg of naloxone in 5–10 ml of normal saline

and give slowly to effect by dosing 0.5–1 ml each minute (Mathews, 2006).

Naloxone is considered quite safe at therapeutic doses. Very high doses have been associated with seizures in a few patients perhaps due to GABA inhibition (Plumb, 2005). It is contraindicated in patients that are hypersensitive to it and should be used with caution in animals with pre-existing cardiac abnormalities due to the potential cardiovascular adverse effects (Plumb, 2005).

## N-acetylcysteine

NAC is used to prevent methemoglobinemia and hepatic necrosis in APAP toxicosis. APAP is metabolized in the liver primarily via glucuronidation and sulfation to non-toxic metabolites. Another minor pathway of metabolism does exist which yields a highly reactive metabolite, *N*-acetyl-para-benzoquinoneimine (NAPQI). NAPQI is produced through the P450 mixed function oxidase system and is normally inactivated through conjugation with glutathione. In a significant exposure, the glucuronidation and sulfation pathways become saturated, leading to increased production of NAPQI.

Initially the excess NAPQI will be detoxified through conjugation with glutathione. However, once glutathione stores in the liver are depleted, the NAPQI metabolite can lead to liver necrosis. NAPQI binds to hepatic cell membranes causing oxidative injury to the bi-lipid layer and potential cellular death and necrosis (Richardson, 2000b). Furthermore, depletion of glutathione in red blood cells (RBCs) allows oxidation of the hemoglobin to methemoglobin which is unable to carry oxygen (Aronson and Drobatz, 1996). While all species can develop both hepatic and RBC effects, dogs more commonly develop hepatic necrosis, whereas cats more commonly develop methemoglobinemia and Heinz body anemia (Taylor and Dhupa, 2000).

There are at least two mechanisms by which NAC prevents methemoglobinemia and hepatic necrosis from APAP. NAC binds directly with NAPQI rendering it inactive and therefore non-toxic. NAC is a precursor for glutathione production. It restores and maintains glutathione levels by increasing synthesis (Richardson, 2000b). NAC administration may also lead to increased serum sulfate which could re-establish the sulfation pathway to non-toxic metabolites (Aronson and Drobatz, 1996) (see Figure 91.2).

The therapeutic dose for dogs and cats is a loading dose of 140 mg/kg per os (PO) or IV (as a 5% solution), then 70 mg/kg PO or IV every 6 h for 7 treatments. The number of additional treatments varies between authors; some recommend up to 17 treatments (Plumb, 2005). Presently, both an injectable form and oral solution are on the market. When given orally, NAC can cause GI irritation, so proper dilution is important.

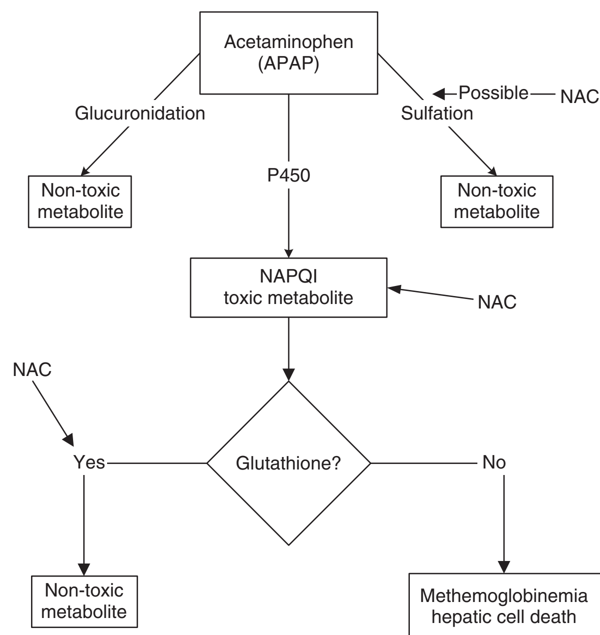


**FIGURE 91.2** Sites of NAC action in the metabolism of APAP.

## Pamidronate disodium

Pamidronate disodium (Aredia®) is a biphosphonate used in the treatment of hypercalcemia associated with malignancy and vitamin $D_3$ toxicity in dogs (Plumb, 2005). Vitamin $D_3$, also called cholecalciferol, is found in some rodenticides. Calcipotriene, which is an analog of cholecalciferol, is found in some human prescription topical antipsoriasis medications (e.g. Dovonex®) (Pesillo *et al.*, 2002). Since vitamin $D_3$ plays an important role in calcium and phosphorus homeostasis, oral exposure to these rodenticides or topical medications can lead to significant elevations in serum calcium and phosphorus levels. Vitamin $D_3$ enhances calcium and phosphorus absorption from the GI tract. In addition, it works with parathyroid hormone to promote calcium reabsorption in the kidney and mobilization of calcium from the bone via osteoclastic-mediated bone resorption (Rumbeiha *et al.*, 2000; Morrow, 2001).

Calcium maintains cell membrane stability and is a second messenger in several cellular responses (Pesillo *et al.*, 2002). Therefore, increasing serum calcium can cause many cellular effects including altered cell membrane permeability, a decrease in cellular energy production and cellular necrosis (Morrow, 2001). Patients poisoned by vitamin $D_3$ and its analogs often present with polyuria, polydipsia, vomiting, anorexia, lethargy, hypercalcemia and hyperphosphatemia (Pesillo *et al.*, 2002). If elevations in serum calcium and phosphorus concentrations go unchecked, the plasma calcium phosphorus product (Ca X P) can rise above 60 leading to soft tissue mineralization (Morrow,

2001). Mineralization of the kidneys and heart can lead to a life-threatening decrease in the function of these organs.

Pamidronate acts to decrease serum calcium levels by binding to hydroxyapatite crystals in the bone to prevent their dissolution and bone resorption. It also interrupts osteoclast activity and induces osteoclast apoptosis (Plumb, 2005). Some authors suggest pamidronate may decrease intestinal absorption of calcium as well (Pesillo *et al*., 2002).

The recommended dose of pamidronate is 1.3–2 mg/kg given as a slow IV infusion over several hours (Plumb, 2005). Pamidronate must be given in saline and should not mix with any IV fluid containing calcium (e.g. Ringer's). In most cases, a single dose will be effective in lowering calcium levels back to normal (Gwaltney-Brant, 2002). However, some patients may require an additional dose 5–7 days after the initial treatment (Morrow, 2001). Calcium levels should be monitored once daily for at least 10 days after they have returned to normal. Pamidronate is most effective if given within 24–36 h of the exposure, prior to development of soft tissue mineralization (Gwaltney-Brant, 2002).

Anemia, thrombocytopenia and granulocytosis have been reported in humans following pamidronate administration (Plumb, 2005). In dogs, hypersensitivity reactions, electrolyte abnormalities including hypomagnesemia and hypocalcemia, arrhythmias and renal toxicity are possible (Plumb, 2005). Slow infusion of the drug over at least 2 h may help in avoiding renal effects. Use of pamidronate in dogs with impaired renal function is controversial since it may confound renal injury (Gwaltney-Brant, 2002).

Pamidronate is expensive but its use may be more cost effective than using salmon calcitonin in vitamin $D_3$ toxicosis. Pamidronate lowers plasma calcium concentrations within 24–48 h of a single IV dose. Calcitonin requires dosing several times daily due to its short half-life. Additionally, with calcitonin therapy, saline diuresis and treatment with diuretics and corticosteroids must also be performed, requiring many days, sometimes multiple weeks, of hospitalization. In most cases treated with pamidronate, treatment can be performed on an out-patient basis once the calcium has returned to the normal range (Morrow, 2001; Pesillo *et al*., 2002). Pamidronate is also preferred to calcitonin since patients may become refractory to calcitonin treatment after 10 days of therapy (Rumbeiha *et al*., 2000; Morrow, 2001).

