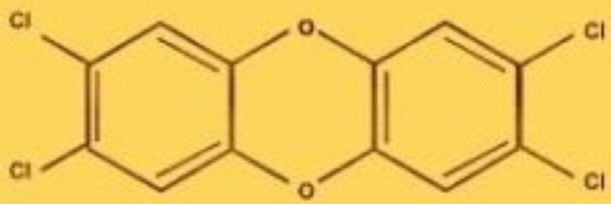
Dioxins and Health

SECOND EDITION



ARNOLD SCHECTER
THOMAS A. GASIEWICZ

DIOXINS AND HEALTH

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SECOND EDITION

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To our families

CONTENTS

Contributors ix

Preface xiii

1. **Overview: The Dioxin Debate** 1
   Thomas F. Webster and Barry Commoner

2. **Production, Distribution, and Fate of Polychlorinated Dibenzo-*p*-Dioxins, Dibenzofurans, and Related Organohalogens in the Environment** 55
   Roger K. Gilpin, Daniel J. Wagel, and Joseph G. Solch

3. **Dioxins and Dioxinlike PCBs in Food** 89
   James R. Startin and Martin D. Rose

4. **Toxicology of Dioxins and Dioxinlike Compounds** 137
   Jeanelle M. Martinez, Michael J. DeVito, Linda S. Birnbaum, and Nigel J. Walker

5. **Health Risk Characterization of Dioxins and Related Compounds** 159
   Linda S. Birnbaum and William H. Farland

6. **Pharmacokinetics of Dioxins and Related Chemicals** 191
   James R. Olson

7. **Dose–Response Modeling for 2,3,7,8-Tetrachlorodibenzo-*p*-Dioxin** 247
   Michael J. DeVito, Amy Kim, Nigel J. Walker, Fred Parham, and Christopher Portier

8. **Immunotoxicology of Dioxins and Related Chemicals** 299
   Nancy I. Kerkvliet

9. **Developmental and Reproductive Toxicity of Dioxins and Related Chemicals** 329
   H. Michael Theobald, Gary L. Kimmel, and Richard E. Peterson

10. Effects of Polychlorinated Biphenyls on Neuronal Signaling **433**
Richard F. Seegal

11. Experimental Toxicology: Carcinogenesis **457**
Justin G. Teeguarden and Nigel J. Walker

12. Ah Receptor: Involvement in Toxic Responses **491**
Thomas A. Gasiewicz and Sang-ki Park

13. Biochemical Responses to Dioxins: Which Genes? Which Endpoints? **533**
J. Kevin Kerzee, Ying Xia, and Alvaro Puga

14. Evolutionary and Physiological Perspectives on Ah Receptor Function and Dioxin Toxicity **559**
Mark E. Hahn

15. Dioxin Toxicity and Aryl Hydrocarbon Receptor Signaling in Fish **603**
Robert L. Tanguay, Eric A. Andreasen, Mary K. Walker, and Richard E. Peterson

16. Exposure Assessment: Measurement of Dioxins and Related Chemicals in Human Tissues **629**
Arnold Schecter, Olaf Päpke, Marian Pavuk, and Rachel E. Tobey

17. Human Health Effects of Polychlorinated Biphenyls **679**
Matthew P. Longnecker, Susan A. Korrick, and Kirsten B. Moysich

18. Epidemiological Studies on Cancer and Exposure to Dioxins and Related Compounds **729**
Lennart Hardell, Mikael Eriksson, Olav Axelson, and Dieter Flesch-Janys

19. Reproductive and Developmental Epidemiology of Dioxins **765**
Sherry G. Selevan, Anne Sweeney, and Marie Haring Sweeney

20. Health Consequences of the Seveso, Italy, Accident **827**
Pier Alberto Bertazzi and Alessandro di Domenico

21. The Yusho Rice Oil Poisoning Incident **855**
Yoshito Masuda

22. The Yucheng Rice Oil Poisoning Incident **893**
Yueliang Leon Guo, Mei-Lin Yu, and Chen-Chin Hsu

Index **921**

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PREFACE

It is sometimes said that there are at least two sides to every story, and that the truth is often somewhere in between. In like manner, the literature on the dioxins is most often dichotomized by interests either directly relevant to human health risks or very focused on the molecular mechanisms by which these chemicals act to affect cellular functions. How we bring all of this information together to actually determine, and not estimate, the true risks to human (and wildlife) populations exposed to these chemicals continues to remain a challenge. There is also a literature on the chemical aspects of dioxins and related synthetic chemicals, and historical events where dioxin contamination was noteworthy and of concern. On the social side there is extensive literature on legal aspects of environmental pollutants and on response of individuals and organizations to incidents posing perceived or actual physical or psychological risks.

During the years since the first edition of this book in 1994, an extensive amount of new policy and scientific literature has been published related to dioxins and dioxin-like chemicals. For the purposes of this text these chemicals include the halogenated dioxins and dibenzofurans, certain polychlorinated biphenyls (PCBs), and other compounds that are structurally and toxicologically similar to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD, or just TCDD), the most extensively studied and most potent member of this group. Much of this literature has continued to refine our understanding of the effects of human exposures, possible effects on human and wildlife populations, dose–effect relationships, and the mechanisms whereby these chemicals work at the molecular level, in particular as mediated by a transcription factor the aryl hydrocarbon receptor (AhR). In several cases, and in part due to new technological advances, substantial new and striking strides have been made in our ability to detect sensitive endpoints of toxicity, to measure, evaluate, and predict with greater accuracy effects of low exposure levels, to examine on a cell- and tissue-specific basis the genes that may be altered following exposure, and to begin to understand the normal function of the AhR. Thus, it appears that the scientific community is at the brink of finally making some connections between the molecular actions of TCDD and those biological and toxic effects in animals and humans. Real issues such as What concentrations are toxic to humans?, What effects are more likely to be observed and at what doses?, and What subpopulations might be most sensitive?, are beginning to be addressed. This text was written to foster further these connections by offering a perspec-

tive as to how recent scientific data may relate to very relevant issues in human and environmental health.

The chapter authors are scientists with international reputation in their particular area of the dioxin arena. Many of these individuals have been responsible for generating the original scientific data that they discuss. They have been asked to address their chapters to an audience of well-educated and intelligent lay persons and professionals who may not necessarily be familiar with the details of the different specialties. As such, the authors have included highlights of their respective fields with a sampling, without being encyclopedic, of important references. Furthermore, each author has been asked to discuss the relevance of these scientific data to possible human exposures and health risks. Through this approach, the book gives a meaningful and accessible presentation to the broadest range of health professionals and nonhealth professionals interested in dioxins, as well as policy makers and the general public.

Since the middle of the 20th century, dioxins have been widespread and persistent synthetic environmental contaminants in the United States and other industrial counties. Because of their ubiquity, persistence, and extreme toxicity in laboratory animals, considerable concern arose regarding their presence in the food chain and in human tissue. The fact that these chemicals have been shown to cause cancer, immune system disorders, reproductive and developmental abnormalities, neurological and endocrine system alterations in laboratory animals at very low doses has further fueled this concern. While there exists controversy regarding extrapolation of laboratory animal data to human risks, more and more data is available that may allow us within the next few years to draw better conclusions about the *actual* risks to human populations.

In their introductory overview of the continuing dioxin debate, Thomas Webster and Barry Commoner provide a summary of some of the major current dioxin controversies. Chapters 2 and 3, by Roger Gilpin, Daniel Wagel, and Joseph Solch, and James Startin and Martin Rose, respectively, provide a perspective on the sources of the dioxins, how they move in the environment, and how and why humans are exposed predominantly by dietary intake.

In separate chapters, several scientists present our current understanding of the information we have obtained, mainly from studies of experimental animals, for the effects of these chemicals on certain health outcomes. Chapter 4, by Jeanelle Martinez, Michael DeVito, Linda Birnbaum, and Nigel Walker, gives a brief overview of the toxicology of the dioxins and the predominant issues that bench scientists are attempting to address.

More detailed considerations of the subdisciplines are presented in subsequent chapters. Chapter 5, by Linda Birnbaum and William Farland, summarizes the approaches used to consider the available animal and human data for possible human health risks. Here, while the good news is presented that human body burdens of these chemicals are, in general, declining in more industrialized countries, an analysis of the toxicity data may suggest that these body burdens are still at or near concentrations where some effects might be expected to occur. In Chapter 6, James Olson summarizes what is known about

the fate of these chemicals in animal and human tissues. These data are particularly important for defining body burdens at different life stages and exposure conditions. One of the major issues that remains to be better characterized is the determination of what body burdens cause what toxic effects. The discussion by Michael DeVito, Amy Kim, Nigel Walker, Fred Parham, and Chris Portier in Chapter 7 indicates that most responses to these chemicals do not have the same dose–response relationships and clearly some responses are more sensitive than others. Notably this conclusion is mirrored in a later chapter where gene responses are discussed. Chapters 8 through 11, by Nancy Kerkvliet; Michael Theobald, Gary Kimmel, and Richard Peterson; Richard Seegal; Justin Teeguarden and Nigel Walker, respectively, discuss in greater detail some of the most consistent and sensitive toxic responses to these chemicals in the immune system, on developing and reproductive tissues, in the nervous system, and for carcinogenesis. Here, much of the focus is on defining the cellular and biochemical alterations that lead to these responses.

Chapters 12, 13, and 14, by Tom Gasiewicz and Sang-ki Park; Kevin Kerzee, Ying Xia, and Alvaro Puga; and Mark Hahn, respectively, are new chapters in this edition. These have been written to summarize the most recent data at the molecular level, indicating that the Ah receptor and its ability to modulate the expression of genes is ultimately responsible for the toxic effects observed. In particular, these studies open avenues to explore the possibility of developing molecular biomarkers of susceptibility and exposure. These chapters also present particularly exciting findings concerning possible normal functions of the receptor, our understanding of which could add much to determining how and at what concentrations the dioxins may be acting to cause toxicity, and why these chemicals elicit such tissue- and species-specific effects. As Robert Tanguay, Eric Andreasen, Mary Walker, and Richard Peterson indicate in Chapter 15, fish have been found to be particularly sensitive to the effects of these chemicals. The newest models using Zebra fish may also be extremely useful in dissecting relationships between molecular actions and effects of the dioxins on several physiological systems.

Since one cannot purposefully dose humans with the dioxins, it is more difficult to come to conclusions on health consequences from studies on people. Yet, epidemiology, the study of human populations and health outcomes, has the advantage of dealing with the human species. A number of chapters discuss what is known from epidemiology, including cancer epidemiology. In Chapter 16 Arnold Schecter, Olaf Päpke, Marian Pavuk, and Rachel Tobey review exposure assessment of dioxins, with special emphasis on high resolution gas chromatography–high resolution mass spectroscopy, the current gold standard of exposure assessment. They note that only in the 1980s did this become possible, and it was only in the 1980s that data showed that all humans carry a body burden of chlorinated dioxins and dibenzofurans.

In Chapter 17 Matthew Longnecker, Susan Korrick, and Kirsten Moysich review the epidemiology of polychlorinated biphenyls or PCBs. In Chapter 18 Lennart Hardell, Mikael Eriksson, Olav Axelson, and Dieter Flesch-Janys

review the epidemiology of dioxins and cancer, including evidence of human carcinogenicity. Chapter 19, by Sherry Selevan, Anne Sweeney, and Marie H. Sweeney, discuss the reproductive and developmental epidemiology of dioxins. In Chapter 20 a major dioxin incident that took place in Seveso, Italy in 1976 is described by Pier Alberto Bertazzi and Alessandro di Domenico, who also noted the health consequences detected to date. Chapter 21 offers a description of the health consequences of Japan rice oil, or Yusho, poisoning with PCBs, dibenzofurans, and small amounts of other chemicals in 1968, by one of the key scientists who studied the incident, Yoshito Masuda. In the last chapter, Chapter 22, Yueliang Leon Guo, Mei-Lin Yu, and Chen-Chin Hsu describe a similar rice oil poisoning—the Yucheng incident—that occurred in Taiwan in 1979 and its consequences on exposed persons.

This book presents policy and science from the molecule to whole animals, and to human epidemiology in a selective fashion within one volume. Hopefully, it will continue the efforts of the first edition in presenting a relatively large but not overwhelming amount of material useful to experts, policy makers, and the general public.

ARNOLD SCHECTER
THOMAS A. GASIEWICZ

CHAPTER 1

Overview: The Dioxin Debate

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1.1 INTRODUCTION

To the general public, dioxin is the archetype of toxic chemicals, a substance that in minute amounts causes cancer and birth defects. Raised to a high level of visibility by the use of Agent Orange in Vietnam, it continues to generate environmental issues that capture public attention: Times Beach, Seveso, Love Canal, herbicide spraying in the United States, waste incineration, and food contamination.

Public fear engendered counter-reactions. Some claimed that dioxin causes no harm to humans other than chloracne, a disfiguring skin disease.[1,2] Others compared the public attitude toward dioxin with witch hunts. Dioxin, they said, is a prime example of *chemophobia*, the irrational fear of chemicals.[3,4] U.S. Assistant Surgeon General Vernon Houk claimed that the evacuation of Times Beach, Missouri had been a mistake.[5,6] Administrator William Reilly of the U.S. Environmental Protection Agency (USEPA) ordered a reassessment of the toxicity of dioxin. He stated: ''I don't want to prejudge the issue, but we are seeing new information on dioxin that suggests a lower risk assessment for dioxin should be applied.''[6]

In our opinion, the public fears are largely justified. The current scientific evidence argues not only that dioxin is a potent carcinogen, but also that the noncancer health and environmental hazards of dioxin may be more serious than believed previously. Indeed, dioxin appears to act like an extremely persistent synthetic hormone, perturbing important physiological signaling systems. Such toxic mimicry leads to a host of biological changes, especially altered cell development, differentiation, and regulation. Perhaps the most

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troubling consequence is the possibility of reproductive, developmental, and immunological effects at the levels of dioxinlike compounds now present in the bodies of the average person. Observation of such phenomena in wildlife suggests that the environment is overburdened with these dangerous compounds.

The pendulum of official opinion has swung back. Contradicting Houk, U.S. Assistant Surgeon General Barry Johnson testified in June 1992 that the evacuation of Times Beach was not a mistake.[7] The USEPA's reassessment, although still incomplete at this writing, indicates that the danger from dioxin may be broader and more serious than thought previously.[8] In this overview, we discuss the basis for this dramatic turnaround and its logical implication: a policy directed toward exposure reduction and pollution prevention.

1.2 DIOXIN AND DIOXINLIKE COMPOUNDS

The polychlorinated dibenzo-*p*-dioxins are a group of 75 structurally related compounds (congeners), including the well-known 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD or, as we shall refer to it, TCDD). Based on toxicity similar to that of TCDD, a wider group of halogenated aromatic compounds have been recognized as dioxinlike. These include certain polychlorinated dibenzofurans (PCDFs), polychlorinated biphenyls (PCBs), polychlorinated diphenyl ethers, polychlorinated naphthalenes, and others. Brominated and chloro–bromo versions of these compounds may be dioxinlike as well.[9]

Membership in the class is defined biologically: dioxinlike compounds produce a similar spectrum of toxic effects thought to be caused by a common mechanism. The key step in the presumed mechanism is binding of the dioxinlike compound to a receptor protein, the Ah (aryl hydrocarbon) receptor (AhR). The molecule's planar shape facilitates binding to the receptor, and its relative potency depends to a large degree on its persistence and how well it fits the receptor. TCDD binds the Ah receptor with a very high affinity and is extremely potent. Other planar molecules of about the same size and shape, including a number of the polyhalogenated dibenzo-*p*-dioxins and dibenzofurans, fit almost as well and are also very active. Although certain types of polychlorinated biphenyls bind to the receptor only weakly, their relative abundance in the environment nevertheless makes them biologically important. PCBs with chlorines in positions that prevent the molecule from assuming a planar position do not bind to the Ah receptor and are not dioxinlike in their biological effects. Some of these PCBs can exert toxicity through other mechanisms, however.[10]

1.3 SOURCES

Large-scale industrial production of the dioxinlike polychlorinated naphthalenes began during World War I. Production of polychlorinated biphenyls

(PCBs) followed in the late 1920s (Table 1.1). The thermal and chemical stability of PCBs, among other properties, led to their widespread use in transformers, capacitors, heat transfer and hydraulic fluids, as well as carbonless copy paper, plasticizers, and numerous other applications. Health and environmental problems led to curbs on their industrial production, but not until decades later. In the meantime, about 650,000 metric tons were produced in the United States and about 1.5 million metric tons worldwide.[11] It is estimated that about 20 to 30% of this amount has entered the environment. Much of the remainder is still in stock or in uses such as capacitors and transformers.[12]

In contrast, the polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) are unwanted by-products. Knowledge of their origins has increased considerably, beginning with the identification of TCDD as an unwanted by-product of the production of certain trichlorophenols and herbicides,[13] in particular, Agent Orange, a 1:1 mixture of the *n*-butyl esters of 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) and 2,4-dichlorophenoxyacetic acid (2,4-D). TCDD formation simply requires combining two molecules of 2,4,5-trichlorophenol under the right conditions.[14] More highly chlorinated PCDDs and PCDFs are formed during the production of pentachlorophenol, a compound still used in the United States and elsewhere as a wood preservative. PCDFs also occur as low-level contaminants of PCBs. Much higher levels are generated by heating PCBs under the right conditions of heat and oxygen.[15] This phenomenon was a major contributor to the Yusho and Yucheng (oil disease) tragedies, where cooking oil was contaminated with PCBs.[16,17] It also occurred in numerous incidents involving capacitor and transformer fires[18,19] and the contamination of Belgian food in 1999.[20]

It was thought for a while that the dioxin problem was limited to a few reactions of closely related chemicals. Unfortunately, this is not the case. PCDD and PCDF were discovered in ash from trash-burning incinerators in 1977 and later in their air emissions.[21] It was not known at first whether the emissions were due to unburned PCDD and PCDF in the fuel, formation from chlorinated organic precursors, or de novo synthesis.[21,22] Based on the observation of fly-ash-catalyzed chlorination of organic residues,[23] it was hypothesized that PCDD and PCDF were synthesized as exhaust gases cooled down in the boiler and air pollution control devices.[24,25] This was soon confirmed by tests conducted at a Canadian incinerator. Little or no PCDD and PCDF were found in the gases leaving the furnace, but these compounds were detected in the cooler stack gases.[26] Laboratory studies indicate significant formation at about 300°C.[27] A number of U.S. incinerators equipped with electrostatic precipitators running at these temperatures were very large dioxin sources: two emitted at rates of roughly 1 kg per year of TCDD equivalents (TEQs),[28] an amount of dioxinlike compounds considered equivalent in toxicity to TCDD.[9] Various mechanisms have been postulated for the synthesis reactions, in which metals play an important role (e.g., Ref. 29). Both organic and inorganic sources of chlorine may contribute to the formation of PCDD and PCDF.[30] Although there undoubtedly exists a connection between total emissions of

TABLE 1.1 Selected History of Dioxin and Dioxinlike Compounds

1897–1899	Chloracne characterized
1918	Outbreaks of chloracne following exposure to chlorinated naphthalenes
1920–1940	Dramatic increase of PCDD and PCDF levels in North American lake sediments (reported 1984)
1929	U.S. commercial production of PCBs begins
1947	X disease described in cattle in the United States
1949–1953	Chemical accidents at Monsanto, Boehringer, and BASF
1957	TCDD identified as unwanted contaminant in the manufacture of trichlorophenol
1957	Chick edema disease outbreak in poultry in southeastern United States
1962–1970	Agent Orange used in Southeast Asia
1965–1966	Holmesburg prison experiments
Mid 1960s	Outbreaks of reproductive and developmental effects in Great Lakes fish-eating birds
1968	Yusho oil disease (Japan)
1971	TCDD found to cause birth defects in mice
	Contamination of Times Beach and other Missouri sites
1972–1976	Ah receptor hypothesis developed
1973	Polybrominated biphenyls accidentally added to cattle feed in Michigan
1974	TCDD detected in human breast milk from South Vietnam
1976	Accident in Seveso, Italy
1977	U.S. commercial production of PCBs halted
	TCDD found to cause cancer in rats
	Discovery of dioxin emissions from trash incinerators
1978	Kociba et al. cancer study of rats exposed to TCDD
1979	USEPA emergency suspension of some 2,4,5-T uses
	Yucheng oil disease (Taiwan)
	Association of soft tissue sarcoma with TCDD and phenoxyacetic acid herbicides
1979–	TCDD found to modulate hormones and their receptors
1980	Evacuation of Love Canal
1981	Transformer fire in Binghamton, New York state office building
1983–1985	General public found to be contaminated with PCDD and PCDF
1985	USEPA health assessment of TCDD
1986	Production of dioxin by chlorine-bleached paper mills discovered, although proposed earlier
1988	First USEPA reassessment of TCDD
	Die-off of Baltic seals
1990	Second Banbury conference on dioxins
1991	NIOSH cancer mortality study of U.S. chemical workers
	Second USEPA reassessment begins
1992	U.S.–Canadian International Joint Commission 6th Biennial Report
1993	AhR is a member of bHLH PAS family
1997	IARC classifies TCDD as a human carcinogen
1998	WHO reduces tolerable daily intake
1999	Food contamination in Belgium
2000	POPs treaty
	USEPA reassessment completed?

these compounds and levels of chlorine in fuel, the nature of this relationship is still not understood completely.[31,32]

Such findings imply that dioxinlike compounds may be formed during virtually any combustion process when chlorine is present. This idea was proposed in the *Trace chemistry of fire hypothesis*, which stated that PCDD formation was a natural consequence of combustion.[33] This led, in turn, to the claim that forest fires and other nonindustrial sources are potentially significant or even the dominant source of dioxin.[34–36] However, this claim is at odds with a number of observations. Levels of PCDD and PCDF are higher in people from industrialized countries than in residents of less industrialized nations.[37,*] Lake sediments from North America and Europe show that PCDD and PCDF levels were very low until approximately 1920–1940.[38] Similarly, the levels of these compounds in ancient mummies and 100- to 400-year-old frozen bodies are far lower than those currently found in the average resident of an industrialized country.[39–41] Hence, although trace amounts of PCDD and PCDF may have been present in preindustrial times, the current levels represent a huge increase over these low values.

In North America, the dramatic increase of PCDD and PCDF in lake sediment matches the beginnings of large-scale industrial chlorine chemistry—and combustion of its products—during the period 1920–1940. Concentrations of PCDD and PCDF in stored vegetation and soil samples from the United Kingdom increased at the turn of the century,[42] perhaps reflecting the advanced development of industrial processes in that country. Coal burning has been suggested as a major contributor; however, large-scale coal burning antedates the increase of PCDD/PCDF in North America sediments. PCDD and PCDF have been detected in the emissions from coal combustion, but at fairly low levels. It is possible that the relatively high levels of sulfur in coal inhibits formation of PCDD/PCDF.[43,44] Low levels of PCDD and PCDF found in British soil and vegetation samples from the mid-nineteenth century may partially reflect even earlier industrial activity. For instance, the Leblanc process for producing alkali, a forerunner of modern industrial chlorine chemistry, first found widespread application in the United Kingdom at this time.[45]

PCDD and PCDF are emitted when other chlorine-containing fuels are burned, including chemical waste, hospital waste, and sewage sludge.[46] Dioxin-containing wastes have not proven as easy to destroy in hazardous waste incinerators as once claimed, perhaps because of resynthesis.[47,†] The exhaust from automobiles burning leaded gasoline contains both chlorinated and mixed halogenated dioxins and dibenzofurans, apparently arising from ethylene dichloride and ethylene dibromide used as lead scavengers. Much lower levels of PCDD and PCDF have been found in exhaust from vehicles

* Levels of PCDD and PCDF, especially TCDD, are elevated in human tissues of southern Vietnam relative to northern Vietnam, reflecting both differences in industrialization and the millions of gallons of Agent Orange sprayed in the south.[37]

† The USEPA's response was that the regulation requiring an incinerator to achieve 99.9999% destruction or removal of dioxin-containing wastes does not actually apply to the dioxin itself.[48]

burning unleaded gasoline, presumably reflecting the low level of chlorine in this fuel.[46]

The identification of additional PCDD and PCDF sources strengthens the connection between these compounds and industrial chlorine chemistry. PCDD and PCDF are formed in the bleaching of pulp and paper with chlorine, not surprising given the rich aromatic content of the lignin found in wood.[49] Hypothesized as early as 1974,[50] this phenomenon was not confirmed until the mid-1980s, when high concentrations of TCDD were found in fish downstream of bleached pulp mills[51,52] and then in the mills themselves.[53,54] Dioxin may also be formed during chlorine regeneration of metal catalysts used in petroleum refining.[55]

Large amounts of PCDD and PCDF are also produced by certain types of metal processing, perhaps reflecting the catalytic properties of a number of metals. PCDD and PCDF are emitted by the burning of scrap metal, such as copper cable coated with PVC plastic insulation.[56] Other sources include aluminum smelting, magnesium and nickel production, scrap metal melting, and iron and steel production.[57–59] In these manufacturing processes chlorine is either used or is contained in cutting oils, plastic, and other contaminants.

Dioxinlike compounds are formed at the heart of the chlorine industry as well. Large amounts of PCDD and PCDF have been found in the sludge from chloralkali plants that used graphite electrodes, once widely employed.* Most modern facilities now use other kinds of electrodes.[60] PCDD/PCDF have been detected in some common chlorinated hydrocarbons.[61] They are formed during the production of ethylene dichloride (EDC).[62–64] EDC is used primarily to produce vinyl chloride, the precursor to polyvinyl chloride (PVC) plastic. About 4.2 million metric tons of PVC were produced in the United States in 1991,[65] making it the single largest use of chlorine in the country.

Octachlorinated dioxin may be formed from pentachlorophenol at near-ambient temperatures in sewage sludge[66] and in the atmosphere.[67] Dioxins have recently been found in ancient clay deposits.[68] Their origin is still mysterious, but they suggest an unknown natural source.

In sum, the range of sources has expanded to the point that virtually all industrial chlorine chemistry can be suspected of generating dioxinlike compounds at some point during production, use, or disposal. The unwanted production of PCDD and PCDF may reflect the relative stability of these compounds. They can be thought of as thermodynamic sinks that are likely to accumulate in reactions involving chlorine and organic materials and may therefore be expected to occur in a very wide range of reactions.

What are the largest sources? National air emissions inventories have been performed in the United States, Canada, Japan, Australia, and a number of European countries.[46,69] Waste incineration—municipal, hospital, hazard-

*The chloralkali process manufactures chlorine and sodium hydroxide (an alkali) from sodium chloride brine via electrolysis. The graphite may provide a source of carbon for the generation of PCDD and PCDF.

ous, and industrial—and metal processing are the largest current estimated sources of emissions to the atmosphere. More difficult to quantify, but potentially important, are releases from pentachlorophenol and 2,4-D as well as "backyard" waste burning.[46] Cases of massive dioxin contamination have been reported in Russia.[70,71] Several groups have attempted to balance emissions against levels found in the environment (e.g., Ref. 72). This is made difficult by the limitations of current inventories and representativeness of environmental samples.[28,46] Nevertheless, environmental levels appear to exceed the level implied by known sources. It is possible that important sources have not yet been accounted for or identified properly.[67]

1.4 ENVIRONMENTAL FATE AND EXPOSURE

Dioxin is primarily a modern problem, a by-product of industrial chlorine chemistry and the combustion of chlorine-containing fuels. The growth of these processes during the twentieth century dramatically increased the levels of these compounds in the environment and in biota. Accumulation also depends on the environmental behavior of dioxinlike compounds, a consequence of their chemical and physical properties: low vapor pressure and water solubility, high lipophilicity, and relative chemical stability.[73] When the metabolic inertness of many congeners is added to the list, the profile is complete: Dioxinlike compounds tend to persist and bioaccumulate.

Combustion sources emit large quantities of dioxinlike compounds into the atmosphere, where they are both dispersed and subjected to selective degradation. The more highly chlorinated compounds tend to adsorb onto airborne particulate at ambient temperature, greatly reducing their rate of degradation.[74] Larger fractions of the lower-chlorinated congeners are found as vapor, making them more susceptible to photolysis and attack by hydroxyl radicals. These phenomena may partly explain the shift toward a preponderance of the more highly chlorinated compounds seen in many abiotic environmental samples relative to the typical pattern found in combustion emissions.[75] Atmospheric residence times of particulate-bound congeners are determined by dry and wet deposition of the particulate.[76]

Thus, many dioxinlike compounds are sufficiently stable to travel long distances in the atmosphere. The ubiquitous presence of dioxins in the environment, even in remote locations such as arctic Canada, is probably due to the cumulative impact of many sources.[77,78] Deposition from the air contaminates soil, water, and vegetation. Deposition of both particulate and vapor onto plants provides a significant entry into the terrestrial food chain.[79–82] Human exposure via milk and beef may be orders of magnitude higher than via inhalation, making it a major issue in the permitting of air emission sources.[79]

Because of their low water solubilities and vapor pressures, PCDD and PCDF tend to partition into soil and sediment. The half-life of TCDD may be on the order of a decade or more in soil and probably longer in sediment.[15] As

a result, these two media can act as reservoirs, leading to recontamination of other media.

In aquatic systems, the highly lipophilic and hydrophobic dioxinlike compounds tend to bioconcentrate from water to aquatic animals and then biomagnify up the multistep food chain.[83–85] Levels of PCBs found in fish-eating birds, animals near the top of the aquatic food chain, can reach concentrations tens of millions of times higher than those of PCBs dissolved in water.[83] The combined effects of bioaccumulation and the action of sediment as a reservoir make direct discharge of these compounds into aquatic systems particularly problematic.

Humans are also high on the food chain, eating the meat and milk of herbivores as well as fish and plants. The average person in an industrial country is thought to be exposed to PCDD and PCDF primarily via these animal products. The average daily dose is about 1 pg/kg per day of TCDD equivalents (TEQs).[8] General contamination of the environment and food sources may explain the relatively similar levels of PCDD and PCDF found in the average residents of industrialized countries.[37]

Dioxinlike compounds accumulate primarily in people's body lipid, especially adipose tissue. Their elimination depends on metabolic degradation—slight or nil for many congeners—and on the rate of excretion that is almost completely via the feces. As a result, the half-life of TCDD in humans is very long, on the order of a decade; OCDD may have a half-life as long as 50 years.[86,87] This biological persistence leads to another route of exposure. After accumulating in the mother over decades, dioxinlike compounds can be passed to the developing fetus in utero—a particularly vulnerable period—or to newborns via lactation.[37] Similarly, birds and fish accumulate these compounds and pass them to the egg.[83,84,88]

Like the increase of atmospheric chlorine caused by chlorofluorocarbons and certain chlorinated solvents, the dioxinlike compounds represent a perturbation of the planet's chemistry. This might have been only a curious and little noticed sidelight of the industrial age if not for an additional factor: the extraordinarily powerful biological effects of the dioxinlike compounds.

1.5 BIOCHEMISTRY AND TOXICITY

1.5.1 Biological Persistence

As noted earlier, the central event that instigates the biological effects of TCDD and dioxinlike substances is thought to be their binding to a receptor protein. This aryl hydrocarbon (Ah) receptor (AhR; see Chapter 12) was first postulated during studies of PCDDs and polycyclic aromatic hydrocarbons (PAHs) such as 3-methylcholanthrene.[89–91] Sequencing of the gene for the AhR revealed a surprise. Rather than being a member of the steroid hormone

receptor family as supposed originally, it belongs to a family of basic helix-loop-helix proteins containing a sequence known as the PAS domain.[92]

Once a compound is bound to the AhR, a series of intracellular processes may ensue, including shedding and binding of other factors [including the aryl hydrocarbon nuclear translocator (ARNT)], migration into the nucleus, and binding of the complex to specific sequences of DNA called *AhR-responsive elements*. By influencing the rate of transcription of specific messenger RNAs, the rate of synthesis of the related proteins is altered. Thus, the binding of an appropriate compound (ligand) to the receptor can change the cellular concentration of certain proteins by regulating the expression of genes governing their synthesis.[93] The presence or absence of cofactors may account for differences in gene expressions by tissue. Other molecular mechanisms are of increasing interest.[92,94]

Among the proteins induced by the Ah receptor are three cytochrome P450 enzymes: CYPIA1, CYPIA2, and CYPIB1. These phase I enzymes oxidize "foreign" (xenobiotic) substances, including PAHs, plant constituents such as flavones, aromatic amines, and some pharmaceutical drugs. One consequence of this metabolic conversion is that the xenobiotic substance, typically lipid- rather than water-soluble, is then subject to further enzymatic conversion. Phase II enzymes add hydrophilic groups, enhancing water solubility and excretion from the body. In this manner, AhR-induced enzymes can reduce the biological effect of some environmental agents by facilitating their metabolic degradation.[95,96]

On the other hand, the oxidative transformation of a xenobiotic compound by the AhR-induced cytochrome P450 enzymes may greatly enhance its biological activity.[95] A classical example is 2-acetylaminoflourene (AAF), a potent liver carcinogen in rats. A number of studies have shown that the proximal carcinogen is not AAF but an oxidized metabolite produced via CYPIA2.[95,97] Preparations containing these enzymes are used in microbial mutagenesis tests to activate otherwise inert genotoxins.

When the oxidative degradation of the xenobiotic compound facilitates its excretion so that the intracellular concentration is reduced, a negative feedback is established: Binding of the compound to the receptor is also reduced, transcription decreases, the level of the cytochrome P450 enzymes diminishes, and the system returns to its initial condition. Other sources of feedback may also be present.[92]

TCDD and related halogenated compounds strongly induce CYPIA1 and CYPIA2 but are not readily oxidized by these enzymes. They are apparently protected from attack by the presence of halogen atoms in certain positions of the molecule. Hence, they are excreted very slowly, resulting in a prolonged and amplified response. In effect, a feedback system that governs behavior of other AhR-binding substances (such as the PAHs) is inoperative in the case of TCDD. Thus, the extraordinary biological potency of dioxinlike substances may be due to the consequence of their unique combination of two properties:

a high affinity for the Ah receptor and biological persistence. The relevance of persistence is evident from a comparison of the behavior of dioxinlike compounds and PAHs.* Although some PAHs bind to the Ah receptor with an affinity almost equal to that of TCDD, their in vivo potency (as measured by enzyme induction) is many orders of magnitude less.[98]

1.5.2 Perturbation of Hormones and Growth Factors

Although the induction of CYPIA1 is the best characterized of the biochemical effects of dioxinlike compounds, it is by no means the only one. The expression of a growing number of genes are thought to be regulated by the Ah receptor.[99,100] This may provide one mechanism whereby dioxinlike compounds perturb the regulation of hormones, growth factors, and other molecular messengers that control growth and differentiation with diverse and potentially devastating impact. Some examples follow.

TCDD may alter the levels of certain hormones through its influence on the enzymes that metabolize primarily xenobiotic compounds. For instance, TCDD induces one form of UDP-glucuronosyltransferase (UDPGT), a phase II enzyme that increases a chemical's solubility by adding glucuronic acid. In addition to xenobiotics, UDPGT also conjugates and enhances the excretion of thyroxine (T4), causing reduced serum levels of this thyroid hormone in rats.[101] Among the resultant complications is a perturbation of an important biological feedback system: The pituitary responds to low T4 with increased secretion of thyroid-stimulating hormone (TSH). When prolonged, this may lead to thyroid tumors,[102] a sensitive endpoint in TCDD-exposed rats.[103]

TCDD does not bind to steroid hormone receptors, and steroid hormones do not bind to the Ah receptor.[104] Nevertheless, TCDD affects steroid hormone regulation in more subtle ways. Thus, TCDD decreases (*downregulates*) the number of estrogen receptors in certain organs of the female rodent, making tissues less responsive to this hormone.[105] This may decrease both fertility and incidence of tumors of these organs, as has been suggested in rats exposed to TCDD postnatally.[106]

TCDD reduces testosterone levels in adult male rats by decreasing the production of testosterone from cholesterol in the testes at a critical rate-limiting step. The pituitary (and/or hypothalamus) normally responds to low testosterone concentration by increasing secretion of luteinizing hormone, causing increased production of testosterone. TCDD interferes with this feedback system, preventing the compensatory increase of luteinizing hormone.[107–110]

TCDD also affects growth factors, a class of extracellular signaling molecules. In the female rat liver, TCDD may increase migration of epidermal

*Recent work shows that changing a single nucleotide flanking the AhR-responsive element can determine whether a gene is transcribed by AhR bound to PAH or TCDD. This finding may have important implications for both understanding molecular mechanisms and extension of TEFs to other classes of compounds [see T. Matikainen et al., *Nature Genetics* **28:** 355–360 (2001)].

growth factor receptors (EGFRs) internally from the cell membrane, providing a stimulus for mitosis.[103,104,111] This effect appears to depend on ovarian hormones; interactions between EGFRs and estrogen receptors have been noticed elsewhere.[112] TCDD may affect EGFRs by increasing the levels of transforming growth factor α (TGFα), a ligand for EGFRs.[103] In mice, TCDD alters the differentiation of certain tissues in the developing palate. This may be caused by perturbation of growth factors and their receptors, including EGFRs. The palatal shelves come into contact but fail to fuse, resulting in cleft palate.[113]

TCDD can lead to increased phosphorylation of amino acids. Protein kinases often play important roles in transducing signals across cell membranes, regulation of growth factor receptors, and cell differentiation.[114] TCDD may alter regulation of the cell cycle.[94,115] TCDD also influences a number of other chemical messengers, including the glucocorticoid hormone receptor, plasminogen activator inhibitor, protein kinase C, interleukin-1β, and other cytokines.[99]

TCDD has been called a persistent *environmental hormone*.[116] One of its molecular mechanisms—binding to a receptor that regulates gene expression—has certain similarities to the action of steroid hormones[117] as well as important differences.[92] It alters cell growth and differentiation. It affects other hormones and growth factors, including altering the levels of their receptors. Finally, like hormones, TCDD causes significant effects at very low doses. This knowledge of dioxin's biochemistry increases our concern over its widespread occurrence in the environment.

Does the body possess some unidentified hormone that binds to the Ah receptor, serving an important but unknown function? Such a situation is not unprecedented. A number of such *orphan receptors* (i.e., receptors without known ligands) have been found.[118] Dioxin may be a case of *toxic mimicry*, possessing a molecular shape similar to that of its putative natural counterpart. The long residence time of TCDD in the body may alter expression of AhR-regulated genes for an inappropriately long period of time. It is also possible that the supposed natural ligand of the Ah receptor might normally function during a specific period of development; TCDD may activate the system at the wrong time.[119] *Knockout mice*—animals without a functional AhR—are viable but show defects in the development of the liver, immune system, and reproduction.[120–122] The question of the normal function of the AhR is a major goal of molecular research in the field.

1.5.3 Toxicity

From the foregoing account it is apparent that TCDD is capable of disrupting a wide variety of biochemical processes which are likely to lead to an equally broad spectrum of macroscopic toxic effects in animals. The latter include acute toxicity, "wasting" and death, atrophy of the thymus, liver damage, epidermal changes, immunotoxocity, birth defects, reduced fertility, endome-

triosis, and cancer.[8,98] It is generally thought that the Ah receptor mediates these effects,[8,9] although there may be exceptions.[123] Hence, it is assumed that other dioxinlike compounds will also cause these effects.

The relative sensitivity of various toxic endpoints appears to vary with tissue and species, implying that humans may be less sensitive than laboratory animals for some effects and more sensitive for others.[8,119] In this review we focus on cancer, reproductive/developmental effects, and immunotoxicity. They have formed the primary basis for regulatory efforts by a number of agencies and are central to the current USEPA reassessment of TCDD toxicity.

1.6 CANCER

1.6.1 Mechanisms

There is no doubt that TCDD causes cancer in animals. This has been shown in both sexes of several species[103,124] (see Chapter 18). The contentious points surround its carcinogenic mechanism(s) and their implications for human exposure at low doses. The development of cancer is generally thought to proceed in several steps: (1) an initial permanent alteration of a cell, typically some kind of genetic damage; (2) clonal proliferation of the altered cell; and (3) another permanent alteration in at least one of these cells, followed by more cell replication.[104] This last step may repeat several times, a process called *tumor progression*.

Two-stage cancer experiments attempt to partially dissect these steps: An animal is given a dose of a DNA-damaging (*initiating*) *agent* followed by chronic exposure to a *promoting agent*. In such an experiment, a classic promoter greatly enhances the number of tumors and precancerous lesions but causes little or no cancer by itself. Its action is considered reversible (i.e., removal of the promoter causes the tumor to regress). Initiation and promotion are operationally defined by this experimental protocol and are not necessarily synonymous with DNA damage and cell proliferation.[103,125]

In two-stage experiments involving rat liver and mouse skin, TCDD is an extremely potent promoter and displays little or no initiating activity.[103,126–128] The latter finding is puzzling given TCDD's ability to generate substantial numbers of tumors in the rat liver (and other organs) in long-term animal experiments (bioassays) when given alone (i.e., without a known initiator).[106] These divergent results explain why some researchers consider TCDD a promoter, whereas others consider it a "complete" carcinogen, able to induce tumors by itself.[124,129] The discrepancy might be related to differences in the food or environment of the test animals or the shorter length of exposure in the two-stage experiments.

Although it is possible that TCDD promotes background initiated cells damaged by some independent process, other evidence suggests that TCDD may act throughout the carcinogenic process (Figure 1.1). TCDD does not directly cause mutations in several common assays and therefore appears to

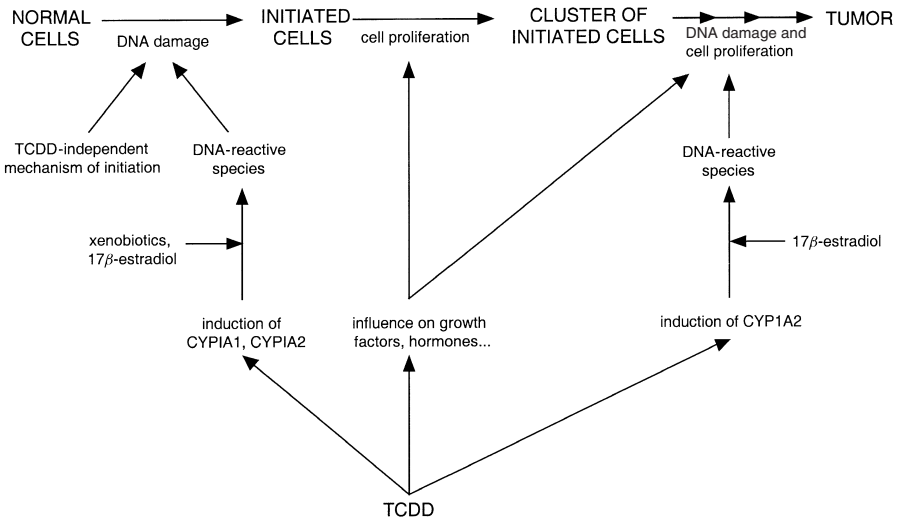

Figure 1.1 Some possible roles of TCDD in carcinogenesis of the female rat liver. (Adapted from Ref. 104.)

lack the direct genetic effect characteristic of an initiator. However, it is possible that TCDD contributes indirectly to DNA damage.[103] For example, by inducing CYPIA1 and CYPIA2, dioxinlike compounds may in some cases increase the conversion of other compounds into mutagens.[130] Lymphocytes from people exposed previously to dioxinlike compounds show increased frequency of sister chromatid exchange—suggesting genetic damage—when cultured with α-naphthoflavone (ANF); ANF is metabolized to an active form by CYPIA1.[131] On the other hand, some experiments show reduced DNA damage and cancer in animals dosed with certain polycyclic aromatic hydrocarbons (PAHs) after exposure to TCDD compared with animals exposed to PAH alone.[132] The balance between metabolic activation and deactivation may depend on the compound and the dosing regime.[103]

Two-stage studies of the female rat liver support the hypothesis that TCDD-induced cell proliferation is involved in promotion, although this may not be the only mechanistic step. Preneoplastic lesions were greatly increased in rats exposed to diethylnitrosamine (DEN) followed by TCDD, but not in animals exposed to TCDD alone. Cell proliferation was greatly increased in animals exposed to TCDD. These elegant studies also demonstrate the involvement of estrogenic hormones. Cell proliferation and preneoplastic lesions were significantly higher in intact rats than in ovariectomized animals.* TCDD may

*In these experiments, lung cancer was seen in ovariectomized females, but not in the intact animals. In long-term bioassays, TCDD significantly increases liver tumors in female but not male rats.[106,133] However, TCDD produces liver tumors in both sexes of mice.[133] The reason for this difference is unknown.

enhance cell proliferation via the epidermal growth factor pathway. Internalization of epidermal growth factor receptor (EGFR), a mitogenic stimulus, is enhanced by TCDD in intact but not ovariectomized animals.[111,128] The initiator DEN is metabolically activated by P450 enzymes other than CYPIA1 and CYPIA2.[134,135] Induction of these enzymes was unaffected by removal of the ovaries.