## Phytonadione (vitamin $K_1$)

Phytonadione, a naphthoquinone derivative identical to naturally occurring vitamin $K_1$, is used in the treatment of anticoagulant toxicosis resulting from anticoagulant rodenticides, the medication warfarin or moldy sweet clover (Plumb, 2005). All household pets, including pocket pets, may be exposed to anticoagulant rodenticides and the medication warfarin. Livestock and horses are more likely to be poisoned by the dicoumarol in moldy sweet clover, but they can also be exposed to rodenticides (Knight, 2004).

Anticoagulants block clotting factor activation. Vitamin $K_1$ is involved in the activation of precursor clotting factors into functional factors. During the activation, vitamin $K_1$ is converted to inactive vitamin $K_1$ epoxide. Normally, vitamin $K_1$ epoxide is reactivated by vitamin $K_1$ epoxide reductase. Anticoagulants work by inhibiting this enzyme and halting the recycling of vitamin $K_1$ (Merola, 2002). As a result, the number of active clotting factors decreases.

The vitamin $K_1$-dependent clotting factors are II, VII, IX and X. These factors are involved in all three pathways of the coagulation system: the extrinsic, intrinsic and common (Means, 2004a). Active bleeding is not expected for 3–7 days after the ingestion because the body has a reserve of active clotting factors. When these degrade naturally or are consumed, clinical signs are possible. If an external source of vitamin $K_1$ is provided, the factors will be activated normally (Merola, 2002).

Bleeding can occur anywhere within the body and the presenting clinical signs will relate to where the bleeding has occurred. The signs may be vague and non-specific. In dogs, dyspnea, coughing, lethargy and hemoptysis are commonly reported due to bleeding into the chest cavity (Merola, 2002). With moldy sweet clover poisoning in livestock, prolonged bleeding in some members following routine surgical procedures, like castration or dehorning, is often the first indication of a problem within the herd (Knight, 2004).

The dose for vitamin $K_1$ in household pets is 1.5–2.5 mg/kg orally twice daily. Pocket pets and other small patients can be given the injectable formulation orally if a suitable tablet size is not available. Small patients should be dosed at the high end of the range and larger patients (e.g. large breed dogs) should be started at the low end (Means, 2004a). Vitamin $K_1$ should be given with a fatty meal to enhance absorption. In dogs, giving the medication with canned food caused a 4–5 fold increase in bioavailability (Plumb, 2005).

Oral administration is preferred since the vitamin $K_1$ will be carried, via the portal circulation, directly to the liver where the activation of clotting factors occurs (Merola, 2002). Additionally, vitamin $K_1$ injections have been associated with anaphylactic reactions and hematoma formation (Plumb, 2005). However, IM administration is recommended by many authors for livestock, perhaps for ease of administration. The recommended dose for cattle, horses, swine, sheep and goats is 0.5–2.5 mg/kg IM (Knight, 2004; Plumb, 2005).

The length of vitamin $K_1$ administration depends on the toxicant. Warfarin exposures generally require a 14-day course. Second generation anticoagulant rodenticides

should be treated for 30 days except for bromadiolone which should be treated for 21 days (Means, 2004a). Moldy sweet clover poisoning should be treated for 1–2 weeks (Knight, 2004). In all cases, a prothrombin time (PT) should be checked 48–72 h after the course of vitamin $K_1$ is complete. If this test is abnormal, resume vitamin $K_1$ therapy for an additional 7 days (Merola, 2002).

Blood transfusions may be required in severely symptomatic patients, since it may take 6–12 h for the liver to produce active clotting factors following initiation of vitamin $K_1$ therapy (Plumb, 2005). Exercise restriction is indicated in all patients during this lag time to prevent active bleeding (Merola, 2002). Substituting vitamin $K_3$, menadione, is not recommended, as it is not as effective and is nephrotoxic in horses (Merola, 2002; Knight, 2004).

## CONCLUSION

Proper client education is crucial to poison prevention. However, when poisonings do occur, the clinician should concentrate on treating the patient and not the poison. The same principles of history collection, physical examination and patient monitoring that are used in other cases should be applied when managing a poisoning patient. Appropriate decontamination and antidotal therapies should be decided on after initial patient stabilization.

## REFERENCES


Albretsen JC (2004) Methylxanthines. In *Clinical Veterinary Toxicology*, Plumlee KH (ed.). Mosby, St. Louis, MO, pp. 322–6.

Allerton JP, Strom JA (1991) Hypernatremia due to repeated doses of charcoal-sorbitol. *Am J Kidney Dis* **17**: 581–4.

American Society of Health System Pharmacists (ASHSP) (2003) *American Hospital Formulary Service Drug Information*. American Hospital Formulary Service, Bethesda, MD, pp. 2421–3.

Aronson LR, Drobatz K (1996) Acetaminophen toxicosis in 17 cats. *J Vet Emerg Crit Care* **6**: 65–9.

Bailey Jr EM, Garland T (1992) Management of toxicoses. In *Current Therapy in Equine Medicine 3*, Robinson NE (ed.). W.B. Saunders Company, Philadelphia, PA, pp. 346–53.

Beasley VR, Dorman DC (1990) Management of toxicoses. *Vet Clin North Am Small Anim Pract* **20**: 307–37.

Brown CM, Bertone J (2001) *The 5-Minute Veterinary Consult Equine*. Lippincott Williams & Wilkins, Philadelphia, PA, pp. 816–17.

Buck WB, Bratich PM (1986) Activated charcoal: preventing unnecessary death by poisoning. *Vet Med* **81**: 73–7.

Buckley NA, Dawson AH, Reith DA (1995) Controlled release drugs in overdose clinical considerations. *Drug Safety* **12**: 73–84.

Burrows GE, Tyrl RJ (2001) *Toxic Plants of North America*. Iowa State Press, Ames, IA, pp. 214–16.

Cantilena Jr LR (2001) Clinical toxicology. In *Casarett and Doull's Toxicology: The Basic Science of Poisons*, 6th edn, Klaassen CD (ed.). McGraw-Hill, New York, pp. 1109–22.

Cheeke PR (1998) *Natural Toxicants in Feeds, Forages, and Poisonous Plants*. Interstate Publishers, Inc., Danville, IL.

Connally HE, Forney SD, Grauer GF, Hamar DW, Thrall MA (1996) Safety and efficacy of 4-methylpyrazole treatment of suspected or confirmed ethylene glycol intoxication in dogs: 107 cases (1983–1995). *J Am Vet Med Assoc* **209**: 1880–3.

Connally HE, Hamar DW, Thrall MA (2002) *Resident Forum Abstract from 8th IVECCS San Antonio*, Texas. Safety and efficacy of high dose fomepizole as therapy for ethylene glycol intoxication in cats. *J Vet Emerg Crit Care* **12**: 191.

Clark R, Curry S, Selden B (1991) Digoxin-specific Fab fragments in the treatment of oleander toxicity in the canine model. *Ann Emerg Med* **20**: 1073–77.

Dalefield R (2004) Ethylene glycol. In *Clinical Veterinary Toxicology*, Plumlee KH (ed.). Mosby, St. Louis, MO, pp. 150–4.

DeClementi C, Bailey KL, Goldstein SC, Orser MS (2004) Suspected toxicosis after topical administration of minoxidil in 2 cats. *J Vet Emerg Crit Care* **14**: 287–92.

Donnelly TM (2004) Rabbits. Basic anatomy, physiology, and husbandry. In *Ferrets, Rabbits, and Rodents Clinical Medicine and Surgery*, Quesenberry KE, Carpenter JW (eds), 2nd edn. W.B. Saunders, St. Louis, MO, pp. 136–9.

Dunayer EK (2004) Hypoglycemia following canine ingestion of xylitol-containing gum. *Vet Hum Toxicol* **46**: 87–8.

Echols S (2005) Collecting diagnostic samples in avian patients. In *The Clinics Collection Veterinary Clinics of North America Exotic Animal Practice*, Rupley AE (ed.). W.B. Saunders Co., Philadelphia, PA, pp. 60–3.

Eubig PE, Brady MS, Gwaltney-Brant SM, Khan SA, Mazzaferro EM, Morrow CMK (2005) Acute renal failure in dogs after ingestion of grapes or raisins: a retrospective evaluation of 43 dogs (1992–2002). *J Vet Intern Med* **19**: 663–74.

Fikes JD (1990) Organophosphorus and carbamate insecticides. *Vet Clin North Am Small Anim Pract* **20**: 353–67.

Fikes JD (1992) Feline chlorpyrifos toxicosis. In *Current Veterinary Therapy XI*, Kirk RW, Bonagura JD (eds.). W.B. Saunders Co., Philadelphia, PA, pp. 188–91.

Gaddy J (2001) Pharm profile fomepizole. *Compend Contin Educ Pract Vet* **X**, 1073–4.

Galey FD (1992) Diagnostic toxicology. In *Current Therapy in Equine Medicine 3*, Robinson NE (ed.). W.B. Saunders Co., Philadelphia, PA, pp. 337–40.

Grossman MR (1993) Amitraz toxicosis associated with ingestion of an acaricide collar in a dog. *J Am Vet Med Assoc* **203**: 55–7.

Gwaltney-Brant SM, Albretsen JC, Khan SA (2000) 5-Hydroxytryptophan toxicosis in dogs: 21 cases (1989–1999). *J Am Vet Med Assoc* **216**: 1937–40.

Gwaltney-Brant SM (2002) Newer antidotal therapies. *Vet Clin North Am Small Anim Pract* **32**: 323–39.

Gwaltney-Brant S (2004) Amitraz. In *Clinical Veterinary Toxicology*, Plumlee KH (ed.). Mosby, St. Louis, MO, pp. 177–8.

Gwaltney-Brant S (2006) Personal Communication.

Horstman CL, Cornell KK, Eubig PA, Khan SA, Selcer BA (2003) Gastric outflow obstruction after ingestion of wood glue in a dog. *J Am Anim Hosp Assoc* **39**: 47–51.

Kittleson MD, Kienle RD (1998) *Small Animal Cardiovascular Medicine*. Mosby, St. Louis, MO, pp. 159–66.

Knight AP (2004) Coumarin glycosides. In *Clinical Veterinary Toxicology*, Plumlee KH (ed.). Mosby, St. Louis, MO, pp. 388–90.

Mathews KA (2006) *Veterinary Emergency and Critical Care Manual*. Lifelearn, Inc., Guelph, Ont., pp. 4–8, 12–17, 85, 630–40, 655–9.

Mealey KL (2006) Adverse drug reactions in herding-breed dogs: the role of P-glycoprotein. *Compend Contin Educ Pract Vet* **28**: 23–33.

Means C (2003) Bread dough toxicosis in dogs. *J Vet Emerg Crit Care* **13**: 39–41.

Means C (2004a) Anticoagulant rodenticides. In *Clinical Veterinary Toxicology*, Plumlee KH (ed.). Mosby, St. Louis, MO, pp. 444–6.

Means C (2004b) Insoluble calcium oxalates. In *Clinical Veterinary Toxicology*, Plumlee KH (ed.). Mosby, St. Louis, MO, pp. 340–1.

Meerdink GL (1989) Organophosphorus and carbamate insecticide poisoning in large animals. *Vet Clin North Am Food Anim Pract* **5**: 375–89.

Meerdink GL (2004) Anticholinesterase insecticides. In *Clinical Veterinary Toxicology*, Plumlee KH (ed.). Mosby, St. Louis, MO, pp. 178–80.

Merola V (2002) Anticoagulant rodenticides: deadly for pests, dangerous for pets. *Vet Med* **97**: 716–22.

Morrow C (2001) Cholecalciferol poisoning. *Vet Med* **96**: 905–11.

Mowry JB, Chylea PA, Furbee RB (1994) Organophosphate insecticides. In *Essentials of Critical Care Pharmacology*, 2nd edn, Chernow B (ed.). Williams & Wilkins, Baltimore, MD, pp. 522–4.

Oehme FW, Mannala S (2006) Paraquat. In *Small Animal Toxicology*, 2nd edn, Peterson, ME, Talcott, PA (eds). Elsevier, Inc., St. Louis, MO, pp. 964–77.

Osweiler GD (2001). Mycotoxins. *Vet Clin North Am Equine Pract* **17**: 547–66.

Pesillo SA, Khan SA, Rozanski EA, Rush, JE (2002) Calcipotriene toxicosis in a dog successfully treated with pamidronate disodium. *J Vet Emerg Crit Care* **12**: 177–81.

Peterson ME (2006) Toxicological decontamination. In *Small Animal Toxicology*, 2nd edn, Peterson ME, Talcott PA (eds). Elsevier, Inc., St. Louis, MO, pp. 127–41.

Plumb DC (2005) *Plumb's Veterinary Drug Handbook*, 5th edn. Blackwell Publishing, Ames, IA, pp. 9–10, 34–5, 69–70, 59–60, 72, 209–10, 314–15, 341, 352–3, 506–7, 532–3, 547–8, 635–7, 647–8, 677–8, 802–6.

Post LO, Keller WC (1999) An update of antidote availability in veterinary medicine. *Vet Hum Toxicol* **41**: 258–61.

Richardson JA (2000a) Permethrin spot-on toxicoses in cats. *J Vet Emerg Crit Care* **10**: 103–6.

Richardson JA (2000b) Management of acetaminophen and ibuprofen toxicosis in dogs and cats. *J Vet Emerg Crit Care* **10**: 285–91.

Richardson JA, Khan SA, Means C, Murphy LA (2001) Managing pet bird toxicoses. *Exotic DVM* **3.1**: 23–7.

Richardson JA, Gwaltney-Brant, SM, Villar D (2002) Zinc toxicosis from penny ingestion in dogs. *Vet Med* **97**: 96–9.

Richardson JA, Gwaltney-Brant SM, Huffman JD, Rosendale ME, Welch SL (2003) Metaldehyde toxicoses in dogs. *Compend Contin Educ Pract Vet* **25**: 376–9.

Rosendale ME (2002) Decontamination strategies. *Vet Clin North Am Small Anim Pract* **32**: 311–21.

Rumbeiha WK, Braselton WE, Fitzgerald SD, Frese KK, Kaneene JB, Kruger JM, Nachreiner R (2000) Use of pamidronate disodium to reduce cholecalciferol-induced toxicosis in dogs. *Am J Vet Res* **60**: 9–13.

Schell MM (2000) Tremorgenic mycotoxin intoxication. *Vet Med* **95**: 283–6.

Shannon M (2003) The demise of ipecac. *J Pediatr* **112**: 1180–1.

Stair EL, Plumlee KH (2004) Blister beetles. In *Clinical Veterinary Toxicology*, Plumlee KH (ed.). Mosby, St. Louis, MO, pp. 101–3.

Taylor NS, Dhupa N (2000) Acetaminophen toxicity in cats and dogs. *Compend Contin Educ Pract Vet* **22**: 160–70.

Volmer PA (1999) Easter lily toxicosis in cats. *Vet Med* **94**: 331.

Volmer PA (2004). Pyrethrins and pyrethroids. In *Clinical Veterinary Toxicology*, Plumlee, KH (ed.). Mosby, St. Louis, MO, pp. 188–90.