Evidence from rat liver studies suggests that TCDD may also play a role in tumor progression. Some precancerous lesions regress when administration of TCDD ends, but others continued to grow in size.[136] One possible explanation is that additional permanent alterations occurred in these lesions making them promoter-independent. TCDD may contribute indirectly to these events by another estrogen-dependent mechanism. Metabolism of the ovarian hormone 17β-estradiol by CYPIA2 can lead to the production of DNA-reactive species.[103,104,111]

Although most of the experimental work has been done on the liver, TCDD alters tumor incidence at numerous sites in long-term bioassays. This finding implies that a number of mechanisms may be involved. As discussed earlier, prolonged secretion of thyroid-stimulating hormone in response to TCDD-induced degradation of T4 may increase thyroid tumors in rats. TCDD decreases the number of estrogen receptors in the uterus and breast; this may be connected to the apparent reduction of tumors in these organs in the rat exposed postnatally.* Interestingly, prenatal exposure of rats to TCDD retarded the development of mammary tissue, making it more susceptible to carcinogen exposure later in life.[137]

TCDD may contribute to cancer in other ways, including interaction with viruses,[138] increased expression of protooncogenes,[139] decreased expression of tumor suppressor genes, oxidative stress,[94] altered regulation of the cell cycle,[115] and suppression of cell-mediated immunity.[140] Epstein–Barr virus, which is widespread in the human population, may cause B-cell proliferation and immortalization. Impairment of cell-mediated immunity by TCDD and other chemicals may allow continued proliferation and development into non-Hodgkin's lymphoma.[141] In one epidemiologic study, the combination of PCBs and Epstein–Barr virus had a strong synergistic effect on non-Hodgkin's lymphoma.[142]

In sum, TCDD may act at a number of steps in the carcinogenic process in conjunction with endogenous hormones, exogenous compounds, and viruses in an organ-specific fashion. Many aspects of its carcinogenic mechanism remain unknown.

1.6.2 Cancer Risk Assessment and Reassessment

Debate over the mechanism of TCDD carcinogenicity has played a central role in regulation of exposure and in the USEPA reassessments of its potency. The

*Reduction in body weight gain is another suggested explanation.[103]

TABLE 1.2 Some Acceptable or Tolerable Daily Doses of TCDD

Organization	Level (pg/kg per day)	Methodology	Basis[a]	Ref.
USEPA (1985)[b]	0.006	LMS model	Kociba	143
ATSDR (1998)[c]	1	Safety factor	Neurotoxicity in rhesus	144
WHO (1998)	1–4	Safety factor and body burdens	Various	145
Canada/Ontario (1985)	10	Safety factor	Kociba, Murray	146
Washington Department of Health (1991)[d]	20–80	Safety factor	Receptor occupancy	147

[a] Older values relied on the rat cancer study of Kociba et al.[106] and/or the rat reproduction study of Murray et al.[148]

[b] For an upper-bound lifetime cancer risk of 10^{-6}. Increase of cancer potency was proposed in draft reassessment.[8]

[c] ATSDR is the U.S. Agency for Toxic Substances and Disease Registry.

[d] Withdrawn after discounting of receptor threshold hypothesis.

general goal of these efforts has been the identification of a ''safe'' or ''acceptable'' daily dose of dioxin. As Table 1.2 shows, the values used by a number of countries and government agencies have ranged from 0.006 to over 20 pg/kg body weight per day, a factor of several thousand. Even the high end reflects the extreme toxicity of TCDD: A picogram is a mere trillionth of a gram.

These risk assessments have generated intense controversy. The average resident of the industrialized countries is exposed to about 1 pg/kg per day of TCDD equivalents (TEQs).[8] If values from the upper end of Table 1.2 are used, the average dose is considered acceptable. On the other hand, average exposure greatly exceeds the lower estimates of acceptable dose, a situation some interpret as requiring remedial action.[149] In practice, the USEPA regulates incremental exposure only from a single source or medium. Nevertheless, some sources fail to meet even these standards.

One can immediately see one reason for the political pressure placed on the USEPA: Its estimate of the acceptable dose is one of the lowest in Table 1.2. A number of dioxin-generating industries and owners of dioxin-contaminated sites have, perhaps not surprisingly, maintained that higher values are more appropriate.

Another fundamental reason for disagreement lies in scientific differences about how to construct an acceptable dose. Practical considerations restrict the number of animals that can be used in a cancer bioassay. This imposes a limit on the ability to detect an increased number of tumors. To avoid false negatives, the doses employed are typically much larger than those commonly experienced by people. Judging the safety of the latter requires extrapolation from high to low dose and from animal to human. Depending on how these

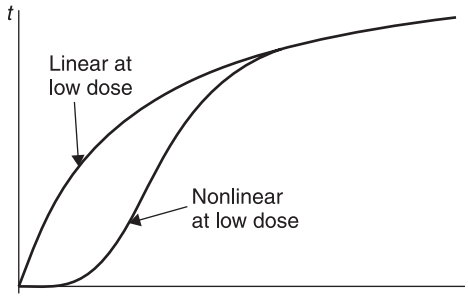

Figure 1.2 Two possible dose–response curves.

extrapolations are carried out, the same data can readily give rise to very different conclusions.

The primary theoretical difference between the high and low estimates of acceptable dose is the shape of the dose–response curve at low doses, in particular the presence or absence of a threshold (Figure 1.2). The high values are derived from the view that there is a dose of TCDD below which there is no effect. They are typically estimated by applying a safety factor to an experimentally defined *no observed adverse effect level* (NOAEL) or *lowest observed adverse effect level* (LOAEL). Such levels depend on both the biology of the phenomenon and the methodology and statistics of the experiment. Thus, an effect might occur at some low dose, but the particular experimental design may not be powerful enough to distinguish it from the control value. Ontario's tolerable average daily intake, 10 pg/kg per day, is based on a presumed no-effect level in animals of 1000 pg/kg per day for cancer and reproductive effects with a safety factor of 100.[146]

In contrast, the low acceptable dose is based on the theory that there is no threshold for cancer. The probability of cancer is assumed to be directly proportional to dose at low dose. Since there is no completely safe dose, the *slope factor* or *potency* of the chemical is used to calculate the dose resulting in a certain lifetime risk of cancer that is regarded as acceptable. The USEPA followed this procedure in its landmark 1985 assessment of TCDD carcinogenicity.[143] The slope factor was estimated from rat tumor data from Kociba et al.[106] using the linearized multistage (LMS) model of cancer. A small additional factor adjusted for possible interspecies differences. According to this estimate, an average daily dose of 0.006 pg/kg per day corresponds to an upper-bound excess lifetime cancer risk of 1 in a million (10^{-6}). Hence, if 1 million people received this level of exposure over their lifetime, less than one additional case of cancer would be expected. The USEPA has considered this level of risk acceptable (*de minimus*) as a matter of policy, although the agency often uses or approves higher values.

Assumptions about the shape of the dose–response curve for cancer depend critically on the mechanism of carcinogenicity. The linear multistage model

(LMS) used by the USEPA in 1985 was derived from the theory that cancer involves a sequence of irreversible stages.[150] There have been numerous criticisms of this approach as it applies to TCDD. One group has argued that TCDD is a promoter, not an initiator, and is therefore subject to a threshold. They contend that the NOAEL/safety factor approach is more appropriate. Others argue that the LMS model is not appropriate because it does not take cell proliferation into account. However, other work indicates that even a pure promoter can act linearly at low dose if its effect is additive to background processes.[151]

A third source of controversy concerns the role of scientific uncertainty in regulatory policy. Whereas some argue that pollution should be allowed until proof of harm is certain, others advocate more precautionary approaches. The USEPA has in the past relied on linear models, assuming that this approach will be more protective of public health.[152]

Some of these arguments were raised during the USEPA's 1988 reassessment of TCDD's slope factor. USEPA concluded that TCDD may cause cancer through a variety of mechanisms and that the LMS model would be retained, in part because there was no adequate alternative model. Nevertheless, the agency's Dioxin Workgroup argued that the 1985 slope factor estimate "is likely to have led to an overestimate of risk."[153] Although the degree of overestimation was unspecified, they proposed raising the acceptable dose to 0.1 pg/kg per day, simply as a matter of policy. This proposal was rejected by the agency's Science Advisory Board because no new scientific evidence had been presented to justify the change in the risk estimate.[154] However, the board expressed concern about the applicability of the LMS model to TCDD and encouraged development of new risk models that would incorporate additional mechanistic research into risk assessment, in particular, receptor mediation of toxicity.

1.6.3 Reassessment II

By the late 1980s a new political factor entered the dioxin arena: the interest of the paper and allied industries. Dioxin had been discovered in effluent and products from pulp mills using chlorine as a bleaching agent. The industry was concerned about possible legal action and impending surface water quality standards. The paper industry began a campaign to "get EPA to 'rethink' dioxin risk assessment."[155,156] The chlorine-producing industry became an ally, presumably because the paper industry consumed a significant fraction of their output.

A new challenge to the USEPA's cancer slope factor came in 1989 during consideration of a water quality standard for the state of Maine. Female liver tissue samples from the 1978 Kociba experiment were reviewed and reclassified based on new criteria for the presence of tumors.[157] Tumors from other sites and bioassays were not examined, although some—male rat thyroid and male mouse liver—also produce high slope factor estimates.[8,129] The revised liver

tumor data might reduce the TCDD cancer slope factor estimate by a factor of about 2 to 3, an insignificant amount in view of the underlying uncertainties.[158] Although some proposed that the liver tumors were a secondary response to hepatotoxicity,[159] a review by the USEPA and Food and Drug Administration disagreed.[103,160]

In October 1990, the Chlorine Institute and the USEPA cosponsored a scientific meeting entitled the "Biological Basis for Risk Assessment of Dioxins and Related Compounds" at the Banbury Center. There was general agreement among the toxicologists and biochemists present at the meeting that the currently known toxic effects of dioxin are mediated by the Ah receptor. Although this was not particularly radical thinking, some participants drew a controversial conclusion: Receptor mediation implied a threshold for the biological effects of dioxin. They stated that a certain number of receptors must be occupied for any biological effect to occur. Rather than being linear at low doses, the dose–response curve was shaped like a hockey stick: flat or increasing very slightly at first and becoming linear only at higher doses. Furthermore, a practical threshold for toxic effects could be determined from the dose of TCDD necessary to induce CYPIA1, the presumed most sensitive endpoint. The resulting rough and rapidly calculated estimate was about 1 to 3 pg/kg per day, much greater than the USEPA's value of 0.006 pg/kg per day.[2,161] A public relations firm hired by the Chlorine Institute went further, claiming in a press packet that the attendees had formally reached consensus on the threshold concept. This was not in fact correct, prompting vociferous protests and creating a minor scandal in the scientific press.[162]

This meeting set the stage for another dioxin reassessment by the USEPA, which was announced in April 1991. The primary focus would be the development of a new biologically based model for dioxin toxicity, developing the ideas from the Banbury conference and the earlier comments of the Science Advisory Board.[163–165] Incorporating the latest scientific findings, the new model would provide an alternative to the safety factor and LMS approaches to risk assessment. News of the reassessment was reported widely, including the notion that TCDD was much less toxic than previously thought.

Meanwhile, the paper industry used the supposed outcome of the Banbury Conference to argue for relaxed TCDD water quality standards in a number of states (e.g., Ref. 166). The Washington State Department of Health issued revised guidelines for fish consumption based on a tolerable daily intake in the range 20 to 80 pg/kg per day. This value was calculated by applying a safety factor to the dose estimated to give 5% occupation of Ah receptors in the rat liver, a level assumed necessary for any biological response. No references to the scientific literature were given for this crucial assumption.[147] The tolerable daily intake was later withdrawn.

There are good reasons to be skeptical of the claim that involvement of a receptor requires a threshold. The simple classic model for receptors predicts a linear relationship between low concentrations of TCDD and the amount of

receptor-bound dioxin.* Biological responses would not have a threshold if they are proportional to the amount of receptor-bound TCDD. Of course, the dose–response curves of more complicated biological responses might deviate from linearity, but this is only a possibility and not a requirement of receptor theory.[207]

The simple threshold model was seriously weakened at the Eleventh International Symposium on Chlorinated Dioxins and Related Compounds, held in North Carolina in September 1991. Studies yielded data on induction of CYPIA1 and CYPIA2 in the rat liver that were consistent with both threshold and nonthreshold (low-dose linear) models. The no-threshold models provided the best mathematical fit. Similar results were found for dioxin-induced loss of EGFR from plasma membrane.[167,†] These results relied on extrapolation from experimental doses, so it is possible that a deviation from linearity may exist at lower doses. However, increased messenger RNA for CYPIA1 has been detected in rats at doses corresponding to background tissue levels in humans.[168,169] Hence, if there is a threshold for this effect, human tissues may already be above it. If the dose–response behaviors of CYPIA1, CYPIA2, and EGFR are used as surrogates of toxicity, the cancer risks posed by TCDD may be as high or higher than previously estimated by USEPA.[167,170] In sum, the model proposed by some at the Banbury meeting is incorrect: Action of TCDD through the Ah receptor does not necessitate a threshold.

The next question, of course, is whether these biochemical markers are reasonable surrogates of cancer or other toxic effects. As noted earlier, CYPIA1, CYPIA2, and internalization of EGFR may be related to cancer in the rat liver. On the other hand, liver cell proliferation and preneoplastic foci show no detectable increase at low doses of dioxin. The dose–response curve for these higher-level biological responses may be nonlinear, but substantial variability among experimental animals clouds the issue.[104,128]

The current dioxin reassessment is producing biologically more realistic models, but these still contain substantial uncertainty, especially with respect to the gap between biochemical markers and more complicated biological responses. Although much of this work has gone into modeling cancer of the rat liver, TCDD causes cancer in other organs as well. Since their mechanistic details appear to differ, they will require additional modeling efforts. Biologically realistic models will also have to address other complications, such as interactions with other compounds and viruses.

*Some people may have been confused by different ways of plotting the fraction of occupied receptors as a function of ligand concentration. Plotted on a linear scale, the curve is linear at low dose; plotted on a log scale the curve looks nonlinear at low doses. The latter version was printed in the *Science* story covering the Banbury conference.[161]

†The difference depends on whether the action of TCDD is additive or independent of existing processes.[167] Heterogeneity of liver cell response suggests a further complication. Low doses of TCDD appear to induce CYP1A1 and CYP1A2 maximally in some cells and little or none in others; increasing the dose "turns on" more cells.[168]

1.6.4 Human Effects

Is dioxin merely a powerful rodenticide? Are humans somehow exempt or less susceptible to the biological effects of dioxin? Is it true that "chloracne is [the] only adverse effect associated with human exposure"?[171] Such arguments are sometimes offered for downgrading the toxicity of dioxin. If correct, the allowable doses of dioxin overstate the real hazard since they are based on animal research.

The reasons for the primary reliance on animal research are well known. First, the underlying biology of animals and humans is generally similar. Second, experimentation on humans is usually considered unethical. Only at Holmesburg prison, Pennsylvania, were people (other than the scientists involved) deliberately exposed to TCDD to test toxicity, in this case the dose necessary to cause chloracne. The fate of most of these unfortunate people is not known.[14,172]

The inadvertent exposure of people to large amounts of dioxinlike compounds began at the turn of the century (see Table 1.1). Chloracne—a severe, persistent acnelike disease—was first described in 1897–1899 in workers handling tarry wastes from the production of chlorine using graphite electrodes.[173] Chloracne cases were observed during World War I following exposure to polychlorinated naphthalenes; these *halowaxes* were used in the production of gas masks.[174] Several of the best known occupational exposures to dioxin occurred around midcentury in facilities manufacturing TCDD-contaminated herbicides. In the Yusho and Yucheng incidents (see Chapters 21 and 22, respectively), people consumed rice oil contaminated with PCBs, PCDFs, and related compounds. Populations were exposed by the use of the herbicide Agent Orange in Southeast Asia, the spreading of dioxin-contaminated wastes in Missouri and the chemical accident at Seveso, Italy (see Chapter 20). Indeed, it was learned in the 1980s that we are all exposed: The general population of the industrialized world carries some quantity of PCDD and PCDF in their bodies.[37,175–180]

Information on the effects of dioxin on humans has been obtained from some of these experiences by comparing the rates of disease in exposed and reference populations. Given the uncontrolled nature of human exposure, it is important to take note of the strengths and weaknesses of these epidemiologic studies. Certain effects, such as cancer, may not occur until many years, even decades, after exposure. Other effects (e.g., subtle neurobehavioral abnormalities in children) may be missed unless specifically looked for. It can be difficult to exclude confounding factors that might contribute to changes in disease incidence. Effects may not be detected unless the exposed population is sufficiently numerous, and the difference in exposure from control groups is relatively large. Estimating who was exposed and at what levels is often quite difficult. As a result, many supposedly negative studies are in reality merely inconclusive. When biologically plausible effects are seen in a number of carefully performed studies, the implications need to be taken very seriously.

Qualitative Evidence for Cancer in Humans Much of the debate about human effects has centered on the question of whether TCDD causes cancer in people. That it causes cancer in animals should not be in doubt. Given similar biology, this strongly suggests that it will cause cancer in humans as well. As of the late 1980s, the human epidemiological evidence was mixed, including both positive and negative studies. Uncertainty was increased by the difficulty of establishing exposure and of separating the possible effects of TCDD from other chemicals to which people were often coexposed (e.g., phenoxyacetic acid herbicides). Some denied any connection between increased human cancer and exposure to phenoxyacetic herbicides and/or their dioxin contaminants.[181–183] Others, such as the Agent Orange Scientific Taskforce—a group of independent scientists on which one of us (B.C.) served—concluded that there was sufficient evidence to legally qualify Vietnam veterans for compensation for several types of cancer and disease,[184,185] a position that the U.S. government later adopted.[186] At that time, the USEPA and the International Agency for Research on Cancer (IARC) rated TCDD as a probable human carcinogen, based on what they considered sufficient evidence in animals and inadequate evidence in humans.[143,187]

The position at that time can be illustrated with two sets of studies. Beginning in the late 1970s, Hardell and others found increased numbers of a very rare cancer, soft tissue sarcoma (STS), in Swedish forestry and agricultural workers exposed to phenoxyacetic acid herbicides and/or chlorophenols which are frequently, but not always, contaminated with dioxins.[188–192] On the other hand, several early studies of chemical workers thought to have been exposed to high levels of TCDD during the manufacture of herbicides and chlorophenols were considered negative. In particular, cancer mortality was not increased in workers exposed following an accident in 1949 at a Monsanto facility in Nitro, West Virginia.[193–195] According to a 1989 report by the World Health Organization, this was one of only two such incidents that "have been adequately followed up epidemiologically with matched control groups."[196] Others have called it a "major source of information about the effects of high-level dioxin exposure."[181] In retrospect, the pioneering work of Hardell et al. on STS has survived criticism better than the Monsanto studies. The latter are at the very least flawed by exposure misclassification.[197,198] Similar controversy surrounds a number of human health studies.[199]

Establishment of exposure played a crucial role in the debates. A technical breakthrough came with the ability to measure PCDD and PCDF in human tissues, first in breast milk,[200] and later in adipose tissue and blood.[37] Since PCDDs and PCDFs are persistent in the body, this provides a useful measure of past exposure (although the absence of elevated levels does not necessarily preclude exposure[184,201]). The U.S. National Institute of Occupational Safety and Health (NIOSH) followed this approach in its landmark retrospective cohort study of male American chemical workers thought to have been exposed to TCDD.[202] Over 5000 workers were included from 12 plants (including Monsanto's Nitro, West Virginia plant). Blood serum from a sample of

workers was used to validate estimates of exposure made on the basis of work history.

NIOSH found a 15% increase [relative risk = 1.15, 95% confidence level (CI) = 1.02 to 1.30] in total cancer mortality in the entire cohort. In a subcohort of 1520 men with more than one year of exposure and over 20 years of latency—a group most likely to show effects—there were increases in overall cancer (RR = 1.46, CI = 1.21 to 1.76) as well as mortality from soft tissue sarcoma (RR = 9.22, CI = 1.90 to 26.95) and respiratory tract cancer (RR = 1.42, CI = 1.03 to 1.92). NIOSH conservatively concluded that these results were "consistent with the status of TCDD as a carcinogen."[202] A follow-up to the NIOSH study has been published.[203] Similar results on lung cancer and all cancers combined have been observed in other occupational studies.[204,205] Certain types of cancer were increased in Seveso as well.[206]

The increase in mortality from all cancers combined noted in several occupational studies is somewhat unusual, as chemical carcinogens are typically associated with a particular organ. In this respect, the human results appear consistent with the animal experiments, in which TCDD causes cancer at multiple sites. There are also some parallels between rodents and humans with respect to certain sites. However, while liver tumors are observed in TCDD-exposed rodents, they are not generally seen in TCDD-exposed people. If TCDD-related liver cancer in humans is dependent on ovarian hormones as it is in rats (but not mice), this may be related to the fact that most studies have examined men.* Primary human liver cancer is very rare outside sub-Saharan Africa and Asia.[208]

The newer studies shifted the weight of evidence toward considering TCDD as a human carcinogen. The agreement of several studies of occupationally exposed men with reasonable checks on exposure provided the strongest evidence thus far. On this basis, IARC revised its qualitative ranking of TCDD upward from inadequate to limited human evidence. In conjunction with sufficient animal evidence and mechanistic considerations (newly added to its classification system), IARC declared TCDD a human carcinogen in 1997.[205] The USEPA proposed a similar reclassification in its draft reassessment.[8]

Quantitative Evidence for Cancer in Humans The publication of the long-awaited NIOSH findings in January 1991 provided part of the scientific rationale for USEPA's reassessment.[165] The study also played a curious role in the public discussion of dioxin. Although it strengthened the qualitative evidence for cancer in humans, it was often portrayed as showing reduced danger from TCDD.[209–211] Some had the story completely wrong, reporting (without qualification) that cancer mortality was not elevated significantly in the cohort.[212,213] Others reported the NIOSH results as indicating that TCDD causes cancer in humans only at very high doses. NIOSH found "statistically

*Ovariectomy increases lung cancer in two-stage experiments with TCDD in the female rat, suggesting a partial hormonal dependence for this tumor.[111]

significant'' increased cancer mortality in the entire cohort as well as in a highly exposed subcohort. They had also analyzed a less exposed subcohort (less than 1 year of exposure and over 20 years of latency). Although cancers of some sites were elevated in this group, none were statistically significant at a 95% level. These results may have been interpreted by some as showing a threshold, in apparent agreement with the alleged consensus of the Banbury conference held only a few months earlier.[214] However, the epidemiological evidence is ambiguous on this point; such results could arise merely from lack of statistical power (i.e., an insufficient number of subjects).[215]

Another argument was that much less cancer was observed in the exposed chemical workers than was expected based on rat experiments. The question of whether TCDD is less potent in humans than in animals was also discussed during USEPA's 1988 reassessment.[216] The NIOSH data presented a better opportunity to test this idea. Several groups estimated the carcinogenic slope factor of TCDD from the increased cancer mortality and the dose these men received, projected from the current serum levels. The results were approximately the same as or higher than those derived by the USEPA in 1985 based on rat data.[217,218] Similar results have emerged from newer studies.[8,219] Indeed, a recent draft of the USEPA reassessment proposed an increase in the cancer potency for TCDD.[8]

Although the epidemiological studies of the last decade have yielded important new evidence for TCDD's carcinogenicity in humans, arguments continue to rage. In the meantime, other signs of danger arose from a completely different direction.

1.7 SENSITIVE NONCANCER EFFECTS

1.7.1 Livestock and Wildlife

Twentieth-century chlorine chemistry exposed livestock and wildlife as well as humans to dioxinlike compounds (Table 1.1). A mysterious cattle malady known as *X disease*, marked by thickened skin (hyperkeratosis), was described in 1947[220]; its cause was later determined to be chlorinated naphthalenes.[221] Polybrominated biphenyls—some with dioxinlike activity—were inadvertently added to cattle feed in Michigan in 1973–1974.[222] This episode has been called the ''most costly and disastrous accidental contamination ever to occur in United States agriculture.''[223] Sickness and death of horses and other animals was one of the first signs of trouble at Times Beach, Missouri, where TCDD was the toxic agent. Chick edema disease was first described in 1957 in the southeastern United States.[224,225] Millions of chickens have since died or had to be killed as a result of consuming feed contaminated with dioxinlike compounds. Several outbreaks were caused by feed containing ''toxic fat'' derived from animal hides treated with chlorophenols.[221,226]

Although these episodes with livestock were due to specific contaminations, a much more ominous phenomenon appeared in wildlife during the

1960s.[227,228] Epidemics of reproductive and developmental problems have since been observed in fish-eating birds from the Great Lakes and elsewhere. There is good evidence that some of these episodes were caused by dioxinlike compounds. The *Great Lakes embryo mortality, edema and deformities syndrome* (GLEMEDS) has very close parallels to chick edema disease. The effects are consistent with the results of laboratory studies with dioxinlike compounds.[227,228] A strong correlation was found between the egg mortality of double-crested cormorants and the levels of dioxinlike compounds found in the eggs, as measured by enzyme induction.[229] Forster's terns from Green Bay hatched fewer eggs in 1983 than those from a less contaminated area. Eggs from the contaminated area had significantly higher levels of dioxinlike compounds. A cross-fostering experiment in which eggs were switched between the two areas showed that parental behavior played a role as well. Adults from the contaminated area took less care of their eggs.[230] The neurotoxic effects of certain nondioxinlike PCBs may have contributed to the latter problem.[10] Dioxinlike compounds may cause bill deformities and embryonic abnormalities in double-crested cormorants and Caspian terns.[231–233]

Although certain locations, such as Green Bay, had a particularly high incidence of GLEMEDS, the problem appeared to be relatively widespread in the Great Lakes. Egg mortality and bill defects of double-crested cormorants were generally more prevalent in Great Lakes birds than in reference areas.[229,231] Persistent dioxinlike PCBs were probably the major problem,[229,230] although outbreaks among Lake Ontario herring gulls in the 1970s were probably caused mainly by TCDD discharged from chemical manufacturing and waste dumps on the Niagara River.[227]

Reproductive and developmental problems have also been observed in lake trout, which are particularly sensitive to TCDD during early development. Failure of restocked fish to reproduce in Lake Ontario during the 1970s was probably related to TCDD and dioxinlike compounds.[84,234] The decreased number of mink and otter found around the Great Lakes may have been connected to PCBs and dioxinlike compounds.[235] Great blue heron chicks living near a pulp mill in British Columbia suffered depressed growth and greater edema than did birds from a less contaminated area.[236] Crossed bills have been observed in cormorants from a number of other North American locations.[231] Concentrations of dioxinlike compounds in the yolk sacs of Dutch cormorants correlated with developmental impairment.[237]

Dioxins, PCBs, and other organochlorines have been linked to increased disease in wildlife. Immunosuppression is strongly associated with PCBs in herring gull and Forster's terns chicks from the Great Lakes.[238] Dioxinlike PCBs may have contributed to the 1988 mass die-off of seals in the Baltic through immune suppression.[239,240]

Developing animals may be particularly sensitive to dioxinlike compounds. Persistent dioxinlike compounds bioaccumulate in the adult and are transferred to the vulnerable developing organism. Wildlife studies imply that entire ecosystems have been overburdened with dioxinlike compounds. This is a pro-

foundly disturbing finding, as it implies that significant problems may be due to general environmental contamination rather than specific accidents or spills. It also serves as a warning of potential hazards to the human population. In this respect, wildlife can be thought of as the modern equivalent of the miner's canary.

1.7.2 Sensitive Noncancer Effects in Laboratory Animals

The dioxinlike compounds perturb hormones and growth factors, powerful regulators of growth and development. This suggests that organisms may be quite susceptible to the effects of these compounds during certain periods of development. Considerable laboratory work has been performed in developmental and reproductive toxicology[241–242] (see Chapter 19). We discuss only some of the most sensitive results (Table 1.3).

Reproductive effects occur in rats at very low doses. Male and female rats exposed to TCDD over three generations showed decreased fertility at 10 ng/kg per day,[148] with indications of effects at a 10-fold lower dose.[251] Some of the older acceptable daily intakes (Table 1.2) were based on this early experiment, published in 1979.

TABLE 1.3 Some Sensitive Endpoints of TCDD (LOAELs)

Species	Effect	Dose	Incremental Body Burden[a] (ng/kg)	Refs.
Rat	Litter size	10 ng/kg per day	290[b]	148
Rhesus	Object learning	~0.160 ng/kg per day	42[c]	243
Rhesus	Endometriosis	~0.160 ng/kg per day	42	244
Rat	Genital malformation (female)	200 ng/kg[d]	73[c]	245
Rat	Immune suppression	100 ng/kg[d]	50[c]	246, 247
Rat	Decreased sperm count	64 ng/kg[d]	28[c]	248, 249
Mouse	Immune suppression (viral susceptibility)	10 ng/kg[d]	10[e]	250
Adult human	Background (TEQ)	1 pg/kg per day	~5	8

Source: Adapted from Ref. 145.

[a] Rodent background body burdens are about 4 ng/kg.

[b] Steady-state body burden in F1 and F2; LOAEL may be lower.[251]

[c] Estimated maternal body burden above background.

[d] Single dose on specific day of pregnancy.

[e] Not used by WHO to set the TDI (see the text).

More recent work has identified more sensitive endpoints. Central nervous system effects were found in rhesus monkeys exposed to TCDD in utero and through lactation. The infant monkeys displayed subtle alterations in certain learning behaviors at chronic maternal doses as low as 0.16 ng/kg per day.[243,252] Prenatal mortality was increased at doses of 0.64 ng/kg per day.[253]

Elevated incidence and severity of endometriosis, a condition characterized by aberrant growth of uterine tissue within the abdominal cavity, was observed in rhesus monkeys chronically exposed to dioxin.[244] Survival of endometrial tissue transplanted to the pelvic cavity was enhanced in cynomolgus monkeys exposed to dioxin.[254] As endometriosis does not occur naturally in rodents; it would not have shown up in early classic reproductive toxicology studies of rats.[148] Endometriosis can be surgically induced in rodents, however. Dioxin promotes surgically induced endometriosis in rats and mice.[255,256]

Strong evidence for the sensitivity of transgenerational effects came from elegant studies of the developing rat. Earlier we mentioned the TCDD-related reduction of testosterone in adult male rats. The dose causing a 50% change (ED_{50}) for this and related effects is about 15 μg/kg.[257] The developing male rat is much more sensitive than the mature animal. The ED_{50} for changes in spermatogenesis, size of secondary sex organs and sexual behavior was only 0.16 μg/kg for a single dose administered to the mother on day 15 of pregnancy. Some effects were seen at the lowest dose tested, 0.064 μg/kg. By day 15 of gestation most organs have been generated and the testis begins to secrete testosterone.[249,258–260] Since chronic dosing of the mother leads to accumulation in her tissue and transfer to the fetus, even lower daily doses would be expected to produce the same results. Genital effects were also found in female rats exposed in utero and lactationally.[245]

TCDD effects the immune systems of laboratory animals in minute doses.[261] It inhibits proper maturation and differentiation of both T-cells and B-cells, important in cell-mediated immunity and the production of antibodies. Exposure of pregnant female rats led to suppression in their offspring of delayed-type hypersensitivity (DTH), a localized inflammatory reaction.[246,247] Captive harbor seals fed Baltic fish had suppressed DTH relative to controls fed cleaner Atlantic fish. The seals' blubber contained 210 ng TEQ/kg lipid versus 62 for controls.[239] Eight-week-old mice treated with 10 ng/kg of dioxin experienced higher mortality when exposed to influenza virus 1 week later.[250] WHO did not include the latter experiment when updating the "tolerable" daily intake in part because of the lack of dose–response in the study.[145] As this experiment is one of the most sensitive adverse effects yet identified, additional research on viral susceptibility is needed.

1.7.3 Noncancer Effects at Near Background Levels: Animal Evidence

Might dioxinlike compounds be causing noncancer effects in the general human population? One approach to this question compares average human exposure

with the levels observed to cause effects in animals. Table 1.3 lists some of the most sensitive effects identified from animal experiments. Several of these are transgenerational, in which the infant, exposed in utero and/or via lactation, is more sensitive than the adult. We assume that these effects are mediated by the Ah receptor and that current TCDD-equivalents factors are applicable.

An "acceptable daily dose" of 10 pg/kg per day for reproductive and developmental effects is used by some governmental bodies (Table 1.2). This value is based on the multigenerational rat fertility experiment of Murray et al.[148] with a presumed NOAEL of 1 ng/kg per day and a safety factor of 100. By this logic, the average human daily background dose of about 1 pg/kg per day of TEQ is considered acceptable. This conclusion may not be sufficiently protective of public health. The data shown in Table 1.3 support an acceptable daily intake of 1 pg/kg per day or less.

There is even less room for optimism when the data are examined in terms of body burdens. Since most dioxinlike compounds are biologically persistent, they accumulate in the body. Measures of internal exposure may be more relevant for many toxic effects than daily dose, a measure of the external rate of exposure.[8,243,253,262] Direct comparison of doses ignores interspecies differences in the half-life of TCDD: on the order of 7 to 10 years in humans, months in monkeys, and several weeks in rats.[87,253] Given the same daily dose, the overall concentration in people at steady state will be considerably higher than that in rats. Ideally, one would want to compare concentrations in target tissues over critical time periods, taking into account differences in distribution, sensitivity, and the biology of the endpoint. For several biochemical endpoints, human cells may be as least as sensitive to dioxin as are rat or mice cells.[263,264]

The simplest comparisons are between the average body burden—average total concentration—found in people and the steady-state body burdens that cause effects in animals. As Table 1.3 shows, effects occur in the monkey at body burdens that are uncomfortably close to the present average human levels in industrialized countries.

The single doses that cause developmental and immunotoxic effects in rodents are less than a factor of 10 above the average human body burden. Although single doses are not equivalent to steady-state body burdens, redistribution to fat takes place quite quickly. This source of uncertainty could be reduced with additional chronic dosing experiments and better knowledge of the biology of the phenomenon, especially critical time periods.

Reproductive and developmental effects may occur at body burdens that are within about a factor of 10 of the average U.S. resident. This provides little or no *margin of safety**: (1) effects may be seen at lower body burdens (the values in Table 1.3 are low effect levels); (2) body burdens vary between individuals, with some members of the general population being higher than average; (3) some people may be more sensitive than others; and (4) the relative sensitivities of humans and animals to these effects are unknown.

*Called a *margin of exposure* in current USEPA terminology.

1.7.4 Noncancer Effects at or Near Background Levels: Human Epidemiology

Highly exposed groups provide evidence for reproductive, developmental, and immunotoxic effects in humans.* Loss of libido was reported early on in chemical workers exposed to TCDD, providing a possible sign of hormonal effects in humans.[196] Reduced testosterone and/or elevated luteinizing hormone was found in occupationally exposed men.[265] Children exposed in utero to a complex mixture of PCDFs, PCBs, and other compounds (Yucheng; see Chaper 22) suffered a number of effects, including developmental and neurotoxic damage, increased respiratory disease and middle ear infections, and reduced penis size at adolescence.[266–268] Children from Seveso with chloracne experienced transient changes in immune parameters, but adverse immunological effects were not observed. The sex ratio of children born in Seveso was altered for several years,[269] but not after Yucheng.[270] So far, there is only limited (and mixed) human epidemiological evidence on endometriosis[204]; a major study of women from Seveso is currently under way.

Neurobehavioral effects and smaller birth size have also been observed in general populations exposed to complex mixtures of dioxinlike and nondioxinlike compounds. Reduced short-term memory was found in infants and 4-year-old children of women who consumed Lake Michigan fish. The effect was more highly correlated with levels of total PCBs in umbilical cord serum than with lactational exposure, implying an in utero effect.[271–273] This interpretation is supported by cross-fostering experiments in rats.[274] A number of potential confounding factors were controlled, including exposure to certain other toxics. However, a contribution by unreported xenobiotics is possible. The most disturbing aspect of the study is the relatively low exposure. The women in the study only consumed, on average, the equivalent of two to three salmon or lake trout meals per month. Cognitive effects in children were associated with prenatal exposure to PCBs in a North Carolina cohort drawn from the general population. However, the effects did not persist as long as in the Michigan group.[275,276]

Effects of dioxins and PCBs on neurodevelopment, the immune system, and thyroid hormone were observed in a cohort of children from the general population of the Netherlands. Many effects tended to be subtle, to diminish over time, and to be more associated with in utero than lactational exposure. Some effects remained at 42 months of age, including small deficits in cognitive ability, increased prevalence of chickenpox and middle ear infections, decreased allergic reactions, and shortness of breath. Altered lymphocyte subsets and levels of thyroid-related hormones (up to 3 months) were associated with levels of PCBs and TEQ, although the changes were within the normal range. PCBs

* For a discussion of cardiovascular disease, diabetes, and other effects, see Ref. 204.

appeared to a more important contributor than TEQ to many effects, although certain endpoints were also related to dioxins.[277–280]

Neurodevelopmental effects were also associated with low-level PCB exposure in a German study.[281] Neurobehavioral studies of Faroese children suggested an interactive effect of PCBs and methyl mercury.[282] Effects of dioxinlike compounds on thyroid hormones and immunological parameters were observed in Japanese children from the general population.[283,284]

Additional evidence for an effect of dioxins in the general population comes from studies of teeth. A study of breast-fed Finnish children found an association between dioxin exposure and hypomineralization defects of permanent teeth.[285] As permanent teeth in humans are mineralized during the first 2 years of life, exposure was estimated from TEQ in the breast milk of mothers multiplied by the length of breast feeding. Severity of the defects was related to this measure of exposure but not to TEQ levels alone or length of exposure alone. These findings suggest the effect is due primarily to lactational exposure. Addition of dioxinlike PCBs did not increase the fit of the model. Teeth defects were observed in the rice oil poisonings. It would be very useful to repeat the Finnish study in other groups of children.

There are some toxicological data to support effects on tooth development. Dioxin caused defects of dental hard tissues in rats.[286,287] Epidermal growth factor receptor may be involved in the mechanism.[288] Rhesus monkeys exposed to PCB suffered dental defects and changes in ameloblasts (enamel-forming cells).[289]

We conclude that there is some evidence for developmental effects in children from the general population. However, as exposure to background levels of dioxinlike and nondioxinlike compounds typically take place together, it is often difficult to sort out their respective effects. The neurodevelopmental effects were more associated with in utero exposure. The dental effects were more strongly related to lactational exposure, a finding consistent with the timing of tooth mineralization in humans. Breastfeeding infants typically receive doses about one to two orders of magnitude greater than adults,[290] although the difference in body burdens is not nearly so large.[8] While breast-feeding infants are a highly exposed group within the general population, their exposure derives from the accumulation of these compounds in mothers. Reduction of environmental levels—and thus exposure to potential mothers and their offspring—is a sensible goal.

Based on epidemiological studies and the animal data in Table 1.3, the World Health Organization recently decreased its tolerable daily intake (TDI) from 10 pg/kg per day to 1 to 4 pg/kg per day of TEQ.[145,*] The TDI was not set at a level judged to make risk unlikely: the committee concluded that subtle effects may be occurring in the general population. Instead, the TDI includes

* For a comparison of WHO, ATSDR, and USEPA on the assessment of noncancer effects, see the discussion in Ref. 291.

a dose of risk management. According to one report, WHO did not want to declare the food supply of the industrialized world adulterated.[292]

1.8 TRENDS OF DIOXINLIKE COMPOUNDS IN THE ENVIRONMENT

Concentrations of TCDD in Lake Ontario sediment peaked in the early 1960s,[234] shortly before the first observations of high embryo mortality in herring gulls.[227] Levels in sediment and herring gull eggs decreased dramatically in the early 1970s, probably as a result of the halting of 2,4,5-trichlorophenol production along the Niagara River.[293] This may have permitted the recovery of the lake's herring gull population from GLEMEDS.[227] However, TCDD levels in herring gull eggs and lake trout changed much less in the following decade. Chemical waste sites such as Love Canal may still be a major source.[293] PCDD and PCDF concentrations reached a maximum in the sediments of other Great Lakes in the 1970s and declined afterward.[38,294] One likely cause was the passage in the early 1970s of the U.S. Clean Air Act and other environmental legislation.[75]

As noted above, the dioxinlike PCBs are thought to be biologically more significant than PCDDs and PCDFs in several Great Lakes wildlife incidents. Concentrations of PCBs in Lake Ontario sediments rose until manufacturing was banned in the 1970s and then began to fall.[295] Concentrations in Great Lakes biota initially declined after the ban, but there were some suggestions of a leveling off in the 1980s and 1990s.[229,296,297] Other studies indicate that environmental levels have continued to fall.[298,299]

As contaminant levels fall, more subtle effects may be noticed. The susceptibility of experimental animals to neurobehavioral and hormonal disturbances suggests the need for an examination of these effects in wildlife.[300] Masking of subtle effects by more overt ones has occurred before. High levels of DDE (a persistent metabolite of DDT) and associated eggshell thinning probably caused populations of double crested cormorants to drop in the 1960s and 1970s. Yet, as DDE levels fell, GLEMEDS emerged.[229]

There may be a downward trend over time in the body burdens of dioxinlike compounds in the general U.S. population. This was suggested by the National Human Adipose Tissue Survey (NHATS) results of 1982 and 1987 and a Veterans' Administration/USEPA analysis of stored NHATS samples from 1971–1982 for males aged 17 to 46. According to the authors, this trend may reflect several factors: real declines in body burdens, advances in analytical methods, and loss of integrity of tissue during storage.[301] Although the NHATS program was discontinued, we can expect U.S. data in the future from the National Health and Nutrition Examination Survey (NHANES). European studies revealed a striking decline in human body burdens and breast milk concentrations. However, levels in Germany may have stabilized in the late 1990s,[302] whereas body burdens in Spain showed a recent increase.[303] Trend

data are lacking for developing countries, but increases would not be unexpected.