Volmer PA (2006) Recreational drugs. In *Small Animal Toxicology*, 2nd edn, Peterson ME, Talcott PA (eds). Elsevier, Inc., St. Louis, MO, pp. 273–11.

Vite CH, Gfeller RW (1994) Suspected albuterol intoxication in a dog. *J Veter Emerg Crit Care* **4**: 7–12.

Ward DM, DeFrancesco TC, Forrester SD, Troy GC (1999) Treatment of severe chronic digoxin toxicosis in a dog with cardiac disease, using ovine digoxin-specific immunoglobulin G Fab fragments. *J Am Vet Med Assoc* **215**: 1808–12.

Wismer TA (2002) Accidental ingestion of alprazolam in 415 dogs. *Vet Hum Toxicol* **44**: 22–3.

Wismer T (2004) Baclofen overdose in dogs. *Vet Med* **99**: 406–10.

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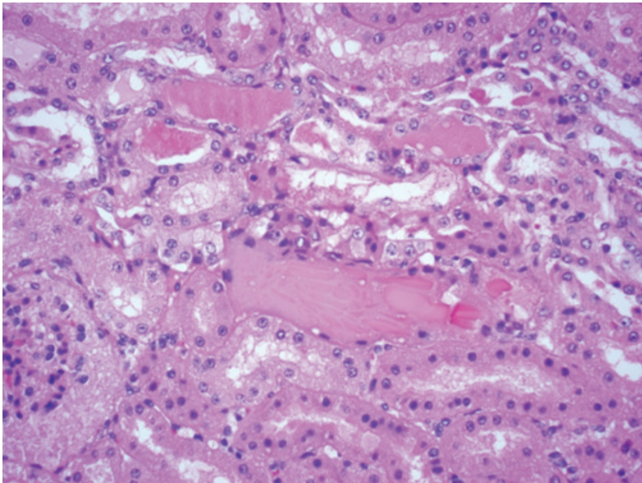
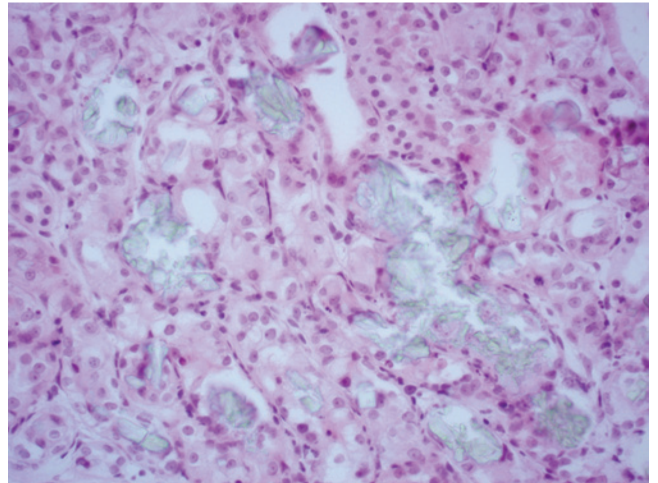
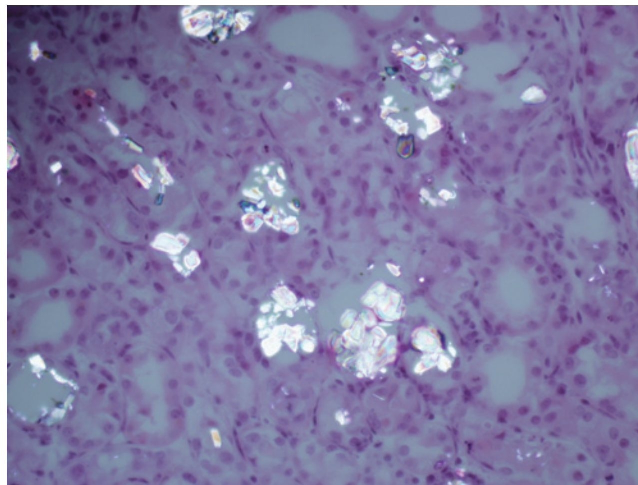


**CHAPTER 11 FIGURE 2** Red maple poisoning, equine kidney, tubules are dilated and lined by degenerate epithelium. The tubular lumen is filled with casts. H&E stain 40×.



**CHAPTER 11 FIGURE 3** Ethylene glycol poisoning, canine kidney, tubules are lined by degenerate epithelium and multiple tubules contain crystalline material. H&E stain 40×.



**CHAPTER 11 FIGURE 4** Ethylene glycol poisoning, canine kidney, multiple tubules contain birefringent crystalline material. H&E stain 40× under polarized light.

**CHAPTER 13** Asclepias syriaca (courtesy of Wikipedia.com).

**CHAPTER 13** *Persea americana* (Courtesy of Wikipedia.com).

**CHAPTER 13** *Digitalis purpurea* (Courtesy of Wikipedia.com).

**CHAPTER 13** *Delphinium elatum* (Courtesy of Wikipedia.com).

**CHAPTER 13** *Aconitum variegatum* (Courtesy of Wikipedia.com).

**CHAPTER 13** *Taxus baccata* (Courtesy of Wikipedia.com).

**CHAPTER 13** *Convallaria majalis* (Courtesy of Wikipedia.com).

**CHAPTER 13** *Zgadenus fremontii* (Courtesy of Wikipedia.com).

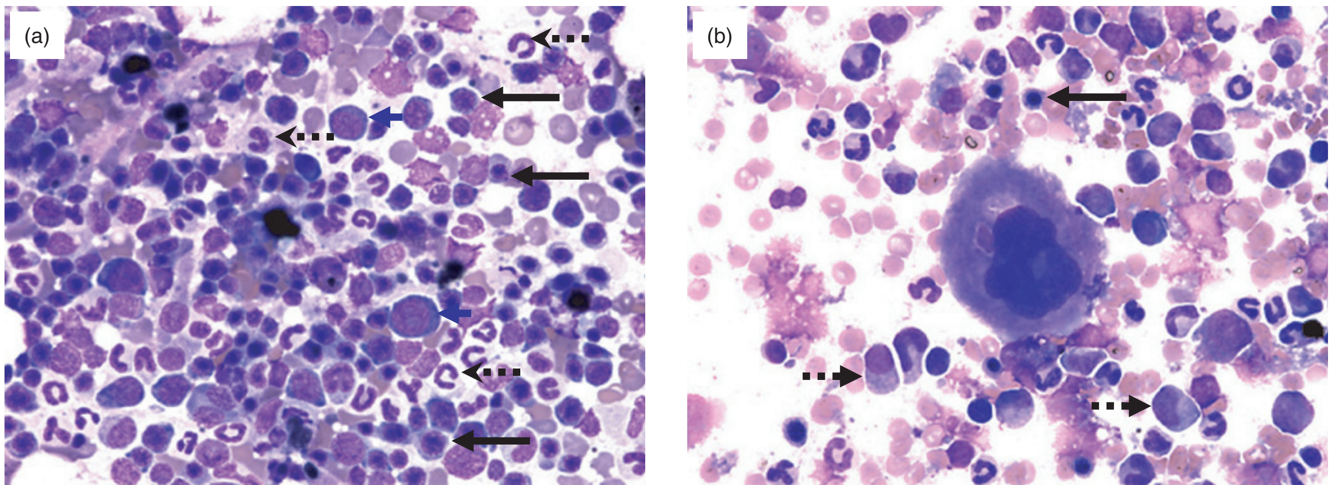
**CHAPTER 13** *Helleborus niger* (Courtesy of Wikipedia.com).

**CHAPTER 13** *Apocynum cannabinum* (Courtesy of Wikipedia.com).

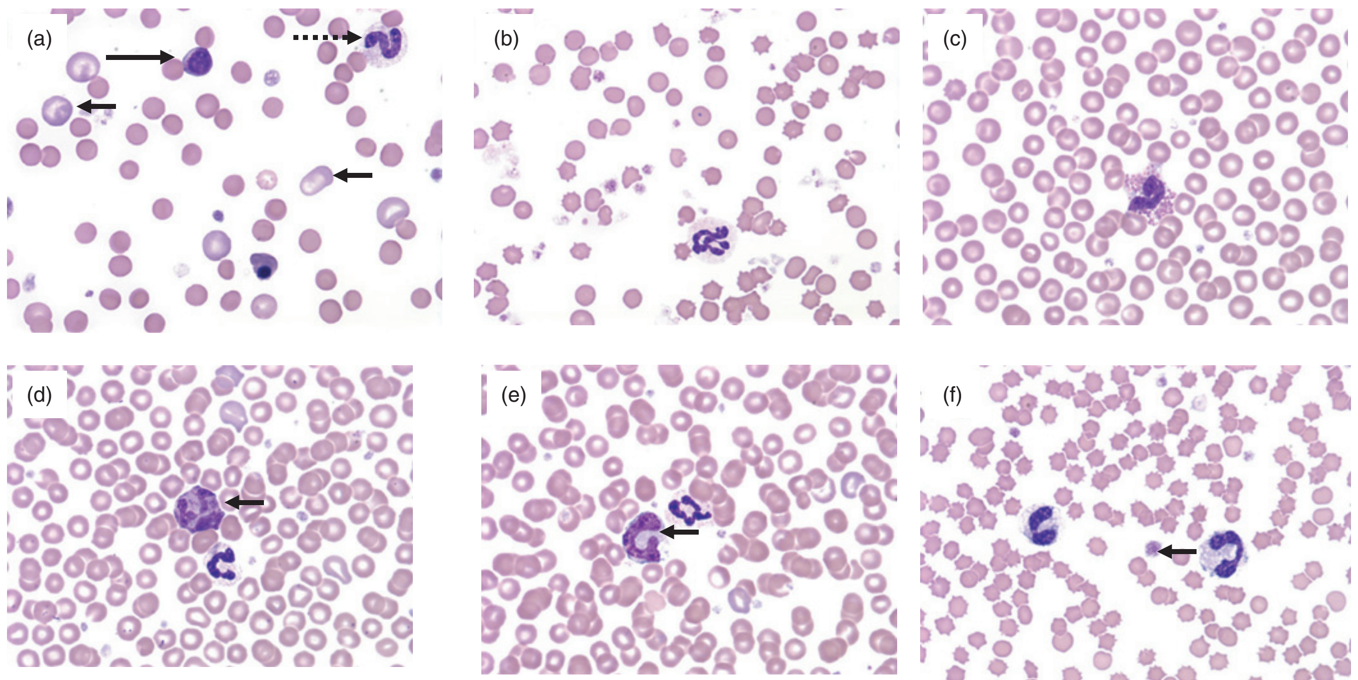
**CHAPTER 13** *Nerium oleander* (Courtesy of Wikipedia.com).

**CHAPTER 13** *Kalmia angustifolia* (Courtesy of Wikipedia.com).

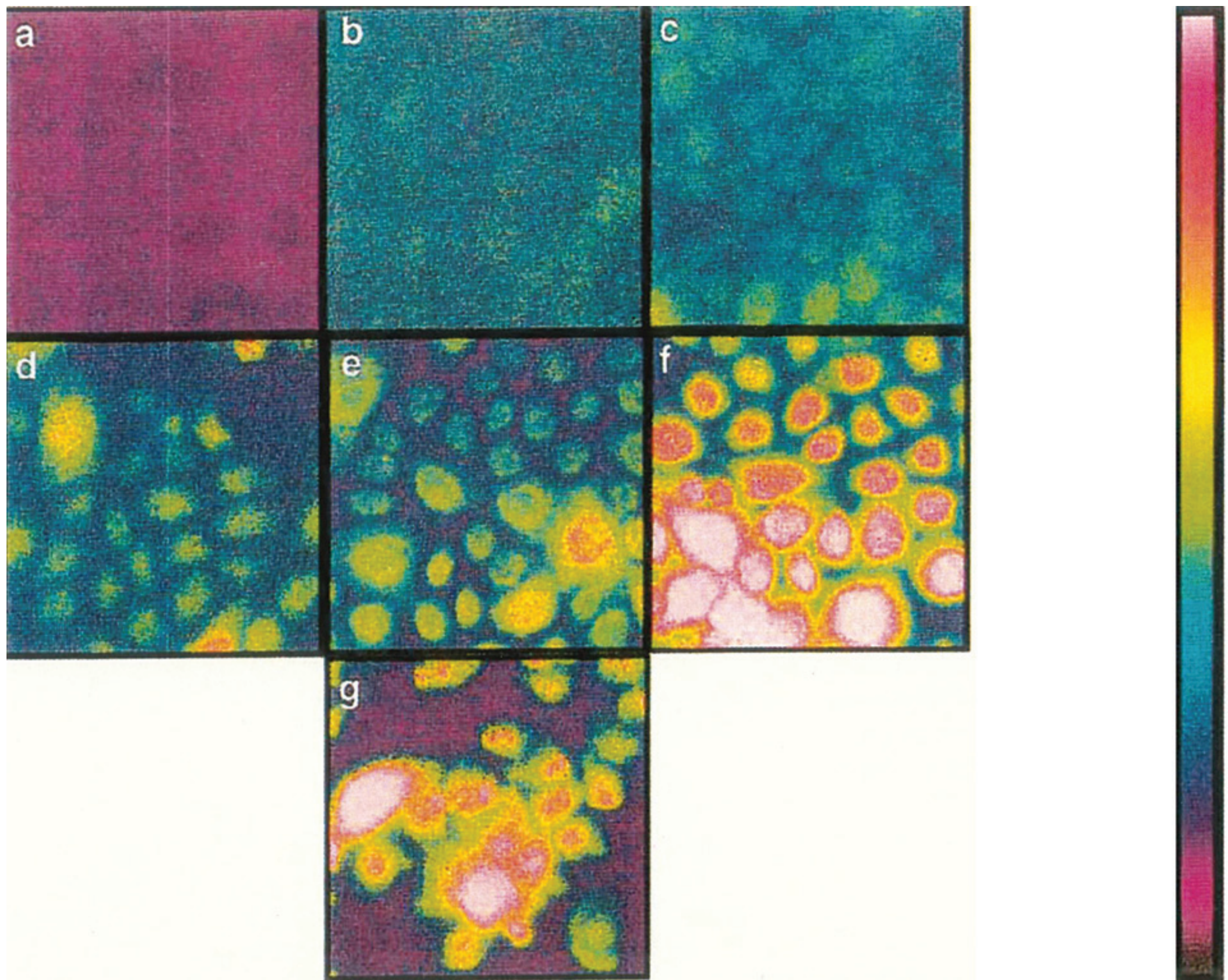
**CHAPTER 13** *Rhododendron ponticum* (Courtesy of Wikipedia.com).