Levels of polybrominated diphenyl ethers (PBDEs) have increased exponentially over the last two decades in Swedish breast milk.[304] Although the toxicology of these widely used fire retardants is still poorly known, they share some characteristics with dioxins and PCBs.[305] It appears that we have not yet learned our lesson.

1.9 CONCLUSIONS

The dioxin debate has centered largely on two issues. First, are humans less sensitive to TCDD than laboratory animals? The weight of evidence indicates that people experience many of the toxic responses observed in animals. TCDD is acknowledged by IARC to cause cancer in humans. The data suggest a quantitative similarity between animal and human responses for some noncancer effects as well.

The second primary issue has been the choice of model for the low-dose effects of TCDD, in particular the appropriateness of a low-dose linear model for cancer. The possibility of an alternative threshold model for biological responses based on receptor mediation formed one of the motivations for the USEPA's most recent reassessment. Since some receptor-mediated biochemical responses appear to be linear at low dose, this simple model was discounted. Given the "background" dose of dioxinlike compounds experienced by residents of industrialized countries, questions about thresholds at lower doses (e.g., whether one molecule can cause effects) are essentially moot. Furthermore, if the mechanism by which TCDD causes cancer is additive to some ongoing process, the cancer dose–response curve for dioxinlike compounds should be regarded as probably linear at low doses.[306]

Gaps clearly remain between our limited biochemical understanding of TCDD and the biology of cancer. A truly realistic model would have to take into account the complexity of the observed phenomenon, which includes Ah receptor mediation, multiple tumor sites, multiple biochemical mechanisms, indirect genotoxicity, timing of exposure, anticarcinogenic effects, hormonal and growth factor involvement, effects on cell proliferation and apoptosis, interactions with other chemicals as well as viruses, and interindividual variation.

Interactions between dioxin and other compounds may pose a particularly difficult modeling problem. TCDD's enzyme inducing ability may significantly affect the toxicity of other exogenous compounds. The result may depend on the specific compound and the dosing regime. Risk assessors typically treat the cancer risks of exposure to multiple carcinogens as being additive. Although it is generally argued that this is a reasonable approximation for low dose exposure, it is unclear that this applies when a promoter is concerned, especially one

that may not have a threshold.[307] Nonadditive effects are also seen in some noncancer endpoints.

Research on the biological mechanism of dioxin-induced effects is an endlessly fascinating scientific undertaking that will no doubt provide enough questions for decades of work. However, the construction of biologically more realistic models—a current goal of USEPA—should not be used as an excuse for delay and inaction. We may aspire to ever closer approximations of reality, but the outcome of the dioxin reassessment so far reinforces something that we have—or should have—known for a long time: Production of dioxin should be avoided. The experience with dioxin does not bode well for regulating other compounds. TCDD is one of the most studied of all toxic compounds. There are thousands of commercial chemicals produced in large quantities for which little or no toxicological information is available.[308,309]

The development of improved cancer models alone will not compensate for an inadequate regulatory structure. One premise of the focus on cancer is the notion that protecting for cancer will also guard against other effects. Yet there is a growing concern that immunotoxicity and reproductive/developmental effects of dioxinlike compounds are more sensitive or important than cancer.[116,227,300,310] At the environmental loads found previously in the Great Lakes, reproductive and developmental effects in wildlife appeared to be a greater problem than cancer.[227,310] But there are subtleties to the sensitivity question. For instance, the relative position of apparent no effect levels depends not only on the biological phenomenon, but also on the measurement techniques and the statistical power of the study. The shapes of the dose–response curves for cancer and other effects remain unclear. The relative importance of various effects at current body burdens and environmental levels is an even more difficult question, akin to asking whether a certain increased cancer risk is more or less important than a certain deficit in cognitive functioning.

However, the premise that standards based on cancer will prevent other effects certainly fails in another respect. Current USEPA regulations of dioxin are based on "acceptable" risks of cancer from incremental exposure (i.e., exposure to one source or through one medium). For instance, combustion sources are evaluated one at a time, ignoring cumulative exposure. Even using traditional methodology, this procedure fails to ensure that total exposure to dioxinlike compounds—incremental plus background—falls below tolerable doses for noncarcinogenic effects.

Indeed, the current background levels of dioxinlike compounds in humans may pose a reproductive, developmental, and immunologic hazard. The problems observed in certain species of wildlife indicate that some ecosystems are overburdened with these compounds. Instead of spending another decade arguing about the shape of the dose–response curve, we believe that the focus of policy needs to shift toward simply reducing exposure. The most sensible approach is pollution prevention: elimination of sources.[311]

The International Joint Commission, the U.S.–Canadian agency with environmental responsibilities regarding the Great Lakes, made a path-breaking

recommendation in this direction in 1992. The Commission called for zero discharge of persistent toxic substances, including PCBs and dioxin, into the Great Lakes ecosystem. These and other persistent toxic compounds—some undoubtedly still unidentified—are products or by-products of industrial chlorine chemistry and the combustion of chlorine-containing fuels. Arguing that chemical-by-chemical regulation has largely failed in this arena, the Commission advocated a precautionary approach: "the Parties, in consultation with industry and other affected interests, develop timetables to sunset the use of chlorine and chlorine-containing compounds as industrial feedstocks and that the means of reducing or eliminating other uses be examined."[312] *Sunsetting* means restriction, phase-out, and eventual banning of the substance. The recently negotiated international treaty on persistent organic pollutants (POPs) calls for curbing releases of dioxins and furans "with the goal of their continuing minimization and, where feasible, elimination."[313]

Due to its notoriety, dioxin remains a target of those who wish to convince the public that environmental contamination is not a problem.[314,315] Much of the media coverage of the dioxin debate has consisted of trying to convince the public that their common sense is wrong and that experts know best. In this case, the public's view has been largely correct. Dioxin is a dangerous and unwanted environmental pollutant. Dioxin policy should strive to eliminate the sources and thereby prevent pollution. Rather than telling the public that certain dioxin risks are "acceptable"—and regarding the resulting opposition as merely hysterical and uninformed—regulators ought to listen to their concerns. The public does not respond according to a one-dimensional measure of risk, but in keeping with a much richer set of criteria. These include, not surprisingly, fairness, democratic choice, and an examination of alternatives.[316]

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CHAPTER 2

Production, Distribution, and Fate of Polychlorinated Dibenzo-*p*-Dioxins, Dibenzofurans, and Related Organohalogens in the Environment

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2.1 INTRODUCTION

As the human population continues to grow and urban, suburban, and agricultural development expands, there is increasing pressure on the environment and our natural resources. Among the more pressing concerns has been the generation and distribution of a group of highly toxic pollutants that generically are referred to as dioxins and dioxinlike compounds. In number, this group of compounds is made up of several hundred chemicals that once produced are extremely stable and remain in the environment for many decades, if not centuries, producing severe health risks and resulting clinical disorders. An example of one of these chemicals is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), which in 1997 was classified as a class 1 carcinogen by the World Health Organization (WHO).

What is most problematic about dioxins and dioxinlike compounds is that they have been and currently are unintentionally produced as by-products by numerous industrial processes that contain chlorine. A few common examples of these include chemical and pesticide synthesis, pulp and paper production, resin and plastic manufacturing, and waste incineration. Because each of these and many other industrial processes, natural events, and noncommercial activities contribute to the production of dioxins and dioxinlike agents in the environment, the health risks from exposure to them are distributed widely. One of the more important emerging problems is "backyard burning," which has

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increased significantly in its relative ranking over the last decade compared to other previously more problematic sources.

The herbicide Agent Orange and the Love Canal dumping site near Buffalo in New York State are perhaps the two most highly publicized examples of national health problems associated with dioxin contamination. However, everyone is affected by the "dioxin problem" in terms of the continued production and accumulation of these types of compounds in dumping sites and *brownfields* (abandoned industrial sites). Similarly, additional compounds are potentially being produced daily by combustion-related activities attributed to waste management. Even the food production industry is affected by the health risks via uptake through the food chain from prolonged lower-level and short-term elevated exposures. As recently as about four years ago, a potential national health concern presented itself as the result of contaminated poultry products resulting from the use of feed containing dioxins.

Unfortunately, dioxin and dioxinlike compounds usually exist in the presence of other potentially toxic chemicals (e.g., polycyclic aromatic hydrocarbons, phthalates, pesticides, etc.) and they are incorporated into complex matrices such as soils, sediments, and biological tissues. The challenge for the assessors is to identify each of these compounds at trace levels (i.e., sub-parts-per-trillion) and then determine the human and ecological risk significance of their presence so that effective remediation and control processes can be implemented. In most cases this involves the use of tedious sample workup steps followed by state-of-the-art measurement methodology that must be operated at high levels of sensitivity that tax the instrument's limits of detection. Compared to many other chemical assays that are performed daily by numerous laboratories, there are relatively few facilities that are adequately equipped and have the expertise to measure dioxin and dioxinlike compounds at the levels of sensitivity and specificity needed. One of the more important practical analytical problems is the development of reliable automated and more efficient sample workup procedures. Unfortunately, although many research groups have addressed important problems related to the chromatographic separation and subsequent mass-spectrometric measurement of dioxin and dioxinlike compounds, much of the front-end isolation and preconcentration steps are carried out using analytical methodologies that were developed more than 40 years ago. The current challenge in this arena is to adapt and, as needed, to develop new, more efficient, high-throughput sample workup procedures that meet the level of reliability needed for assuring regulatory compliance.

An earlier version of the current book was published in 1994, and the environmental sources, distribution, and fate of polychlorinated dibenzodioxins, dibenzofurans, and related organochlorines were discussed in detail.[1] The reader is referred to this work for information in the field prior to the mid-1990s. Clearly, the concern about the dioxin problem has continued to expand, and since that review, a large number of primary literature sources have appeared that deal with all aspects of the topic. Similarly, a recent reassessment of the impact of dioxin and dioxinlike compounds has been carried out by the

U.S. Environmental Protection Agency (USEPA). In view of the continuing interest in the environmental impact of these compounds and improvements in analytical methodology to measure them, the primary focus of the current chapter is on more recent information related to the sources and fate as well as measurement methodology not covered earlier.[1]

2.2 PHYSICAL AND CHEMICAL CONSIDERATIONS

Polychlorinated dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs), and dioxinlike biphenyls (PCBs) are three highly toxic classes of compounds with long half-lives, and if they are present in nature, will bioaccumulate in mammals. In total, there are 419 of these compounds that vary in degree of chlorination and isomeric substitution. The numbers of congeners at each level of chlorination are summarized in Table 2.1. Some of these compounds are viewed to be environmentally more problematic because of their higher toxicity and longer environmental persistence, which leads to greater health risk due to mammalian uptake during prolonged exposure. As noted above, TCDD has been the compound of most concern followed by related compounds that contain this parent structure.

The WHO has recommended that the daily dietary intake of PCDD/Fs should be less than 10 pg/kg body weight as evaluated by the dioxin toxic equivalency factor (TEF) scale that uses as its basis of comparison 2,3,7,8-TCDD.[2,3] The latter compound has received the most attention because it is considered to be one of the most toxic organochlorines and is a powerful carcinogenic agent. In various elimination studies carried out in hamsters, rats, and guinea pigs, its half-live is relatively short and has ranged from about 2 weeks up to 3 months.[4–7] However, in humans it has a significantly longer half-life.

TABLE 2.1 Number of Halogenated PCDD/F and PCB Congeners

Number of Halogen Substitutions	Number of Congeners		
	Dibenzo-*p*-Dioxins	Dibenzofurans	Biphenyls
Mono	2	4	3
Di	10	16	12
Tri	14	28	24
Tetra	22	38	42
Penta	14	28	46
Hexa	10	16	42
Hepta	2	4	24
Octa	1	1	12
Nona	—	—	3
Deca	—	—	1
Total	75	135	209

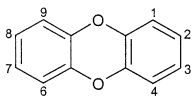
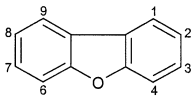
Although there have been measured differences among investigators there is general agreement that the half-life of 2,3,7,8-TCDD in humans ranges between about 7 and 9 years. In one study that examined heavily exposed Vietnam veterans a value of approximately 9 years has been reported[8] and in another study a value of approximately 7 years has been given.[9] The toxicological activity of 2,3,7,8-TCDD as well as other related PCDD/Fs is attributed to the binding of them to the aryl hydrocarbon (Ah) cytoplasmic receptor protein (see Chapter 12). Because the various PCDD/Fs as well as certain PCB congeners are believed to act by a common biochemical mechanism, they are usually assessed and regulated as a class of compounds. Additional information in terms of toxicity, mechanism of action, and pharmacokinetics as well as the implications of exceeding recommended daily intake can be found elsewhere[2,3,10] (see also Chapters 4–7 and 12).

2.2.1 Important Properties

Shown in Table 2.2 are some of the more biologically relevant and highly studied isomers of PCDDs and PCDFs, along with their corresponding Chemical Abstract Services (CAS) numbers and toxic equivalency factors (TEFs). Similar information for the important dioxinlike PCBs is given in Table 2.3. The parent structures for these compounds are dibenzo-*p*-dioxin, dibenzofuran, and biphenyl. The polychlorinated members in these three families of compounds vary only in the degree of chlorination and isomeric arrangement of the chlorine substituents. A common structural property of all the compounds is their planar configuration, which is believed to be an important molecular feature in Ah receptor binding affinity and hence their higher toxicity. As groups of compounds, the order of toxicity is PCDDs > PCDFs > dioxinlike PCBs with the congeners with chloro subsitituents at the 2,3,7,8 positions highest. There are 17 PCDD/Fs that fall into the latter category (i.e., those in Table 2.2).

The TEF concept has been proposed as a means of assessing the toxicity of mixtures of PCDD/Fs and dioxinlike PCBs and uses knowledge of structural similarities among the compounds to generate a weighting factor for a particular congener relative to 2,3,7,8-TCDD.[2] This factor multiplied by the weight of the compound is used to calculate the TEQ, the toxic equivalency quantity, for an individual compound. As an example, according to the TEF approach, on a weight basis it would take 10 times as much 1,2,3,4,7,8-hexachlorodibenzo-*p*-dioxin as 2,3,7,8-TCDD to produce the equivalent toxicological effect. Whereas 1,2,3,7,8-pentachlorodibenzo-*p*-dioxin is equivalent in toxicity to 2,3,7,8-TCDD by WHO estimates. A few of the compounds where the degree of chlorine substitution can influence their planar structure (e.g., PCB isomers with dichloro substitution at the ortho positions in both aromatic rings) have been withdrawn by the WHO based on this structural consideration as well as toxicological information[2] as discussed below. It is important to recognize that in using the TEF to predict the toxicity of the various congeners one assumes

TABLE 2.2 Nomenclature and Toxic Equivalent Factors for Important PCDDs and PCDFs

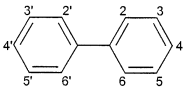
	Description	CAS Number	WHO TEFs	I-TEFs
Polychlorinated dibenzo-*p*-dioxins (PCDDs or CDDs) 	2,3,7,8-TCDD	1746-01-6	1	1
	1,2,3,7,8-penta-CDD	40321-76-4	1	0.5
	1,2,3,4,7,8-hexa-CDD	39227-28-6	0.1	0.1
	1,2,3,6,7,8-hexa-CDD	57653-85-7	0.1	0.1
	1,2,3,7,8,9-hexa-CDD	19408-74-3	0.1	0.1
	1,2,3,4,6,7,8-hepta-CDD	35822-46-9	0.01	0.01
	Octa-CDD	3268-87-9	0.0001	0.001
	Total TCDD	41903-57-5		
	Total-penta-CDD	36088-22-9		
	Total-hexa-CDD	34465-46-8		
	Total-hepta-CDD	37871-00-4		
Polychlorinated dibenzofurans (PCDFs or CDFs) 	2,3,7,8-TCDF	51207-31-9	0.1	0.1
	1,2,3,7,8-penta-CDF	57117-41-6	0.05	0.05
	2,3,4,7,8-penta-CDF	57117-31-4	0.5	0.5
	1,2,3,4,7,8-hexa-CDF	70648-26-9	0.1	0.1
	1,2,3,6,7,8-hexa-CDF	57117-44-9	0.1	0.1
	1,2,3,7,8,9-hexa-CDF	72918-21-9	0.1	0.1
	2,3,4,6,7,8-hexa-CDF	60851-34-5	0.1	0.1
	1,2,3,4,6,7,8-hepta-CDF	67562-39-4	0.01	0.01
	1,2,3,4,7,8,9-hepta-CDF	55673-89-7	0.01	0.01
	Octa-CDF	39001-02-0	0.0001	0.001
	Total-TCDF	55722-27-5		
	Total-penta-CDF	30402-15-4		
	Total-hexa-CDF	55684-94-1		
	Total-hepta-CDF	38998-75-3		

Source: WHO TEFs from Ref. 2; I-TEFs from NATO and USEPA, Refs. 11–13.

that (1) the pharmacokinetic effect of a particular compound in vivo is equivalent to 2,3,7,8-TCDD, and (2) the effects among compounds are additive (i.e., there are no synergistic or antagonistic effects).

In practice, the TEF/TEQ approach is useful in estimating the relative toxicity of a complex mixture of compounds by rendering it as a single numerical value and is commonly used in evaluating potential human health safety risks of individual samples/contamination sites. However, in doing this, the approach has several limitations: (1) it can underestimate potential synergistic effects of nondioxinlike compounds and metals that also may be present, (2) it does not consider potential interactions among different classes of compounds, and (3) it neglects other toxic agents that may be found at higher levels, including non-2,3,7,8-substituted PCDD/Fs. Since 1993 the relative toxicity of dioxin and dioxinlike compounds that meet the criteria for inclusion in

TABLE 2.3 Nomenclature and Toxic Equivalent Factors for Important Dioxinlike PCBs

	Congener	IUPAC Number	CAS Number	WHO TEFs
Polychlorinated biphenyls (PCBs) 	3,3′,4,4′-tetra-CB	PCB 77	32598-13-3	0.0001
	3,4,4′,5-tetra-CB	PCB 81	70362-50-4	0.0001
	2,3,3′,4,4′-penta-CB	PCB 105	32598-14-4	0.0001
	2,3,4,4′,5-penta-CB	PCB 114	74472-37-0	0.0005
	2,3′,4,4′,5-penta-CB	PCB 118	31508-00-6	0.0001
	2′,3,4,4′,5-penta-CB	PCB 123	65510-44-3	0.0001
	3,3′,4,4′,5-penta-CB	PCB 126	57465-28-8	0.1
	2,3,3′,4,4′,5-hexa-CB	PCB 156	38380-08-4	0.0005
	2,3,3′,4,4′,5′-hexa-CB	PCB 157	69782-90-7	0.0005
	2,3′,4,4′,5,5′-hexa-CB	PCB 167	52663-72-6	0.00001
	3,3′,4,4′,5,5′-hexa-CB	PCB 169	32774-16-6	0.01
	2,3,3′,4,4′,5,5′-hepta-CB	PCB 189	39635-31-9	0.0001

the WHO TEF scheme have been stored in a database at the Karolinska Institute in Stockholm, Sweden.[2] The intent of the WHO group that oversees this task is to expand the TEF concept to include additional polyhalogenated compounds that display dioxinlike activity. Some of these are naphthalenes, diphenyl ethers, diphenyl toluenes, anthracenes, fluorenes, and a host of other planar chlorinated and brominated aromatics. A more complete list of these compounds can be found elsewhere.[2]

The 2,3,7,8-chlorine substitution pattern and the planar configuration of PCDD/Fs are the important molecular features that contribute to the toxicity of these compounds. In PCBs, the two phenyl rings can rotate around the central linkage, the 1,1′-carbon single bond. When there are no chlorine substitutions adjacent to the central linkage (i.e., ortho-position substitutions), the PCB molecule can assume a planar configuration and produce toxic effects that are similar to the PCDD/Fs. This is reflected in the relatively high TEF value of 0.1 that is assigned to the 3,3′,4,4′,5-penta-CB (PCB 126) congener. The presence of a chlorine substitution at one of the ortho ring positions, as in the case of 2,3,3′,4,4′,5-hexa-CB (PCB 156), sterically hinders the rings from forming a completely planar configuration. This is reflected in the significantly lower TEF value of 0.0005, which indicates the properties of this congener are significantly less dioxinlike. Besides this feature, there are other electron donating and withdrawing considerations that lead to additional differences. These subtle differences and their effect on receptor site binding and toxicity are less well understood and hence less predictable.

In general, the PCDDs, PCDFs, and PCBs are highly nonpolar and tend to concentrate in hydrophobic matrices. When they are present in aquatic streams, lakes, and reservoirs, they partition into the corresponding sediments with increasing degrees of chlorination with approximately 90% of the

TABLE 2.4 Physical Properties of Selected PCDDs and PCDFs

Congener	Boiling Point (°C)	Vapor Pressure (Pa)	log K_{ow}[a]	Solubility (mg/m^3)
2,7-di-CDD	373.5	1.2×10^{-4}	5.75	3.75
1,2,4-tri-CDD	375	1.0×10^{-4}	6.35	8.41
1,2,3,4-TCDD	419	6.40×10^{-6}	6.60	0.55
2,3,7,8-TCDD	446.5	2.00×10^{-7}	6.80	0.0193
1,2,3,4,7-penta-CDD	464.7	8.80×10^{-8}	7.40	0.118
1,2,3,4,7,8-hexa-CDD	487.7	5.10×10^{-9}	7.80	0.00442
1,2,3,4,6,7,8-hepta-CDD	507.2	7.50×10^{-10}	8.00	0.0024
Octa-CDD	510	1.10×10^{-10}	8.20	0.000074
2,8-di-CDF	375	3.9×10^{-4}	5.44	14.5
2,3,7,8-TCDD	438.3	2.00×10^{-6}	6.1	0.419
2,3,4,7,8-penta-CDF	464.7	3.50×10^{-7}	6.5	0.236
1,2,3,4,7,8-hexa-CDF	487.7	3.2×10^{-8}	7.0	0.00825
1,2,3,4,6,7,8-hepta-CDF	507.2	4.7×10^{-9}	7.4	0.00135
Octa-CDF	537	5.0×10^{-10}	8.0	0.00116

Source: Data from Ref. 14.

[a]Octanol/water partitioning constants.

dichlorodibenzo-*p*-dioxins and 97, 98, and 99% of the corresponding tri, tetra, and octa compounds associated with the sediments, respectively.[14] Summarized in Table 2.4 are log K_{ow} values and other related physical data for several PCDD/F congeners. The octanol/water partitioning distribution constant, K_{ow}, is a useful relative measure of a compound's hydrophobic character and hence its affinity to associate with a nonpolar matrix. In using this approach, it should be recognized that it ignores secondary effects that may influence solid-phase sorption such as geometrical/structural features in the molecule. Often, there are not large differences in octanol/water partitioning within a congener group based on the degree of substitution as occurs when these compounds partition between water and a solid matrix. This problem is exacerbated when the solid matrix materials contain an appreciable number of higher-energy sorption sites (e.g., polar silanol groups associate with siliceous materials, the nonpolar highly planar sites found in graphitic carbons, etc.).

PCDD/Fs are highly stable compounds. They are resistant to chemical and biological breakdown, and once produced they remain in the environment for long periods of time, especially when they are associated with solid matrices. When the PCDDs are considered as a congener group based on the degree of chlorination, the average longevity has been reported to increase with increasing degree of chlorination.[14] However, caution should be exercised in accepting non-structural-based generalizations of this type since they ignore or at least minimize important chemical considerations. In a recent study carried out on sediment core samples that covered a period from the late nineteenth century to the mid-1980s, large differences within a given congener group were

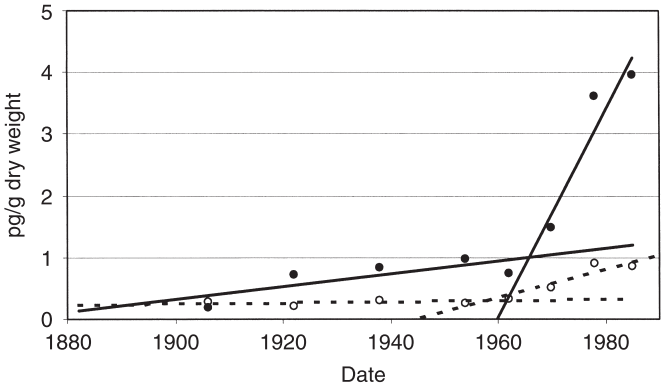


Figure 2.1 Change in measured amounts of selected TCDD congeners in sediment core samples with time. Solid data points and solid lines are composite data for the 1,2,3,8-TCDD, 1,2,3,7-TCDD, 1,2,4,9-TCDD, 1,2,4,6-TCDD, and 1,2,3,4-TCDD and open data points and dashed lines are for 1,2,6,9-TCDD.[15] The solid and dashed lines are linear regression fits of the data separated into two distinct chronological regions.

observed (i.e., factors of 3 or more in half-lives).[15] For example, the half-lives for the 1,2,3,8-TCDD, 1,2,3,7-TCDD, 1,2,4,9-TCDD, 1,2,4,6-TCDD, and 1,2,3,4-TCDD were measured to be about 65 years, whereas the half-lives for 1,2,4,7-TCDD, 1,2,4,8-TCDD, 1,3,6,9-TCDD, 1,2,6,8-TCDD, and 1,2,6,9-TCDD were nearly 200 years. Similar variability also has been observed for the penta, hexa, and hepta congeners.

Shown in Figure 2.1 are plots of concentration versus time for the short-lived compounds listed above and for one of the long-lived compounds. The short-lived compounds are included as a single plot because the original experimental data are reported as composite information for these compounds.[15] A similar plot is shown in Figure 2.2 for a group of hexa-CDDs. The plots in Figures 2.1 and 2.2 show two distinct trends, a slow rate of change prior to about 1940–1960, and a rapid rate in more recent times that presumably results from the increasing influence of the chlorine age production of PCDDs that distort the half-life information erroneously to lower than actual half-life values. An additional complication for both earlier and later trends in the data is PCDD input from noncommercial combustion created by an increasing population. Although the latter trend is less problematic in terms of distorting the half-life determinations, it still makes the determination of accurate half-lives under long-lived natural conditions extremely difficult. These same arguments also hold for similar half-life measurements for PCDFs and dioxinlike PCBs.

Some of the more relevant chemical characteristics of PCDDs, PCDFs, and PCBs are a high level of thermal and photolytic stability and they are

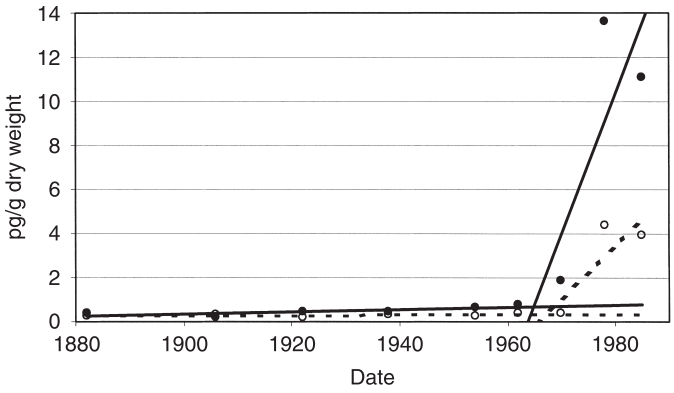


Figure 2.2 Change in measured amounts of selected hexa-CDD congeners in sediment core samples with time. Solid data points and solid lines are composite data for the 1,2,3,6,7,8-hexa-CDD, and open data points and dashed lines are for 1,2,3,4,7,8-hexa-CDD.[15] The solid and dashed lines are linear regression fits of the data separated into two distinct chronological regions.

resistant to degradation under acidic conditions. During the sample extract cleanup procedures, concentrated sulfuric acid is used to remove interfering matrix materials prior to carrying out gas chromatographic–mass spectrometric PCDD/F measurements. Under typical daylight conditions, photolysis in near-surface waters has been reported to be an important elimination pathway with a half-life of slightly less than 2 days reported for TCDD.[16] In terms of thermal degradation, laboratory tests have shown that only about 50% of TCDD is oxidized at 700°C, but at higher temperatures (i.e., greater than 800°C) over 99.5% of it will react within less than 30 s. Based on these properties, incinerator-operating conditions of at least 1000°C are recommended to reduce the times for complete oxidation to less than 2 s.[17]

Strong bases are known to cause degradation of the higher chlorinated dioxins.[18] In one reported study, both laboratory and field tests have shown that PCDD/Fs can be dechlorinated using polyethylene glycol (PEG) treated with potassium hydroxide.[19] The reaction mechanism in this process is believed to involve the formation of potassium alkoxide, which reacts with PCDD/F to form corresponding ethers following displacement of chlorine. Presumably, as the reaction proceeds, the higher-chlorinated congeners are sequentially converted to lower-chlorinated forms. Thus, a limitation of this process is that if the reaction is stopped too soon, the observed levels of TCDD may be larger than are present in the starting contaminated matrix if appreciable levels of congeners containing greater than tetra substitution are present. Reaction temperatures greater than 100°C and reaction times over several hours are typical. To date, the PEG process been used most often for smaller-scale decontamination problems.

2.2.2 Production of Dioxin and Dioxinlike Compounds

With the advent of the chlorine age and the numerous industrial processes that use chlorine, there has been an increase in the levels of dioxin and dioxin-related compounds produced. According to USEPA information, this trend hit a high in the late 1980s and has since decreased. This improvement is related to reductions in a targeted industrial process discussed later in the chapter. In some instances the chemistry leading to dioxin and dioxinlike compounds is well understood. A good example of this is the production of 2,3,7,8-TCDD as a by-product in the commercial manufacture of the herbicide 2,4,5-trichlorophenoxyacetic acid (2,4,5-T). Historically, 2,4,5-T has been used as an effective agent for controlling woody plant growth. When it was mixed with 2,4-dichlorophenoxyacetic acid, it produced a product that has become known as Agent Orange or Herbicide Orange, which was applied widely as a defoliant during the Vietnam conflict.

The synthesis of 2,4,5-T is illustrated in the reaction scheme appearing in Figure 2.3. The starting material in this reaction is 1,2,4,5-tetrachlorobenzene, which is converted to the corresponding trichlorophenol under basic conditions. To increase the efficiency of the reaction, it is carried out at elevated temperatures and pressures. In one patented process, water is employed as the reaction solvent and temperatures between 225 and 300°C and pressures between 400 and 1500 psi are used. Unfortunately, under these aqueous alkaline conditions, significant amounts of 2,3,7,8-TCDD can be formed from the condensation of two equivalents of Na-2,4,5-TCP. When Firestone et al. studied this reaction,[20] they found that the concentrations of 2,3,7,8-TCDD in 2,4,5-TCP samples ranged from 0.07 to 6.2 ppm.

Although the process described above is a well-known producer of dioxins and is no longer used, in other instances these compounds are formed inadvertently as by-products and decomposition products of numerous manu-

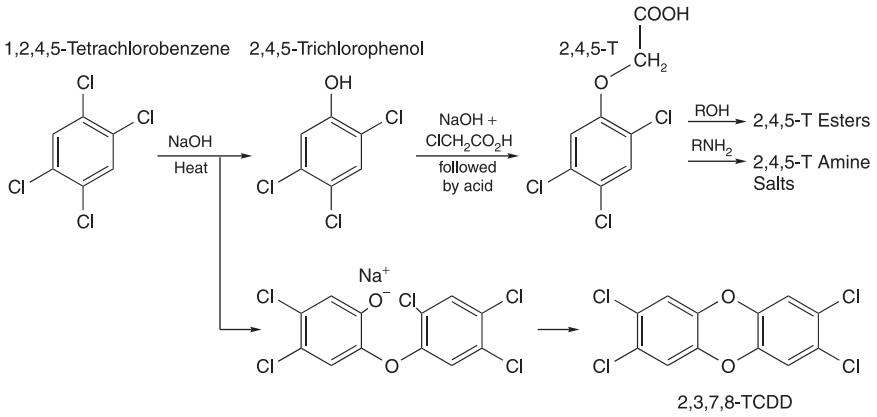


Figure 2.3 Reaction scheme used to produce 2,4,5-trichlorophenoxyacetic acid and the inadvertent production of 2,3,7,8-TCDD.

TABLE 2.5 Proposed Mechanisms for the Formation of PCDD/Fs during Incineration

Incinerator Zone	Reaction Conditions	Formation Mechanisms
Preflame	$T = 200$–$1000°C$ $t_r \ll 1$ s $[O_2] \sim 50\%$ excess air	1. Concerted molecular elimination 2. Complex radical-molecular pathways 3. Recombination and association reactions
Flame	$T = 1000$–$1800°C$ $t_r \leq 0.01$ s $[O_2] \sim 50\%$ excess air	1. Complex radical-molecular pathways 2. Concerted molecular elimination 3. Recombination and association reactions
High-temperature thermal	$T = 600$–$1100°C$ $t_r = 1$–10 s $[O_2] = 50$–100% excess air	1. Recombination and association reactions 2. Complex radical-molecular pathways 3. Concerted molecular elimination
Gas quench	$T = 80$–$600°C$ $t_r \sim 10$ s $[O_2] = 3$–9%	Recombination and association reactions
Surface catalyst	$T = 150$–$500°C$ $t_r = 10$ s to 10 min $[O_2] = 3$–9%	Surface-catalyzed synthesis

facturing and waste disposal processes, including incineration. In the latter case, the exact synthetic routes and mechanisms involved still are not defined completely or are debated vigorously in the literature. Irrespective of the exact mechanistic details, generally speaking, the initial process involves the production of polychlorinated benzenes via low-temperature thermal combustion of organic waste containing chlorine. In a review of the subject, Taylor has suggested that the optimum temperature for the thermal production of PCBs is about 300°C.[21] Further, it has been suggested that the vast majority of the pollutants observed in the stack effluent of incinerators are produced outside the flame in the lower-temperature postflame zones, downstream in quenched gases, or as the result of surface-catalyzed reactions.[21–24] An overview of these postflame processes is summarized in Table 2.5.

As can be seen from an inspection of the information summarized in Table 2.5, the overall processes involved in the formation of PCDD/Fs during incineration are chemically complex and are believed to involve several types of conversion pathways: (1) concerted molecular elimination reactions, (2) complex radical-molecular pathways, (3) recombination and association reactions, and (4) surface-catalyzed synthesis. The importance of each of these is dependent on the various reaction conditions, including temperature, resident time, oxygen concentration, and the presence or absence of catalytic surfaces. Additional details concerning the reaction mechanisms can be found elsewhere.[21,25,26] Irrespective of the numerous models as well as variations on these, incineration of organic materials in the presence of trace levels of inorganic or organic chlorine leads to the various classes of organochlorine compounds.

Polyvinyl chloride (PVC) plastics are a good example of materials that, when burned, lead to the production of significant levels of airborne polychlorinated aromatics. However, they are not the only important sources since chlorocarbons are used widely as cleaning agents, solvents, and starting materials in many manufacturing applications, including the production of herbicides, pesticides, and non-PVC polymers. Once polychlorinated aromatics are produced, they are believed to be converted to corresponding polychlorinated phenols via solid-state base rich conditions, such as those found on the surface of particulates formed and released during incineration. Kaune et al.[27] have suggested that chlorinated benzenes and chlorinated phenols are the most likely precursors to PCDD/F since these classes of compounds are always found together with PCDD/F in the effluents of incinerators. Chlorinated benzene concentrations are typically approximately three orders of magnitude higher than PCDD/Fs in incinerators. Unlike the production of polychlorinated benzenes, the optimum temperature for the formation of polychlorinated phenols is at slightly lower temperatures (i.e., according to Taylor,[21] 250°C is optimum). It is reasonable to speculate that the final step in the formation of PCDDs under incineration conditions probably involves a condensation reaction similar to that shown in the reaction scheme above for 2,4,5-T, which occurs at superbasic sites on the surface of fly ash or other inorganic oxide particles, such as silica.

After carefully removing organic contaminants from municipal solid waste incinerator (MSWI) fly ash, Vogg et al.[28] added known concentrations of isotopically labeled PCDD/Fs to the matrix. The MSWI fly ash was then heated for 2 h in a laboratory furnace at varying temperatures. The treated fly ash was exposed to increasing temperatures in 50°C increments in the temperature range 150 to 500°C. Figure 2.4 summarizes these data. Because the relative

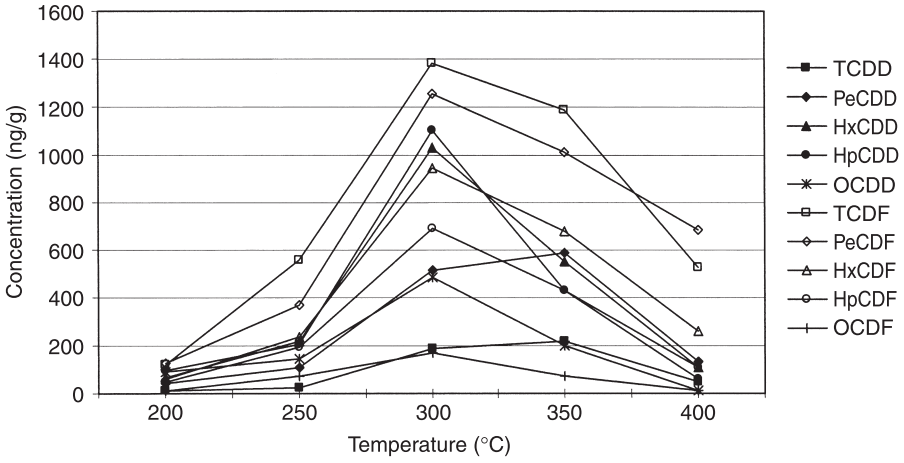


Figure 2.4 Temperature dependence of PCDD/F formation on municipal incinerator fly ash.

concentration of PCDD/Fs were observed to vary with temperature, it was concluded that the temperature of the combustion gas was crucial to promoting the formation of PCDD/Fs on the surface of fly ash. Within the temperature range 200 to 450°C, the concentration of PCDD/Fs increases to their maxima; outside this range, the concentration diminishes.

The relative concentrations of the PCDD/F congeners are frequently used to determine the source of the emissions. Table 2.6 summarizes the relative concentrations of the 2,3,7,8-substituted PCDD/F isomers from several different sources. The final column in the table lists the amount of PCDD/Fs that are released for each weight or distance from the different sources. Figure 2.5 gives a graphical presentation of the different patterns of the 2,3,7,8-substituted isomers that are produced by MSWIs, medical waste incinerators, and cement kilns that are burning hazardous wastes. The large difference in the overall pattern between the three different sources is clearly apparent and can serve as a useful fingerprint in identifying sources of emissions.

2.2.3 Transport and Accumulation

The general transport of PCDDs, PCDFs, and PCBs are from air (i.e., mostly as sorbed species on airborne particulates) to water and from water to soil and sediments. Their mean half-lives increase from 1 to 3 weeks in air to 2 to 5 years in sediments. A more detailed summary of this information is included in Table 2.7 for several representative compounds with increasing degree of chlorination. More detailed compilations for 2,3,7,8-TCDD half-life information in air, water, and soil/sediment appear in Table 2.8. The differences between the data in Table 2.8 versus Table 2.7 reflect the difficulty in making these types of measurements. One of the more significant problems is the influence of compound input. As has been noted by Kjeller and Rappe,[15] calculated half-life times contain at least two components, the true degradation component and a suggested component that arises from changes in output flux from the source (i.e., input flux to the soil/sediment). The latter component contributes in such a fashion that it can make the measured values for half-lives appear to be much shorter than the actual value. The reader is referred to Figures 2.1 and 2.2 and the corresponding discussion for additional details.

2.2.4 Mechanisms of Degradation and Elimination

Potentially, there are several modes of degradation in nature; however, none of these are kinetically fast. One of these is atmospheric photo degradation via exposure to ultraviolet radiation. However, when PCDD/Fs are strongly adsorbed onto particulate surfaces, this process is less effective.[39] Similarly, photo-degradative mechanisms are less efficient for the higher chlorinated congeners compared to the less chlorinated congeners because of greater chemical stability, lower volatility, and increased adsorption. The sorbed compounds are believed to have lifetimes that are about 10 times those that are found in the

TABLE 2.6 Percent of Each Congener in the 2,3,7,8-Substituted Emissions from Different Sources and the Corresponding Rate of Production of PCDD/Fs from Them

Description	2,3,7,8-TCDD	1,2,3,7,8-penta-CDD	1,2,3,4,7,8-hexa-CDD	1,2,3,6,7,8-hexa-CDD	1,2,3,7,8,9-hexa-CDD	1,2,3,4,6,7,8-hepta-CDD	Octa-CDD	2,3,7,8-TCDF	1,2,3,7,8-penta-CDF	2,3,4,7,8-penta-CDF	1,2,3,4,7,8-hexa-CDF	1,2,3,6,7,8-hexa-CDF	1,2,3,7,8,9-hexa-CDF	2,3,4,6,7,8-hexa-CDF	1,2,3,4,6,7,8-hepta-CDF	1,2,3,4,7,8,9-hepta-CDF	Octa-CDF	Sum 2378-Substituted Congeners
Pulp and paper mills: wood residue/sludge incinerators	1.5	2.5	4.1	4.3	11.8	14.3	29.7	16.0	3.3	3.6	2.4	1.6	1.4	0.3	1.6	0.4	1.1	4.4 ng/kg
Municipal solid waste incinerator																		
Mass burn, water wall with dry scrubber and fabric filter	0.3	0.8	0.6	2.3	2.8	20.1	28.8	5.1	2.2	3.1	4.9	3.0	0.3	4.1	11.8	2.2	7.5	13 ng/kg
Mass burn, water wall with ESP	0.3	1.7	1.6	1.9	3.5	11.1	14.5	2.8	3.0	4.3	11.1	4.8	1.3	6.4	22.8	2.8	6.4	6900 ng/kg
Refuse-derived fuel with dry scrubber and fabric filter	0.5	0.7	1.1	1.3	1.1	10.8	43.2	4.8	2.3	2.7	4.5	1.0	0.6	1.4	9.1	1.0	13.9	5.1 ng/kg

Hazardous waste incinerators	0.2	0.2	0.3	0.4	0.8	2.8	6.0	4.3	3.7	4.0	15.5	6.3	0.5	4.3	26.6	2.7	21.5	63 ng/kg
Medical waste incinerator																		
With no APCD	0.1	0.6	0.8	1.1	1.6	7.9	11.9	0.6	2.2	2.9	7.8	5.8	0.5	6.7	22.4	4.1	22.9	30,000 ng/kg
With wet scrubber, bag house, and fabric filter	0.0	0.3	0.5	1.1	1.9	11.3	20.6	0.4	5.5	1.6	3.3	2.3	3.8	2.8	15.3	2.8	26.5	2300 ng/kg
Sewage sludge incinerator	0.2	0.4	0.1	0.2	0.5	4.3	22.9	44.7	3.3	11.5	3.7	1.3	0.1	2.1	2.5	0.3	2.0	59 ng/kg
Secondary copper smelters	1.9						20.1	40.5									37.5	6700 ng/kg
Diesel truck	0.5	0.4	0.6	1.2	2.1	14.1	64.9	1.3	0.5	1.1	1.9	0.9	0.5	1.2	3.9	0.4	4.5	6800 pg/km
Unleaded auto with catalytic converter	0.9	0.4	0.4	0.9	0.5	6.5	51.7	3.0	1.5	1.1	1.2	1.3	0.4	1.5	13.3	0.4	15.1	52 pg/km
Industrial wood combustion	0.0	0.3	0.3	0.5	0.5	5.6	37.1	4.1	4.9	4.6	4.7	5.8	2.1	2.8	15.4	1.6	9.8	16 ng/kg
Utilities and industrial boilers, coal combustion	0.4	0.0	0.0	0.3	0.3	12.4	29.6	6.2	0.5	4.3	6.3	0.9	0.9	3.1	20.3	5.6	9.1	1.7 ng/kg
Cement kilns																		
Burning hazardous waste	0.9	2.6	2.8	3.2	4.5	10.4	3.1	13.0	7.8	19.5	12.0	5.1	1.0	7.5	4.8	1.0	0.7	140 ng/kg
Not burning hazardous waste	0.4	1.1	0.9	1.4	1.6	14.1	22.7	23.9	3.3	7.3	6.1	1.8	0.2	2.7	4.8	0.2	7.7	3.3 ng/kg

Source: Data from Ref. 29.