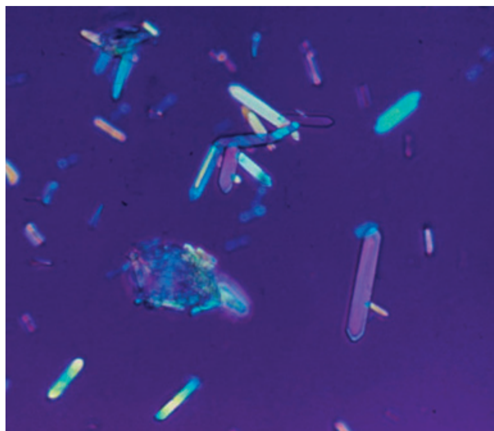

**CHAPTER 17 FIGURE 2** (a) Examples of erythroid precursors and granulocytic precursors (arrowheads) in the bone marrow of a dog. 60×, Diff-quik stain. Early erythroid precursors (arrow heads), late erythroid precursors (solid arrows) and granulocytic precursors (dotted arrows) are shown. (b) A mature megakaryocyte in the bone marrow of a dog is shown in the center of the picture. Erythroid and myeloid precursors are also noted (arrows). 60×, Modified Wrights stain.




**CHAPTER 17 FIGURE 3** *Normal and few abnormal erythrocyte and leukocyte morphology.* (a) Two large basophilic polychromatophilic erythrocytes or reticulocytes (small solid arrow) are present in the blood from a dog with IMHA. A normal neutrophil (dotted arrow), a metarubricyte and a lymphocyte (long arrow) are also present. 100×, Modified Wrights Stain. (b) Heinz bodies in the blood from a cat appearing as pale "spots" within erythrocytes with a modified Wrights stain, 100×. (c) Eosinophil with round granules in the blood from a dog. 100×, Modified Wrights stain. (d) Basophil (arrow) and neutrophil in the blood from a dog. 100×, Modified Wrights stain. (e) Monocyte (arrow) and neutrophil in the blood from a dog. 100×, Modified Wrights stain. (f) Toxic neutrophils in the blood from a cat demonstrating Döhle bodies, cytoplasmic vacuolation, and basophilia. A platelet (arrow) is also identified in the field 100×, Modified Wrights stain.




**CHAPTER 20 FIGURE 3** Changes in intracellular redox states of human cells after treatment with a tobacco extract. Cells (70% confluent) were treated with the tobacco extract for 24 h. After 24 h, medium was replaced with fresh medium containing 5 μm 2,7-dichlorofluorescein diacetate and fluorescence intensity was measured 5 m in later at 513 nm with a confocal laser scanning microscope. a, Control; b, 100 μg/ml; c, 150 μg/ml; d, 200 μg/ml; e, 250 μg/ml; f, 300 μg/ml; and g, 3 mM hydrogen peroxide (positive control).

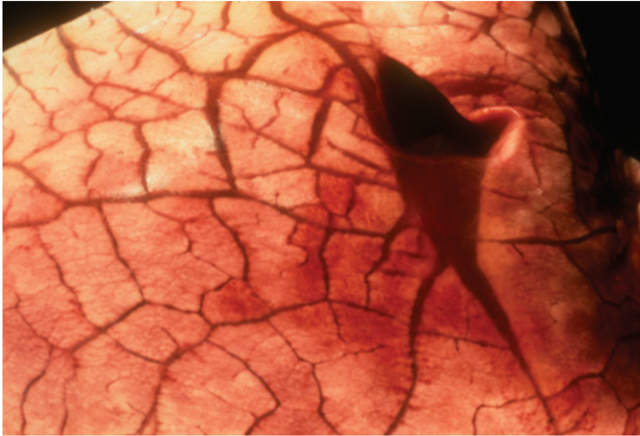


**CHAPTER 53 FIGURE 1** Calcium oxalate monohydrate crystals (polarized light) from a dog with EG toxicosis.

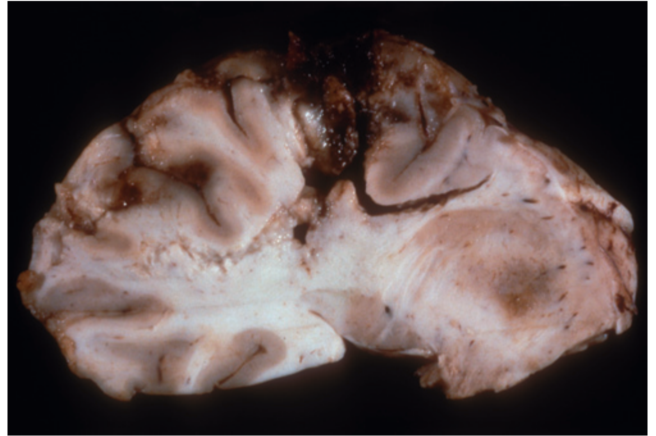
**CHAPTER 63 FIGURE 1** Late instar ETCs in nest.

**CHAPTER 64 FIGURE 1** An adult striped blister beetle (*E. vittata*). (Photo courtesy of James E. Appleby, University of Illinois.)

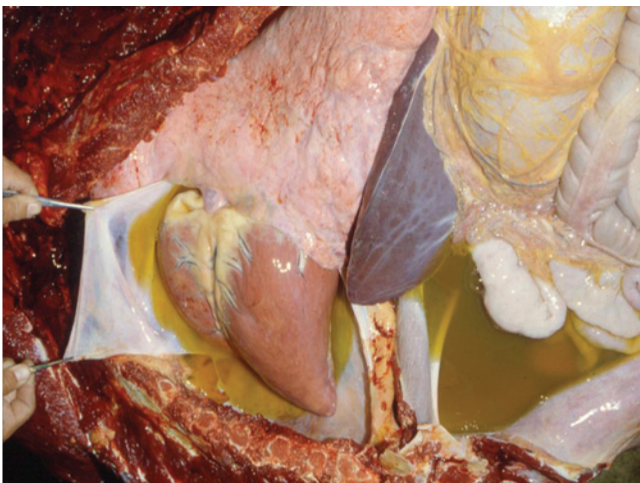
**CHAPTER 64 FIGURE 2** An adult blister beetle (*Epicauta*), with hemolymph droplets containing cantharidin. (Photo courtesy of Dr. Maria Eisner, Cornell University.)



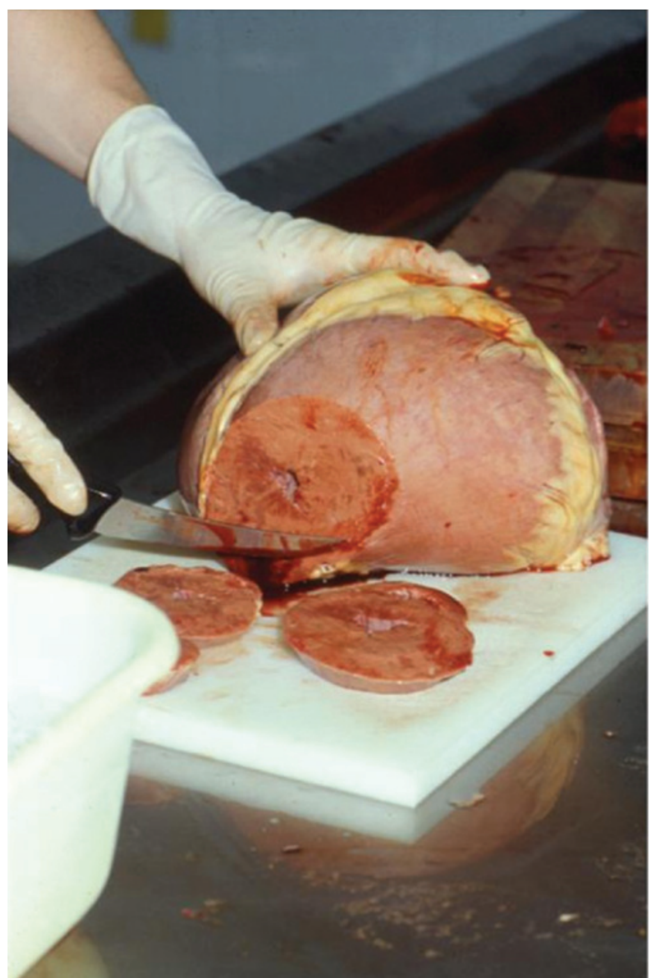
**CHAPTER 78 FIGURE 4** Lung from a pig fed fumonisin-containing culture material at a dose of 20 mg fumonisin $B_1$ per kg of body weight for 4 days. Pulmonary edema is characterized by severe widening of the interlobular septa (reprinted with permission from Smith and Constable, 2004).