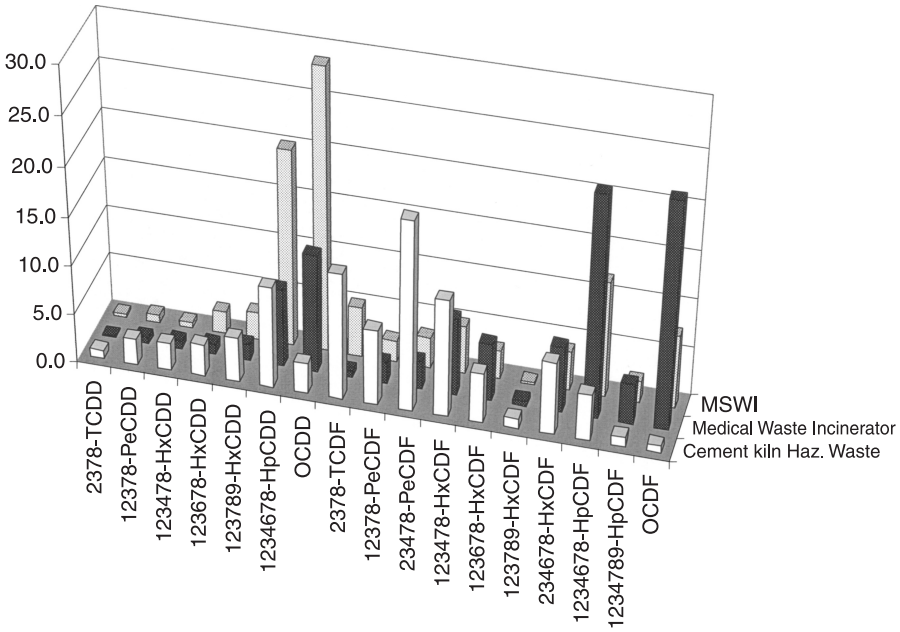


Figure 2.5 Comparison of 2,3,7,8-substituted congener profiles for emissions from MSWIs, medical waste incinerators, and cement kilns burning hazardous wastes. (Adapted from Ref. 29.)

TABLE 2.7 Mean Half-Lives for PCDD/Fs in Air, Water, Soil, and Sediment Summarized from Information Reported Elsewhere

	Mean Half-Lives for Congeners in Environmental Compartments (weeks)			
Congener	Air	Water	Soil	Sediment
2,7-di-CDD	1	1	33	100
1,2,4-tri-CDD	1	1	33	100
1,2,3,4-TCDD	1	3	100	330
2,3,7,8-TCDD	1	3	100	330
1,2,3,4,7-penta-CDD	3	3	100	330
1,2,3,4,7,8-hexa-CDD	3	10	330	330
1,2,3,4,6,7,8-hepta-CDD	3	10	330	330
Octa-CDD	3	33	330	330
2,8-di-CDF	1	3	33	100
2,3,7,8-TCDD	1	3	100	330
2,3,4,7,8-penta-CDF	3	3	100	330
1,2,3,4,7,8-hexa-CDF				
1,2,3,4,6,7,8-hepta-CDF	3	10	100	330
Octa-CDF	3	33	330	330

Source: Data from Ref. 14.

TABLE 2.8 Half-Life of 2,3,7,8-TCDD in Air, Water, and Sediment/Soil Compiled from the Literature

Half-Life	Environment	Refs.
	Air Half-Lives	
200 h	Estimated OH radical oxidation of fraction in vapor phase	30
22.3–223 h	Estimated photo-oxidation by hydroxyl radicals	31, 32
288 h	Estimated with respect to gas-phase reaction with OH radical in troposphere	33
< 1 h	Photolysis of fraction in vapor phase	16
	Water Half-Lives	
600 days	Model surface water environment	34
32 days	Calculated volatilization from pond and lake surface water	30
16 days	Calculated volatilization from river surface water	30
118 h (winter) 27 h (spring) 21 h (summer) 51 h (fall)	Calculated sunlight photolysis in water at 40° latitude	30
40 h	Photolysis in near-surface waters	16
1.15–1.62 y	Estimated unacclimated aqueous aerobic biodegradation in surface water	32
2.29–3.23 y	Estimated unacclimated aqueous aerobic biodegradation in groundwater	32
	Soil and Sediment Half-Lives	
1.15–1.62 y	Soil die-away test data for two soils	35
10–12 y	Degradation in soil	36
9–15 y	Surface soils	37
25–100 y	Subsurface soils	37
> 10 y	PCDD/Fs in sewage sludge applied to land	38
> 97 y	Sediment core data	15

unabsorbed state.[40] Another mode of degradation is via reaction with hydroxyl radical in the troposphere.[41] Although the concentration of this species is very small, it is very effective at breaking down certain organochlorines. A third mechanism for the elimination of PCDD/Fs is through reactions that occur in water,[14] and finally, there are biological transformation processes that lead to their elimination. In the latter case, a number of studies have been carried out over the last decade that have examined the application of various microorganisms as a means of remediating PCDD/Fs and PCBs from contaminated sites. Some examples of recent work in this area can be found in Refs. 42 and 43.

In terms of commercial means of degradation, one of the more effective means of eliminations of PCDD/Fs and dioxinlike PCBs is high-temperature

TABLE 2.9 Proposed Mechanisms for the Decomposition of PCDD/Fs during Incineration

Incinerator Zone	Reaction Conditions	Decomposition Mechanisms
Preflame	$T = 200$–$1000°C$ $t_r \ll 1$ s $[O_2] \sim 50\%$ excess air	1. Concerted molecular elimination 2. Bond fission 3. Bimolecular radical attack
Flame	$T = 1000$–$1800°C$ $t_r \leq 0.01$ s $[O_2] \sim 50\%$ excess air	1. Bimolecular radical attack 2. Bond fission 3. Concerted molecular elimination
High-temperature thermal	$T = 600$–$1100°C$ $t_r = 1$–10 s $[O_2] = 50$–100% excess air	1. Concerted molecular elimination 2. Bond fission 3. Bimolecular radical attack
Gas quench	$T = 80$–$600°C$ $t_r \sim 10$ s $[O_2] = 3$–9%	1. Concerted molecular elimination 2. Bond fission
Surface catalyst	$T = 150$–$500°C$ $t_r = 10$ s to 10 min $[O_2] = 3$–9%	Surface-catalyzed decomposition

combustion. As discussed previously, temperatures in excess of 1000°C have been recommended in order to effectively remove/break down PCDD/Fs and related compounds. Summarized in Table 2.9 are the proposed mechanisms that are believed to be responsible for the effective decomposition of PCDD/Fs and related compounds.

2.3 TRENDS AND PERSPECTIVES ON THE PROBLEM

2.3.1 Earlier Considerations and Recent Reassessment

The major sources of PCDDs, PCDFs, and PCBs can be grouped into three broad categories: naturally occurring, commercially produced, and non-commercial activities such as the ''backyard burning'' of trash. In terms of overall importance, municipal solid waste incineration was by far the biggest producer of dioxin and dioxinlike compounds, accounting for 63.4% of the TEQs in the mid-1980s.[29] At this same time, next in importance were medical waste incineration and secondary copper smelting, which contributed 18.5 and 7.0%, respectively. In 1987, backyard burning was ranked fourth at 4.3%. More recently, the overall production of dioxinlike compounds in terms of TEQs has dropped significantly, and in 1995 it was estimated to be only about 23% of that produced in the preceding decade. However, an important trend has been in the redistribution of the principal sources for the compounds. This can be seen from the information summarized in Table 2.10. Where it ranked fourth at 4.3% in 1987, backyard burning had moved to second place in 1995.

TABLE 2.10 Comparison of PCDD/F Emissions in 1987 and 1995 and the Relative Contribution from Different Sources[a]

		1987 Emissions		1995 Emissions	
Emission Source	Impacted System	g TEQ_{df} WHO_{98}/yr	Percent of Total	g TEQ_{df} WHO_{98}/yr	Percent of Total
Municipal solid waste incineration	Air	8,877	63.4	1,250	38.4
Backyard refuse barrel burning	Air	604	4.31	628	19.3
Medical waste/pathological incineration	Air	2,590	18.5	488	15.0
Secondary copper smelting	Air	983	7.02	271	8.33
Cement kilns (hazardous waste burning)	Air	118	0.842	156	4.80
Municipal wastewater treatment sludge	Land	76.6	0.547	76.6	2.35
Residential wood burning	Air	89.6	0.640	62.8	1.93
Coal-fired utilities	Air	50.8	0.363	60.1	1.85
Diesel trucks	Air	27.8	0.199	35.5	1.09
Secondary aluminum smelting	Air	16.3	0.116	29.1	0.894
2,4-D	Land	33.4	0.239	28.9	0.888
Iron ore sintering	Air	32.7	0.234	28.0	0.860
Industrial wood burning	Air	26.4	0.189	27.6	0.848
Bleached pulp and paper mills	Water	356	2.54	19.5	0.599
Cement kilns (non-hazardous waste burning)	Air	13.7	0.098	17.8	0.547
Sewage sludge incineration	Air	6.1	0.044	14.8	0.455
EDC/vinyl chloride	Air	NA	—	11.2	0.344
Oil-fired utilities	Air	17.8	0.127	10.7	0.329
Crematoria	Air	5.5	0.039	9.1	0.280
Unleaded gasoline	Air	3.6	0.026	5.6	0.172
Hazardous waste incineration	Air	5.0	0.036	5.8	0.178
Lightweight aggregate kilns (hazardous waste burning)	Air	2.4	0.017	3.3	0.101
Commercially marketed sewage sludge	Land	2.6	0.019	2.6	0.080
Kraft black liquor boilers	Air	2.0	0.014	2.3	0.071
Petroleum refining catalyst regeneration	Air	2.24	0.016	2.21	0.068

(Continued)

TABLE 2.10 *(Continued)*

Emission Source	Impacted System	1987 Emissions		1995 Emissions	
		g TEQ_{df} WHO_{98}/yr	Percent of Total	g TEQ_{df} WHO_{98}/yr	Percent of Total
Leaded gasoline	Air	37.5	0.268	2.0	0.061
Secondary lead smelting	Air	1.29	0.009	1.72	0.053
Paper mill sludge	Land	14.1	0.101	1.4	0.043
Cigarette smoke	Air	1.0	0.007	0.8	0.025
EDC/vinyl chloride	Land	NA		0.73	0.022
Primary copper	Air	0.5	0.004	0.5	0.015
EDC/vinyl chloride	Water	NA		0.43	0.013
Boilers/industrial furnaces	Air	0.78	0.006	0.39	0.012
Tire combustion	Air	0.11	0.001	0.11	0.003
Drum reclamation	Air	0.08	0.001	0.08	0.002
Carbon reactivation furnace	Air	0.08	0.001	0.06	0.002
Total		13,998		3,255	

Source: Data from Ref. 29.

[a] TEQ, toxic equivalent quantities = $\sum$ (measured amounts of individual congeners × their corresponding TEFs); TEQ_{df} WHO_{98} from Ref. 2.

Although the absolute amount increased only about 4%, the large jump on a relative basis is attributable to lower emission in other sectors. The most significant improvements have been made in municipal solid waste incineration. Other significant reductions were in medical waste incineration, secondary copper smelting, and bleached pulp and paper manufacturing.

In addition to manmade pollutants, currently, a major source of uncontrolled production of dioxin and dioxinlike compounds are inadvertent grassland, forest, and landfill fires. Although the quantitative information for these sources is much less reliable than the information reported in Table 2.10, in 1995 it was estimated that these sources contributed over 1200 g TEQ/yr to the environment. The most significant of these sources is landfill fires followed by forest and brush fires.

2.3.2 Recent Occurrences and Problems

A survey conducted in 1997 by the U.S. Department of Agriculture and the USEPA found elevated levels of dioxin in chickens raised in the southern United States. The source of the contamination was traced to ball clay that was added as a processing aid to soybean meal in feed for chickens and farm-raised catfish.[44,45] Analyses showed that the raw and processed ball clay contained average PCDD/F TEQs (WHO-TEFs) of 1513 and 977 pg/g dry weight, respectively. The unique PCDD/F congener pattern found in the chicken and fish was consistent with the pattern found in the ball clay. The use of ball clay

in animal feeds has been discontinued, but large amounts of ball clay (1 million metric tons in 1998) are still mined for ceramic products. Although PCDD/Fs have not been detected in the finished ceramic products, additional studies are being considered to examine the potential releases of PCDD/Fs to the atmosphere that may occur during ball clay processing and firing.[46]

Several factors support the hypothesis that the PCDD/Fs in the ball clay have a natural origin. The samples were collected from undisturbed sediments deposited approximately 40 to 45 million years ago. Although anthropogenic sources cannot be ruled out, the PCDD/F congener patterns for the ball clay do not match the patterns of known sources. The high relative proportions of 2,3,7,8-TCDD and 1,2,3,7,8,9-hexa-CDD and the low concentrations of PCDFs relative to PCDDs are distinguishing features of the PCDD/Fs in the ball clay.[44] Investigations of the PCDD/F concentrations and congener patterns in areas surrounding the clay deposits will help determine if anthropogenic sources can account for the clay contamination. As early as 1980, Bumb et al.[47] proposed a natural origin for PCDD/Fs in the trace chemistries of fire. More recently, a biological mechanism for the formation of chlorinated phenols and PCDD/Fs in the soil of a Douglas fir forest has been reported.[48] A natural origin for the PCDD/Fs in the clay deposits would help explain the discovery of similar patterns of contamination in clay deposits located in Kentucky and Germany.[44]

2.4 ANALYTICAL METHODOLOGY

2.4.1 Cleanup and Preconcentration

Shown in Figure 2.6 is a flowchart of the sample extraction and cleanup steps that are typically used in published regulatory methodology. The procedures, which involve extraction, followed by acid–base back extractions, and column cleanup steps are complicated, lengthy, and require significant quantities of highly pure reagents. Soxhlet, Soxhlet with the Dean–Stark modification, liquid–liquid, and sonication are the most commonly employed extraction methods.[49,50] Once the extraction and acid cleanup steps are completed, column cleanup protocols using large-capacity silica, alumina, carbon, and florisil columns are used to remove interfering matrix materials. The volumes of solvents employed in all of these operations require significant and time-consuming concentrations.

2.4.2 Measurement Methods

Current USEPA methods (Table 2.11 and 2.12) generally use one of four techniques for sample extract analysis: immunoassay, high-resolution gas chromatography (HRGC), HRGC with low-resolution mass spectrometry (HRGC/LRMS), and HRGC with high-resolution mass spectrometry

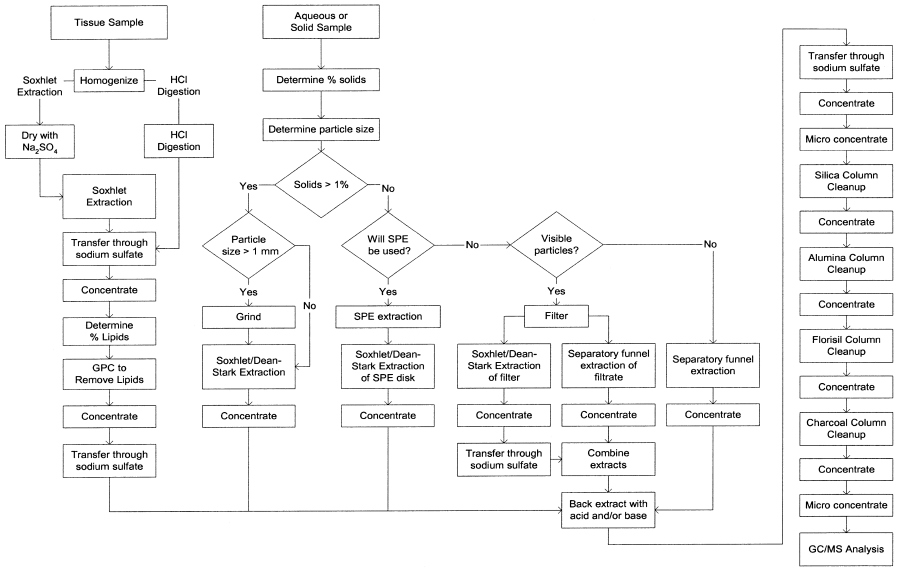


Figure 2.6 Example sample preparation scheme for isolating PCDD/Fs from tissue, aqueous, and solid samples.

(HRGC/HRMS). Screening techniques employ immunoassay, which is currently validated for a limited number of matrices,[51,52] electrochemical,[51] ultraviolet,[58] and GC/HRGC.[53–57] A few regulatory methods allow HRGC/LRMS,[51,59] but the definitive methods for dioxin/furan analyses in the United States use HRGC/HRMS.[49–51,59–65] An example of a typical output from a HRGC/HRMS analysis is shown in Figure 2.7.

The top tracing in Figure 2.7 is a reconstructed ion profile for a high-resolution gas chromatograph/high-resolution mass spectrometer (HRGC/HRMS) USEPA 1613 analysis for chlorinated dioxins and furans. Most current methods require the addition of $^{13}C_{12}$-isotopically enriched standards for each 2,3,7,8-substituted dioxin and furan to the samples prior to extraction to improve quantitation through the use of isotope dilution techniques. The ion profile displayed at the top is a sum of the ions monitored for the native compounds and does not include the ions from the added $^{13}C_{12}$-labeled standards. The displayed 20- to 58-minute portion of the analysis on a J&W DB-5 column contains the Cl_4–Cl_8 dioxins and furans.

The expanded portion of the chromatogram shown in Figure 2.7 covers the critical 2,3,7,8-TCDD region and serves to illustrate that isomers are often analyzed at concentrations that are close to the noise level. The expanded section shows the native 2,3,7,8-TCDD peak at 25:19 min (m/z 320 and m/z 322) and the added $^{13}C_{12}$-labeled 2,3,7,8-TCDD standard peak at 25:18 min (m/z 332 and m/z 334). Currently, there are only a limited number of commercially

TABLE 2.11 Description of Methods Used Currently in the United States to Measure PCDD/Fs

Method Number	Chemical or Method Description	USEPA Reference Number	Quantitation	Analytes	Matrices[a]	Date Issued
0023A	PCDDs and PCDFs: stationary sources sampling[51]	SW-846 Ch 10	HRGC/HRMS	PCDD/PCDF	SS	12/1/1996
1613B	Dioxins, tetra- through octa-(CDDs), and furans (CDFs)[49]	821/B-94-005	HRGC/HRMS	PCDD/PCDF	OS, SE, SL, SO, T, W	9/15/1997
4425	PCBs, dioxins/furans PAH by immunoassay[52]	SW-846, update IVB	Immunoassay	Total PAHs + PCBs + PCDDs + PCDFs	S, SE, T, W	11/1/2000
8280B	PCDD and PCDF by HRGC/LRMS[50]	SW-846, update IVA	HRGC/LRMS	PCDD/PCDF	C, FA, FO, S, SB, SL, W	1/1/1998
8290A	PCDD and PCDF by HRGC/HRMS[50]	SW-846, update IVA	HRGC/HRMS	PCDD/PCDF	C, FA, FO, F, H, O, SB, SE, S, SL, W	1/1/1998
CLP-SOW	Dioxins and furans DLM01.4[62]	DLM01.4	HRGC/HRMS	PCDD/PCDF	AS, O, OM, S, SE, SL, T, W	8/29/2001
Fish	PCDF and PCDD in fish[63]	600/3-90-022	HRGC/HRMS	PCDD/PCDF	F	3/1/1990
Tissue	PCDD and PCDF in human adipose tissue[64]	560/5-86-020	HRGC/HRMS	PCDD/PCDF	H	10/1/1986
TCDD	Dioxin-2,3,7,8-TCDD[65]	600/3-85-019	HRGC/HRMS	2,3,7,8-TCDD	S	5/1/1986
TO-09A	PCDDs/PCDFs, PBDDs/ PBDFs, BCDDs/ BCCDFs in ambient air[59]	625/R-96-010-B	HRGC/HRMS	PCDD/PCDF	A	1/1/1999

[a]A, ambient air; AS, ash; C, chemical waste; F, fish tissue; FA, fly ash; FO, fuel oil; H, human adipose; O, oil; OM, oily matrices; OS, other samples; S, soil; SB, still bottoms; SE, sediment; SL, sludge; SS, stack samples; T, tissue (no human tissue); W, water.

TABLE 2.12 Description of Methods Used Currently in the United States to Measure PCBs

Method Number	Chemical or Method Description	USEPA Reference Number	Quantitation	Analytes	Matrices[a]	Date Issued
505	Organohalide pesticides and PCB products in water[54]	821/R-93-010-B	HRGC	PCBs as Arochlors	W	1/1/1989
508A	PCBs screening by perchlorination and GC[55]	600/4-88-039	HRGC	PCBs as Arochlors	W	1/1/1989
608	PCBs and organochlorine pesticides[53]	40CFR Part 136	GC	PCBs as Arochlors	W	10/26/1984
617	Pesticides, organohalide, and PCBs in wastewater[56]	821/R-93-010-A	HRGC		W	6/1/1993
1668A	Chlorinated biopheny congeners by HRGC/HRMS[60]	EPA 821/R-00-022	HRGC/HRMS	209 PCBs with > 150 specific congeners	S, T, MP, W	12/1/1999
4020	PCBs screening by immunoassay[51]	SW-846 Ch 4.4	Immunoassay	Total PCBs	NA, S	12/1/1996
4425	PCBs, dioxins/furans PAH by immunoassay[52]	SW-846, update IVB	Immunoassay	Total PAHs + PCBs + PCDDs + PCDFs	S, SE, T, W	9/1/1999
8082A	PCBs by GC[52]	SW-846, update IVB	HRGC	PCBs as Aroclors, 19 specific congeners	S, T, W	5/1/1999

8275A	PAHs and PCBs in soils/ sludges by TE/GC/MS[51]	SW-846 Ch 4.3.2	Thermal extr. HRGC/LRMS	19 PCB specific congeners	S, SL, SW	12/1/1996
9078	PCBs in soil screening test[51]	SW-846 Ch 4.5	Electrochemical	Total PCBs	S	12/1/1996
9079	PCBs in transformer oil screening test[51]	SW-846 Ch 4.5	Electrochemical	Total PCBs	O	12/1/1996
Field	PCBs: field screening by SPE membranes[58]	540/R-94-519	SPE/Ag/UV	Total PCBs	W	10/1/1994
PCB	PCBs in transformer fluid and waste oils[57]	600/4-81-045	GC	PCBs as Arochlors	O	9/1/1982
PCBs	Polychlorinated biphenyls (PCBs) in fish[61]	600/3-90-023	HRGC/HRMS	PCBs by congener group	F	3/1/1990
TO-04A	Pesticides and PCBs by high-volume PUF[59]	625/R-96-010-B	HRGC LRMS or HRMS	PCBs as Arochlors	A	1/1/1999
TO-10A	Pesticides and PCBs by low-volume PUF[59]	625/R-96-010-B	HRGC LRMS or HRMS	PCBs as Arochlors	A	1/1/1999

[a]A, ambient air; AS, ash; C, chemical waste; F, fish tissue; FA, fly ash; FO, fuel oil; H, human adipose; MP, multi phase; NA, nonaqueous liquids; O, oil; OM, oily matrices; OS, other samples; S, soil; SB, still bottoms; SE, sediment; SL, sludge; SS, stack samples; SW, solid wastes; T, tissue (no human tissue); W, water.

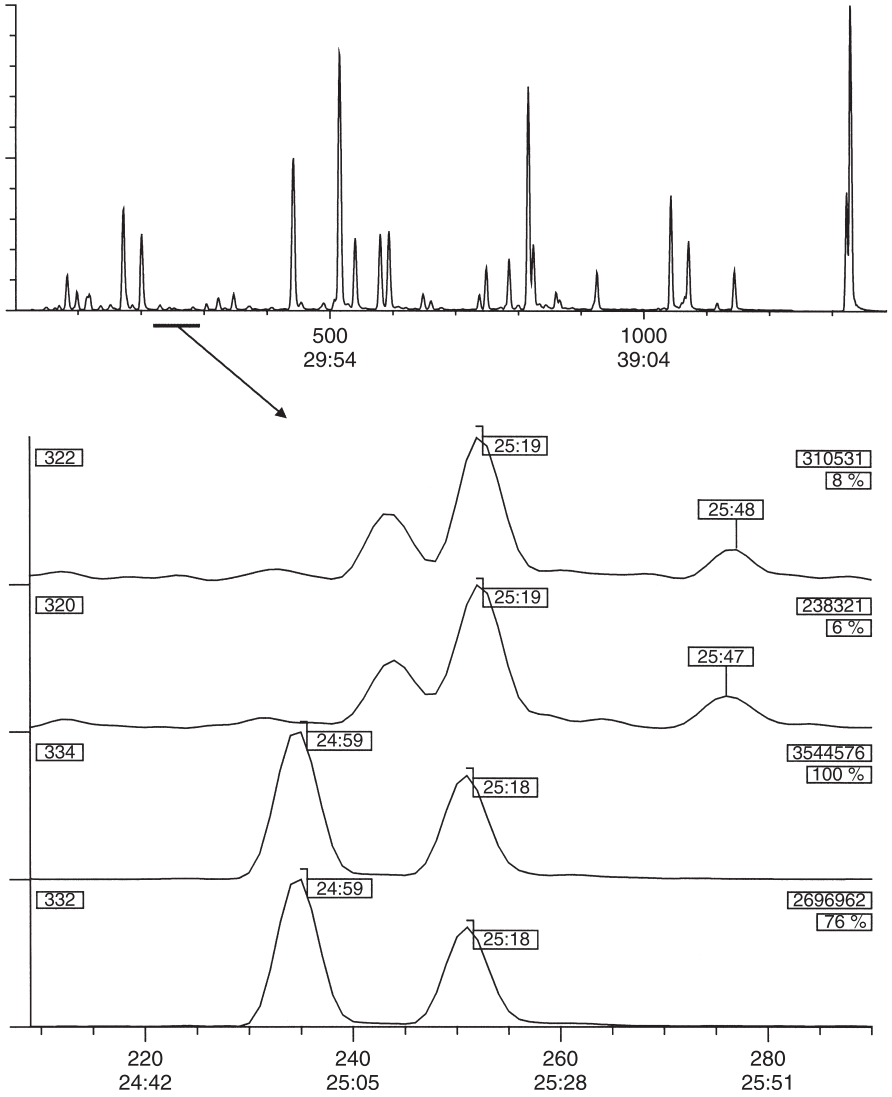


Figure 2.7 Reconstructed ion profiles for a USEPA 1613 HRGC/HRMS analysis for PCDD/Fs.

available columns that provide adequate resolution of the PCDD/F congener groups and 2,3,7,8-TCDD. The need for high chromatographic resolution is illustrated by the occurrence of another TCDD isomer peak at approximately 25 : 09 min. This peak (a coelution of 1,2,3,7- and 1,2,3,8-TCDD) elutes immediately before the native 2,3,7,8-TCDD peak. The chromatographic separation requirements specified by current regulatory methods (less than or equal to

25% valley between the 2,3,7,8-TCDD peak and any other isomer or contaminant peak) typically require an 60-m capillary column for HRGC/HRMS analysis. In addition, the mass spectrometer resolution requirements specified by current state-of-the-art regulatory methods (m/z resolutions greater than 10,000) only can be achieved using high-resolution magnetic sector instruments. Not discussed here, but covered in detail in the USEPA HRGC/HRMS methods,[49,50] are numerous other mandated requirements. These include items such as absolute and relative retention times, fragment ion ratios, and measurements for interferences.

2.4.3 Problems and Limitations of Existing Techniques

Although some USEPA HRGC/HRMS methods permit the use of a single column for analysis, most laboratories find it necessary to use two columns to meet mandated resolution requirements. Typically, a 95% methyl/5% phenyl phase column (i.e., a J&W Scientific, Folsom, California, DB-5 column or equivalent) is used for the primary analysis because this phase has good stability. Samples having measurable levels of the 2,3,7,8-TCDF isomer must have a separate confirmation analysis run on another column, since the 95% methyl/5% phenyl columns cannot uniquely separate this isomer. The lack of a stable column that meets official requirements for dioxin/furan analyses without the need for confirmation analyses is a significant limitation of current HRGC/HRMS methods. In the case of PCBs, analyses are complicated by the need to separate 209 congeners. EPA 1668A[60] specifies the Supelco SPB-Octyl column, which can resolve approximately 150 of the 209 PCB isomers. Recently, we have studied the performance of this column and found that retention stability is a major limitation.[66] In addition to these problems, current method sensitivity is limited by the trade-off between adequate concentration of the target analytes and interferences from large amounts (10^6 to 10^9) of co-extracted matrix materials. Although larger sample sizes can be extracted in some cases, this does not address the problem of chemical noise, which can vary widely between sample types. Especially problematic are samples that contain high levels of nonpolar aromatics.

2.4.4 Improving Existing Analytical Approaches

Dioxin and related compounds usually exist in the presence of other potentially toxic chemicals (e.g., polycyclic aromatic hydrocarbons, phthalates, pesticides, etc.) that are at significantly higher concentrations. Similarly, they often are incorporated into complex matrices such as soils, sediments, and biological tissues. The challenge in improving methodology is to effectively separate and identify the analytes at increasingly lower levels (i.e., sub-parts-per-trillion). This mandates the use of highly efficient sample workup steps followed by state-of-the-art measurement methodology that is operated at levels of sensitivity that often tax an instrument's limits of detection. Currently, there are relatively few

analytical laboratories that are adequately equipped and have the expertise to measure dioxin and dioxinlike compounds at the levels of sensitivity and specificity needed. Three of the more important and practical analytical problems in improving methodology are (1) the development of reliable automated and more efficient sample workup procedures, (2) improvements in capillary gas chromatographic columns that will enhance their performance in terms or resolution and stability, and (3) the application of emerging mass spectrometric technology that will improve instrument performance and reduce operational costs. Although many research groups have addressed important problems related to the chromatographic separation and subsequent mass-spectrometric measurement of dioxin and dioxinlike compounds, most of the initial isolation and concentration steps in the sample workup are carried out using analytical methodology that was developed several decades ago. These procedures, which are viewed as the ''gold'' standards in terms of reliability, often require many hours to perform and require substantial volumes of highly purified solvents.

The current method improvement challenge is to adapt and as needed to develop new, more efficient high-throughput sample workup procedures. Typically, liquid–liquid extraction is used for aqueous samples, and Soxhlet extraction is used for solid and biological matrices. Although Soxhlet extractors can run unattended, the sample loading and washing procedures are labor-intensive and they limit the sample turnaround time. Advances in automated solid-phase techniques for aqueous samples, microwave-assisted extraction, and the use of high-temperature and high-pressure extraction for solid samples have the potential to improve efficiency and reduce solvent consumption. Current sample cleanup protocols employ high-capacity silica, alumina, carbon, and florisil gravity-flow columns that require constant attention from the analyst. These columns provide the capacity that is required to separate the analytes from interfering matrix materials. Automated chromatography systems have been developed which are very efficient for handling samples that contain predictable and consistent levels of target analytes. The problems of carryover from a high-level sample to a low-level sample make these systems impractical in laboratories that process samples with highly variable levels of analytes. Automated systems that reduce the use of reusable hardware to direct the sample through the apparatus may show promise for automating the sample cleanup procedures.

As noted above, there have been few advances in the electron impact HRGC/HRMS analysis techniques in recent years. One emerging alternative is time-of-flight mass spectrometry (TOF-MS). The equipment for this analysis technique is less expensive than the high-resolution magnetic sector instrumentation that is currently employed for environmental analyses. Continued improvements in the mass resolution of the TOF systems will be necessary for this to be a feasible alternative. The multiple analytical capillary GC columns that are required for PCDD/F analyses and the limitations of the current analytical column for the PCB analysis are examples of the difficulties that analysts currently face. This is an area where columns with improved selectivity and

ruggedness would increase the efficiency and reduce the cost of PCDD/F and PCB analyses. For any of these improvements to be deemed acceptable, the appropriate analytical and regulatory communities must approve their use. Overcoming this hurtle is a formidable task. This will require that the new methods provide accurate and precise measurements that are rugged and cost-effective and that they provide the same level of performance and reliability as existing methods.

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CHAPTER 3

Dioxins and Dioxinlike PCBs in Food

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3.1 INTRODUCTION

Polychlorinated dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs) (dioxins) are ubiquitously present in human tissues when there is no history of occupational or accidental exposure. Although exposure could occur through inhalation of air, dermal absorption, consumption of drinking water and consumption of food, there is no doubt that the latter is the predominant route for the background population.

Only the 17 congeners that contain chlorine at the 2, 3, 7, and 8 positions persist and accumulate in animal tissues, even though these congeners form only a small proportion of the total output from many sources and of the environmental load. It is also these 2,3,7,8-substituted congeners that are regarded as significantly toxic and are thus the focus of interest. They are highly lipophilic and are thus found primarily in fatty tissues. In 1987, Travis concluded from modeling calculations that dietary intake of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) accounted for 98% of human exposure to this compound.[1] Over the last 15 years many measurements have been made of the full range of PCDD/Fs in foods, and many estimates of exposure have been made which all lead to the conclusion that well over 90% is from food.[2,3] It has also become widely accepted that some biphenyls (PCBs) also bind to the aryl hydrocarbon (Ah) receptor and elicit dioxinlike biochemical and toxic responses, so that assessment of the health risks of exposure to dioxinlike chemicals must consider these PCBs as well as PCDD/Fs. The amount of information available on dioxinlike PCBs in foods is somewhat less than for PCDD/Fs themselves but is growing rapidly. PCBs have

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a variety of other biological effects, however, and although consideration of dioxins is incomplete without the inclusion of dioxinlike PCBs, this treatment is certainly not a sufficient response to PCBs in general.

In contrast to the selectivity exhibited for Ah-active PCDD/Fs, other PCBs also accumulate in animal tissues and the dioxinlike compounds form only a small proportion of the total PCB concentration. For example, in a duplicate diet study of PCBs in a group of 20 subjects consuming a typical Italian diet, it was found that the mean intake of total PCB was 3.72 μg/day. The diortho congeners 18, 138, and 153 constituted, respectively, 11.4, 10.9, and 13.8% of the total, while the nonortho congeners 77, 126, and 169 amounted to 0.5% of the total.[4]

Since the first edition of *Dioxins and Health*[5] over 200 new papers and accessible reports have appeared that deal with levels of dioxinlike compounds in foods and dietary intake. Much of the information on PCDD/Fs in foods and in human milk that was available in 1996 was included in a compilation published in Volume 69 of the *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans*.[6] Comprehensive reviews of data available from Member States of the European Union, including those for dioxinlike PCBs, are also available[7,8] and give more detail than can be accommodated in this chapter. Much information and discussion of PCDD/Fs and PCBs is also included in reviews of persistent organic pollutants in food[9] and of environmental contaminants in food.[10]

In this chapter we discuss the availability of recent data and the levels currently present in foods from different parts of the world, and also the resulting intakes, geographical variations, trends over time, and the pathways by which PCDD/Fs and PCBs are transferred from the environment to the food chain. For some of these topics there is now an abundance of published work, and the references given are a personal selection. In particular we do not discuss or reference many earlier studies, choosing to emphasize more recent work, as this is most likely to be representative of current levels and intakes. Earlier data are introduced mostly where they are important to the consideration of trends over time. We also discuss in some detail a number of factors that influence the accuracy and comparability of analytical data, and thus of intake assessments. We believe proper consideration of these to be crucially important to the correct interpretation of the data.

3.2 TOXIC EQUIVALENCY FACTORS

Because of the need to assess the risk from complex mixtures of PCDD/Fs, an approach has been adopted that assigns relative potency factors to each congener, based on a comparison with the potency of 2,3,7,8-TCDD.[11] Each chemical is assigned a toxic equivalency factor (TEF) relative to 2,3,7,8-TCDD. The total toxic equivalency of a mixture is the sum of the TEF $\times$ concentration products for each compound in the mixture, but there is some

variation in the terminology used by different authors; total TEQ, summed TEQ, and $\sum$TEQ are self-explanatory, while the unqualified acronym, TEQ, is used by different authors to refer either to the total or to the TEF $\times$ concentration product of an individual compound.

Although consideration of total TEQs is essential, it is regrettable that many data are easily available only in this form. As discussed below, total TEQ figures can have serious deficiencies, and their interpretation is greatly facilitated by inspection of the underlying congener-specific data, especially when data from different laboratories are compared. The contribution of specific congeners to the total is also of great value for source identification.

There remains some uncertainty about the accuracy with which TEF values reflect actual effects on humans. It should be noted that TEF values are given only to the nearest one-half order of magnitude, and thus the range within which the "true" TEF lies is right skewed, from 0.5 to 5 times its stated value. Treatment of data using probabilistic statistics techniques, which take into account uncertainty associated with assignment of TEFs, has shown that the TEQ can increase by 1.5 to 2 times compared to deterministic estimates using fixed values for the TEFs.[12,13]

3.2.1 PCDDs and PCDFs

During the 1980s, a rather large number of different TEF schemes were used. International toxic equivalency factors (I-TEFs) for PCDD/Fs were set in 1990[14] and were adopted by almost all scientists and regulatory authorities. A more recent system of TEFs, set by the World Health Organization in 1997 (WHO-TEFs),[15,16] has been accepted by most authorities and is coming into wider use, but much of the recent reporting of data for PCDD/Fs has continued to use the 1990 scheme. In the WHO-TEF scheme the TEF for 1,2,3,7,8-penta-CDD was doubled, from 0.5 in the I-TEF scheme to 1, while the TEFs for octa-CDD and octa-CDF were reduced by factors of 10, from 0.001 to 0.0001. The net effect of these changes for most food samples is an increase of around 15 to 20% in the calculated result for total TEQ level of PCDD/Fs.

3.2.2 PCBs

The WHO system also sets TEFs for those PCBs that bind to the Ah receptor and elicit dioxinlike biochemical and toxic responses. In 1994, TEFs were set for three nonortho PCBs (IUPAC Nos. 77, 126, and 169), eight monoortho PCBs (105, 114, 118, 123, 156, 157, 167, and 189), and for the diortho PCBs 170 and 180.[17] In the 1997 WHO scheme[15] PCBs 170 and 180 were removed, PCB 81 added, and the TEF for the nonortho PCB 77 reduced by a factor of 5. For most food samples these changes make a negligible difference to the total TEQ attributable to PCBs.

The term *coplanar PCB* is often used to refer to the three nonortho compounds, and sometimes to these and the eight monoortho PCBs above. In

many food samples the TEQ contribution made by PCBs may equal or, especially in fish, exceed that made by PCDD/Fs. In comparing or interpreting data expressed as TEQs, it is of paramount importance to note whether or not PCBs are included in the total.

3.3 ACCURACY AND COMPARABILITY OF ANALYTICAL DATA

Anyone who uses analytical data on dioxinlike chemicals or the intake assessments derived from them needs to remain aware of various issues that may influence the accuracy and comparability of the results. Even in some recent studies there are undoubtedly issues related to the representativeness of sampling and to the accuracy of measured concentrations. Furthermore, interpretation of reported data is complicated by the differing ways in which compounds that are not actually detected and measured are represented in summations or averages. There are a disturbing number of statements in the literature about differences between foods or between locations that probably reflect simply differences in analytical performance and data assessment methodologies.

3.3.1 Analytical Accuracy and Precision

All analytical measurements have associated with them a measurement uncertainty. Unfortunately, information on the accuracy and precision of measurements is often absent from reports of data on PCDD/Fs and dioxinlike PCBs. The analysis of these substances in foods is particularly challenging because of the very low concentrations that are involved. Although bioassay tests[18,19] are becoming important, most data on dioxinlike compounds are obtained by a rather lengthy series of extraction, cleanup, and concentration steps followed by high-resolution gas chromatography coupled to high-resolution mass spectrometry, involving instruments that require great skill in maintenance and operation.

Several studies have shown that good agreement can be achieved by highly expert laboratories,[20–22] but the number of laboratories engaged in these analyses has increased dramatically in the last few years and expertise is not necessarily acquired instantly. In the fourth round of WHO-coordinated interlaboratory quality assessment studies, only three of 11 laboratories participating met all the quality criteria for the determination of PCDD/Fs in human milk,[23] a less challenging task than is presented by cows' milk and some other foods. At the same time, in measurements of fat content, less than half of the participants achieved deviations of less than 10% from the accepted value; many food data are expressed on a fat basis, the final result being calculated from measurements of the target compounds on a whole sample basis and separate measurement of the fat content.

More recent interlaboratory studies have been organized by the Norwegian

Institute of Public Health. In 2000, the study included chicken, butter, and fish, results being supplied by 37 laboratories from 15 countries.[24] In 2001, matrices were beef, cod's liver, and human milk, results being supplied by 55 laboratories from 24 countries.[25] Analytes included PCDD/Fs and dioxinlike PCBs, but not all laboratories determined both classes. In the second study it was shown that for levels of PCDD/Fs of 4 to 5 pg TEQ/g fat, some 30 of the participating laboratories could determine the compounds with "good accuracy" ($\pm$20% of the consensus TEQ), but that for samples with a lower content of PCDD/Fs (about 0.5 pg TEQ/g fat), considerably fewer laboratories (12) reached this standard. It was noted that because of the large number of nondetects, it was difficult to establish the true concentration and that the consensus value used as a target may actually have been an overestimate.

For example, taking the consensus levels for the beef test material studied, in which the total TEQ (PCDD/Fs and PCBs) was 1.0 pg/g fat, 2,3,7,8-TCDD, 1,2,3,7,8-penta-CDD and 2,3,4,7,8-penta-CDF together account for about 73% of the total TEQ for PCDD/Fs, and PCB 126 accounts for 73% of the total TEQ for PCBs. For 2,3,7,8-TCDD, 29 of 52 reported values were nondetects. Limit of detections were between 0.02 and 0.86 pg/g fat (an LOD of 10 was also reported but from a laboratory attempting the analysis with equipment that most analysts would regard as unsuitable). Positive results varied from 0.026 to 0.30 pg/g fat with a consensus of 0.1 pg/g fat. For 2,3,4,7,8-penta-CDF, for which the consensus value was 0.31 pg/g fat, there were only seven nondetects, but results still ranged from 0.11 to 0.76 after exclusion of a grossly outlying result of 27. For PCB 126, with a consensus value of 3.4 pg/g fat, results ranged from 0.3 to 8.1, although both these were identified as outliers and most results were within the range 1.0 to 5.8.

3.3.2 Limits of Detection

It has been stated that in studies of food contaminants the analytical LODs should be sufficiently low that further reduction of the LOD does not result in additional reduction in estimates of exposure.[26] For PCDD/Fs this criterion is met in only a very few laboratories, and even then, not for every food under consideration. It is common for the concentrations of some or all of the individual dioxins and PCBs to be below the LOD. Inevitably, the sensitivity of analyses in different laboratories varies and so does the method of assessing and reporting the LOD. Frequently, quantitative results are reported for any congener giving a gas chromatograph/mass spectrometer peak with a signal-to-noise ratio (S/N) of, say, 3 or more. If no peak is detected, an estimate is made of the concentration that would have produced this S/N. LODs so estimated vary between congeners and between different analyses. Some laboratories work, instead, to a *limit of quantitation* (LOQ), which, strictly, should be defined as the lowest concentration at which a specified measurement precision is achieved, as demonstrated in method validation studies, but which is, instead, sometimes taken to be a multiple of the LOD. Alternatively, some data

are reported after applying an arbitrary but consistent *reporting limit*. Different laboratories analyzing the same samples, or even interpreting the same raw data, may arrive at differing conclusions about which congeners are present at measurable concentrations, and at quite different LOD values for other congeners, even though their positive results may be in good agreement.

When data include nondetects, at least three different methods are in use for the calculation of total TEQ levels. These are the representation of nondetected congeners in subsequent calculations by (1) a concentration of 0, (2) a concentration equal to the LOD, or (3) a concentration equal to one-half of the LOD. Even this may expand to five different methods if LODs and LOQs are differentiated.

The terms *lower bound* and *upper bound* are often used to refer to the first two of these methods, which correspond, respectively, to the minimum concentration *known* to be present and to a larger concentration that *might* be present. The $\mathrm{ND} = \frac{1}{2}\mathrm{LOD}$ calculation, for which the term *median bound* is sometimes adopted, gives a result that lies midway between the upper and lower bounds. This often gives the appearance of better comparability, and may be preferable to the use of either lower or upper bound alone. However, if the upper- and lower-bound totals are far apart, as is frequently the case, the midpoint is not necessarily any closer to the true total TEQ concentration than is either extreme. Use of any single convention in calculations from data in which the LOD itself varies between analyses can be particularly misleading.

Further complications may arise when results for specific congeners are averaged across a number of samples, with the total TEQ summation made using the average concentrations. In addition to use of all of the substitution approaches above, some workers average only detected values to generate a statistic that is meaningless unless the frequency of detection is also taken into account.

All of these approaches have utility in some circumstances if applied consistently. Upper-bound estimates are favored by the European commission for regulatory monitoring, while the use of median bound is currently proposed for intake estimation and risk assessment purposes. In all cases, comparisons of total TEQ data representing different detection limits and calculation methods can be extremely misleading. There have undoubtedly been occasions when comparisons of total TEQs have been made and interpreted when nothing at all has been detected and measured, differences in the totals arising simply from differences in detection limit.

3.3.3 Representativeness

The most accurate laboratory analysis can only give a result that represents the sample taken for analysis. How that sample is related to the broad food supply depends on both the quality and the intent of the sampling scheme. For use in an estimation of intakes, sampling should be designed to take account of many factors, some of which are as follows: (1) the proportion of each food that

is imported, and variation in the countries of origin; (2) seasonal variation (since supplies available to the consumer may be different throughout the year, imports may be more prevalent at different times, or food may have been stored for longer periods at out of season times); and (3) regional variation, as local food production may be affected by differences in climate, by local pollution sources or urbanization, or by regional variation in available brands. Food prepared (takeout meals) or eaten outside the home (in restaurants, for example) also needs to be considered.

Sampling to assess compliance with limits, whether statutory or guidelines, may not be appropriate for estimation of average intakes. For example, compliance monitoring may be concentrated on domestic food production and exclude imports, or aim for broad geographical coverage without weighting by food production statistics. Any indication of localized contamination usually leads to more intensive sampling in the same location; small but significantly contaminated locations may therefore make a disproportionately large contribution to average concentrations if these are based on all available data.

For most foodstuffs, achievement of a representative result inescapably necessitates coverage of a large number of samples. However, because of the cost and difficulty of analysis, most of the older studies from the late 1980s and early 1990s, and some completed more recently, were based on rather limited numbers of samples, sometimes even single examples of a commodity or food product. One solution to this problem is to pool samples to form composites representing a specific category of foodstuff. This approach has been used in a number of national studies: for example, in Finland, the Netherlands, New Zealand, and the United Kingdom. The use of composite samples is a cost-effective way of obtaining robust measures of average concentrations, but it does not furnish any information on the width and shape of the distribution of concentrations in the individual samples. It may also have the disadvantage of placing great reliance on single analyses.

Total diet study (TDS) schemes have often been used as the source of samples. In the U.K. TDS, a total of 121 categories of food and drink are purchased fortnightly from 24 randomly selected locations representative of the United Kingdom as a whole. Samples are prepared and cooked as for consumption and then combined into composite samples representing 20 defined food groups. The quantity and relative proportions of the foods that make up each composite are based on data from the National Food Survey (a continuous survey that provides information on the types and quantities of foods purchased by households) and are updated annually.[27] Other total diet studies vary considerably in their geographical range, in the number of individual samples taken, and in the number and timing of samplings, which vary from a single occasion to repeated sampling over a year.

Differences in food classification can also lead to lack of comparability between studies. In the U.K. TDS, the meat group is not segregated by animal species and includes beef, mutton, and pork. In many studies some classes, such as meat products, fruit products, and cereals, are differently defined and not

comparable. Cereal, for example, may refer simply to grain and flour, or may include cereal products encompassing breads, cakes, and pastries prepared with animal fats, and sometimes various breakfast foods.

3.3.4 Fat Weight and Whole Weight Reporting

PCDD/Fs and PCBs are lipid-soluble and results for most food types containing over about 2% fat are reported on a fat weight basis. This gives more consistency for comparisons of samples such as milk, which show more variability with respect to dioxins on a whole weight basis than on a lipid basis. For some samples, however, reporting on a fat weight basis can lead to confusion. Fish can show seasonal variations in fat content, which can result in fat weight results giving an illusion of variation, even if the body burden with respect to dioxins remains constant. For low-fat samples such as fruit and vegetables, the reported fat content can reflect organic coextractives rather than true fat values. The amounts of these coextractives is small and negligible for samples with higher fat content, but become significant when the fat content is very low. Results for these samples should be considered only on a whole weight basis.

Reporting of results on a fat weight basis can also lead to different analytical approaches. Some laboratories isolate fat from samples (or ask for submission of isolated fat) before analysis starts. Internal standards are then added to the fat, and the dioxins are determined on the fat sample. Other laboratories will add internal standards to the whole sample before isolation of fat or will analyze the whole sample without fat isolation and convert results to a fat basis using the results of off-line determination of fat. Where there are inaccuracies in fat measurement or inefficient recoveries, these can lead to analytical differences.

3.4 METHODS FOR ESTIMATING DIETARY EXPOSURE

The simplest method of assessing dietary intake is the analysis of duplicate diets, a method that has been applied to assess the intake of PCDD/Fs in Germany by adults[28–30] and by small children,[31] of PCDD/Fs and PCBs in the Netherlands,[32,33] and of PCBs from the Italian diet.[34] This is probably the most accurate way of establishing the intake of an individual or small group of people over a short time period, but gives no information on the relative contribution of different foods and may not include enough people to give a result representative of the population.

The more common method is to multiply the average concentrations found in each type of food by consumption estimates, and to add together the contributions from various components of the diet. Much of this chapter is concerned with the data from which the average food levels are established. Several methods can be used to assess individual food consumption, including 24-h recall, dietary records, food frequency surveys, and dietary history.[35] Popula-

tion averages can also be obtained from household budget studies.[36] Dietary records (food diaries) have been used most commonly in surveys used to estimate dioxin intakes, sometimes augmented by other methods. The period over which records are kept by each person in a survey may be important,[37] although it has been shown that survey duration does not influence mean total population intakes.[38]

An important attribute of such individual-oriented surveys is that the distribution of consumption of different foods can be established so that the intake associated with high level consumers of individual foods can be calculated. Various percentile points have been used, including the 90th, 95th, and 97.5th percentiles. Most of the contribution to the total TEQ for a sample comes from a limited number of congeners, and because of this, it has been proposed that dietary estimates of exposure could be made by analyzing a reduced congener set. This would, however, result in an incomplete picture of congener profiles, so that identification of specific sources of PCDD/Fs would become more difficult and could result in contamination events which involve only congeners not included in the reduced set being overlooked.

3.5 TOLERABLE INTAKES

During the 1980s and early 1990s, a number of countries performed risk assessments and derived tolerable daily intakes (TDIs) of dioxins in the range 1 to 10 pg/kg body weight, as reviewed by Larsen et al.[39] A TDI is the maximum amount of a contaminant that can be eaten every day over an entire lifetime without incurring appreciable risk to health. As the data on aspects of the toxicology of PCDD/Fs and PCBs have become more extensive and of better quality, views about the appropriate value of a TDI have changed and values resulting from different assessments have become more consistent. Thus, while in 1990, WHO established a TDI of 10 pg/kg body weight for TCDD, in 1998 an expert consultation concluded that the TDI was in the range 1 to 4 pg TEQ/kg body weight.[40] At the end of May 2001, the Scientific Committee on Food (SCF), an expert committee that advises the European commission, decided that the tolerable intake should be expressed on a weekly rather than a daily basis and set a tolerable *weekly* intake (TWI) of 14 pg WHO-TEQ/kg body weight.[41] In June 2001, the WHO/FAO Joint Expert Committee on Food Additives (JECFA) established a provisional tolerable monthly intake (PTMI) of 70 pg/kg body weight per month.[42]

3.6 LEGISLATION

Although regulatory limits for dioxins in food have been set on an ad hoc basis by various authorities in the past, the European Union (EU) is the first body to set extensive and comprehensive limits for these compounds.[43] This regula-

tion came into force in July 2002 and includes limits for PCDD/Fs in food and animal feed. There is an intention to review the limits by December 31, 2004 with a view to include dioxinlike PCBs. This regulation will be supported by a monitoring plan, which is to be implemented by all member states, and by strict performance criteria for analytical methods that are used. Upper-bound results are to be used for monitoring purposes (see Section 3.3.2).

3.7 REGIONAL STUDIES AND INTAKE ASSESSMENTS

In this section we summarize the more recent data representing background levels of PCDD/Fs and dioxinlike PCBs in foods from different countries, and where they exist, the various national estimates of dietary intake.

3.7.1 Europe

No data appear to be available to represent the greater part of Eastern Europe, Greece, Luxembourg, Portugal, and Iceland, and the only data available for Switzerland are from early studies of cows' milk.[44,45] Data exist for most other West European countries, and very large data sets have been assembled by some of them. An extensive review of human exposure data in the EU was compiled in a report presented by AEA Technology.[7] More recently, a compilation of data provided by 10 European countries was assembled as a result of the program of Scientific Co-operation on Questions relating to Food (SCOOP) Task 3.2.5.[8] This gives some 500 different total TEQ results, many being averages based on very large numbers of individual samples, representing measurements performed on samples of different foodstuffs collected in the period 1982–1999, including data for human milk. Many of these results are also available in the open scientific literature, but a significant amount of information is included which otherwise appears to be unpublished. The handling of nondetects remains that chosen by the originators of the data and is inconsistent; the method is indicated, but the detection limits themselves are not.

Austria Published data are limited. PCDD/Fs have been determined in pork (19 samples) and chicken (5 samples) from the Styrian region of Austria.[46] One pork fat sample showed 7.5 pg TEQ/g fat, and the remainder < 2 pg TEQ/g fat.

Belgium In Belgium data on PCDD/Fs in cows' milk have been obtained annually since 1994,[8,48] and additional data on long-life milk from one region has been reported.[48] Data from the analysis of 150 samples (meat, milk products, prawns, and trout) have also been reported.[49] The SCOOP report includes PCB data from a wider range of foods.[8] Food consumption data also appear to

be limited to a 7-day food record study of adolescents in the city of Ghent performed in 1997.

Denmark Food consumption data are available for 1985 and 1995.[8] Apart from data on cows' milk sampled in 1999,[8] the only available information on food levels is from a small survey for PCDD/Fs in dairy products, beef, and fish that was conducted in 1987.[8,50] From the latter, Liem et al.[3] calculated the dietary intake for Danish consumers to be 290 pg I-TEQ/day. Fish consumption was responsible for about half of this intake. The reported concentrations are somewhat higher than in other, contemporaneous studies.

Finland Data supplied to the SCOOP project[8] were gathered mostly in the early 1990s.[51] New data have recently been obtained from analysis of samples collected in the period 1998–2000.[52] Commodities included cows' milk, pork, beef, eggs, trout, leafy vegetables (lettuce and cabbage), fruit vegetables (cucumber, tomato, onion sweet pepper), potatoes, and flour, obtained from various locations in Finland. Samples were pooled by production area and analyzed for PCDD/Fs and dioxinlike PCBs. For intake calculations, contributions from Baltic herring and other fish were included, based on unpublished concentration data. Consumption data were from a 1997 24-h dietary recall survey of the adult population. The lower- and upper-bound estimates of intake of PCDD/Fs were 46 and 65 pg I-TEQ/day and those of dioxinlike PCBs were 53 and 54 pg TEQ/day (using 1994 WHO TEFs).

France Published studies of milk and dairy products[53–56] are supplemented in the SCOOP report[8] by data for PCDD/Fs (but not for PCBs) in fish (56 samples), seafood, meat (50 samples), meat products (15 samples), offal (6 samples), eggs (5 samples), fruits and vegetables (40 samples), and cereals (13 samples, including cereals, bread, rice, and pasta). These were all sampled in 1998–1999. Food consumption data from 7-day records of 3003 consumers from age 2 upward were obtained in 1998–1999. The upper-bound estimated intake of PCDD/Fs is 97.1 pg TEQ/day (1.45 pg TEQ/kg body weight/day).[8]

Germany Data representing many thousands of samples, most analyzed for PCDD/Fs only, are summarized and referenced in the SCOOP report,[8] which gives references to many publications and reports. Data are included from over 1400 samples collected between 1993 and 1996 in the southwestern part of Germany as part of the official food inspection program.[57] Some data on foods and canteen meals are available from southern Germany.[58] Other studies include milk,[59,60] meat,[61,62] fish,[63] and seafood.[64]

The food consumption data available are for 1985–1989 and are expressed as the fat intakes from various foods. Using these figures, the average intake of PCDD/Fs in Germany in 1994–1995 was estimated by Fürst and Wilmers[65] to be 69.6 pg I-TEQ/day or 1.0 pg/kg body weight/day. Using the same food

consumption figures with food data for the period 1993–1996, Malisch calculated the PCDD/F intake to be 61.3 pg I-TEQ/day (0.88 pg/kg body weight/day).[57] For the period 1996–1998, a mean daily intake of 50.9 pg I-TEQ/day has been estimated.[8] All of these estimates use the ND = $\frac{1}{2}$LOD substitution.

Duplicate diet studies in 1994–1995 indicated a daily intake in the range 23 to 96 pg I-TEQ/day with a mean of 49 pg I-TEQ/day (0.72 pg/kg body weight/day),[28] or, alternatively, a mean of 61.5 pg I-TEQ/day (0.85 pg/kg body weight/day) with intakes by women and men of 54 pg I-TEQ/day and 69 pg I-TEQ/day, respectively.[29,30]

Ireland Some data for PCDD/Fs in cows' milk sampled in 1995 are available[66] and are also included in Ref. 7, but not in the SCOOP report. Concentrations found were in the range 0.13 to 0.51 pg I-TEQ/g fat. In a further study in 2000 of 24 samples, levels of PCCD/Fs were slightly lower and data for dioxinlike PCBs, found to contribute about equally to the total WHO-TEQ, were also obtained.[67] Recent data from analyses of fish and fish oil are reported on a World Wide Web site.[68]

Italy Food consumption data are available from 7-day records of 3000 people in 1200 households, collected in 1994–1996. Data, for PCDD/Fs only, in milk and dairy products, meat, eggs, fish, and shellfish are included in the SCOOP report together with a mean intake estimate of 45.1 pg TEQ/day (0.74 pg/kg body weight/day) made using the ND = $\frac{1}{2}$LOD method of calculation.[8] A study of PCDD/Fs in foods collected in Venice in 1997 has been published.[69] Only three individual samples of some foodstuffs, such as beef, poultry, and butter, were analyzed, and especially since the ranges of concentrations found were rather large, the representativeness of the averages is doubtful. For fish and shellfish from the Lagoon of Venice, sampling was more comprehensive. Using the extremes of the range of concentrations for each foodstuff to calculate intakes resulted in a range for the latter of 15 to 128 pg I-TEQ/day. The average intake, calculated using concentrations for eggs taken from U.K. data, was 42 pg I-TEQ/day. Fish and dairy products accounted for the bulk of intake. In a duplicate diet study of a group of 20 subjects consuming a typical Italian diet, the intake of dioxinlike PCBs was found to range from 4.6 to 119 pg TEQ/day in 18 subjects, but the remaining subjects were reported to have intakes of 2.1 and 4.6 ng/day.[4]

The Netherlands Comprehensive studies of foodstuffs collected in the first half of the 1990s are summarized in the SCOOP report[8] and presented in greater detail in the dissertation of Liem and Theelen.[32] A very detailed report has been published on a similarly comprehensive study using samples collected in 1999.[70] In both studies foods were selected and intakes calculated based on consumption statistics from over 6000 subjects, assessed by 2-day dietary records distributed equally over the 7 days of the week and over a year,

TABLE 3.1 Estimates of Average/90th Percentile Age-Specific Dietary Intake of PCDD/Fs and PCBs in the Netherlands

	Intake (pg TEQ/kg body weight per day)		
Compounds	2 yr	10 yr	40 yr
PCDD/Fs	1.5/2.2	0.80/1.2	0.60/0.87
PCBs	1.4/2.2	0.73/1.1	0.53/0.81
Total	3.0/4.4	1.5/2.3	1.1/1.7

Source: Data from Ref. 70.

in 1987–1988 and 1997–1998, respectively. Samples were collected in four (first study) or five (1999) different regions in the Netherlands, two sets being collected in each region, combined into 10 regional sets, and finally, into two nationally representative sets of 18 food categories.

For the earlier period,[32] the median intake of the general Dutch population (1 to 70 years of age) was estimated to be 65 pg I-TEQ/day for PCDD/Fs and 70 pg WHO-TEQ/day for nonortho PCBs. Milk, dairy products, and beef contributed 50% of this intake. From the more recent data, average intakes were estimated as 45 pg WHO-TEQ/day for PCDD/Fs and 46 pg WHO-TEQ/day for PCBs. Meat products contributed 23% to the total, dairy products 27%, fish 16%, eggs 4%, vegetable products 13%, and industrial oils and fats 17%. Average and 90th percentile age-specific intakes are summarized in Table 3.1. All of these estimates are based on lower-bound TEQ levels. Both lower- and upper-bound totals for each foodstuff, together with full congener-specific data, are included in the report[70] and differ by only a few percent for foods making important contributions to intake. Pooled samples of duplicate diets collected by adults over 24-h periods in 1978, 1984–1985, and 1994 have also been analyzed.[32,33] The resulting intake estimates are given in Table 3.2.

Norway Food consumption data are available from a quantitative food frequency survey conducted in 1997. Data on levels in food are available

TABLE 3.2 Dietary Intakes of PCDD/Fs and Dioxinlike PCBs in the Netherlands Obtained from Duplicate Diet Studies

	Daily Intake (pg TEQ/kg body weight per day)		
Compounds	1978	1984–1985	1994
PCDD/Fs	4.2	1.8	0.53
PCBs	6.8	2.2	0.92
Total	11	4.0	1.5

Source: After Ref. 32.

for PCDD/Fs and PCBs, but some derive from sampling dates from the late 1980s and early 1990s.[8,71,72] Composite samples of 18 different food categories, covering meat, fish, eggs, and dairy products, were formed from 20 to 25 individual samples of seafood, or 10 to 15 of other foods and several separate pools of each category were analyzed. In reference 71, lower- and upper-bound average weekly intakes for PCDD/Fs are given as 354 and 592 pg I-TEQ/week (51 and 84 pg I-TEQ/day) and for PCBs as 605 and 743 pg WHO-TEQ/week (86 and 106 pg WHO-TEQ/day), with nearly half of the intake of both PCDD/Fs and PCBs from fish. Upper-bound average intake estimates quoted in the SCOOP report are inconsistent with these figures, being 29 and 110 pg TEQ/day for PCDD/Fs and PCBs, respectively.

Russia PCDD/Fs have been determined in samples from the Republic of Bashkortostan[73,74] dating from 1996. Intake estimates are 2.31 pg TEQ/kg body weight/day for residents of industrial cities such as Ufa, and 1.15 pg TEQ/kg body weight/day for the rural population. The congener-specific data[74] show relatively high proportions of 2,3,7,8-TCDD and 2,3,7,8-TCDF in packed milk, which might indicate continued use of cartons manufactured from chlorine-bleached pulp. Similar indications are contained in data provided by McLachlan for a number of milk samples from the Irkutsk Oblast in 1997, where one sample gave a level as high as 10.3 pg TEQ/g fat.[75] In the same study, raw milk, chicken, pork, and beef gave total TEQs for PCDD/Fs and PCBs which are similar to those found in most other countries.

Other data, for PCDD/Fs only, relate to single samples of cows' milk from the Chuvash Republic, which contained 1.1 pg TEQ/g fat[76], and of chicken flesh and butter from the Sverdovsk region, which contained 2.5 and 1.4 pg TEQ/g fat,[77] levels broadly similar to those reported from many other countries. Some other data from a number of regions have been reported.[78] A rather high level of 173 pg TEQ/g fat has been reported for PCDD/Fs in freshwater fish from Syktyvkar in the Republic of Komi.[79]

Spain There are no data from Spain in the SCOOP report. Data have been reported for PCDD/Fs based on 35 food samples obtained in 1996 from a town in Catalonia.[80] The daily intake was calculated to be 210 pg I-TEQ/day, of which 8% and 23%, respectively, was from vegetables and cereal products. The levels found in some foods, especially in milk and in vegetables, are higher than many reported elsewhere. In another study,[81] composite samples representing three typical daily food intakes were analyzed for PCDD/Fs and PCBs 77 and 169, but data for PCB 126, which would usually be the dominant contributor to the PCB TEQ, were not obtained. The average lower- and upper-bound intakes were 81 and 142 pg TEQ/day. An estimate of 84 to 128 pg I-TEQ/day has also been made for the Basque region.[82] Data on cows' milk,[83] butter,[84] milk powder,[85] and fish oil dietary supplements[86] have been reported, and PCBs have been determined in soya infant formulas.[87]

Sweden From Sweden a comprehensive report is available on a 5-year survey of PCDD/Fs and PCBs begun in 1988,[88] while data from 1997–1998 are most readily accessed through the SCOOP report.[8] Food consumption data from 7-day records for adults aged 18 to 74 relating to 1997–1998 were used to calculate upper-bound mean intakes, which are 78 pg TEQ/day (1.06 pg/kg body weight/day) for PCDD/Fs and 63 pg TEQ/day (0.85 pg/kg body weight/day) for PCBs, giving a total of 141 pg TEQ/day (1.91 pg/kg body weight/day).[8] A market basket study in which meat, fish, eggs, milk, and milk products were added to composite samples in proportion to average consumption gave very similar upper-bound intake estimates of 79 and 58 pg TEQ/day for PCDD/Fs and PCBs, respectively.

United Kingdom From the United Kingdom, results from analysis of the U.K. TDS samples from 1997 are available,[89,90] updating earlier data from samples collected in 1982 and 1992.[91–93] Only the earlier data are included in the SCOOP report.[8] Food consumption data were obtained in 1986–1987 from a 7-day weighted diary study of 2197 adults (16 to 65). Statistics from a separate 7-day diary study of 3367 schoolchildren also relate to 1986–1987. A 4-day study of children aged 1.5 to 4.5 years was performed in 1992–1993. Upper-bound estimated intakes are summarized in Table 3.3. In addition, results from surveys of retail cows' milk,[94,95] farmed trout,[96] marine fish,[97] free-range eggs,[98] and fish oil dietary supplements[99] are available, and these results are discussed in sections on specific foods.

3.7.2 New Zealand

In a recent survey of New Zealand Foods for PCDDs, PCDFs and PCBs, 19 food-type composites were made from 53 foods purchased in April 1997 at retail outlets in five locations.[100,101] Foods that would normally be cooked prior to consumption were cooked in a manner consistent with the methods commonly used by New Zealanders. In this survey, most compounds in most of the foods were below the limit of detection, due to the low levels of contamination rather than to analytical limitations. Intake calculations were based on food concentrations calculated using one-half of the detection limit for nondetected compounds as well as with lower-bound (ND = 0) values.

The estimated intake of PCDD/Fs for an adult male consuming 10.8 MJ/day is 14.5 pg I-TEQ/day (ND = $\frac{1}{2}$LOD) or 3.72 pg I-TEQ/day (lower bound), equivalent to 0.18 and 0.047 pg/kg body weight per day, respectively. The estimated intake of dioxinlike PCBs is 12.2 pg WHO-TEQ/day (ND = $\frac{1}{2}$LOD) or 7.83 pg WHO-TEQ/day (lower bound), equivalent to 0.33 and 0.15 pg/kg body weight per day respectively. For an adolescent male the estimate intake of PCDD/Fs is 30.6 pg I-TEQ/day (ND = $\frac{1}{2}$LOD) or 9.82 (lower bound), corresponding to 0.44 and 0.14 pg/kg body weight per day, and of PCBs is 22.7 pg WHO-TEQ/day (ND = $\frac{1}{2}$LOD) or 14.3 pg WHO-TEQ/day (lower bound), equivalent to 0.76 and 0.34 pg/kg body weight per day.

TABLE 3.3 Upper Bound (ND = LOD) Estimates of Average and 97.5th Percentile Dietary Intake of PCDD/Fs and PCBs in the United Kingdom

	Dietary Exposure (pg WHO-TEQ/kg body weight per day)								
	1982			1992			1997		
Age Group	PCDD/Fs	PCBs	Total	PCDD/Fs	PCBs	Total	PCDD/Fs	PCBs	Total
1.5–2.5	15/34	7.9/16	23/49	5.0/8.9	2.6/5.0	7.5/14	2.6/5.2	2.6/4.9	5.1/10
2.5–3.5	12/27	6.6/14	19/41	4.2/7.5	2.1/4.0	6.3/11	2.3/4.3	2.2/4.1	4.4/8.4
3.5–4.5 male	11/22	5.9/11	17/33	3.7/6.0	1.9/3.3	5.6/9.2	2.1/3.6	1.9/3.4	4.0/6.9
2.5–4.5 female	11/24	5.8/11	17/34	3.7/6.6	1.9/3.2	5.6/9.6	2.1/3.8	1.9/3.4	4.0/7.2
Schoolchildren	5.6/10	3.0/5.2	8.6/15	2.0/3.2	1.0/1.6	3.0/4.7	1.2/1.9	1.0/1.7	2.2/3.5
Adults	4.6/8.3	2.6/4.6	7.2/13	1.6/2.8	0.9/1.6	2.5/4.3	0.9/1.6	0.9/1.6	1.8/3.1

Source: Data from Ref. 90.

3.7.3 North America

The most recent data for Canada are for PCDD/Fs and nonortho PCBs in fatty food composites from the Canadian Total Diet Programme collected from Toronto in 1992 and Montreal in 1993.[102] Foods for which data are reported, although with few detail, are beef, pork, poultry, freshwater and marine fish, milk and dairy products, and cooking fats and salad oil. Levels of PCDD/Fs in ground beef, butter, wieners, and shellfish are compared with data for the same commodities and locations obtained in 1988 and show no evidence of a decrease. For milk a decrease of around 80% was noted and attributed to reduced migration from paperboard packaging.

From the United States, results from statistically based national surveys of PCDD/Fs and dioxinlike PCBs in beef,[103–105] pork,[106] poultry,[107] and milk[108] from the mid-1990s are available; these results are discussed in sections on specific foods. Following a preliminary study of 18 dairy, meat, and fish samples from a supermarket in upstate New York,[109] a more comprehensive and representative study of retail foods was undertaken by Schecter et al.[110,111] This was based on 110 food items purchased in 1995 from supermarkets in five regions of the United States. A total of 20 samples of fruits, vegetables, legumes, and cereal products were combined into a single composite to simulate a vegan diet. Other samples were pooled into the following categories: beef, chicken, pork, meat products, marine fish and shellfish, freshwater fish, butter, cheese, milk, ice cream, and eggs. Data were used with consumption figures for 1989–1991 from the U.S. Department of Agriculture's Continuing Survey of Food Intakes by Individuals to calculate the estimated daily intakes shown in Table 3.4. These estimates used one-half of the limit of detection to represent nondetects. Detection limits were somewhat higher than in many recent studies, and the lower-bound (ND = 0) contributions from meat, marine fish, butter, and cheese would be roughly half of those given by the ND = $\frac{1}{2}$LOD method and upper bound about 50% greater. Lower-bound contributions from milk, eggs, and vegetables would be only 10 to 20% of those with ND = $\frac{1}{2}$LOD, and upper bound 80 to 90% greater. Results have also been reported from analysis for PCDD/PCDF of 43 foodstuff samples obtained in 1994 from local supermarkets in southern Mississippi.[112] Additional data for fish and dairy foods have also been reported.[113]

3.7.4 Japan

Intakes derived from analysis of TDS samples of 14 food groups from 16 locations in Japan, collected in 1999 and 2000, are available,[114] but the levels in the foods are not given. The lower bound and ND = $\frac{1}{2}$LOD estimates of mean daily intake for an adult weighing 50 kg are 2.25 pg TEQ/kg body weight per day and 3.22 pg TEQ/kg body weight per day. Fish and shellfish made the greatest contribution to intake (53.9% with ND = $\frac{1}{2}$LOD), followed by meat and eggs 11.7% at ND = $\frac{1}{2}$LOD). The dioxinlike PCBs accounted for about

TABLE 3.4 Estimated Daily Intakes of PCDD/Fs and Dioxinlike PCBs in the United States (ND = $\frac{1}{2}$LOD)

Age	Gender	Daily Intake (pg TEQ)						Mean Body Weight (kg)	Intake (pg TEQ/kg body weight)
		Meat	Fish	Dairy	Eggs	Vegetables	Total		
1–11	Male	32.8	4.8	74.8	5.9	25.6	144	23	6.3
	Female	32.8	5.4	71.0	5.2	27.5	141	23	6.1
12–19	Male	61.3	4.8	81.9	6.6	35.9	191	55	3.5
	Female	41.5	4.2	57.7	4.5	25.0	133	50	2.7
20–79	Male	61.7	14.9	49.1	9.5	36.4	171	70	2.4
	Female	38.8	10.8	36.9	5.9	28.5	121	55	2.2
80+	Male	38.9	3.0	38.7	8.6	36.3	126	70	1.8
	Female	25.5	11.4	43.2	4.5	26.6	111	55	2.0

Source: Data from Ref. 111.

50% of the total TEQs. A number of other recent studies of Japanese foods have been reported only in the Japanese language.[115–117] Additional data on fish, shellfish, and crabs from the Tokyo Bay area are also available.[118]

According to Liem et al.,[3] the Ministry of Health and Welfare of Japan obtained results from the analysis of TDS samples collected in 1996 from three districts of Japan and arrived at estimated dietary intakes of PCDD/Fs in the range 22.1 to 37.4 pg I-TEQ/day with a mean of 31.4 pg I-TEQ/day. Of this, 67.5% was from fish consumption and 5.7% from green vegetables. In the same study, the intake of dioxinlike PCBs was estimated as 48.3 pg TEQ/day, but the PCB congeners included and the TEF scheme applied are not specified. Fish accounted for 88.9% of this PCB-TEQ intake.

In another study, daily intakes of PCDDs, PCDFs, and co-PCBs in 1977 and 1998 were estimated from data obtained from pooled total diet samples (13 food group composites) from one district of Japan.[115] The lower bound and ND = $\frac{1}{2}$LOD estimates of intake of PCDD/Fs in 1977 are 3.75/4.68 pg TEQ/kg body weight per day (187.5/234 pg TEQ/day for a 50-kg person), and in 1998 are 0.92/1.79 pg TEQ/kg body weight per day (46/89.5 pg TEQ/day). For the same years the intake of dioxinlike PCBs was estimated to be 4.43/4.72 pg TEQ/kg body weight per day and 1.80/2.06 pg TEQ/kg body weight per day, respectively. Again, it is not clear which TEF scheme was applied.

3.7.5 Korea

Until recently no published data were available for Korean foods, but data on a variety of foodstuffs were presented at the Dioxin 2001 conference that was held in that country.[119–122] Kang et al.[119] analyzed a variety of fish and meat types and found the mean PCDD/F concentrations on a wet weight basis to be 0.1 to 0.89 pg WHO-TEQ/g for different fish species; 0.16 pg WHO-TEQ/g for beef; 0.03 pg WHO-TEQ/g for pork and 0.04 pg WHO-TEQ/g for chicken. Dioxinlike PCBs were also determined for the meat samples and found to make a slightly higher contribution than PCDD/Fs to the total TEQ. Daily intake from fish and meat was calculated to be 42 pg WHO-TEQ/day using 1994 Korean food consumption data. The contribution from fish was about 10-fold higher than that from meat, despite the omission of the contribution of dioxin-like PCBs to the latter. Ok et al. arrived at a much lower estimate of daily intake.[121] Some data on fast foods are also available.[123,124]

3.7.6 Other Regions

Santillo et al. have reported data for PCDD/Fs and PCBs in single samples of butter from 24 different countries,[125] and Weiss et al. for 67 samples of butter from a 39 countries.[126] Although these samples cannot be nationally representative, these data do provide a broad global comparison and include samples from several regions from which no other data are available. Some of these are discussed in Section 3.9.1.

Santillo reported a total of 0.84 pg TEQ/g fat in butter from Australia with 0.56 pg TEQ/g fat contributed by PCDD/Fs. In another study nine samples of butter from producers in various states in Australia were analyzed for PCDD/Fs,[127] and the highest level found was 0.46 pg TEQ/g fat, with a mean 0.19 pg TEQ/g fat. Some data are also available on PCDD/Fs in butter available in Egypt.[128] In 33 samples collected between 1994 and 1996, the mean level was 7.7 pg I-TEQ/g fat with a range of 0.41 to 28.9 pg TEQ/g fat.

Very little information is available on levels in foodstuffs in China. Evidence of high environmental and human tissue levels of PCDD/Fs, resulting from use of sodium pentachlorophenate to control snailborne schistosomiasis, suggests that relatively high levels might also occur in foods.[129–132] However, the single sample of butter reported by Santillo[125] contained a total of 1.79 pg TEQ/g fat, which is similar to levels in Europe and many other regions.

Some data on retail cows' milk from Taiwan have recently been reported[133] with the mean of nine samples being 0.94 pg WHO-TEQ/g fat. PCBs including nonortho congeners have been determined in a total of 146 samples of five species of fish purchased from markets in Taiwan.[134] Results from determination of PCDD/Fs and dioxinlike PCBs in a few samples of fish and of chicken, lamb, and goat fat from India have been reported.[135] The totals found were between 1.9 and 18 pg TEQ/g fat in fish and between 1.4 and 5.3 pg TEQ/g fat in meat.

3.8 VEGETABLES, FRUITS, PULSES, AND GRAIN

Growing plants may be exposed to PCDD/Fs and PCBs via soil, groundwater, and the air. With the exception of the Cucurbitaceae family (which includes zucchini or courgette), in which some uptake from soil has been demonstrated,[136] absorption of dioxinlike compounds through plant roots and subsequent translocation does not occur to any significant extent. The outer layers of roots crops may become contaminated by direct contact with soil particles, but this will normally be removed by peeling or washing.[137,138] Contamination of the aboveground part of plants is considered to result largely from retention of airborne PCDD/Fs and PCBs, which may include absorption from the vapor phase by the waxy cuticle and retention of particulate-bound contaminants.[139] The highest levels are therefore expected when a convoluted surface of high surface area is combined with a pronounced waxy cuticle.

Although contamination of plant material consumed by food-producing animals is a major part of the pathway from primary source to human dietary exposure, levels in vegetables, fruits, and grains consumed by humans are very low, immeasurably so for many laboratories. Many otherwise broadly based food surveys have omitted analysis of fruits and vegetables, either because of the assumption that their contribution to intake would be insignificant compared with that of fatty foods, or because of the very real analytical difficulties presented by the low concentrations of contaminants present. A number of

intake assessments do, however, include quite large contributions for fruits and vegetables.

Data that have been reported need to be viewed with particular care because of issues of representativeness, detection limit, and accuracy. In the latter context, in contrast to animal tissues in which only the 2,3,7,8-substituted PCDD/Fs are found, many other congeners will be present in deposits on vegetation, at higher concentrations than those that contribute to the TEQ. Obtaining the required congener specificity in the analysis becomes much more difficult in this case.

In a Dutch study, two separate nationally representative composites of vegetables were analyzed.[70] The vegetables were not washed or cleaned before being added to the composite samples. All of the laterally substituted PCDD/Fs other than 2,3,7,8-TCDF were measurable, as were PCBs 77 and 126. The average total TEQ concentration was 58 pg WHO-TEQ/kg wet weight to which dioxinlike PCBs made a negligible contribution. In a very welcome discussion of the uncertainties associated with the data, the authors note that a significant contribution from laboratory contamination could not be excluded and that overall uncertainty could be as high as 50%.

In their earlier studies, Liem and Theelen[32] measured PCDD/Fs in curly kale obtained from three locations in the Netherlands, where elevated levels had been found in cows' milk and local point sources were identifiable, and from two background locations. Almost all of the TEQ contributors were measurable, giving an average total of 130 pg I-TEQ/kg. There was little difference between samples from background and contaminated locations. Much lower concentrations of PCDD/Fs were found by Malisch in a total of 57 samples of fruits and vegetables obtained in southwestern Germany between 1993 and 1996.[57] Here the average total TEQ was 13 pg I-TEQ/kg wet weight.

Higher concentrations have been reported for composites of vegetables (lettuce, chard, spinach, chickpea), pulses (lentil, bean), and fruits (orange, banana, apple) purchased in Tarragona, Spain in 1996. Most relevant PCDD/F congeners were measurable in the vegetable composite, giving a total of 140 pg I-TEQ/kg wet weight.[80] Other entries in the SCOOP report, representing data from Finland, the United Kingdom, and France, fall within the range 10 to 90 pg TEQ/kg whole weight for PCDD/Fs (ND = LOD) and are typically negligible for PCBs.[8]

Nuts form a separate category of plant product foods, for which there are few data. In the Dutch survey, PCDD/Fs were not detected, nor were the important dioxinlike PCBs, although some other PCBs were found.[70] Nuts were analyzed as a food group in the U.K. 1997 total diet study and were found to contain 0.57 ng WHO-TEQ/kg fat.[90]

Schecter et al.[111] reported analysis for PCDD/Fs and PCBs of a single composite containing fruit, vegetables, pulses, and grain products from Binghampton, New York, simulating a vegan diet. The total TEQ calculated from one-half of the detection limits was 86 pg TEQ/kg wet weight, but the lower-bound value was only 8 pg TEQ/kg.

Although Tsutsumi et al.[114] lists vegetables and rice as contributing about 2.5 pg TEQ/person per day to the intake associated with an average Japanese diet, this seems also to be derived from summations of detection limits. PCDD/Fs and PCBs have been detected in the Japanese green leafy vegetable Komatsuna,[140] where 58 pg TEQ/kg was found in unwashed produce, and 26 pg TEQ/kg after washing with tap water.

The limited number of results available and the uncertainty associated with them makes it difficult to reach any reliable conclusion about the intake derived from fruits and vegetables. It is clear that these commodities contribute only a small proportion of dietary exposure for any diet that includes a substantial amount of animal products or fish, but they may contribute significantly to exposure for some diets and must comprise the main source for the vegan diet. It is therefore notable that the concentrations of PCDD/Fs and PCBs measured in the blood of persons in the United States following a vegan diet were extremely low.[141]

3.9 ANIMAL PRODUCTS

The main route for exposure of most food-producing animals to PCDD/Fs and PCBs is through their feed. Considerable progress has been made in understanding and modeling agricultural food chain accumulation, in which, for background contamination, the dominant pathway is that of atmospheric distribution and deposition onto vegetation.[142–146] There is abundant evidence that proximity to point sources of atmospheric PCDD/Fs, such as incinerators and metal reclamation sites, can lead to markedly raised levels in milk and meat.

There have been a number of recent examples of high levels of contamination in manufactured feeds being carried over into foods. The main incidents are discussed in a later section. In Europe much analytical and regulatory attention is now focused on animal feeds, but little information is available on the relative importance, under background conditions, of manufactured feeds versus atmospheric distribution.

There is some possibility that the use of sewage sludge for soil amendment could result in increased exposure of livestock,[147–149] but recent experimental evidence indicates that carryover of PCDD/Fs entering the feed as a result of sewage sludge fertilization is not significantly different from that for feed containing background levels of PCDD/Fs of atmospheric origin.[150] The use of pentachlorophenol-treated timber in animal housings has occasionally been found to result in increased levels of PCDD/Fs in cattle.[151,152]

3.9.1 Milk and Milk Products

Excretion of PCDD/Fs and PCBs in milk is the main elimination pathway for these compounds in lactating cows. Since the first reports of the detection of

PCDD/Fs in cows' milk, by Rappe et al.[44] and Beck et al.[153] in 1987, many surveys have been reported and probably more data have been generated on milk than on any other foodstuff. Since milk from many animals is usually blended together on the farm and then mixed with milk from other farms at the dairy, difficulties with representativeness of sampling are less than with other foods, and by sampling individual farms or individual dairies, data representing differently sized areas can be obtained.

PCDD/Fs and PCBs are contained entirely in the milk fat. When expressed on a fat basis, milk products will contain the same concentrations as the milk from which they were produced. Differences between milk and butter or cheese, reported in several surveys, may arise partly from issues of accuracy and representativeness but may also occur because milk products are exported and imported to a greater extent and over greater distances than milk itself. Consequently, locally representative data for milk is not necessarily representative of milk products.

A number of studies from the late 1980s and early 1990s demonstrated a marked localized influence on levels in milk produced in the vicinity of some incinerators and other point sources. In surveillance carried out in 1989–1990 around incinerators in Holland, levels up to 13.5 pg TEQ/g fat were found. The highest dioxin concentrations were usually found within about 2 km of the source. The Dutch authorities adopted an action level of 6 pg TEQ/g fat, and milk from the area found to exceed this was withdrawn from the public supply.[154]

Riss et al.[155] investigated the contamination caused by a metal reclamation plant at Brixlegg in Austria and found PCDD/F levels in cows' milk in the range 13.5 to 37.0 pg TEQ/g fat (using the German scheme of toxic equivalent values, which gave a somewhat lower total than the I-TEF scheme), while a contemporaneous control sample gave 3.6 g TEQ/g fat.

As part of a study on levels of PCDD/Fs in milk in the United Kingdom, samples of cows' milk were collected from farms in Derbyshire in 1990 and 1991. The concentrations of PCDD/Fs in milk samples from two farms in the Bolsover area of Derbyshire were found to be significantly elevated, with total concentrations of 40 and 42 pg I-TEQ/g fat, compared with the emerging normal range for PCDD/Fs in milk in the United Kingdom at that time, which was 1.1 to 7.1 pg I-TEQ/g fat. Shortly afterward, milk from a suckler herd produced a result of 56 pg I-TEQ/g fat.[156] These farms were in the vicinity of the Coalite chemicals plant, which had manufactured organochlorine chemicals since the 1960s and where a chemical waste incinerator was operated. The incinerator was closed in late 1991, and subsequent monitoring of milk showed that levels declined.[157]

Subsequently, analysis of milk has often been undertaken more for the purpose of environmental monitoring than for investigating dietary intake, and the emphasis in many studies has been on potentially contaminated areas; of the approximately 80 European surveys of cows' milk reflected in the SCOOP report,[8] less than half are classified as intended to be representative.