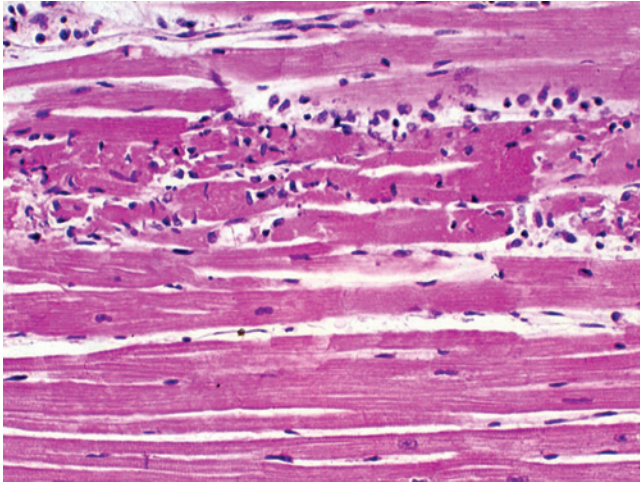
**CHAPTER 78 FIGURE 5** A cross-section of a cerebral hemisphere from a horse demonstrating liquefactive necrosis of the white matter typical of ELEM (reprinted with permission from Smith and Constable, 2004).



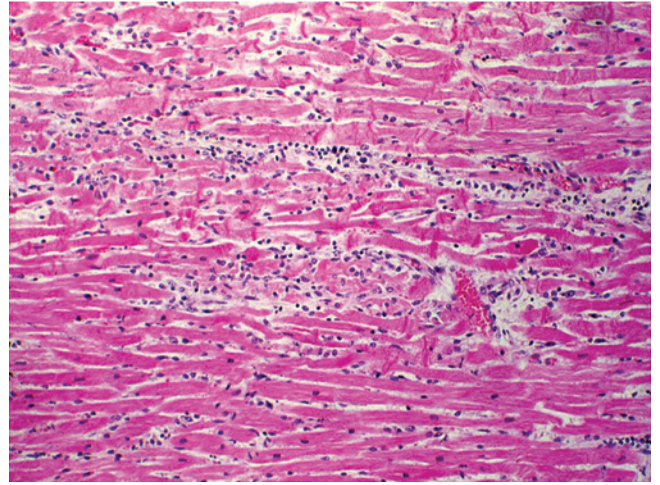
**CHAPTER 83 FIGURE 5** Thoracic and abdominal viscera from a mare that died 7 days following a single gavage dose of 1.65 mg monensin/kg body weight. Note the fluid accumulation in the body cavities and the pale areas and epicardial hemorrhages on the heart.



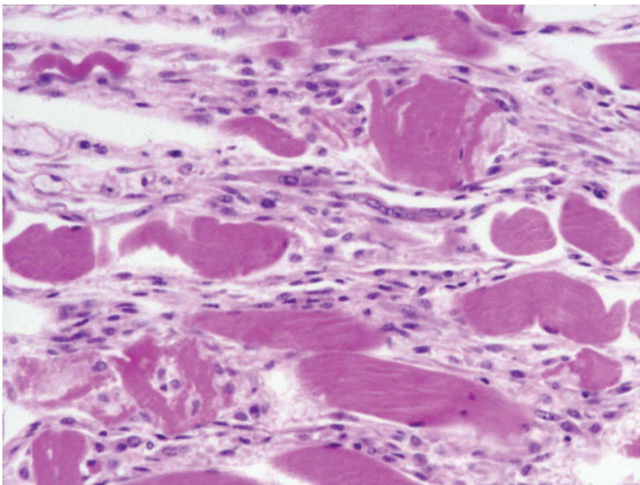
**CHAPTER 83 FIGURE 6** Diffuse pallor in cross sections of the heart from the same mare described in Figure 83.5 legend.



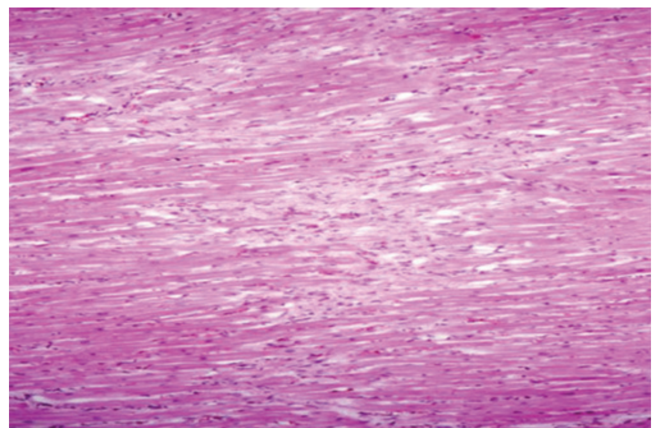
**CHAPTER 83 FIGURE 7** Early necrotic focus in the left ventricle of a gelding that died 20 h following gavage with 2.5 mg monensin/kg body weight. Necrotic muscle fibers have sparse infiltration of neutrophils and lymphocytes. H&E. Original magnification ×64.

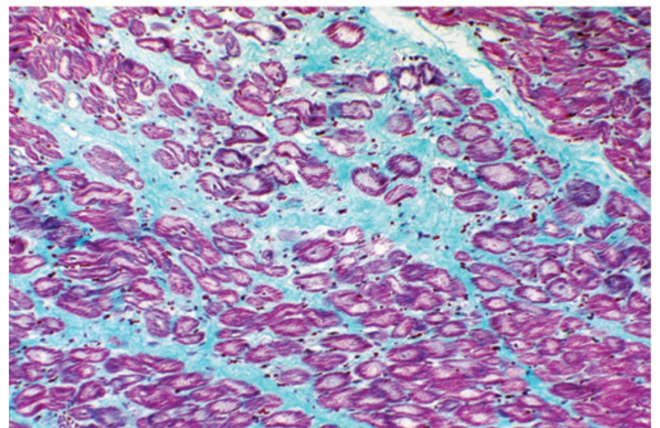


**CHAPTER 83 FIGURE 8** Larger foci of myofiber necrosis with contraction bands in the interventricular septum from a horse euthanized 72 h following a single oral dose of 1.65 mg monensin/kg body weight given by gavage. More cellular infiltrates with lymphocytes, macrophages, and few eosinophils are present. H&E. Original magnification ×10.

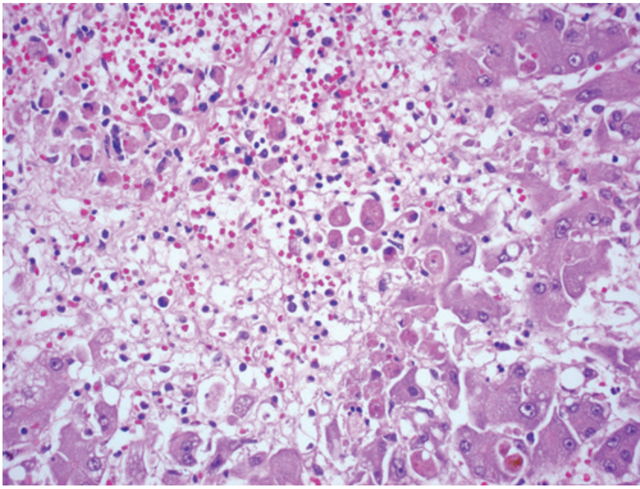


**CHAPTER 83 FIGURE 9** Skeletal muscle from a steer that died 6 days after a gavage dose of 39.8 mg monensin/kg body weight. Notice the fragmented and regenerating fibers. H&E. Original magnification ×80.