In a national survey of long-life half-skimmed milk from France which was conducted in 1998, the mean concentration of PCDD/Fs was 0.65 pg TEQ/g fat.[56] In cheese and butter sampled in 1996, mean levels were 1.11 and 1.01 pg TEQ/g fat, respectively.[54] Previously, in 1994, data had been obtained from sites specially selected as being in the vicinity of municipal waste incinerators when the average was 1.74 pg TEQ/g fat.[53]

In the late 1980s, contamination of milk as a result of migration from paperboard packaging was found to occur,[158–161] the presence of 1,2,7,8-TCDF and of raised levels of 2,3,7,8-TCDF providing a highly characteristic signature. Following revision of the pulp bleaching process to reduce PCDD/F formation dramatically, this contribution to milk contamination is generally regarded as negligible. Data from Canada[102] shows a four- to eightfold decrease in the total TEQ content of milk between 1988 and 1992–1993, ascribed to reduction of this source of contamination. It is, however, noteworthy that in the 1998 New Zealand survey,[100] only 1,2,7,8-TCDF and 2,3,7,8-TCDF could be detected in milk, other PCDD/Fs being below the detection limits. It appears that if background levels are low enough, the influence of packaging may still be not only significant but dominant. There are also indications that migration from paperboard packages could be an important source of milk contamination in Russia, as discussed earlier.

The SCOOP report summarizes the range of recently determined national averages for milk and dairy products in the EU as being 0.3 to 2.1 pg I-TEQ/g fat for PCDD/Fs and 0.2 to 1.8 pg WHO-TEQ/g fat for dioxinlike PCBs.[8] If data available from the rest of the world are included, the global range extends farther downward, because of the exceptionally low levels found in New Zealand. It may extend farther upward if rather high levels in some packaged milk from Russia, discussed above, are representative. Apart from these extremes, the range of means is not much greater than that which could result from lack of representativeness in sampling combined with differences in methodology, analytical performance, and data handling. In terms of contribution to dietary intake, the estimated contribution from milk varies from about 15 to 40%.

In the United Kingdom, a survey of retail milk was conducted in 1995.[94] A total of 105 samples of full fat milk (approximately 4% fat) were purchased at various shops in each of 12 geographical regions. All the samples were packaged in glass bottles. The samples from each region were pooled prior to analysis. Upper-bound PCDD/F concentrations ranged from 0.67 to 1.4 ng TEQ/kg fat with a mean of 1.01 pg TEQ/g fat. Dioxinlike PCB concentrations were in the range 0.75 to 2.3 pg TEQ/g fat with a mean of 1.8 pg TEQ/g fat. The mean combined total TEQ was thus 2.81 pg TEQ/g fat with a range for individual pools of 1.4 to 3.5 pg TEQ/g fat. The ratio of PCB-TEQ to PCDD/F-TEQ in individual pools varied between 1.5 and 2.4. Subsequently, the milk composite from the 1997 U.K. TDS gave levels of PCDD/Fs and PCBs of 0.83 and 0.74 pg TEQ/g fat, respectively.[89]

In the Baden–Württemburg region of Germany, the mean PCDD/F level in 448 full-cream milk samples taken between 1993 and 1996 was 0.72 pg

I-TEQ/g fat with a range of 0.24 to 3.13 pg I-TEQ/g fat. Of these samples, 90 were from retail outlets and the remainder direct from farms or collection tankers. In butter (196 samples) the mean concentration was 0.63 pg I-TEQ/g fat, with a range of 0.34 to 2.00. From 99 samples of cheese the mean was 0.66 pg I-TEQ/g fat (range 0.06 to 2.48).[57]

In another German study of PCDD/Fs in butter, 204 samples were taken throughout Germany in March 1995. The range of PCDD/F levels was 0.28 to 1.19 pg I-TEQ/g fat, with a mean of 0.68 pg I-TEQ/g fat.[59] In butter from the Republic of Bashkortostan, the PCDD/F content has been reported to be 0.43 pg TEQ/g fat.[73] In the work on butter from different countries reported by Santillo,[125] the lowest measured total TEQ concentrations were from the Philippines and New Zealand (0.18 and 0.19 pg TEQ/g fat), and the highest from Tunisia and Spain (3.8 and 5.7 pg TEQ/g fat). In a sample from the Netherlands, the level found was 2.7 pg TEQ/g fat, while in composite samples from the recent Dutch study the upper-bound total was 1.7 pg TEQ/g fat.[70] A sample from the United Kingdom gave a total of 1.25 pg TEQ/g fat, whereas in the U.K. TDS milk products category from 1997, the total was 2.0 pg TEQ/g fat.[90]

From the New Zealand[101] study, the lower- and upper-bound total concentrations of PCDD/Fs in milk (recalculated from the reported data) were 0.019 and 0.30 pg I-TEQ/g fat. Lower- and upper-bound totals for dioxinlike PCBs were 0.027 and 0.273 pg TEQ/g fat (using 1994 WHO TEFs).

In the United States, composite samples of milk were taken at 3-month intervals during 1996 and 1997 through sampling stations in 41 U.S. states, Panama, and Puerto Rico to provide 48 samples. The sampling and pooling scheme was designed to investigate both geographical and temporal trends as well as to provide an estimate of the average concentrations of PCDD/Fs and dioxinlike PCBs in the U.S. milk supply. National averages were 0.82 pg TEQ/g fat for PCDD/Fs and 0.50 pg TEQ/g fat for PCBs, calculated using $ND = \frac{1}{2}LOD$. There was little difference between lower- and upper-bound values. There was some evidence of geographical differences but not of temporal trends.[99]

3.9.2 Meat

Meat is a more heterogeneous group than milk and milk products, and there are considerable differences in the species consumed by different ethnic groups and individuals. The meat products classification included in a number of studies is poorly defined in terms of both the type and proportion of meat included, and the representativeness of the resulting data is sometimes questionable. As illustrated below, most of the available data indicate concentrations of PCDD/Fs and dioxinlike PCBs to be similar with reported averages for each falling in a range of roughly 0.25 to 2.5 pg TEQ/g fat. Data from the United States suggest similar concentrations in beef and pork. This appears not to be the case in most of Europe, where as various examples below illus-

trate, pork has usually been found to have considerably lower levels of both PCDD/Fs and PCBs than those of beef. This difference between the United States and Europe presumably reflects differences in animal husbandry and feeding practices. Poultry meat typically has been found to contain slightly higher levels. There is limited information on meat from other species. From samples of goat and horse collected in the Netherlands in 1990, levels of around 12 and 40 pg TEQ/g fat were reported for the total of PCDD/Fs and PCBs,[32] and about 34 pg TEQ/g fat in game. More recent analyses of game in Germany indicate levels of PCDD/Fs comparable to other meats.[8]

In a U.S. survey of beef, samples of back fat were obtained from 65 animals (2 bulls, 33 steers, 18 heifers, 6 dairy cows, and 6 beef cows), proportional to total U.S. beef production in 1993. These were analysed separately for PCDD/Fs[104] and coplanar PCBs.[105] The mean concentration of PCDD/Fs was 0.89 pg I-TEQ/g fat when calculated with ND = $\frac{1}{2}$LOD, and the lower-bound average was 0.35 pg I-TEQ/g fat (from these figures the upper-bound average can be calculated to be 1.43 pg I-TEQ/g fat). For PCBs the mean was 0.51 pg WHO-TEF/g fat with no difference between lower and upper bounds.

In a similar study of pork fat,[106] a total of 78 sample were analyzed. The mean concentration of PCDD/Fs was 1.3 pg I-TEQ/g fat when calculated with ND = $\frac{1}{2}$LOD, and the lower-bound average was 0.46 pg I-TEQ/g fat (upper bound 2.14 pg I-TEQ/g fat). The range of individual results (ND = $\frac{1}{2}$LOD) was 0.61 to 23 pg I-TEQ/g fat. For PCBs the mean was 0.06 pg I-TEQ/g fat (ND = $\frac{1}{2}$LOD) with a range of 0.02 to 1.7 and upper and lower bounds of 0.04 and 0.08 pg I-TEQ/g fat. Five of the animals were boars 2 or more years old. The highest concentrations of both PCDD/Fs and PCBs came from this group, in which the mean concentrations (ND = $\frac{1}{2}$LOD) were 6.5 pg I-TEQ/g fat for PCDD/Fs and 0.54 pg I-TEQ/g fat for PCBs.

In Schecter's data from foods collected in 1995,[110] the combined total for PCDD/Fs and PCBs, expressed as pg WHO-TEQ/g fat for ND = $\frac{1}{2}$LOD, and with lower- and upper-bound concentrations in parentheses, were 3.03 (1.24 to 4.82) in beef, 4.26 (1.32 to 7.20) in pork, and 6.30 (2.66 to 9.94) in chicken. Detection limits were relatively high and the ND = $\frac{1}{2}$LOD calculation may represent an overestimate of true concentrations.

In a study of foods collected in southern Mississippi,[112] in which there were very few nondetects and little difference between upper- and lower-bound concentrations, levels of PCDD/Fs in three samples of ground beef were 0.63, 1.1, and 0.53 pg I-TEQ/g fat, in chicken meat 0.78, 0.71, and 0.61 pg I-TEQ/g fat, and in chicken liver 0.75, 0.88, and 1.3 pg I-TEQ/g fat.

In a recent Finnish study[52] the lower-bound concentrations of PCDD/Fs in beef and pork were 0.29 and 0.051 pg I-TEQ/g fat and of PCBs were 0.31 and 0.024 pg TEQ/g fat. In data from the Netherlands, given in Table 3.5, concentrations are higher than those reported in the Finnish study, but again are lower in pork than in beef.

In samples from Catalonia, Spain in 1998, concentrations of PCDD/Fs were reported to be as pg I-TEQ/g fat, 1.76 in beef and beef products, 0.90 in pork

TABLE 3.5 Lower-Bound Concentrations of PCDD/Fs and Dioxinlike PCBs Found in Nationally Representative Composite Samples of Meat in the Netherlands

	Concentration (pg WHO-TEQ/g fat)		
Food Group	PCDD/Fs	PCBs	Total
Beef	0.82	1.24	2.06
Pig	0.24	0.23	0.47
Poultry	1.06	1.72	2.78

Source: Data from Ref. 70.

and pork products, 1.15 in chicken and chicken products, and 1.76 in lamb.[80] Similarly, in samples collected in 1997 and 1998 in Bavaria (Germany), the lowest PCDD/F levels were found in pork with a mean of 0.27 pg I-TEQ/g fat while poultry gave about 0.5 pg I-TEQ/g fat and beef 0.7 to 0.8 pg I-TEQ/g fat.[58] In another study in Germany, mean levels of PCDD/Fs in pg I-TEQ/g fat in beef were 0.46, in poultry 0.22, in various sausages 0.21 to 0.13, and in pork 0.07. The highest dioxin concentrations were observed in meat from the western parts of Germany, and the lowest in the south.[62]

Results from 1997 U.K. TDS samples are given in Table 3.6. In the U.K. TDS the meat group is not segregated by species, although poultry meat is a separate group. As this shows, offal tends to contain much higher concentrations of PCDD/Fs, often 10 times more than from carcase meat from the same species, presumably because PCDD/Fs and to a lesser extent, PCBs, are sequestered in the liver.

3.9.3 Eggs

Although the 2,3,7,8-substituted congeners predominate in eggs, other congeners can be observed to a greater extent than in other animal-derived foods. It has been shown that PCDD/F concentrations in eggs depend on the type of

TABLE 3.6 Upper-Bound Concentrations of PCDD/Fs and Dioxinlike PCBs Found in Meat Samples from the 1997 U.K. Total Diet Study[a]

	Concentration (pg WHO-TEQ/g fat)		
Food Group	PCDD/Fs	PCBs	Total
Carcase meat	0.80	1.07	1.87
Offal (organ meat)	6.29	2.47	8.76
Meat products	0.77	0.61	1.38
Poultry	1.01	1.31	2.32

Source: Data from Ref. 90.

[a]Lower-bound concentrations were almost identical.

housing; elevated wire cages gave the lowest concentrations, while access to soil gave higher concentrations,[162] as borne out in data reported by Malisch,[57] who found a mean concentration of PCDD/Fs in eggs collected in Germany between 1993 and 1996 of 2.1 pg I-TEQ/g fat (range 0.17 to 22.8 pg I-TEQ/g fat). The total of 218 samples included eggs from chickens housed in elevated wire cages, from chickens kept on the ground, and from free-range birds in which the means levels were, respectively, 1.28, 1.51, and 4.39 pg I-TEQ/g fat. This apparently results from intake of soil organisms such as insects and annelids in which PCDD/Fs accumulate.[163]

In a small survey of eggs from noncaged poultry in the United Kingdom in which samples were obtained between 1994 and 1996, 29 hen eggs gave total levels of PCDD/Fs and PCBs in the range 1.1 to 22 pg WHO-TEQ/g fat, and 16 duck eggs totals in the range 1.9 to 49 pg WHO-TEQ/g/fat.[98] For comparison the egg composite from the 1997 U.K. TDS study contained 1.4 pg WHO-TEQ/g fat, with similar contributions from PCDD/Fs and PCBs.[90] In the Netherlands the total found for PCDD/Fs and PCB was 2.4 pg WHO-TEQ/g fat[70] with about 63% from PCDD/Fs. The levels found in Finland were 0.52 pg WHO-TEQ/g fat for PCDD/Fs and 0.12 pg WHO-TEQ/g fat for PCBs. In Sweden, upper-bound levels of 1.03 and 1.45 pg WHO-TEQ/g fat have been reported for PCDD/Fs and PCBs, respectively.

Rather few data are available from the United States. Investigations of PCDD/F contamination have been conducted in an area of pentachlorophenol contamination in a rural area of northern California,[164] and as a comparison, five samples from chickens foraging in uncontaminated areas and of commercial eggs from local stores were analyzed. These gave, respectively, 0.15 and 0.03 pg I-TEQ/g whole sample. Since the fat content of egg is typically 10%, these are equivalent to levels of 1.5 and 0.3 pg I-TEQ/g fat. Schecter's data for retail eggs, converted to a fat basis from the reported fat content of 14.7%, shows 2.3 pg WHO-TEQ/g fat with ND $= \frac{1}{2}$LOD but a rather wide divergence of lower- and upper-bound levels of 0.49 to 4.18 pg I-TEQ/g fat.

3.10 FISH

Although PCDD/Fs and PCBs are usually present in aquatic systems only at very low concentrations, bioaccumulation can result in significant concentrations in fish. As with animals, the 2,3,7,8-substituted PCDD/F congeners dominate the pattern found in fish, although this is not necessarily true of crustaceans and shellfish.[112,165] Fish comprise by far the most inhomogeneous food group, due to the large number of different species used as food, with widely varying fat contents, different trophic positions, and the great variety of fishing grounds. In general, concentrations of chemicals such as PCDD/Fs and PCBs in fish depend on their fat contents, the extent to which the fish migrate, the number of times they spawn, and their ages, size, and feeding habits.[166] For example, plaice are bottom-feeding fish and therefore may be more ex-

posed to PCDD/Fs and PCBs bound to sediment. Herring has a relatively high fat content and is nonmigratory, which renders it more subject to localized contamination sources.[167] Consequently, the concentrations of PCDD/Fs and of PCBs are very varied. Because of the large seasonal variation in fat content of most fish, data are often expressed on a whole product basis, but units based on fat are also used, particularly when different species with different average fat contents are to be compared.

Certain fish species originating from the Baltic region are recognized as containing a high concentration of PCDD/Fs and PCBs. A significant proportion of fatty fish from this region such as Baltic herring and Baltic salmon are unlikely to comply with the EU limit for PCDD/Fs of 4 pg WHO-TEQ/g fresh weight introduced in July 2002, and this fish would therefore be excluded from the Swedish and Finnish diet. There are indications that such exclusion would have a negative health impact in Sweden and Finland, and consequently, there is a local exemption to compliance with the legislation. Sweden and Finland have in place a system that informs consumers about the dietary recommendations about the consumption of fish from the Baltic region in order to avoid potential health risks.[43]

A great variety of data from Europe are contained in the SCOOP report.[8] In a study in Germany, 161 samples of fish from 1997–1998 showed a mean level of PCDD/Fs of about 0.5 pg I-TEQ/g wet weight,[64] and a similar average was also reported by Malisch.[57] In the 1997 U.K. TDS composite sample, PCDD/Fs were found at 2.4 pg WHO-TEQ/g fat and PCBs at 4.53 pg WHO-TEQ/g fat. The fat content was 8.15%, and on a whole product basis these levels become 0.20 and 0.37 pg WHO-TEQ/g.[90]

In the United Kingdom a further study of marine fish was carried out using samples collected in 1995 and 1996.[97] For this survey 108 samples of marine fish species (30 cod, 26 haddock, 13 plaice, 14 whiting, 2 red fish, 10 herring, and 13 mackerel) and 10 salmon were sampled to reflect geographical and seasonal variations in fish landed and imported into the United Kingdom and were analyzed individually. Concentrations of dioxins and PCBs found varied with the species of fish, the fat content, and the month of sampling. Concentrations of dioxins and PCBs on a fat basis were significantly higher in herring, red fish, and plaice than in the other species and were significantly lower in haddock and mackerel. Concentrations were also significantly lower in fish samples collected in February 1996 than in those collected in November and May 1996, presumably reflecting seasonal variation. The concentrations found are summarized in Table 3.7. The mean levels found in composites in the Netherlands study of 1999 are given in Table 3.8.

In the United States, Schecter reported lower- and upper-bound averages for the total of PCDD/Fs and dioxinlike PCBs of 0.16 and 0.62 pg WHO-TEQ/g on a whole product basis for marine fish (12 and 44 pg WHO-TEQ/g fat), and of 1.6 and 1.9 pg WHO-TEQ/g whole product for freshwater fish (33 and 39 pg WHO-TEW/g fat).[110] Farmed fish are often fed feed that contains fish meal. This has led to concerns that there is a possibility of biomagnification

TABLE 3.7 Lower-Bound Concentrations of PCDD/Fs and Dioxinlike PCBs Found in Nationally Representative Composite Samples of Fish in the Netherlands

	Concentration (pg WHO-TEQ/kg)			
Food Group	PCDD/Fs	PCBs	Total	Fat (%)
Fish products	0.10	0.16	0.27	10
Fatty fish	0.92	2.24	3.16	14.3
Lean fish	0.18	0.41	0.59	0.5
Crustaceans	0.44	0.88	1.35	1.75

Source: Data from Ref. 70.

of PCDD/Fs and PCBs which are prevalent in fish, although there is no clear evidence that concentrations of these are higher in farmed fish compared with wild fish of the same variety.

A survey of 40 samples of edible trout flesh, each consisting of several fillets of muscle taken from different fish of similar age and size at each location, obtained during the period October–December 1995 from trout farms across England and Wales has been reported.[96] PCDD/F concentrations were in the range 2.1 to 13 (mean 5.1) pg TEQ/g fat, or 0.06 to 0.67 (mean 0.24) pg

TABLE 3.8 Upper-Bound Concentrations of PCDD/Fs and Dioxinlike PCBs Found in Fish in the United Kingdom[a]

	Concentration (pg WHO-TEQ/g fat)					
	PCDD/Fs		PCBs		Total	
Fish	Mean	Range	Mean	Range	Mean	Range
U.K.-landed						
Cod	9.0	2.1–24	17	3.3–76	26	7.2–98
Haddock	6.9	1.1–14	7.4	2.2–22	14	5.5–24
Plaice	25	3.6–43	42	9.5–55	67	13–90
Whiting	8.3	2.0–20	23	2.4–91	32	4.4–110
Herring	24	13–38	59	12–110	83	26–140
Mackerel	3.8	1.0–9.0	14	2.5–31	17	3.4–40
Salmon	6.5	4.6–11	19	12–30	25	16–38
Trout	5.7	2.4–14	18	8.7–50	24	12–61
Imported						
Cod	6.1	1.4–18	9.7	2.0–32	16	6.3–50
Haddock	4.6	1.9–8.5	5.4	1.9–12	10	4.2–19
Plaice	20	16–27	33	21–57	54	37–84
Salmon	3.4	3.4	12	12	16	16
Red fish	14	12–16	43	42–44	57	57–57

Source: Data from Ref. 90.

[a] Lower-bound concentrations were almost identical.

TABLE 3.9 Levels of PCDD/Fs and PCBs in Eel Samples from the Netherlands

Samples (Eels)	*n*	$\sum$ 7 PCBs (μg/kg wet weight)	PCBs (pg TEQ/g wet weight)	PCDD/Fs (pg TEQ/g wet weight)	Total (TEQ)
Freshwater	39	13–1740	0.8–44	0.3–7.9	1.0–52
Farmed	10	21–58	3.9–7.7	0.9–3.1	4.6–11
Smoked	5	21–52	1.8–6.4	0.5–2.2	2.3–8.6
Imported	15	<1.1–65	0.3–6.8	0.2–3.0	0.5–9.8

Source: Data from Ref. 168.

TEQ/g fresh weight. PCB concentrations were in the range 8.9 to 51 (mean 19) g TEQ/g fat, or 0.22 to 2.4 (mean 0.87) g TEQ/g fresh weight. The combined dioxins and PCBs concentrations were in the range 12 to 60 (mean 24) pg TEQ/g fat, or 0.29 to 3.1 (mean 1.1) pg TEQ/g fresh weight. Measured fat contents of the samples were in the range 1.8 to 8.6%.

In the SCOOP report,[8] several other results for farmed trout are reported, including levels of PCDD/Fs of 0.44 pg TEQ/g fish in Bavaria and 0.07 pg TEQ/g fish in Lower Saxony. A survey of eels carried out in the Netherlands showed much greater variation of PCDD/F and PCB content in freshwater eels than in farmed, smoked, or imported varieties (Table 3.9). Six out of 39 freshwater eels exceeded the EU limit.[168]

3.11 MISCELLANEOUS OILS AND FATS

Additional intake of fats arises from their use in cooking or for direct consumption. The PCDD/F and PCB content of vegetable oils, as expected, has been found to be small but not negligible. For example, in the Netherlands study of 1999, the mean total of PCDD/Fs and PCBs was 0.18 pg WHO-TEQ/g fat.[70] In a composite of margarine, low-fat margarine, cooking fat, frying fat, and French fries, the mean concentration of PCDD/Fs was 0.12 pg WHO-TEQ/g fat (lower bound) and that of PCBs was 0.18 pg WHO-TEQ/g fat. However, frying potatoes in lard has been shown to result in considerably high levels of PCDD/Fs in the edible product than in raw potato, as a result of fat absorption.[169]

3.12 FOOD PROCESSING AND COOKING

PCDD/Fs and PCBs are stable chemicals that require temperatures far in excess of those encountered in cooking for their destruction. Decreases in concentration in meat during cooking have been observed. Thus in one study, pan-frying of hamburger patties was found to reduce the amount of

PCDD/Fs actually consumed by 40 to 50% if the pan fats and juices were discarded.[170] In another, beef with PCDD/F incurred in an animal feeding trial was fried, grilled, barbecued, roasted using conventional and microwave ovens, stewed in an open pan, and pressure cooked. Concentration changes, on a whole product basis, were observed between raw and cooked product. However, in all cases these could be explained simply through changes arising from loss of water and elimination of PCDD/Fs with released fat. The total amounts of PCDD/Fs present in the system (including released fat) remained unchanged.[171] Dietary intake of PCDD/Fs could therefore be reduced by the removal of visible fat from meat before cooking and by discarding any fat released from foods during the cooking process.

Reduction in the levels remaining in the tissue has also been reported for fish.[172–174] It has also been demonstrated that removal of the skin from carp reduces the concentration of PCBs in the carp by around 50%.[175] In a study of smoked meat products produced in Germany, it was found that a small proportion of samples contained very high levels of PCDD/Fs with a maximum of 85 pg I-TEQ/g fat, although the majority were not significantly different from untreated samples.[176]

3.13 TIME TRENDS

In view of the great efforts made to identify and control or eliminate sources of PCDD/Fs, it would be both surprising and disappointing if a decrease in levels in foods was not evident, and such a decrease is readily apparent in data from several European countries. A reduction, from a peak in the late 1960s to early 1970s, is also evident in human milk and human blood as well as in environmental samples.[177] Levels of dioxinlike PCBs are also declining, but probably at a slower rate that those of PCDD/Fs, so that the proportion of the total TEQ intake attributable to PCBs is increasing. This presumably stems from the fact that PCB production ceased many years before effective control of PCDD/F formation in incineration, pulp bleaching, and other processes was achieved.

In the Netherlands, levels of PCDD/Fs, as total TEQs, in pooled samples of milk, butter, cheese, beef, pork, and eggs collected in 1999 were 33 to 59% of those from 1991, and nonortho PCBs 24 to 61%. A smaller decrease is evident in levels in fish.[70] In Germany a similar decrease since the late 1980s is evident in the levels of PCDD/Fs in milk and meat.[8] In the United Kingdom, the availability of data for TDS samples from 1982, analyzed at the same time as 1992 samples,[93] allows the trend in food levels to be traced back a little further. A comparison of the levels of PCDD/Fs and PCBs found in some food groups in 1982, 1992, and 1997 is given in Table 3.10.

Obviously, a decrease in intake can involve two contributions: (1) this decrease in the levels in foods themselves, and (2) a shift in food consumption habits. Clear evidence of a decrease in food levels is furnished by data from the Netherlands, Finland, Germany, and the United Kingdom. For the latter

TABLE 3.10 Concentrations of PCDD/Fs and Dioxinlike PCBs in U.K. TDS Food Group Samples

	1982			1992			1997		
Food Group	PCDD/Fs	PCBs	Total	PCDD/Fs	PCBs	Total	PCDD/Fs	PCBs	Total
Meat	3.16	1.88	5.04	1.15	0.86	2.01	0.80	1.07	1.87
Poultry	5.89	2.29	8.18	1.85	0.89	2.74	1.01	1.31	2.32
Milk	5.21	2.68	7.88	2.38	1.23	3.61	0.83	0.74	1.57
Eggs	8.93	2.20	11.12	1.97	0.94	2.91	0.77	0.64	1.41
Fish	5.83	11.24	17.07	3.14	4.60	7.75	2.40	4.53	6.93

Source: Data from Ref. 90.

two countries the more recent intake estimates have continued to rely on food consumption data compiled in the second half of the 1980s, so that any contribution from changes in consumption habits are not reflected in the intake estimates, which for the United Kingdom are shown in Table 3.3.

In the Netherlands the estimated average reduction in intake between 1990–1991 and 1998–1999 is 50% for PCDD/Fs and 60% for PCBs,[70] and evidence from duplicate diet studies (Table 3.2) suggests that intakes may have decreased by nearly one order of magnitude over the last 24 years. In Finland the recent PCDD/F intake estimate was 46 to 65 pg I-TEQ/day (lower and upper bound, respectively), based on 1997 consumption data and 1998–2000 food levels. The estimated intake in 1992 was 95 pg Nordic-TEQ/day (total I-TEQ would be only slightly different), again a reduction of about 50%. When, for comparison, 1992 food consumption data were used with the more recently determined food levels, the intake of PCDD/Fs was calculated to be 70 pg I-TEQ/day (lower bound).[52]

Although data which would allow recent trends in the United States to be assessed are not available, Winters et al. have reported the analysis of 14 samples of preserved meat products[178] from earlier decades. In samples from the 1950s–1970s, total TEQs due to PCDD/Fs were several times higher than those found in recent studies, while those due to PCBs were an order of magnitude higher. As noted previously, data from Canada for TDS samples from 1988 and 1992/93 do not indicate a decrease in levels, except for paperboard packaged milk.[102]

3.14 ANIMAL FEEDSTUFFS

Relatively little attention has been paid to the study and monitoring of commercially distributed animal feedstuffs, apart from cases where their contamination has been discovered through food monitoring or other consequences. However, contamination of feedstuffs has resulted in several recent incidents that have precipitated considerable regulatory action in the EU.

In the United States, unexpectedly high levels of PCDD/Fs were found in farmed catfish in 1995, the major source being traced to the feed.[179,180] Subsequently, high levels in chickens in the same area were also traced to feed, the contamination originating in ball clay, a mined mineral used as a minor component in the feeds.[181] PCDD/Fs have also been found in caolinitic clay mined in Germany.[182,183] The PCDD/F pattern in clays from Mississippi and from Germany are similar, and a similar pattern has also been found in sediments from Australia.[184] This contamination is thought to result from natural formation of PCDD/Fs.[183,185]

In 1997, food control laboratories in Germany noticed a reversal of the downward trend in PCDD/Fs levels in milk, with average concentrations increasing over a short period from around 0.6 pg TEQ/g fat to 1.41 pg TEQ/g fat. An individual sample of milk was found to contain 7.86 pg TEQ/g fat. This

increase was traced back to contaminated feed, and within the compound feed to the incorporation of Brazilian citrus pulp pellets.[186] In the production of these pellets about 2% lime is added and the most probable source of contamination was traced to use of a particular lime product formed as a by-product in a chemical process.[184]

The Belgian incident, which has been described in more detail by Fiedler,[184] Dujardin,[187] van Larebeke,[188] and Bernard,[189] gained international notoriety. In March 1999 reduced hatch rates and increased mortality were observed in chickens. Some two months later, analysis showed high levels of PCDD/Fs in feedstuffs and hens. It was eventually discovered that these were a concomitant of PCB contamination of fat. It has subsequently been established that about 25 L of PCB-containing transformer oil somehow became a contaminant of about 100 tons of animal fat that was being recycled. Most of the fat was used to produce poultry feed and some other animal feeds. After incorporation into feeds, this affected some 20,000 tons of poultry feed, 6000 tons of pig feed, and 400 tons of cattle feed, the former being contaminated at 811 ng WHO-TEQ/kg product. In two egg samples, concentrations as high as 266 and 713 pg WHO-TEQ/g fat were measured.

A further example of feed contamination, also discovered in Germany, was traced to the use of PCDD/F contaminated sawdust as a carrier for the incorporation of choline chloride into the feed.[190]

3.15 CONCLUSIONS

PCDD/Fs and dioxinlike PCBs are present in foods throughout the world. Dietary intake is associated primarily with animal fats and with fish, and in a number of exposure estimates the role of plant products may have been overestimated, partly as a result of the difficulty of obtaining accurate analytical data. The spread of concentrations in different examples of most animal products is rather disperse. However, regionally representative averages are usually somewhat similar. For fish the dispersion of concentrations is extremely large and the averages from different surveys much less uniform.

Most people consuming an "average" mixed diet receive with it an intake of PCDD/Fs and dioxinlike PCBs of about 1.2 to 3.0 pg/kg body weight per day.[41] This is close to the tolerable daily intake currently recommended by WHO, and a proportion of the population exceeds the TDI. In the Netherlands this proportion is estimated to be 8% of the population.[70] In Europe the levels of these compounds in food have fallen by about 50% since the early 1990s, and by a much larger factor over the last 25 years. It is clear that many adults will have had, for part of their lives, intakes well above current recommendations.

Regulation of primary sources of PCDD/Fs probably accounts for a significant proportion of the gradual decrease in the amount of these compounds in the environment, and the consequent downward trend of concentrations in food. Continued vigilance in the control of these emissions should help to

ensure a sustained reduction in exposure. However, there have been several specific incidents in which PCDD/Fs and PCBs have entered the food supply as a consequence of the use of contaminated animal feed. There could quite possibly have been incidents that have gone unnoticed because of the limited monitoring and surveillance programs that have been in place for these compounds. At the same time, the use of animal fat in feed can result in the recycling and biomagnification of dioxins and PCBs in food. Regulation of animal feeds, supported by surveillance programs, is therefore of considerable importance if dietary intakes are to be reduced to a minimum.

People who wish to reduce their dietary intake of PCDD/Fs and PCBs can do so by reducing their consumption of animal fat, a choice that is well known to have several health benefits. However, regular consumers of fatty fish may receive a considerable part of their exposure to these compounds, possibly most of it, from this part of their diet, which also has well-known benefits for health, and it is necessary to put any negative effects from exposure to dioxins and PCBs in context with the health benefits offered by the consumption of fish rich in unsaturated oils.

A holistic approach to food regulation will be needed increasingly in the future. Toxicologists, nutritionists, environmental scientists, risk assessors, epidemiologists, analytical chemists, and others must all cooperate to examine the overall picture before decisions on regulation of foods are made.

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CHAPTER 4

Toxicology of Dioxins and Dioxinlike Compounds

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4.1 INTRODUCTION

Polychlorinated dibenzo-*p*-dioxins are toxic compounds that are found in the environment worldwide and considered highly hazardous. They are released mostly through emissions from the incineration of municipal and chemical wastes, in exhaust from automobiles using leaded gasoline and from the improper disposal of certain chlorinated chemical wastes. These chemicals are lipophilic and degrade slowly, so they bioaccummulate in the food chain. Consequently, food is the major source ($> 90\%$) of human exposure to chlorinated dibenzo-*p*-dioxins.[1]

Chlorinated dioxins belong to a family of chemicals designated polyhalogenated aromatic hydrocarbons (PHAHs). Although the PHAHs are a large group of compounds that have a diverse array of health effects, in this chapter we focus on those that have dioxinlike properties. These include the polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans

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(PCDFs), and polychlorinated biphenyls (PCBs). Depending on the position and number of chlorines, the structure of these chemicals allows for 75 chlorinated dioxins, 135 chlorinated dibenzofurans, and 209 chlorinated biphenyls.[2] The public concern from these chemicals is depicted by the description of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) as one of the most potent ''humanmade'' toxicants ever. Not all of these compounds (PCDDs, PCDFs, and PCBs) induce dioxinlike toxicities. Those that do will be referred to as ''dioxins'' because they produce similar toxicities in the same manner as does TCDD.

Dioxin compounds are extremely potent at producing a variety of toxic effects in experimental animals. TCDD and its congeners produce a wide spectrum of toxic effects, including reproductive alterations, immunotoxicity, teratogenicity, carcinogenicity, and lethality. The interest in dioxins and its congeners in the scientific community have evolved because of their pleiotropic toxic effects. Toxicity of dioxins is often associated with alterations of growth regulatory genes or drug-metabolizing enzymes from a variety of different cell types present in different organs.[3]

4.2 TOXICITY

Effects that occur following acute administration versus those that occur following chronic administration often categorize the toxic effects of a chemical. For many chemicals, the acute effects are often quite different from those following chronic treatment. For example, an acute high dose of volatile organic hydrocarbons will cause lethality because of its narcotic effects, while chronic exposure to lower doses may produce a completely different lethal pathology. For dioxins, categorizing the effect as either acute or chronic is problematic. In animals, the toxic effects of dioxins appear to be independent of the route (i.e., oral or intraperitoneal administration). Instead, the toxicity of these chemicals is dependent on body burdens.[4] Body burdens for lipophilic chemicals are dependent on the weight and body fat of an animal, which are highly variable. Thus, TCDD has a half-life of approximately 10 to 15 days in mice and 12 to 31 days in rats.[5,6] In humans, the half-life for TCDD has been estimated to be 5.8 to 11.3 years.[7,8] In cases of acute TCDD toxicity, olestra, a nondigestible fat substitute, can increase the excretion rate of TCDD.[9] Many dioxinlike compounds have shorter half-lives, yet, these chemicals still have estimated half-lives on the order of months or years in humans. Therefore, a single exposure to dioxins results in an exposure duration that is dependent on the chemicals' half-life. The discussions in this chapter focus on the effects of dioxins that one would expect to find shortly after a high-dose exposure (Table 4.1). Long-term effects due to low-dose exposure, including immunotoxicology, carcinogenicity, and teratogenicity and/or reproductive/developmental effects, are covered extensively in chapters elsewhere in this volume as well as in other excellent reviews.[10,11]

TABLE 4.1 Short-Term Toxicity of Dioxins

Species	Strain (Gender)—Route of Exposure	Acute LD_{50} (μg/kg)	Ah Receptor Sequence Notes	Clinical Toxic Effects
Human	—	—	—	Nausea and anorexia[20,92] Chloracne[92,93] Red, irritated eyes, eyelid inflammations, and cysts; hyperpigmentation, hirsutism, actinic elastosis, hepatomegaly Increase in γ-glutamyl transferase (GGT) levels, increase in serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels[94]
Rabbits	New Zealand white (both genders)—dermal	275[89]	—	Body weight loss, acnenegic, eye irritation[89]
	New Zealand white (both genders)—oral	115[89]		Hypertriglyceridemia, decrease adipose tissue lipoprotein lipase (LPL), hyperlipidemia[74] Severe liver necrosis[13]
Mice	D2A/2J (male)—oral	2.570[95]	Resistance of DBA vs. B6D2F1 attributed to a point mutation in the LBD of the AhR[61,62]	Body weight loss, wasting syndrome[89,95]
	D2—intraperitoneal	620[96]		Serum hypoglycemia, hepatic hypertriglyceridemia[95]
	B6D2F1—intraperitoneal	300[96]		Hepatic vitamin A depletion,[78] ethoxyresorufin *O*-deethylase (EROD) activity induced in liver,[97] liver lesion, decreased activity in liver gluconeogenesis enzymes; phosphoenolpyruvate carboxykinase (PEPCK), and glucose-6-phosphatase (G6P)[98]
	B6DF1 (male)—oral	296[95]		
	B6 (male)—oral	182[95]		
	B6—intraperitoneal	132[96]		Inhibition of bone marrow hematopoiesis

(Continued)

TABLE 4.1 *(Continued)*

Species	Strain (Gender)—Route of Exposure	Acute LD_{50} (μg/kg)	Ah Receptor Sequence Notes	Clinical Toxic Effects
Mice				Reactive oxidative species (ROS), increases in liver superoxide anion production and thiobarbituric acid reactive substances[98] Changes in the mature B-cell subpopulation[99]
Monkey (primates)	Rhesus monkey (male)—single oral dose Rhesus monkey (female)—single oral dose	≈ 25[100] ≈ 70[29]	—	Body weight loss, anemia, blepharitis, hirusitism, cutaneous lesions, thymic atrophy, increases in serum tryglycerides, wasting syndrome (depletion of body fat storage)[29,35] Increase in serum glutamic pyruvate transaminase (SGPT) and sorbitol dehydrogenase (SDH), liver fat infiltration[100]
Chicken	White leghorn—single oral dose	< 25	—	Body weight loss, stunted growth, dyspnea, edema, sudden death[31,101]
Hamsters	Syrian golden—intraperitoneal Syrian golden (male)—oral	3000[90] 5051[102]	Resistance of hamster vs. guinea pig attributed to altered transactivation domain in C-terminus of the AhR[103]	Body weight loss, liver and thymus alterations[104] Ileitis and peritonitis[102] Hepatic vitamin A depletion[78]
Rats	Han/Wistar (Kuopio; H/W; both genders)—intragastric administration Long–Evans (strain Turku AB; L–E; female/male)—intragastric administration	> 9600[63] ≈ 10[63]	Resistance of H/W vs. L–E attributed to a critical point mutation in the transactivation domain of the AhR[105]	Anorexia,[34] body weight loss, wasting syndrome[89,106] Thymic atrophy Lung hemorrhage, infection, and perivascular edema[31]

	Fischer (F/334N) and CD (male)—oral	303/297[32]		Hepatic vitamin A depletion,[78] liver lesions,[31] decreased activity in liver gluconeogenesis enzymes; phosphoenolpyruvate carboxykinase (PEPCK), and glucose-6-phosphatase (G6P)[35]
	Sherman, Spartan (male)—oral	22[89]		
	Sherman, Spartan (female)—oral	45[89]		
	Sprague–Dawley (male and female)—oral	25–50[13]		Porphyria, impaired normal growth of the skull and incisor tooth[107] TCDD dose dependency interferes with bone growth, modeling, and mechanical strength[108] Decreased serum melatonin,[109] increased tryptophan brain levels[110] Serum hypoglycemia, hypertriglyceridemia, and hypercholesterolemia[32]
Mink	*Mustela vison* (male)—single oral dose	4.2[111]	—	Wasting syndrome, ulceration of stomach and/or duodenum[111] Decreased T3, T4, and plasma cortisol Hypotriglceridemia[13]
Guinea pig	Hartley (male)—oral	0.6–2.1[89]	Homology of the AhR is most similar to human[112]	Body weight loss,[89] hypertriglceridemia[113] Atrophy of the thymus and lymphatic tissues[113] Hepatic vitamin A depletion[78] Adverse contraction of atrial muscle[115] TCDD causes a rise in protein-tyrosine kinases[116]

4.2.1 Wasting Syndrome

Species-, strain-, age-, tissue-, and dose-specific differences in the biologic and toxic effects of dioxins are widely documented.[12,13] However, a hallmark of dioxin intoxication followed by lethality in most animals is a wasting syndrome. Wasting syndrome is characterized by anorexia (loss of appetite) followed by diminishment of muscle mass and adipose tissue. Acute lethality caused by high-dose exposure to dioxins takes weeks to manifest rather than days, with the time to death being characteristic of the species examined. Sublethal doses of dioxins also produce dose-dependent decreases in weight and body weight gain. Homeostatic functions, including body weight and food intakes, are regulated by the hypothalamus, which if altered can result in anorexia or hypophagia. Although the exact mechanism of the wasting syndrome is undetermined, there is substantial evidence that dioxins decrease the "set point" for body weight (reviewed by Peterson et al.[14]). Thus, weight loss observed from an animal given an acute or sublethal dose of dioxin is maintained and defended against dietary challenges.

Weight loss can arise from a decreased energy intake, increased energy expenditure, or a combination of both. When one controls for decreased feed intake by using pair-fed controls, weight loss is similar but lethality is greater in TCDD-treated animals.[15] In studies where weight loss is prevented in rats and guinea pigs, lethality still occurs.[16,17] Hence, while weight loss is definitively associated with the wasting syndrome, other causative factors are involved. Contributing factors linked to TCDD-induced wasting include those involved in intermediary metabolism. Increased fat utilization and mobilization as well as decreased gluconeogenesis as causal mediators are reviewed nicely by Pohjanvirta and Toumisto.[13] Evidence that TCDD causes a negative fat balance is supported by rodent studies using dietary manipulations. When the diet had increased protein or carbohydrates, the time to TCCD-induced lethality was prolonged while increased fat consumption reduced it.[18] Alterations in other metabolic enzymes may play a role in TCDD wasting, since TCDD-induced wasting syndrome has characteristics very similar to those found in wasting syndrome induced by vitamin A deficiency in rats.[19] Dioxin disregulation of metabolism may be due in part to direct or indirect toxic effects on glands like the thyroid or the hypothalamic–pituitary axis. Cytokines may also play a role in wasting syndrome since the acute toxicity of TCDD was reduced substantially in mice that were treated with antibodies against tumor necrosis factor α (a cytokine linked to protein catabolism). Finally, the interplay between TCDD and circadian feeding rhythm is shown in a study where the feeding pattern was mimicked and the animals treated with TCDD had no wasting syndrome but still died. Wasting syndrome is a complex pathophysiologic condition where multiple factors need to be taken into account.

In humans there are no reported cases of a wasting syndrome leading to death following acute or chronic exposure to dioxins. However, symptoms such as appetite suppression and weight loss that are associated with the wasting syndrome have been seen in human populations exposed to high levels

of dioxin.[20] Humans exposed to high TCDD levels accidentally, exhibit appetite suppression as well as other toxic gastrointestinal effects, including epigastric pain, nausea, and vomiting. The lack of a wasting syndrome lethality in humans does not necessarily demonstrate that humans are a species insensitive to the toxic effects of dioxins; rather, that human exposure has simply not approached acute lethal levels.

4.2.2 Thymic Atrophy

The most frequently observed gross response to TCDD immunotoxicity is the induction of thymic atrophy, which occurs in a wide range of mammalian species.[13] The thymus is a small organ composed of an inner medulla and an outer cortex and is surrounded by a thin covering called the *capsule*. The thymus reaches its maximum weight during puberty, then decreases slowly in size during adulthood and is gradually replaced by fat tissue. During fetal development and childhood, the thymus is involved in the production and maturation of T lymphocytes, a type of white blood cell important in the immune system. T lymphocyte precursor cells are produced in the bone marrow and transported to the thymus, where they proliferate and develop into T cells. T cells then travel to lymph throughout the body and help the immune system protect the body from bacteria, viruses, fungus, and other types of infections. The thymus contains two main types of cells, thymic epithelial cells and lymphocytes. Lymphocytes, whether in the thymus, spleen, or lymph nodes, can become malignant and develop into cancers, including Hodgkin's disease and non-Hodgkin's lymphomas.

TCDD-mediated thymic atrophy is characterized by lymphocyte depletion and disruption of epithelial cells in the thymic cortex.[21] Thymocyte precursors from bone marrow are also sensitive to the effects of TCDD[22] and are dependent on aryl hydrocarbon receptor (AhR) activation.[23] Rodents exposed to dioxins perinatally or neonatally are more sensitive than adults to dioxin-induced thymic atrophy. Immune suppression is associated with thymic atrophy in immature animals exposed to dioxins. In adult animals, thymic atrophy is still one of the most sensitive TCDD targets. However, associations with immune suppression are debatable since immunotoxic effects take place even in the absence of thymic atrophy, suggesting other targets in the immune system. Immunotoxic responses observed in animal studies include decreased host resistance to infectious disease and suppressed humoral and cell-mediated immune responses.[10,24] In addition, alterations in autoimmunity because of chemically mediated thymus toxicity have not been ruled out. The immunotoxicology of dioxin is discussed in depth in Chapter 8.

4.2.3 Dermal Toxicity

It was first discovered by Kimmig and Schulz (1957) that TCDD was a by-product in the manufacture of trichlorophenols (used in herbicides and pesticides), responsible for chloracne.[25] Chloracne is a distinct type of acne

caused specifically by PHAHs.[26] This occupational-associated acne is distinct from juvenile acne because it can affect follicles (also known as *pores*) all over the body. A follicle is a canal consisting of a layer of cells (keratinocytes) that allow a sebaceous gland surrounding a fine hair to empty its oily product (sebum) onto the skin surface. Diagnosis of chloracne is based on exposure history, time of onset in relation to age, and similar factors. This disease involves both hyperplastic and hyperkeratotic changes in the skin and alterations in pigmentation, resulting in a form of cystic acne. Chloracne has been seen in humans, monkeys, cows, hairless mice, and on rabbit ears following either dermal or systemic exposure. Chloracne is a high-dose response in animals and humans. In animals, thymic atrophy and wasting syndrome accompany this response. In humans, only loss of appetite is reported to accompany chloracne. Clearly, chloracne in humans is proof of PHAH exposure; however, lack of chloracne does not indicate that exposure has not taken place.[27]

As mentioned previously, high levels of exposure to dioxin are required for dermal toxicity. For example, a recent human TCDD-intoxication accident indicated that keratosis punctata palmaris et plantaris (KPPP) may be another dermal manifestation of dioxin toxicity.[28] Interestingly, the palms and soles of TCDD-treated rhesus monkeys were also affected with symptoms of atopic dermatitis.[29] Another dermal lesions associated with dioxin is porphyria cutanea tarda,[30] which is an inherited metabolic disorder due to a defective enzyme (uroporphyrinogen decarboxylase) in the liver that results in an increase in porphyrins in the skin. Increase in porphryins leads to photosensitivity where the skin is damaged by sunlight. Symptoms include pigmentation, light sensitivity causing rashes and blisters, sores, fragile skin, cysts on sun-exposed areas such as the hands, neck, and forearms, facial hair and patches of baldness, and elevated sugar. Similar symptoms have been documented in different animal species and humans. The time required to display these dermal effects is usually weeks to months and is often associated with chronic exposure since long-term low-dose effects of dioxins can be similar to high doses.

4.2.4 Other Toxic Endpoints

In animal studies, the liver is widely documented to be a target organ for dioxin. Liver lesions from dioxin treatment are both short term and long term, depending on the endpoint examined. Short-term changes include increased liver weights, biochemical changes, and histological alterations.[31–34] Like other TCDD-target organs, there are species differences in hepatotoxic effects. For example, the degree of liver necrosis is greatest in the rabbit and minimal to undetectable in the guinea pig,[35] while hepatomegaly is seen consistently in all species examined.[13]

Historically, the liver is recognized as a cancer endpoint for dioxin exposure in chronic rodent bioassays.[38,39] This, combined with evidence of carcinogenicity from studies in humans involving a combination of epidemiological and mechanistic information, indicates a causal relationship between exposure

to TCDD and human cancer. Hence, in addition to the International Agency for Cancer Research (IARC), the Ninth Report on Carcinogens from the National Toxicology Program has classified TCDD as a "known human carcinogen."[38,39] In addition to cancer, dioxins have a number of other effects, including immunotoxicity, teratogenicity, and reproductive/developmental toxicity, that are covered in detail in other chapters of this volume.

4.3 MOLECULAR MECHANISMS

Multiple lines of evidence indicate that practically all toxic effects of dioxins are mediated by the AhR.[40–44] The AhR is an intracellular protein that can act as a ligand-activated transcription factor that is involved in the regulation of a large number of genes. The primary structure of the AhR is linked to differences in acute toxicity to dioxin. Briefly, the AhR has three major functional domains: (1) the N-terminus, which contains a basic helix-loop-helix (bHLH) motif, is involved with DNA binding and dimerization; (2) the PAS domain, which is involved in ligand binding and dimerization with ARNT; and (3) the C-terminus, which is involved with transcriptional activation and transformation.[45]

TCDD is the most potent dioxin and binds with the highest affinity to the AhR. Chemicals that bind to the AhR produce the same effects as TCDD but require higher doses because they do not bind as well to the AhR. Structure–activity relationships demonstrate a direct relationship between binding affinity to the AhR and the potency of the chemical to induce CYP1A1, thymic atrophy, or weight loss in animals.[46,47] In general, the greater the binding affinity of a chemical for the AhR, the more potent it is at producing these toxic effects.

Toxicity of dioxins are initiated by both direct and indirect AhR molecular mechanisms. How TCDD induces gene transcription for metabolic enzymes, particularly cytochrome P4501A1 (CYP1A1), is a well-characterized event involving direct ligand-receptor binding. Briefly, the AhR protein[41,48,49] exists in the cytosol as a complex associated with other cytosolic proteins, including heat shock protein 90 (HSP90), c-SRC,[50] AIP1 (also known as ARA9 or XAP2),[51] and p23.[52] Dioxin binds to the AhR in the cytosol, then is transferred into the nucleus, where it forms a heterodimer with another bHLH protein, the aryl hydrocarbon nuclear translocator (ARNT). The AhR/ARNT complex binds to the DNA xenobiotic responsive element [XRE, also known as the AhRE or dioxin response element (DRE)] in the promoter region of AhR responsive genes. Response elements are regions of DNA that bind to specific proteins. On binding to the response element, the AhR bends the DNA so that a conformational change occurs to allow access for other transcription factors to bind to DNA. The result is an increase in transcription of genes that contain the XRE. In general, most genes that are responsive to AhR agonists have been classified as growth regulatory genes or drug-metabolizing enzymes.[53–55]

It is becoming increasingly clear that the role of the AhR is more than just a ligand-activated transcription factor. In addition, not all genes altered by exposure to dioxin necessarily have XREs in their DNA. The AhR is involved in a number of complex protein–protein interactions. For example, the AhR can sequester ARNT, preventing other known ARNT-dependent intracellular events to occur,[56] or it can bind directly to other proteins that interact with other transcription factors.[56–60] A characterization of AhR and its interactions with other proteins are discussed thoroughly in Chapter 12.

4.3.1 Sensitivity to Dioxin

Differences in receptor-ligand binding affinity are known for the C57BL/6 and DBA/2 prototypic sensitive and resistant strains of mice, respectively. Like other rodents, their sensitivity to biochemical and toxic effects of dioxin congeners segregates with the Ah locus. The AhR gene is encoded by several different alleles of the same locus (i.e., the position in a chromosome of a particular gene). A gene may have several different variants, called *alleles*. To demonstrate the role of the AhR, congenic mouse strains have been bred. Congenic strains are genetically identical except at a particular allele that is located at a certain location on the DNA. In mice congenic at the Ah locus, one strain has an AhR that has a high binding affinity for dioxins while the other strain has a receptor with a low affinity for dioxins. Animals with a high-affinity receptor require lower doses of dioxins to produce a toxic effect compared with animals with low-affinity receptors that require higher doses. Molecular cloning and sequencing of the AhR cDNA alleles have found that the low binding affinity found in the DBA mouse strain, compared to the higher binding affinity seen in the C57BL strain, is attributed to a specific DNA mutation. This mutation changes the amino acid composition of the AhR protein. Specifically, the Ala375 is changed to Val with an elongated carboxyl-terminal sequence due to a T-to-C mutation at the first letter of the termination codon of C57BL AhR.[61,62]

Other AhR allele differences are related to transactivation capabilities. In rats the prototypic sensitive strain is the Long–Evans (L–E), while the resistant strain is the Han/Wistar (H/W).[63] The AhR of H/W rats is smaller (about 98 kDa) than the receptor in other rat strains (106 kDa). The smaller size is due to a deletion/insertion type of change at the 3′ end of exon 10 in the receptor cDNA. A single point mutation at the first nucleotide of intron 10 results in altered mRNA splicing. At the protein level, this mutation leads to a total loss of either 43 or 38 amino acids toward the carboxyl-terminal end in the transactivation domain of the AhR. H/W rats also harbor a point mutation in exon 10 that will cause a Val-to-Ala substitution in codon 497, but this occurs in a variable region of the AhR. These findings suggest that a relatively small region in the AhR transactivation domain may be capable of providing selectivity to its function.[64]

4.3.2 AhR-Null Studies

The most significant evidence for active participation of the AhR in acute toxicity of dioxin comes from studies involving mice designed to be devoid of a functional AhR (AhR-null mice). These mice exhibit a number of phenotypic abnormalities but are viable animals that live up to 12 months or longer. These AhR-null mice lack expression of the AhR protein, and the AhR-regulated genes CYP1A1 and CYP1A2 are not transcriptionally activated by dioxins. A marked resistance is detected in the dose required for TCDD toxicity. A dose greater than 10 times the effective dose in sensitive mice mediates only minor effects. Direct AhR-mediated effects were found in the liver and thymus, causing these AhR-null mice to be resistant to the TCDD-induced wasting syndrome, immune suppression, thymic atrophy, cortical lymphocyte depletion, and lipid accumulation within hepatocytes.[65–68]

4.4 BIOCHEMICAL EFFECTS

There are a large number of biochemical alterations induced by dioxins; however, some of the most sensitive biochemical effect of dioxins is on metabolic enzymes. The induction of the phase I cytochrome P4501A1 (CYP1A1) is a biochemical hallmark of exposure to dioxin and other PHAHs. Other drug-metabolizing enzymes under direct transcriptional control of the AhR and expressed in a cell-specific manner include CYP1A2 and CYP1B1.[3,49] CYP1A2 is a constitutively expressed liver enzyme regulated by both AhR-dependent and AhR-independent mechanisms.[69] In addition, CYP1A2 is a hepatic binding protein that contributes to the hepatotoxicity of dioxins by sequestering it in the liver. By using CYP1A2 knockout mice, Smith et al. have shown that CYP1A2 is mostly responsible for TCDD hepatotoxicity, particularly uroporphyria, inhibition of uroporphyrinogen decarboxylase (UROD) activity, and hepatocellular damage.[70] Like CYP1A1, CYP1A2, and CYP1B1, there are a number of other phase II metabolic enzymes activated transcriptionally by the AhR binding to their DRE (i.e., *ALDH4* and *NQO1*).[71]

Wasting syndrome is a complex interplay caused by reduced nutrient intake (i.e., glucose) and metabolic disturbances (i.e., increased fat mobilization and protein catabolism). Dioxin causes many biochemical alterations involved in metabolism that are linked to physiological responses. For example, the loss of appetite associated with wasting syndrome results in a fasting behavior. When fasting, the body's glucose needs are met by gluconeogenesis, primarily in the liver. The activity of essential regulatory enzymes involved in gluconeogenesis [i.e., phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6P)] is decreased following TCDD exposure.[34] Reduced blood glucose is also affected by levels of glucose transporters found in the liver and adipose tissue.[72] That glucose transporters are reduced, hence uptake of glucose is also reduced, is thought to play a role in the regulation of lipoprotein lipase.[73] Lipo-

protein lipase (LPL) aids in the conversion of dietary fat to free fatty acids that can be stored in adipose tissue or muscle. Decreased LPL activity due to TCDD exposure has been detected in a number of species, including rabbits,[74] guinea pigs,[75] monkeys,[76] and immature chickens.[77] A decreased level of LPL is an effect also detected in starvation and aids in the mobilization of free fatty acids. The increase in free fatty acids in the blood competes with amino acids such as tryptophan for binding sites of albumin. Increased levels of tryptophan in the blood facilitate its transfer to the central nervous system, where it acts as a precursor to the neurotransmitter serotonin. Serotonin, found in and around the hypothalamus, may play a role in eating disorders. Many examples of physiological responses and changes in metabolic enzymes are reported, but the exact relationship between biochemical alterations and toxicity of dioxins is not clear.

Part of the actions of dioxins can be explained by alterations of enzymes involved in cellular metabolism involving hormones and/or their receptors. Vitamin A is acquired only from dietary means and acts as a hormone. It is a possible biomarker for animals exposed to TCDD, since a decrease in body weight gain observed following exposure to sublethal doses of TCDD in several rodents is correlated to a decrease in hepatic vitamin A.[78] This depletion of hepatic vitamin A is observed following exposure to a variety of dioxins and correlates with the chemicals' binding affinity to the AhR.[79–83] In AhR-null mice, increased liver levels of retinoic acid, retinol, and retinyl palmitrate were measured.[84] Altered vitamin A homeostasis has been observed in all species tested, but the rat is the best characterized and exhibits decreased levels of vitamin A in the liver, increased levels in the kidney, and altered serum and tissue levels as a result of acute dioxin exposure.[85] A decrease in retinoic acid catabolism as a result of lowered amounts of a P450 enzyme controlled directly or indirectly by the AhR found in AhR-null mice is thought to be responsible.[67] Decreases in circulating thyroxine are related to increases in UDP-glucuroyltransferase, an enzyme involved in its metabolism.[86] Another hormone affected that regulates carbohydrate and fat metabolism is insulin. Changes in insulin receptor concentrations following dioxin treatment have been detected in human breast cancer cells.[87]

Regulation of cell growth/differentiation controlled by steroid hormones and growth factors is often disrupted by dioxins. In addition to hormones, dioxins alter the levels of both growth factors and their receptors. Growth factors are proteins that also regulate apoptosis (a mechanism of regulated cell death) by interacting with specific membrane receptors that act as signal transducers. Inappropriate proliferation and alterations in differentiation can lead to deleterious effects such as developmental abnormalities and cancer. Alterations in cell growth regulation, such as hyperplasia, hypoplasia, metaplasia, and dysplasia, are generalized responses to dioxin exposure in animals. *Hyperplasia*, an increase in cell number, occurs in the gastric mucosa and bile duct in monkeys in response to dioxins.[88] Guinea pigs develop urinary bladder hyperplasia following exposure to dioxins.[88] Hyperplasia of hepatic and dermal tissue occurs

in several species. *Hypoplasia*, a decrease in number of cells, occurs in the lymphoid tissues in all species exposed to dioxins. *Metaplasia* is the transformation of one cell type to another. Squamous metaplasia occurs in the meibonian glands of the eyelid and the ceruminous glands of the ears, producing waxy exudates, of monkeys exposed to dioxins.[88] *Dysplasia*, the abnormal growth or development of an organ, occurs in ectodermal tissue in humans and primates, resulting in alterations in teeth and nails following exposure to dioxins. Thus, many of the toxic actions of dioxins are related to their ability to disrupt normal growth processes, and uncontrolled growth of aberrant cells can lead to tumors.

TCDD is a known tumor promoter that modifies the normal cellular proliferation–differentiation process, which is linked to altered regulation of gene expression mediated by gene-signaling cascades. With the advent of microarray or cDNA analysis, techniques that enables simultaneous expression of thousands of genes altered by a toxicant, the number of known dioxin-regulated genes is increasing daily.[58] Thus, although the AhR mediates the mechanism by which dioxins produce the majority of their effects, it is becoming increasingly clear that multiple pathways are affected after AhR activation. For a thorough discussion of AhR-regulated genes, the reader is referred to other chapters in this volume, especially Chapters 12 and 14.

4.5 SPECIES DIFFERENCES

Many reports in the literature demonstrate large species differences in the doses needed to produce lethal effects of dioxins (see Table 4.1). For example, the most sensitive species to the acute lethal effects of TCDD is the guinea pig, where the LD_{50} (i.e., the dose of a chemical required to kill 50% of the animals treated) is approximately 0.6 μg/kg.[89] The hamster is approximately 1000 to 10,000 times less sensitive than the guinea pig to the lethal effects of dioxins ($LD_{50} > 3000$ μg/kg).[90] However, in many other species, such as monkeys, rabbits, rats, mice, and dogs, the LD_{50} is generally between 100 and 300 μg/kg. In contrast to the lethal effects on adult animals, doses of TCDD that are fetotoxic are similar across several species and are lower than those of adult animals. A more accurate description of the species differences in susceptibility to dioxins is that for every toxic endpoint there are either extremely sensitive or resistant species; however, most species respond to the toxic effects of dioxins at similar doses. The species differences in toxic effects do not appear to be causally related to the amount of AhR present in a particular tissue. The species differences may be explained, in part, by differences in the size and binding affinity of the AhR. In addition, there may be inherent differences in the actions of this receptor between species. Pharmacokinetic differences between species may also play a role in the differences in sensitivity.

One of the difficulties in extrapolating the animal data to humans is determining whether humans are a sensitive or resistant species. In the early

years of dioxin research, chloracne was the only documented response in humans, and it occurs only after exposure to very high doses of dioxins. Currently, a significant body of evidence indicates that humans are sensitive to biochemical effects of dioxins. Functional AhRs have been found in many human tissues, including lymphocytes, liver, lung, and placenta. Human CYP1A1 is inducible in lung and placenta, indicating that human cells are a responsive species and their sensitivity to certain dioxin biochemical effects is similar to the range seen for other species.

4.6 SUMMARY

Dioxins are members of a class of compounds that share several features. They are chlorinated aromatic hydrocarbons that are persistent in both environmental and biological samples and produce a similar spectrum of acute toxicity mediated by interaction with the AhR. Pleiotropic toxic endpoints are induced by dioxins that are species, strain, age, tissue, and dose specific. The toxic effects of these chemicals can best be described by their actions in disrupting normal homeostatic processes, including metabolism, cellular growth, and differentiation. While disruption in these processes produces a variety of toxicities and pathologies, the hallmark toxicities of dioxin exposure include wasting syndrome, thymic atrophy, and chloracne. The available data demonstrate that humans are responsive to these chemicals and for effects that have clearly been associated with dioxins, such as chloracne and induction of CYP1A1, humans and animals respond at similar body burdens.[91]

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CHAPTER 5

Health Risk Characterization of Dioxins and Related Compounds

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5.1 INTRODUCTION

Environmental policy to prevent, eliminate, or manage environmental risks is based on a variety of inputs to the decision-making process. Scientific information in the form of a risk characterization is factored in with information on economic, legal, social, political, and engineering considerations to effect *risk management*. Risk management policy can range from a decision to take no action at all; to manage risks through a campaign of public information; to institute national or local standards, mandating use of control devices and emission limitations on equipment and facilities; to outright bans on processes or products that are responsible for environmental releases/exposures of chemicals of concern; or to clean up or regulate access to situations that might represent significant exposure. The process used to organize much of scientific input into the risk management process is called *risk assessment*. *Risk characterization* is the product of the process of risk assessment.

The process of risk assessment as described by the National Research Council[1] is divided into four steps. Although individual steps may be conducted in isolation, they are best thought of as overlapping and interdependent efforts. The four steps were identified as hazard identification, dose–response assessment, exposure assessment, and risk characterization.[1] More recent descriptions of the risk assessment process have recognized the complex rela-

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tionships among these four steps and the importance of a comprehensive characterization at each step in the process.[2,3] Hazard characterization involves determining the potential toxicity of a chemical, process, or stressor. This is not only a qualitative effort to describe a potential response, but involves elucidation of the mechanistic determinants of the response. Dose–response characterization involves the qualitative and quantitative description of the relationship between the dose, which may be expressed in different dose metrics. Examples of useful dose metrics include daily administered dose, lifetime average daily dose, steady-state body burden, dose to the target tissue, or cumulative dose. Dose–response relationships may vary for different effects. Exposure characterization involves an understanding of how concentrations in the environment are translated into a dose to the organism. Risk characterization is the integrative step within the risk assessment process, in which the information on hazard, exposure, and dose is compiled in a transparent manner with a discussion of accompanying assumptions and uncertainties. This is the step in which the likelihood of a response under given conditions for a specific individual or population is described. Risk characterization is the scientific integration of the entire risk assessment process and is often a key determinant of risk management decision making.

The health risk characterization for dioxin and related compounds represents a synthesis of an extremely large and complex database of studies on the exposure and effects of these compounds in the laboratory and in human populations. Studies of fate and transport in the environment and of effects on nonhuman species in the environment also play a large role in understanding and characterizing human health risk.

5.2 CHARACTERIZING COMPLEX MIXTURES OF DIOXINLIKE COMPOUNDS

2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; dioxin) is the prototype for a family of structurally related compounds with a common mechanism of action and a common spectrum of biological responses. Furthermore, TCDD is never found alone but is always found as part of a complex mixture both environmentally and in animal tissues.[4] In fact, in most instances, TCDD is only a small part of the total chemical mass. However, it is the compound for which the most information, chemically and biologically, is available. This family of related compounds is often referred to as *dioxins*. From a chemistry perspective, there are potentially 75 polychlorinated dibenzo-*p*-dioxins (PCDDs; dioxins), 135 polychlorinated dibenzofurans (PCDFs; furans), and 209 polychlorinated biphenyls (PCBs; biphenyls). Structurally similar brominated and mixed chlorobromo congeners also exist. In addition, there are other classes of polyhalogenated compounds, such as the polychlorinated/polybrominated naphthalenes (PCNs/PBNs), and the polyhalogenated azo- and azoxybenzenes, among others (e.g., quatraphenyls, terphenyls), which although less similar

structurally, show some functional similarities to dioxins. All told, there is the potential for the existence of thousands of polyhalogenated aromatic hydrocarbons (PHAHs) which might be considered to be dioxins. However, not all of these congeners will have TCDD-like properties. To truly be a dioxin, the chemical must have at least three halogen atoms in the lateral positions. Thus, only 7 of the PCDDs, 10 of the PCDFs, and 11 of the PCBs are appropriately considered to be dioxins.[5]

The existence of environmental mixtures of a broad spectrum of dioxinlike chemicals with unknown toxicity but with a striking structural relatedness leads to consideration of three regulatory approaches for assessing the risk of such complex mixtures.[6] The first would be to treat all the dioxinlike PHAHs in the mixture as if they all were equitoxic to TCDD. This is not very satisfactory since there is ample information to indicate a wide range in the toxicity of some of the congeners in this class, most of which are significantly less toxic than TCDD, the prototype. The second approach might be to ignore all the dioxinlike PHAHs for which toxicological information is lacking. This is also not acceptable because the limited information that does exist for many of the congeners indicates that many of the other dioxinlike PHAHs are toxic, but perhaps simply to a lesser extent than the prototype. The third and preferred approach is to develop a relative potency-ranking scheme that utilizes existing data and scientific judgment. This has led to the development of the toxic equivalency (TEQ) approach, an approach endorsed by state, national, and international organizations as the method of choice, at least at this time, to estimate the potential toxicity of complex mixtures of dioxinlike chemicals.[5,7–9] For chemicals to be included in the dioxinlike TEQ approach, they must meet four criteria: structural relatedness; binding to the Ah (aryl hydrocarbon, dioxin) receptor; elicitation of dioxin-specific responses and effects; and persistence.[10,11] Toxic equivalency factors (TEFs) for individual related chemicals (congeners) are order-of-magnitude estimates of the toxic potency of a given congener relative to the toxicity of TCDD, based on all the data available.[4] Data from individual studies are assigned relative potency factors (REPs). The TEFs are consensus estimates based on evaluation of the available REPs. A tiered approach is used for the weighting of REP values.[5] Chronic/long-term studies, such as those of carcinogenicity, tumor promotion, and reproductive effects, are given the highest weighting. This is followed by short-term studies examining developmental and immunotoxic effects as well as biochemical responses. Acute studies in which endpoints examined include thymic atrophy, body weight loss, and even lethality fall into the third category. In vitro studies involving enzyme induction or differentiation assays of cultured cells, as well as measures of binding of the individual congeners to the Ah receptor, carry the least amount of weight in determination of the TEFs, but for a number of congeners, they are the only information available. The TEQ of the entire mixture is the sum of the product of the mass of each congener times its TEF value. From data described in detail by the USEPA in its dioxin reassessment effort,[12–14] it is clear that the chemicals that contribute approxi-

mately 80% to the total human TEQ (2,3,7,8-TCDD, 1,2,3,7,8-PCDD, 1,2,3,6,7,8-hexa-CDD, 2,3,4,7,8-PCDF, and PCB 126) are well studied, and the TEFs assigned provide reasonable estimates of the relative potency of these chemicals. In contrast, while there are some chemicals in the TEF methodology that have minimal data sets to assess their relative potency reliably, these chemicals do not contribute substantially to the human TEQ. Thus, TEFs have been developed for risk assessment as an interim approach by which to interpret the complex toxicity database on and relative concentrations derived from analysis of samples containing mixtures of dioxinlike PHAHs.

Although a number of uncertainties associated with the TEF concept have been identified (e.g., nonadditive interactions with nondioxinlike PCBs; natural ligands for the Ah receptor[15]; low-dose linearity of REP responses), the 1997 World Health Organization (WHO) expert meeting decided that an additive TEF model remained the most feasible risk assessment method for complex mixtures of dioxinlike PHAHs.[5] The TEQ approach assumes dose additivity.[4] This is an appropriate assumption and has been well demonstrated for congeners whose sole mechanism of action is the same as that of TCDD and involves binding to the Ah receptor. However, the complex mixtures present in the environment and in biological samples often involve chemicals that do not meet the criteria for inclusion in the TEF scheme. In such complex mixtures, nonadditive interactions have been observed, usually at high dose levels. Both antagonistic and synergistic interactions have been noted. In most cases the magnitude of the impact of these interactions are smaller than the uncertainties already present in the TEF values. However, these interactions are also highly species-, response-, and dose-dependent. For example, nondioxinlike PCBs have been shown to antagonize the immunosuppressive effects of TCDD at extremely high doses.[16,17] However, at lower doses, they have been shown to dramatically enhance (>1000 times) the induction of hepatic porphyrins by dioxin.[18,19] As discussed earlier, only a small subset of the PCBs are dioxinlike. The majority of the PCBs have their own inherent and distinct toxicities, which appear to fall into multiple and often overlapping structural classes.[10,20] While the PCB mixtures always have dioxinlike PCBs present, dioxins could be present in the absence of PCBs.

Presently, there are several limitations to our understanding of the importance of naturally occurring AhR ligands, such as physiologically derived chemicals like bilirubin or dietary components like indole derivatives, versus the dioxinlike chemicals included in the TEF methodology. First is the limited data available on the dioxinlike toxicities of the natural compounds. In addition, there is a lack of data on the interactions between these classes of chemicals. Few studies, if any, on mixtures of natural AhR ligands and PCDDs or PCDFs examining a toxic response have been published. Many of the natural AhR ligands have multiple mechanisms of action that presently cannot be accounted for in the TEF methodology. For example, indole-3-carbinol (I-3-C) has anticarcinogenic properties in tumor promotion studies, and these effects may or may not be mediated through the dioxin receptor (AhR) mechanisms.[21]

The lack of data and the role of non-AhR mechanisms in the biological effects of these chemicals prohibit a definitive conclusion on the role of natural versus anthropogenic dioxins in human health risk assessment.[14] Although it is important to address these issues, the available data do not lend themselves to an assessment of the quantitative impact on the assessment of dioxin and related compounds.

5.3 SOURCES OF DIOXINLIKE CHEMICALS

PCDDs and PCDFs were never produced intentionally, except for small amounts for laboratory purposes. They are unwanted by-products of industrial and combustion processes.[5] There has been a shift in the major sources over the past 30 years,[14] in large part due to the success of the regulatory agenda and to focused, voluntary efforts. In the past, production of chlorinated herbicide and biocides was associated with relatively high levels of contamination with dioxins and furans. Many of these products, such as the herbicide 2,4,5,-T and the biocide hexachlorophene, have been banned. Combustion of leaded gasoline in motor vehicles was also a significant source. Bleaching of paper and pulp products using free chlorine in kraft mills led to the production of dioxins. Most of these mills have been shut down or use alternative bleaching processes that lead to reduced or no dioxin production.[14] The major known sources of dioxins and furans today involve combustion processes. The fact that natural processes such as volcanoes and forest fires can produce trace amounts of dioxins has been known for a decade or more, but it is generally thought that this source accounts for only a relatively small amount of the new emissions in recent years.[14] However, as best available technologies are implemented, the amount of emissions from industrial sources decreases. Old incinerators or improperly maintained incinerators are problems with regard to air emissions; new, state-of-the-art incinerators are not. The same is true for hazardous waste incinerators which are held to even higher standards than are medical and municipal waste incinerators. Although municipal and medical waste incineration were once major sources, voluntary action and regulatory compliance have and will continue to reduce their contributions to current emission inventories by over 90%. Uncontrolled burning and a collection of small sources are the most significant sources of new dioxin emissions today. The characteristics of combustion that are associated with generation of PCDDs and PCDFs are fairly well understood and involve temperature, oxygen, and source material. Barrel burning of trash has been shown to be a significant contributor to annual dioxin emissions, representing a worst-case scenario for PCDD/PCDF generation as well as production of PCBs.[22] In fact, the emissions are several orders of magnitude higher than for controlled combustion in a modern incinerator. Certain metal refining and iron sintering processes also appear to lead to generation of dioxin. These contributions to a contemporary inventory will need to be assessed further.

The other classes of dioxinlike compounds, such as the PCBs/PBBs, PCNs, and PCABs/PCAOBs (polychlorinated azobenzenes/polychlorinated azoxybenzenes), are major industrial chemicals that have been produced commercially in large volumes. Production of PCBs was banned in the United States in 1977, but not in Russia until 1999. Use in closed systems continues, and many old transformers and capacitors are loaded with PCBs. The concentration of PCDFs, which are unwanted contaminants in the production of PCBs, increases upon heating, so that used PCBs are more heavily contaminated by PCDFs than are fresh samples. It is estimated that 70% of the PCBs ever produced are still in circulation.[23] PCDDs are not contaminants of PCBs, but can be present in polychlorinated benzene preparations. The PBBs were used as flame retardants and have largely been replaced by the polybrominated diphenyl ethers (PBDEs), which may have similar toxic as well as industrial properties. It is interesting to note that the PBBs were themselves contaminated with PBNs. The PCNs, commercially known as *halowaxes*, are still in use. The PCABs/PCAOBs are still key industrial intermediates used in closed systems.

5.4 FATE AND TRANSPORT IN THE ENVIRONMENT

The dioxinlike PHAHs are ubiquitous in the environment.[14] Much of this is due to their resistance to physical, chemical, and biological degradation. Once produced, these chemicals are largely distributed by means of atmospheric transport, either through volatilization or trapped on particles. They then settle out onto either the land or over water, where they are ingested by living organisms and subjected to bioaccumulation up the terrestrial or aquatic food chain, concentrating largely in the fatty portions of animals. Reentrainment of previously emitted dioxinlike PHAHs (e.g., from volatilization, windblown dust, or flooding of contaminated sediments) also contributes to the potential for exposure from old sources. This leads to recycling of PHAHs throughout the environment and the development of secondary or reservoir sources. The estimates of environmental releases are often presented in terms of TEQs. This is done for convenience in presenting summary information and to facilitate comparisons across sources. For purposes of environmental fate modeling, however, it is important to use the individual CDD/CDF and PCB congeners values rather than TEQs. This is because the physical/chemical properties of individual dioxin congeners vary and will behave differently in the environment. For example, the relative mix of congeners released from a stack cannot be assumed to remain constant during transport through the atmosphere and deposition to various media. There is also evidence of a decline in both emissions and environmental levels of dioxinlike PHAHs over time.

The most compelling supportive evidence of a general decline in environmental levels for CDD/CDFs and PCBs comes from dated sediment core studies. CDD/CDF and PCB concentrations in sediments began to increase

around the 1930s and continued to increase until about 1970. Decreases began in 1970 and have continued to the time of the most recent sediment samples (about 1990). Additionally, sediment studies in lakes located in several European countries have shown similar trends.

It is reasonable to assume that sediment core trends should be driven by a similar trend in emissions to the environment. The period of increase generally matches the time when a variety of industrial activities began rising, and the period of decline appears to correspond with growth in pollution abatement. Many of these abatement efforts should have resulted in decreases in dioxin emissions (i.e., elimination of most open burning, particulate controls on combustors, phase out of leaded gas, bans on PCBs, 2,4,5-T, and hexachlorophene, and restrictions on the use of pentachlorophenol). Also, the national source inventory described by the USEPA[14] documented a significant decline in emissions from the late 1980s to the mid-1990s.

5.5 HOW DOES EXPOSURE TO DIOXINS OCCUR?

The vast majority of exposure ($> 95\%$) occurs through the ingestion of minute amounts of dioxins in animal fat contained in food.[14] This includes meat, fish, and dairy. Small amounts of exposure occur through breathing ambient air or inadvertent ingestion of small amounts of soil. Exposure to this class of compounds through drinking water is usually minimal, due both to their low solubility and to their limited mobility to move through soil to reach sources of groundwater.

Exposure can be estimated by knowing the levels of dioxinlike compounds in environmental media (air, soil, water) and in food and the ranges of rates of contact or ingestion levels. A number of studies around the world have compiled these data and average and high-end values based on an increasing set of information have been estimated, although the studies were generally not designed to estimate national averages. The most comprehensive information comes from a few industrialized countries, including the United States. These data are discussed in detail in Chapters 2 and 16.

Food consumption accounts for a current intake of dioxins of approximately 1 pg $TEQ_{DFP\text{-}WHO98}$/kg body weight per day. This intake, along with previous daily exposures at somewhat higher levels, results in current levels in the general population in the United States of approximately 20 to 30 ppt TEQ in the serum on a lipid-adjusted basis. This is equivalent to a body burden of approximately 5 to 8 ng TEQ/kg. However, this is the average. USEPA[14] estimates that there are significant numbers of people (1 to 5%) within the background population who have levels two to three times higher than the mean. This is reflective of their consumption of two to three times more animal fat in their diet than for the average consumer. The mean and upper-bound values are consistent with observations of serum levels in limited studies of the general population.[13] These values are also similar to those seen in other industrialized

countries, such as those in western Europe. People in less developed countries tend to lower values, on average. However, because of localized sources of contamination, there are populations that have much higher concentrations. For example, the indigenous peoples of the Arctic, such as the Inuit in Greenland, have concentrations of PHAHs that are as much as 20 times, on average, that of people in southern Canada.[24] This is due largely to their subsistence dietary practices of eating sea mammals, such as whale, seal, and polar bear, which have high levels of contamination, due to their high position on the food chain.

It is quite possible that the major contributors of dioxin to food for the general population in industrialized countries may not be those sources that represent the largest fractions of total emissions. The geographic locations of sources relative to the areas from which much of the beef, pork, milk, and fish are produced are important to consider. In the United States, for instance, most of the agricultural areas that produce dietary animal fats are not located near or directly downwind of the major sources of dioxin and related compounds.[25]

Based on this observation, the contribution of reservoir sources to human exposure is likely to be significant.[14] Several factors support this finding. First, human exposure to the dioxinlike PCBs is thought to be derived almost completely from reservoir sources. Because approximately one-third of general population TEQ exposure is due to PCBs, at least one-third of the overall risk from dioxinlike compounds comes from reservoir sources. Second, CDD/CDF releases from soil via soil erosion and runoff to waterways appear to be greater than direct deposition to water from the primary sources included in the inventory. CDD/CDFs in waterways can bioaccumulate in fish, leading to human exposure via consumption of fish. This suggests that depending on consumption values for freshwater fish, a significant portion of the CDD/CDF TEQ exposure could be due to releases from the soil reservoir. Finally, soil reservoirs could have vapor and particulate releases that deposit on plants and enter the terrestrial food chain. The magnitude of this contribution, however, is unknown. Collectively, these three factors suggest that reservoirs are a significant source of current background TEQ exposure, perhaps contributing half or more of the total.[14]

As discussed earlier, background exposures to dioxinlike compounds may extend to levels at least three times higher than the mean. This upper range is assumed to result from the normal variability of diet and human behaviors. Exposures from local elevated sources or exposures resulting from unique diets would be in addition to this background variability. Such elevated exposures may occur in small segments of the population, such as people living near discrete local sources, which have resulted in higher-than-average levels of contamination. Nursing infants represent a special case. For a limited portion of their lives, these people may have elevated exposures to dioxin and related compounds on a body weight basis compared with nonnursing infants and adults. Intakes of dioxinlike PHAHs are also higher on a body weight basis by approximately a factor of 3 for young children as compared to adults.[14]

Dioxin contamination incidents involving the commercial food supply have occurred in the United States and other countries.[14] For example, in the United States, contaminated ball clay was used as an anticaking agent in soybean meal and resulted in elevated dioxin levels in some poultry and catfish. This incident, which occurred in 1998, involved less than 5% of the national poultry production and has since been eliminated. Elevated dioxin levels have also been observed in a few beef and dairy animals, where the contamination was associated with contact with pentachlorophenol-treated wood.[26] Evidence of this kind of elevated exposure was not detected in the national beef survey.[14] Consequently its occurrence is likely to be low, but it has not been determined. These incidents may have led to small increases in dioxin exposure to the general population. However, it is unlikely that such incidents have led to disproportionate exposures to populations living near where these incidents have occurred, because in the United States, meat and dairy products are highly distributed on a national scale. If contamination events were to occur in foods that are distributed predominantly on a local or regional scale, such events could lead to highly exposed local populations.

Elevated exposures associated with the workplace or industrial accidents have also been documented. U.S. workers in certain segments of the chemical industry had elevated levels of TCDD exposure, with some tissue measurements in the thousands of parts per thousand TCDD.[27,28] There is no clear evidence that similar, elevated exposures from occupational sources are currently occurring among U.S. workers. Documented examples of past exposures for other groups include certain Air Force personnel exposed to Agent Orange during the Vietnam conflict and people exposed as a result of industrial accidents in Europe and Asia.[14,29] The implications of the potential for higher exposures are discussed further below. These implications depend on the timing and duration of exposure, with regard both to windows of increased vulnerability and the impact on lifetime average body burdens.

5.6 EXPOSURE TO DOSE

Although the primary route of exposure to dioxinlike compounds for most people is through food consumption, the route of exposure has little influence on the response,[30] since the effects of dioxin (see below) involve multiple cells, tissues, and organs. TCDD is well absorbed via the pulmonary or gastrointestinal tracts. Dermal absorption is more limited, although it does occur slowly. Once absorbed into the body, there are three major determinants of the behavior of the dioxins: metabolism, lipophilicity, and hepatic sequestration. Metabolism is the major determinant of elimination, although some nonmetabolized dioxin may be excreted via transluminal passage across the gut wall.[31] The extreme persistence of the dioxins is due to their low rate of metabolism, thus limiting their detoxification and elimination. The dioxins are all highly lipophilic, resulting in their partitioning into fatty tissues. Animals

with a higher fat content will tend to retain the dioxins for longer times because there is a larger volume for their distribution. The third major determinant of their pharmacokinetic behavior is hepatic binding due to the dose-dependent induction of a hepatic binding protein, recently identified conclusively as CYP1A2.[32] The induction of this protein is under the control of the Ah receptor. This results in a shift in the relative concentrations of TCDD in the liver and adipose tissue as the dose of dioxin increases.

The persistence and bioaccumulating nature of the dioxins raises the issue of the appropriate dose metric. Pharmacology has long taught that the dose to the target tissue is the most relevant measure of dose. For dioxins, the tissue dose is a function of the past history of exposure as well as the current exposure. It is also known that for reversible responses, the response is associated with the tissue dose at the time the response is measured.[33] The same is true for developmental exposure,[34,35] where there are windows of susceptibility. Understanding the pharmacokinetic behavior of the dioxins, it is easy to correlate tissue dose with body burden. Since most of the dioxin is associated with the lipid fraction of the tissue (other than in the liver), if the dioxin concentration is expressed on a lipid-adjusted basis (i.e., normalized), the concentration in any tissue of the body, or of the entire organism, can be estimated if the lipid content is known. The development of physiologically based pharmacokinetic models also allows the prediction of body burdens/tissue concentrations at steady state if the daily exposure is known.[36] The pharmacokinetic models can also be used to predict what the daily exposure would be to result in given steady-state body burdens. The use of body burdens as the dose metric allows the incorporation of pharmacokinetic differences across species and between individuals. The USEPA[14] has recommended the use of body burden as a "default" dose metric, replacing previous approaches that used intake values, corrected to represent human equivalent dose.[37]

5.7 MODE OF ACTION OF DIOXINLIKE COMPOUNDS

The mode of action for all the effects of dioxin appears to involve interaction of dioxin, or related congeners, with a specific cellular protein, known as the Ah or dioxin receptor[38] (see Chapter 12). Support for the essential role of the Ah receptor comes from several lines of research. The first, already mentioned, is the robust structure–activity relationships in which there is a parallel relationship between the activity of a chemical and its ability to bind to the Ah receptor. Second, genetic studies have been conducted both using cell lines and in mice, demonstrating a clear role of the Ah receptor in dioxin's effects. Strains of mice exist that are relatively sensitive or resistant to the effects of TCDD. This differential sensitivity has been shown to segregate with the sensitive versus resistant alleles at the Ah locus.[39] As discussed in Ref. 14, more recent studies demonstrated that these alleles resulted in a protein with a single amino acid substitution in the ligand-binding domain of the Ah receptor. Cell lines

from mouse liver have been created that have low numbers of the Ah receptor and are less sensitive to the effects of dioxin. The most powerful evidence to date comes from the construction of mice in which the gene for the Ah receptor has been knocked out. Few, if any, adverse effects of TCDD have been observed in any of the three lines of transgenic mice created,[40–42] supporting the key role of this protein in all of the responses of organisms to TCDD. There is, however, limited and unconfirmed evidence for non-receptor-mediated responses from a few studies.