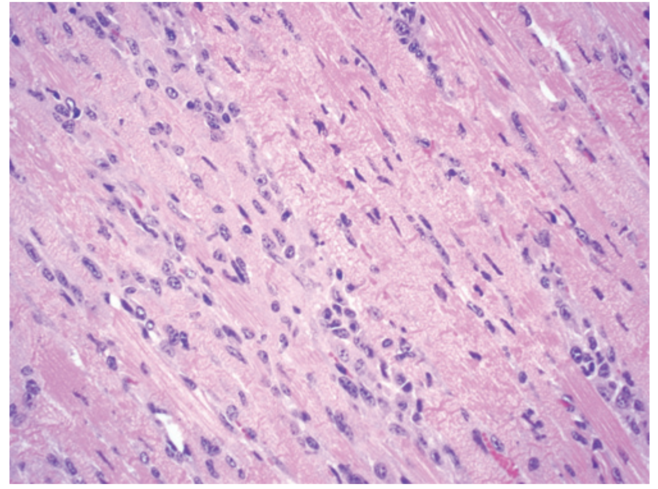




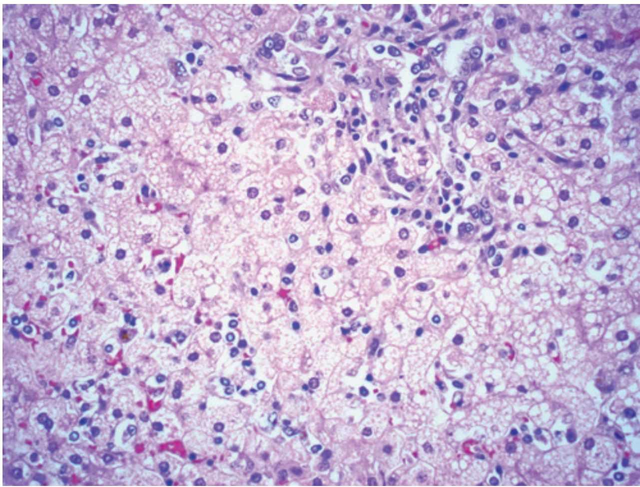
**CHAPTER 83 FIGURE 10** Hearts from animals that survived acute ionophore toxicity. Top: section of left ventricle with focal interstitial fibrosis from another steer, cohort of that described in Figure 83.9 legend, euthanized 28 days after a gavage dose of 39.8 mg monensin/kg body weight. H&E. Original magnification ×20. Bottom: interstitial fibrosis in the left ventricle from a gelding that survived a gavage dose of 2 mg monensin/kg body weight for 4 months. Masson's trichrome. Original magnification ×32.



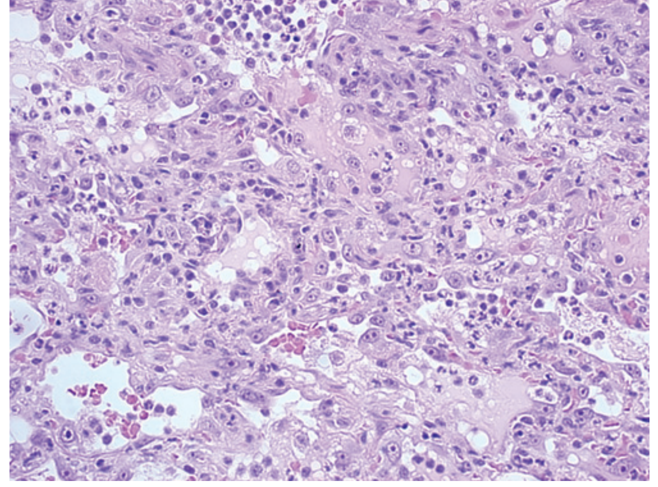
**CHAPTER 90 FIGURE 1** NSAID-induced toxicity, liver, canine, hepatocyte necrosis and degeneration in the centrolobular areas with marked biliary stasis, H&E stain, 403.



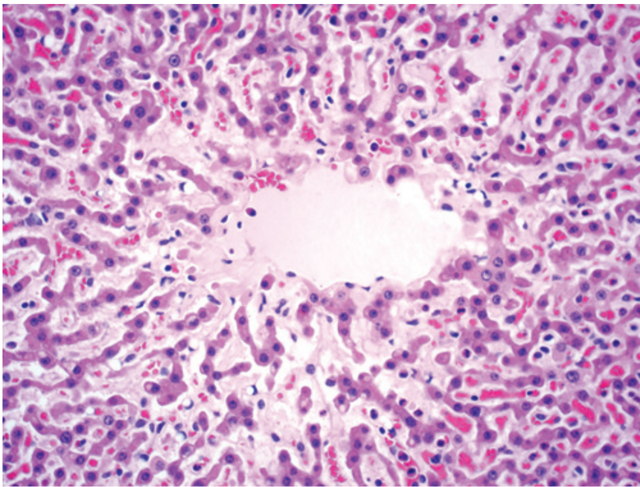
**CHAPTER 90 FIGURE 2** Monensin toxicity, heart, bovine, cardiac myocytes show degeneration and some areas myocytes replaced and infiltrated by lymphocytes and histiocytes, H&E stain, 40×. (Courtesy Dr. ME Hines, College of Veterinary Medicine, University of Georgia.)



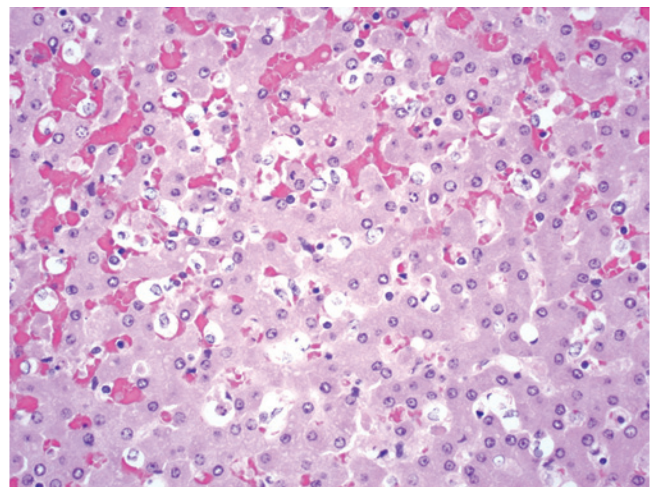
**CHAPTER 90 FIGURE 3** Aflatoxicosis, liver, canine, hepatocellular vacuolation with biliary hyperplasia, H&E stain, 40×. (Courtesy Dr. S Newman, College of Veterinary Medicine, University of Tennessee.)



**CHAPTER 90 FIGURE 4** Paraquat poisoning, lung, canine, diffuse interstitial pneumonia with type II pneumocyte hyperplasia, H&E stain, 40×. (Courtesy Dr. ME Hines, College of Veterinary Medicine, University of Georgia.)



**CHAPTER 90 FIGURE 5** Blue green algae poisoning, liver, bovine, centrilobular cord atrophy with dilated sinusoids and necrosis, H&E stain, 40×. (Courtesy Dr. K Frazer, Philadelphia.)



**CHAPTER 90 FIGURE 6** Mushroom poisoning, liver, canine, hepatocyte dissociation and degeneration, H&E stain, 40×. (Courtesy Dr. A Ligget, College of Veterinary Medicine, University of Georgia.)