The Ah receptor is a member of the PAS superfamily of basic helix-loop-helix proteins which function as key regulatory molecules.[43] The PAS proteins are defined by the presence of a PAS (Per, AhR, ARNT, and Sim) domain, which is a stretch of 200 to 250 amino acids containing two 51-amino acid repeats which are involved in dimerization. Per and Sim are *Drosophila* proteins which are involved in circadian rhythms and neural differentiation, respectively. The *Drosophila* homolog of ARNT known as Tango, pairs with both Sim and Trachealess, another *Drosophila* PAS protein. Mammalian forms of Sim have been identified and are involved in the development of the nervous system. In addition, ARNT pairs with HIF-1, which is involved in response to hypoxia. Other mammalian PAS proteins identified, out of a rapidly expanding list, include the SRC-1 (steroid receptor coactivator-1), AIB-1 (amplified in breast cancer), EGAS (endothelial PAS proteins), several MOPS (mammalian orphan PAS proteins), NPAS 1,2 (neuronal PAS domain proteins), and AINT-1. This family of proteins is present throughout the animal kingdom, and related proteins even exist in plants. They all tend to function as heterodimers. The Ah receptor is the only known member of this class to require ligand binding for dimerization. However, it is interesting to note that this property of the Ah receptor exists only in vertebrates. In fact, much of the sequence of the Ah receptor is highly conserved, and homologous sequences, which do not bind TCDD, are found in invertebrates such as *Caenorhabditis elegans* and in *Neurospora* (see Chapter 14).

The best studied role of the Ah receptor is as a ligand-activated transcription factor controlling the expression of a battery of genes involved in biotransformation reactions.[44] In this function, the ligand bound form of the Ah receptor binds to one molecule of the related PAS protein, ARNT. (*Note:* There are at least two forms of ARNT. Also, ARNT appears to have the ability to heterodimerize with multiple partners, whereas the only ''partner'' of the AhR receptor appears to be ARNT.) The TCDD/AhR/ARNT complex then undergoes binding to specific sequences in the DNA, known as *dioxin response elements* [DREs, or *xenobiotic response elements* (XREs), or *aryl hydrocarbon response elements* (AhREs)]. This multimeric protein/DNA complex is then bound by additional coactivators or corepressors and interacts with multiple components of the transcriptional complex to enhance the expression of specific structural genes.

A second role of the Ah receptor is becoming recognized and involves protein–protein interactions. The non-ligand-bound form of the Ah receptor

does not exist free in the cell but is in fact bound to two molecules of heat shock protein 90. In addition, recent studies have indicated that another protein is also present in this multimeric protein complex. This molecule has been variously called p37, XAP2 (originally called p50 or p43), and ara 9. It appears to be related to the immunophilins. In addition, recent studies have indicated that at least in certain tissues, other regulatory proteins can be associated with the non-ligand-bound form of the Ah receptor. These include c-SRC, rel, and Rb. Mice deficient in c-SRC do not express the full spectrum of TCDD toxicity.[45] One of the early events of TCDD appears to be activation of the src-related tyrosine kinases, leading to enhanced phosphorylation of proteins. Rel is a key component of the NF-κ B complex, which controls apoptosis (programmed cell death). Rb, the retinoblastoma protein, is the key to control of the cell cycle. Thus, the Ah receptor may also play a key role in regulating signal transduction other than by its role as a transcriptional enhancer. Dioxin has long been known to increase protein phosphorylation[46–48] and to affect calcium signaling pathways,[49] key regulatory mechanisms in the cell. Thus, the Ah receptor appears to be necessary, but not sufficient for all the effects of the dioxins. It is a bHLH/PAS protein which appears to have at least two mechanisms of action. When bound to TCDD or a related congener, it acts as a ligand-activated transcription factor. When no ligand is present, it acts as a negative regulator of key regulatory proteins involved in tyrosine phosphorylation, cell cycling, and apoptosis. A third possibility is that the ligand-bound form of the receptor ties up ARNT, which is then not available to participate in its key role as a partner of other PAS proteins involved in differentiation, oxygen stress, and development.[50] This may, in fact, be the endogenous role of the AhR receptor, its role in control protein/protein interactions. The relatively subtle adverse effects seen in the Ah receptor knock-out mice, such as premature senescence, decreased fertility and viability, suggest a key role during developing and aging.[51] In addition, the pronounced circadian rhythmicity of the Ah receptor and of ARNT[52] suggest that there may be a role for these proteins in control of daily rhythms, as they are highly homologous to other clock proteins.

Given (1) that the Ah receptor, and its partner ARNT, are present in essentially all vertebrates[53]; (2) that the effects of dioxins are broad spectrum (multiple effects in both sexes of multiple species throughout the life span), and associated with alterations in differentiation and proliferation, homeostasis, and metabolism; (3) that most effects are seen in most species at some stage; (4) that a given species can be an outlier for any given response; and (5) that nearly all vertebrates examined, from fish through mammals, wildlife and laboratory animals, respond to dioxin, what about people? Human beings are an animal species. People have the Ah receptor, ARNT, and the other members of the Ah receptor signaling complex. Human cells and organs respond in culture to dioxins in a manner similar to that seen with other species. Biochemical responses, such as the induction of CYP1A1, a key enzyme involved in metabolism of many xenobiotics as well as steroid hormones, have been seen in

exposed people. Clearly, toxic effects such as chloracne have been seen in highly exposed people. Cancer has been associated with occupational exposures. More subtle effects have been suggested from studies within the general population. The real question is not can people respond to dioxins, but at what doses they respond with subtle or frank effects.

5.8 CHARACTERIZING THE EFFECTS OF DIOXINLIKE COMPOUNDS

Environmental contamination by the dioxinlike PHAHs has been clearly associated with adverse effects on various species of wildlife in different parts of the world. The disappearance of lake trout from Lake Ontario has been attributed to the contamination of the Great Lakes with dioxin. The lack of reproduction of wild mink around Lake Michigan may be due to the heavy contamination of their food supply, Great Lakes fish. Birth defects such as crossed bills and decreased hatching success of birds in the Great Lakes are likely due to the dioxinlike PHAHs. A major die-off of seals in the Baltic was suggested to be due to immunosuppression, resulting in massive infections by a distemperlike virus and was associated with the dioxins in their food supply.[54] Most of the effects on wildlife have involved developmental and/or reproductive effects and effects on the immune system. There is also a suggestion of increased incidence of cancer in Beluga whales in the St. Lawrence River. Adverse effects on domestic animals have also been reported, usually following poisoning episodes. Inadvertent contamination of cattle feed with PBBs in Michigan led to deaths of cows and other domestic animals. A PCB poisoning in Belgium, often called the Belgium dioxin poisoning, led to deaths of chicken. In Times Beach, Missouri, waste oil contaminated with dioxin that was spread on a horse arena led to the death of horses. Other domestic species, such as sheep, have also been affected. These purported ecological effects of exposure to dioxinlike compounds have heightened concern for and aid in the characterization of health risk for humans.

A large number of different species of laboratory animals have been shown to exhibit adverse effects to dioxin.[14] These range from fish to birds to mammals. Fish species include both freshwater and saltwater species. Poultry as well as birds of prey have been studied. Mice, rats, guinea pigs, hamsters, rabbits, and dogs have all been used in laboratory studies of dioxins. In addition, several species of nonhuman primates, including rhesus and squirrel monkeys and marmosets, have been examined in the laboratory for the adverse effects of dioxins. One of the key findings is that essentially all vertebrate species tested appear to be sensitive to some of the effects of dioxins. While a given species may be relatively resistant, or sensitive, to a given effect, in general all vertebrates are susceptible.[55] Dioxin is not the kind of toxicant that causes a single effect in one tissue of one sex at one developmental stage of one species of animal. Effects range from those that might be considered adaptive and represent biochemical alterations, to those that are clearly toxic. Biochemical changes

include induction of a battery of enzymes involved in metabolism of drugs and xenobiotics, as well as endogenous compounds. These include the phase I biotransformation enzymes, such as the cytochromes P450, specifically members of the CYP1A family (CYP1A1 and CYP1A2 in mammals) and CYP1B1. In addition, several phase II conjugation enzymes, such as specific isoforms of the glucuronyl transferases, glutathione transferases, and aldehyde dehydrogenases, as well as DT-diaphorase, are induced by dioxin. Other proteins involved in DNA synthesis and transcriptional control may also be induced.

The toxic effects of dioxin range from lethality at relatively high doses to multiple target organ system toxicity. Dioxin-induced death involves a delayed lethality, preceded by severe wasting. Time to death is species specific, ranging from approximately 2 weeks in guinea pigs to 6 or more weeks in monkeys.[30] At doses below those which produce severe wasting, atrophy of the gonads and lymphoid tissue is seen. In addition, both hyperplasia and metaplasia are seen in a number of tissues. For example, the ceruminous glands of the ear canal start to produce wax, as do the Meibomian glands lining the base of the eyelids. These effects demonstrate that dioxin can alter both proliferation and differentiation. Chloracne, a severe acneform condition that is often called the hallmark of dioxin toxicity in humans,[56] has also been seen in domestic animals, nonhuman primates, rabbits, and hairless mice. Chloracne is both a hyperkeratotic and hyperproliferative disorder, showing altered differentiation and proliferation.

Dioxins are potent endocrine disruptors, resulting in alterations in every endocrine system examined in at least some tissue of some species at some developmental stage.[57] This is true of both steroid and protein hormones. Dioxin can act at the level of the hormone receptor, the synthesis or breakdown of the hormone itself, or at the level of transport via the blood. For example, TCDD can upregulate expression of the glucocorticoid receptor in the developing palate,[58] but downregulate the same receptor in the liver.[59] Dioxin can decrease circulating melatonin levels by increasing the metabolism of this hormone. In addition to disturbing multiple components of the traditional endocrine systems, dioxins perturb multiple growth factor systems, including vitamin A and other retinoids (both metabolism and receptors), TGFs and the EGF receptor, and multiple cytokines (e.g., IL-1, IGF-1R).[30,55]

Dioxins are also potent developmental and reproductive toxicants[60] (see Chapter 19). In fact, dioxins are developmentally toxic in every animal species examined.[61] In most, cases, dioxins cause thymic atrophy and fetotoxicity. In some species, such as the mouse, dioxins induce a distinct syndrome of developmental malformations, including hydronephrosis and cleft palate. These effects occur at doses well below those where any maternal toxicity, or overt fetoxicity, are observed. In other species, these terata are not observed except at fetotoxic doses. However, recent studies have demonstrated that low dose exposure to the dam can result in permanent alterations in the developing pup.[62] These effects include permanent decrease in sperm count in the male offspring[63,64] and structural alterations of the external genitalia in the female

pups.[65] Changes have also been reported in the male sex accessory glands,[66] in the core body temperature,[67] and in the developing immune system.[68,69] In fact, the immune system is a key target of dioxin in multiple species (see Chapter 11). Immunosuppression, especially the reduced ability to mount a primary antibody response, has been seen at low doses in mice and nonhuman primates. Both T and B lymphocytes have been affected. Other studies have indicated a shift from the humoral antibody response to the generation of autoantibodies. Enhanced mortality from influenza virus has been observed at extremely low doses.[70] Developmental exposure also appears to target the nervous system, affecting learning, hearing, and behavior.[14] The adult nervous system appears relatively resistant to the toxic effects of dioxins.

The liver is a common target organ for the adverse effects of dioxins.[30] Hypertrophy is seen in many species in association not only with the induction of metabolizing enzymes, but with the change in lipid metabolism, leading to a fatty liver. Both necrosis and apoptosis have been reported in hepatocytes following dioxin exposure. Increases in hepatic porphyrins have been associated with an induction of enzymes involved in pathways of heme metabolism. The cardiovascular system also appears to be a target in several species, especially nonmammalian animals, in which the effects on the vasculature are preeminent. High doses of dioxin in guinea pigs have also been reported to affect heart rhythms, although animals are clearly showing other effects of dioxin toxicity.[71] Dioxins are also carcinogens in every animal tested: mice, rat, hamsters, fish (see Chapter 11). Mechanistically, dioxins appear to be tumor promoters, in that TCDD and related congeners are not directly mutagenic and do bind to DNA. Dioxins have been shown to be tumor promoters in multiple in vitro systems and in the liver, lung, and skin.[14]

Thus, the effects of TCDD and related chemicals are not limited to a single tissue or species. These related chemicals induce multiple effects in multiple tissues at multiple developmental stages of both sexes of multiple species throughout the vertebrate kingdom. Molecular changes lead to biochemical alterations. Induction, or repression, of metabolism leads to cellular effects. Alterations in proliferation and differentiation results in effects at the levels of the tissues and organs. And changes in hormones and growth factors lead to alterations in homeostasis which can eventually lead to overt toxicity, wasting, and death.

What effects have been seen in people that have also been seen in animals? The best described is chloracne. However, this is a relatively high-dose response. There also appears to be differential susceptibility among people, so that some respond at the same body burden, whereas others do not.[55] This is in agreement with the defined genetic polymorphism that has been described in mice. Mice must be homozygous at the hr locus, the allele associated with hairlessness, in order for them to respond to dioxin with chloracne. Chloracne has not only been seen in children and adults following high levels of exposure, such as have occurred occupationally, but has also been observed in infants who were exposed in utero.[72] In fact, a syndrome of ectodermal dysplasia has

been described in these prenatally exposed children. Not only did they have acneform eruptions, they also had altered pigmentation and problems with their teeth and nails. Porphyria has also been reported in highly exposed people as well as rats and mice. However, rodent studies have led us to suggest that females might be more sensitive, and the response is enhanced by the presence of additional dietary iron. Recent studies in an occupational cohort have shown that elevated urinary porphyrins are associated with exposure to dioxins in combination with nondioxinlike PCBs.[73] Similar synergism has been seen in rats and mice[18,19] and appears to involve multiple mechanisms that have an impact on the heme biosynthetic pathway: induction of uroporphyrin decarboxylase and CYP1A2 by dioxins, and increased entry into the pathway by means of the induction of aminolevulenic acid synthetase by the nondioxin-like PCBs. Cardiovascular disease has also be seen in several human epidemiological studies, with an increase in ischemic heart disease.[36] There are also indications of alterations in lipid metabolism. Similar effects have been seen in several animal studies.

Induction of enzymes involved in biotransformation of drugs, endogenous compounds, and xenobiotics is one of the most common and best studied effects of TCDD and related compounds.[14] The mechanism for induction of cytochrome P4501A1 has been studied in great detail, both in vivo, in cultured cells, and in isolated constructs. Much of the understanding of the molecular mechanisms involved in the transcriptional activator function of the ligand-bound Ah receptor comes from studies of CYP1A1 induction. These mechanisms are common among fish, birds, laboratory animals, and people. The body burdens associated with expression of CYP1A1 in extrahepatic tissues (CYP1A1 is essentially noninducible in vivo in human liver; it can be induced in vitro in human hepatocytes in culture) is similar to that required in rodents for induction. Similarly, CYP1A2, the inducible hepatic binding protein that results in sequestration of dioxins in the liver, is induced in parallel in humans and in rodents. Recent studies with a third cytochrome P450, which is under Ah receptor control, have shown that the induction of CYP1B1 appears similar across species. Less in vivo work has been carried out with other enzymes in this Ah receptor–controlled gene battery. However, in vitro studies have demonstrated that humans have the other enzymes involved in phase II metabolism and that their inducibility is controlled similarly to that of rodents.

The EGF receptor is a key member in a major mitogenic signaling pathway. In animal studies, dioxins have been shown to downregulate the expression of the EGF receptor in the liver, prostate, and placenta.[14] In contrast, it appears to increase the expression of this protein in the developing palate and urinary tract. This regulation is at the level of gene transcription. It is interesting that the decrease in the EGF receptor has been seen in human placentas from the Yucheng rice oil poisoning episode. In contrast, and in further agreement with the animal data, organ cultures of human embryonic palate demonstrate a similar increase in EGF receptors in response to TCDD as do cultured mouse and rat palates, both in vivo and in vitro.

Recent studies have demonstrated an association of elevated levels of dioxins with diabetes in several human populations.[74] This association was first noted in the early 1990s when a decrease in glucose tolerance was seen in the NIOSH cohort.[75] This was followed by a report of an increase in diabetes in the Ranch Hand cohort.[76,77] An increase in diabetes in other occupational cohorts,[78,79] as well as the Seveso population[80] (see Chapter 20 for details of the Seveso incident) has also been reported. There was not a significant increase in diabetes in the NIOSH mortality study, although 6 of the 10 most highly exposed workers did have diabetes.[81] However, it is well understood that mortality studies are limited in their ability to assess risk from diabetes mellitus. A paper by Longnecker and Michalek[77] found a pattern suggesting that low levels of dioxin may influence the prevalence of diabetes. However, these results did not show an exposure–response relationship. Because it is the only study of its type to have been published, additional population-based studies are warranted to validate its findings. The most recent update of the Ranch Hand study shows a 47% excess of diabetes in the most heavily exposed group of veterans.[76]

Most of the data suggest that the diabetes is type II, or adult-onset diabetes, rather than insulin dependent, or type I. Aging and obesity are the key risk factors for type II diabetes. However, dioxins may shift the distribution of sensitivity, putting people at risk at younger ages or with less weight. Dioxin alters lipid metabolism in multiple species, including humans.[75,82] Dioxin also alters glucose uptake into both human and animal cells in culture.[45,83] Mechanistic studies have demonstrated that dioxin affects glucose transport,[45] a property under the control of the hypoxia response pathway.[84] A key regulatory protein in this pathway is the partner of the AhR, ARNT (also known as HIF-1β).[44,85] Activation of the AhR by dioxin may compete with other pathways, such as the hypoxia-inducible factor (HIF) pathway, for ARNT.[86] Dioxin has also been shown to downregulate the insulin growth factor receptor.[87] These three issues—altered lipid metabolism, altered glucose transport, and alterations in the insulin signaling pathway—all provide biological plausibility to the association of dioxins with diabetes.

Effects on the endocrine-related disorders have also been observed in other hormone systems of people.[14] In both an occupational cohort and in the Ranch Hand veterans, a decrease in circulating testosterone levels have been observed. Although this decrease is slight and the men are still within the normal range of testosterone concentrations, it raises the issue of effects on a population basis and whether more men would be at risk of having low testosterone in association with elevated dioxins. In contrast to the studies in people, acute exposure to experimental animals has only shown a decrease in circulating androgens at relatively high levels of exposure. In both people and animals, however, thyroid homeostasis has been shown to be perturbed. Exposure to dioxins results in decreases in circulating thyroxine (T4) levels in rodents. This effect is probably due to the induction of a specific isozyme of glucuronyl transferase which conjugates thyroxine with glucuronic acid and enhances its rate of elimination. The

same enzyme has also been shown to exist in people. Prenatal exposure has been shown to cause decreases in circulating T4 levels in human infants shortly after birth, accompanied by an elevation in thyroid-stimulating hormone (TSH).[88] Whereas the decrease in T4 disappears after several months, the rise in TSH persists, suggesting continued insult of the thyroid system. Elevations of maternal TSH, but still within the normal range, have recently been shown to be associated with decreases in IQ scores in the children. Increases in TSH have recently been shown in association with elevated dioxin in the Ranch Hand cohort.

The other endocrine-related disorder that has recently been associated with dioxin exposure in humans is endometriosis. Endometriosis appears to involve localized production of estrogens and alterations in the immune system. The incidence has been increasing and the age of onset decreasing. Epidemiological studies in Europe first suggested the possible association between elevated organochlorine levels in women and endometriosis. TCDD was shown to increase both the incidence and severity of spontaneous endometriosis in rhesus monkeys in a dose-related manner.[89] Recent studies with surgically induced endometriosis in cynomologus monkeys have also seen an increase in endometriosis.[90] TCDD has also been shown to promote the growth of surgically induced lesions in both rats and mice.[91] This effect appears to be mediated by the AhR based on a preliminary structure/activity study.[92] Prenatal exposure of mice to TCDD results in enhanced sensitivity to promotion of surgically induced endometriosis in the adult offspring by TCDD.[93] Growth of human endometrial cells injected into nude mice is enhanced by dioxin.[94] Since dioxin induces IL-1β and TNFα, both of which have been shown to be induced in endometriosis, the relationship between TCDD and endometriosis is biologically plausible. Recent cohort studies involving surgically diagnosed endometriosis and dioxins have been seen in women in Israel[95] and Belgium.[96] However, future studies are needed to confirm this relationship.

Effects on the immune system, an extremely sensitive target in many species of animals, have not been observed clearly in adult humans. Some changes have been seen in an occupational cohort in Germany in different types of white blood cells in highly exposed men. However, most of the responses that are targets in adult mice and nonhuman primates have not been examined in people. Suppression of the primary antibody response is a key marker of dioxin-induced immune suppression. Recent data following prenatal exposure demonstrates that children whose mothers are at the high end of the background distribution have lower antibody titers following vaccination than do those whose mothers were less exposed. These children also have an increased incidence of otitis (ear infections), and a higher incidence of chickenpox.[97] Increases in respiratory disease and otitis have also been reported in more highly exposed populations. In addition, persistent changes in lymphocyte subsets have been reported in the children. The sensitivity of the developing immune system to perturbation has also been observed not only in mice and nonhuman primates which are also sensitive as adults, but in rats in which the

adults are relatively resistant, but the developing rat is extremely sensitive to an immune insult following low doses perinatally.[98]

In fact, the developing organism appears to be especially susceptible to the adverse effects of dioxin exposure. Low-dose exposure to rodents is associated with permanent effects on both the male and female reproductive system, the immune system (see above), and the central nervous system. Studies have shown that prenatal exposure to low doses of dioxin results in permanent suppression of the core body temperature in the offspring.[65] This is controlled by a set point in the hypothalamus. Effects on the developing male reproductive system have been seen in rats, hamsters, and mice and include a permanent decrease in sperm count as well as changes in the development of the male sex accessory glands, such as the prostate and seminal vesicle. Adverse effects on the developing female offspring include actual malformations of the external genitalia (vaginal thread and cleft phallus) as well as premature reproductive senescence. In addition, prenatal exposure in mice appears to sensitize the female offspring to dioxin-mediated surgically induced endometriosis in the adult. In humans, recent studies from Seveso have indicated a change in sex ratio in the most heavily exposed families, with the number of girls being born exceeding the number of boys.[99] Particularly intriguing in this evaluation is the observation that exposure before and during puberty is linked to this sex ratio effect. In the Yucheng cohort, not only are the children small for their age and may be immune compromised, the boys do not develop normally at puberty and have small penises. Recent reports indicate that the morphology and motility of their sperm are also abnormal.[100]

Among the endpoints of greatest concern are those involving developmental neurotoxicity. Several populations have demonstrated decreased neurooptimality, decreased psychomotor ability, cognitive deficits, and behavioral alterations in children exposed prenatally to mixtures of dioxins and PCBs. These effects have been seen in the children involved in the Asian rice oil poisonings as well as in background populations in the United States, the Netherlands, and Japan. A 4-point IQ deficit was detected in 42-month-old Dutch children in association with their mother's blood levels.[101] In the United States, a 6-point IQ deficit was present at 11 years of age in children whose mothers were at the high end of PCB concentrations in the general population.[102] It is unclear whether these learning effects are due to dioxins, PCBs, or the combination of both Ah receptor–mediated and non-Ah-mediated effects. What is important to note is that PCBs never occur without dioxinlike PCBs. The converse can be true at high exposure scenarios, such as in the Seveso cohort, which involves only TCDD superimposed upon the general dioxin/PCB background. Hearing deficits were also noted in the Yucheng population.[103]

Although noncancer effects have been detected at the high end of the background population, an increase in cancer has not been observed at background exposure levels. This is expected, however, given what is predicted regarding potency and the power of epidemiologic studies to detect an effect when background cancers are high. However, dioxin does appear to have the potential

to cause cancer in people as well as in experimental animals. In 1997, the International Agency for Research on Cancer[29] reached consensus that TCDD be considered as a known human carcinogen. The USEPA[14] and the U.S. Department of Health and Human Services[104] followed suit, with similar descriptive conclusions based on their respective criteria. This was based on clear evidence of animal carcinogenicity in all four animal species tested (rats, mice, hamsters, *Medaka*). Dioxin was positive in both sexes and in multiple tissues. The evidence on human carcinogenicity was considered limited at that time. In the past few years, additional positive studies of both occupational cohorts and the Seveso population, including women, have been published, and the evidence of a dose–response relationship strengthened.[14,105–108] The consensus of "known human carcinogen" was also based on a mechanistic understanding of the common role of the Ah receptor in people and animals and the fact that dioxin is a clear tumor promoter in animals, in line with the increase in all cancers seen in the highly exposed occupational cohorts. Lung cancer has also been seen in several populations. Other sites that have been suggested include soft tissue sarcoma, non-Hodgkins's lymphoma, breast cancer, and the gastrointestinal tract.

5.9 DOSE–RESPONSE RELATIONSHIPS

The key question in the risk characterization of dioxin is one of dose. Since body burden is the most appropriate dose metric to compare across species, in order to account for pharmacokinetic differences as well as the persistent and bioaccumulative nature of the dioxins, it is helpful to examine the body burdens (in ng/kg body weight) associated with effects.[14,30] Clearly, adverse effects have been seen with body burdens in the range 10 to 100 ng TCDD/kg. In adult animals, endometriosis has been associated with body burdens of approximately 40 ng/kg in rhesus monkeys, while enhanced mortality due to influenza virus was seen in mice with body burdens of less than 10 ng/kg. Developmental effects include learning deficits in monkeys associated with maternal body burdens of about 40 ng/kg, decreased sperm counts, and genetic malformations in pups from about 30 to 100 ng/kg in rodents, and permanent immune suppression at approximately 50 ng/kg in the dam. Biochemical responses occur at body burdens that are an order of magnitude lower than those which are frankly adverse. Induction of mRNA for CYP1A1, down-regulation of mRNA for the EGF receptor, and measures of oxidative stress have been detected in body burdens in rodents of 3 ng/kg. Induction of mRNA for CYP1A2 and IL-1β have been seen at 10 ng/kg. Increases in enzymatic activity of CYP1A1 and 1A2 have been measured at incremental increases of 2 ng/kg above the usual body background, which has been measured at approximately 4 ng TEQ/kg in age-matched mice (about 5 months of age).[109] Another study[110] has suggested neurobehavioral impacts on adult rats exposed perinatally at levels that yield body burden ED_{01} values below current average

human body burdens and as low as the lowest noncancer effects previously evaluated.

To model the dose–response relationships, body burden is used, as this is a more appropriate dose metric than daily dose. This accounts for the great difference in half-life between species, and should be used for any persistent bioaccumulative toxicant. In a recent effort to fit the data for over 50 studies to either a linear or a nonlinear model, about half of the studies appeared linear and the other half were best fit by a nonlinear model.[14] The majority of the biochemical responses appeared to be linear. Most of the toxic responses were better fit by a nonlinear model; however, nearly 40% of the adverse effects appeared to have a linear dose–response relationship. Comparisons across multiple endpoints, multiple species, and multiple experimental protocols are too complicated to be made on the basis of the full dose–response curve. Comparisons of this sort can be made either by choosing a given exposure and comparing the responses or by choosing a particular response level and comparing the associated exposures. The USEPA[14] used a benchmark dose approach in the modeling in order to incorporate all of the available information. An effective dose to attain a 1% response (ED_{01}) was used as the point of comparison. The ED_{01} was either within or close to the experimental data. Thus, the dose associated with a 1% response rate was usually an interpolation of the available data, not an extrapolation. For the noncancer effects in animals, empirical modeling resulted in the lowest ED_{01} values, ranging between burdens of 1 and 11 ng/kg. Empirical dose–response modeling attempts to find a simple mathematical model that adequately describes the pattern of the data available and allows comparisons across individual data sets. In contrast to empirical modeling, mechanism-based modeling attempts to use an understanding of the mechanistic relationship between exposure and multiple endpoints to describe the observed response simultaneously. Mechanism-based modeling can be a powerful tool for understanding and combining information on complex biological phenomena.[111] Development of a mechanistically based model suggested the lowest ED_{01} at 0.2 ng/kg. ED_{01} values in the range 1 to 10 ng/kg are in agreement with the LOAEL values observed for biochemical effects (see above). Empirical modeling of the cancer data in animals resulted in an ED_{01} ranging from 14 to 1190 ng/kg (most estimates were in the range 14 to 500 ng/kg). Mechanistic modeling of the liver tumor response in female Sprague–Dawley rats resulted in an ED_{01} value of 2.7 ng/kg. The results from animal modeling are in excellent agreement with the estimated ED_{01} values from a log-linear fit of human occupational data of 6 to 62 ng/kg.[13]

Assuming that the average body burden in the general population of the developed world is approximately 5 ng TEQ/kg, what do the modeling results mean? Then the excess risk of cancer to the background population may exceed 1/1000. This analysis is based both on the liver tumors in female rats and the increase in all cancers in exposed workers.

It is not necessary to use mathematical models to characterize the risk to dioxin and related chemicals. One approach that can be helpful in decision

making is determination of the *margin of exposure* (MOE), defined as the ratio of the exposure dose of interest divided by the dose associated with a sensitive effect. For the dioxins, the dose is expressed as the body burden. The average body burden is approximately 5 ng TEQ/kg body weight. However, approximately 5% of the population has body burdens twice this concentration, and 1% have body burdens of 15 ng/kg.[13] Biochemical effects (e.g., enzyme induction, cytokine induction, oxidative stress) have been observed in experimental animals between 3 and 10 ng/kg. Developmental neurotoxicity, reproductive toxicity, and immunotoxicity have been seen between 10 and 100 ng/kg. Adult reproductive and immunological effects have been seen at body burdens between 10 and 50 ng/kg. Thus, clearly adverse responses in animals are seen within an order of magnitude of the current mean background body burden in people. Cancer has been seen in people with body burdens between 10 and 100 times those of the background population. However, type II diabetes and alterations in glucose tolerance and insulin metabolism have been associated with dioxin levels within a factor of 10 of the general population. Several different cohort studies of children have indicated that developmental neurotoxicity, immunotoxicity, and hormonal effects are occurring within the high end of the background populations.

5.10 CONCLUSIONS

Releases of dioxins to the environment from sources that have been characterized have decreased significantly over the last decade and are expected to continue to decrease. Other sources are still poorly characterized, and an environmental reservoir of dioxins from both humanmade and natural sources has been recognized. Human body burdens have also declined, but their relationship to contemporary sources or reservoirs is uncertain.

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (dioxin) is highly toxic to many animal species, producing a variety of noncancer and cancer effects. Other 2,3,7,8-substituted polychlorinated dibenzo-*p*-dioxins and dibenzofurans, and coplanar polychlorinated biphenyls (PCBs), exhibit similar effects albeit at different doses and with different degrees of confidence in the database. The similarities in toxicity between species and across different dioxin congeners stem from a common mode of action via initial binding to the aryl hydrocarbon (Ah) receptor. This common mode of action is supported by consistency in effects evident from multiple congener databases, although uncertainty remains, due to data gaps for some congeners. The databases supportive of dioxinlike toxicity, both cancer and noncancer, are strongest for those congeners that are the major contributors to the risk to human populations. This has led to an international scientific consensus that it is prudent science policy to use the concept of toxic equivalency factors (TEFs) to sum the contributions of individual PCDD, PCDF, and coplanar PCB congeners with dioxinlike activity. In addressing receptor-mediated responses resulting from complex mixtures of

dioxinlike congeners, this assessment has provided a basis for the use of integrated measures of dose, such as average lifetime body burden, as more appropriate default metrics than average lifetime daily intake. While average body burdens over a lifetime appear to be the most useful dose metric for chronic effects, average body burden during the window of sensitivity may be the most appropriate metric for developmental effects. In fact, the final choice of the appropriate metric may depend on the endpoint under evaluation.

Dioxin and related compounds have been shown in multiple animal species to be developmental, reproductive, immunological, endocrinological, and cancer hazards, among others. There is no reason to expect, in general, that humans would not be similarly affected at some dose, and indeed there is a growing body of data supporting this assumption. Based on the animal data, current margins of exposure are low, especially for more highly exposed human populations. The human database supporting this concern for potential effects near background body burdens is less certain. Occupational and accidentally exposed cohorts exposed at higher levels show correlations with exposure for cancer and a number of noncancer effects, consistent with those seen in the animal studies.

For cancer outcomes, the epidemiological evidence provides consistent findings of statistically significant elevations, with some showing dose–response trends for all cancers combined and lung cancer risk in occupational cohorts, along with evidence of possible additional tissue-specific cancer rate elevations. Given this substantial, yet still nondefinitive epidemiological data, the positive cancer bioassays at multiple sites and in all animal species tested, and mechanistic considerations common to animals and humans for dioxin carcinogenicity, USEPA characterizes 2,3,7,8-tetrachlorodibenzo-*p*-dioxin as ''carcinogenic to humans.'' On the basis of similarities of response in animal and mode of action studies and consistent with the concept of toxicity equivalence, complex mixtures of dioxin and related compounds are considered highly potent, ''likely'' carcinogens. The calculated body burdens of dioxin and dioxinlike substances leading to an estimated 1% increase (ED_{01}) in the lifetime risk of cancer in the two occupational studies with the best exposure information fall within a 10-fold range, and those calculated from the animal bioassay data fall slightly above that range. The ED_{01} values for all cancers combined from the two occupational cohorts range from 2 to 20 ng TCDD/kg body weight, depending on the study and the model used. By comparison, current background body burdens in the United States are approximately 5 ng TEQ/kg body weight, suggesting little to no margin of exposure at today's body burden levels. From these same occupational and animal cancer studies, EPA estimates that an upper bound on the lifetime risk of all cancers combined might exceed 1×10^{-3} pg TEQ/kg per day. This cancer slope factor is based on a statistical estimate of risks from occupational exposures, principally to healthy adult male workers and must be coupled with a recognition that a small number of people may be both more susceptible and consume up to three times the average level of fat per day (the principal exposure pathway for dioxins in the general popu-

lation). Using best available estimates of cancer risks, the upper bound on general population lifetime risk for all cancers might be on the order of 1 in 1000 or more. Upper-bound risk estimates allow calculation of the high end of the probability of cancer risk in the population. This means that there is greater than a 95% chance that cancer risks will be less than the upper bound and could be as low as zero in some people.

For the characterization of noncancer effects, USEPA generally calculates a reference dose (RfD/RfC) value that represents an estimate (with uncertainty spanning perhaps an order of magnitude) of a daily exposure to the human population (including sensitive subgroups) that is likely to be without an appreciable risk of deleterious effects during a lifetime. The current estimated average dose to the U.S. population (about 1 pg TEQ/kg per day) is greater than RfD/RfC dose values that might be calculated given the data reviewed in this characterization, and therefore RfD/RfC values would be uninformative for safety assessment. EPA has chosen rather to characterize the margins of exposure (MOEs) for noncancer endpoints in order to inform risk management decisions. MOE is the ratio of the human body burden to the effect level in the comparison species (ED_{01} or low effect level), animal or human. For the most sensitive endpoints identified, MOEs range from, for example, less than 1 for enzyme induction in mice, through 2.6 to 15 for enzyme induction in rats, less than 3 for developmental effects, and 5 for endometriosis in nonhuman primates. In evaluating MOEs, consideration should be given to uncertainties in distinguishing between adaptive biochemical changes and adverse effects, on both an individual level and as these changes affect the entire populations. Children's risks from dioxin and related compounds may be greater than for adults, but more data are needed to address this issue fully.

These results suggest that there is little or no margin of exposure. Body burdens in the general population are at or near the concentration where effects might be expected to occur. WHO[112] and other international organizations have concluded that current intakes are tolerable, but suggested that all efforts should be made to continue to reduce exposures. However, environmental concentrations and body burdens have been decreasing over the past few decades, from their high in the late 1960s–early 1970s. Only 10 years ago, the estimated average body burden was approximately 10 ng TEQ/kg, as opposed to the data from the present time. The decrease in body burdens is a reflection of the decrease in exposure resulting from a decline in emissions to the environment. Regulation of the major sources of this unwanted contamination in the past, including chlorine bleaching, chlorinated herbicide and biocide production, and incineration, has led to large declines in these sources. Similarly, bans on the production of PCBs has led to decreases in PCB emissions. Thus, the regulatory agendas are successfully reducing the sources, emissions, and exposures to this class of chemicals. Although many aspects of the characterization of the health risks of dioxinlike chemicals remain controversial, several international and national advisory groups have concluded their reviews of dioxin sources, exposure, and toxicity with similar recommendations: Continue

efforts to further reduce exposures and reevaluate the situation periodically as new information becomes available. This seems like prudent advice.

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CHAPTER 6

Pharmacokinetics of Dioxins and Related Chemicals

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6.1 INTRODUCTION

Polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and polychlorinated biphenyls (PCBs) are structurally and toxicologically similar persistent environmental contaminants. PCDDs and PCDFs are formed as by-products in various chemical and combustion processes, while PCBs were produced for a wide range of commercial applications. These agents are now global environmental contaminants. The environmental persistence and lipophilic properties of these compounds have lead to effective transport of these chemicals into the food chain, with pronounced accumulation at higher trophic levels, including humans. As a result, there is concern regarding possible adverse effects of these contaminants on human health and the environment.

PCDDs, PCDFs, and PCBs elicit a broad spectrum of congener/isomer-, species-, and tissue-specific biological and toxicological responses, with the induction of hepatic cytochrome P450 (CYP) 1A1, 1A2, and 1B1 and extrahepatic CYP 1A1 and 1B1 representing some of the most sensitive responses associated with exposure to these compounds. Chlorine substitution of PCDDs and PCDFs at the 2, 3, 7, and 8 positions is generally considered necessary for dioxinlike activity, with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD; referred to herein simply as TCDD) representing the most potent and extensively studied of these compounds. Dioxinlike PCBs include the coplanar PCBs, which have no chlorine substitution at ortho (2,6,2′,6′) positions. The coplanar PCBs include PCB 77 (3,3′,4,4′-chlorine substituted), PCB 81 (3,4,4′,5), PCB 126 (3,3′,4,4′,5), and PCB 169 (3,3′,4,4′,5,5′). The congener-

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dependent binding of 2,3,7,8-substituted PCDDs and PCDFs and coplanar PCBs to the cytosolic Ah receptor (aryl hydrocarbon or dioxin receptor) is generally considered the initial event necessary for the expression of the dioxin-like activity of these compounds. Congener-specific affinity of PCDDs, PCDFs, and coplanar PCBs for the Ah receptor and congener-specific pharmacokinetics (absorption, metabolism, distribution, excretion) are two major factors that contribute to the relative in vivo potency of a given dioxinlike compound in a given species.

The pharmacokinetics of PCDDs, PCDFs, and coplanar PCBs is congener-, dose-, and species-specific, with urinary and biliary excretion being dependent on the metabolism of these compounds. For dioxinlike PCDDs, PCDFs, and coplanar PCBs, it is generally considered that the parent compounds are the causal agents, with metabolism and subsequent elimination of these compounds representing a detoxification process. In this respect, pharmacokinetics plays a significant role in determining the overall toxicity of these compounds. The disposition and pharmacokinetics of TCDD and related compounds have been investigated in several species and under various exposure conditions. There are several reviews on this subject that focus on TCDD and related halogenated aromatic hydrocarbons.[1–5] In this chapter we review the disposition and pharmacokinetics of these agents in humans and selected laboratory animals and identify congener- and species-specific factors that may have an impact on the dose-related biological responses of these compounds.

6.2 ABSORPTION AND BIOAVAILABILITY FOLLOWING EXPOSURE

The gastrointestinal, dermal, and transpulmonary absorption of these compounds are discussed herein because they represent potential routes for human exposure to this class of persistent environmental contaminants.

6.2.1 Absorption Following Oral Exposure

A major source of human exposure to TCDD and related compounds is thought to be through diet.[6,7] Experimentally, these compounds are commonly administered in the diet or by gavage in an oil vehicle. Gastrointestinal absorption is usually estimated as the difference between the administered dose (100%) and the fraction of the dose that was not absorbed. The unabsorbed fraction is estimated as the recovery of parent compound in feces within 24 to 48 h of a single oral exposure by gavage.

In Sprague–Dawley rats given a single oral dose of 1.0 μg [^{14}C]TCDD/kg body weight in acetone : corn oil (1 : 25, v/v), the fraction absorbed ranged from 66 to 93%, with a mean of 84%.[8] With repeated oral dosing of rats at 0.1 or 1.0 μg/kg per day (5 days/week for 7 weeks), gastrointestinal absorption of TCDD was observed to be approximately that observed following a single oral expo-

sure.[8] Oral exposure of Sprague–Dawley rats to a larger dose of TCDD in acetone:corn oil (50 μg/kg) resulted in an average absorption of 70% of the dose administered.[9]

Absorption was also investigated in the guinea pig and hamster, the species most sensitive and most resistant to the acute lethality of TCDD, respectively. TCDD in corn oil was generally well absorbed from both species, with 50 and 75% of the dose absorbed from the guinea pig and hamster, respectively.[9,10] Poiger and Schlatter[11] investigated the absorption of TCDD in a 42-year-old man after ingestion of 105 ng [^{3}H]TCDD (1.14 ng/kg body weight) in 6 mL of corn oil and found that more than 87% of the oral dose was absorbed from the gastrointestinal tract. The results from several species suggest that TCDD is effectively absorbed following oral exposure in an oil vehicle.

The relative absorbed dose or bioavailability of the brominated analog of TCDD, 2,3,7,8-tetrabromodibenzodioxin (2,3,7,8-TBDD), was estimated in the rat at 78, 82, 60, and 47% after a single oral exposure at 0.001, 0.01, 0.1, and 0.5 μmol/kg, respectively. These results suggest nonlinear absorption at the higher doses, with maximal oral absorption at an exposure of $\leq$ 0.01 μmol/kg (5 μg/kg).[12]

The absorption of 2,3,7,8-tetrachlorodibenzofuran (TCDF) has been investigated after oral exposure by gavage. Approximately 90% of the dose administered (0.1 and 1.0 μmol/kg) of 2,3,7,8-TCDF in Emulphor:ethanol (1:1) was absorbed in male Fischer 344 rats.[13] [Emulphor EL-620 is a polyethoxylated vegetable oil preparation (GAF Corp., New York, NY)]. Similarly, more than 90% of the dose administered (0.2 μmol/kg, 6 μg/kg, and 1 to 15 μg/kg) of 2,3,7,8-TCDF in Emulphor:ethanol:water (1:1:8) was absorbed in male Hartley guinea pigs.[14,15] Thus, 2,3,7,8-TCDF appears to be absorbed almost completely from the gastrointestinal tract. This may be related to the greater relative solubility of 2,3,7,8-TCDF compared to that of TCDD or 2,3,7,8-TBDD.

The oral bioavailability of 2,3,4,7,8-penta-CDF (2,3,4,7,8-pentachlorodibenzofuran) in corn oil is similar to that of TCDD.[16] Furthermore, 2,3,4,7,8-penta-CDF absorption was independent of the dose (0.1, 0.5, or 1.0 μmol/kg). Incomplete and variable absorption of 1,2,3,7,8-penta-CDD in corn oil was reported in rats, with 19 to 71% of the dose absorbed within the first 2 days after oral exposure.[17] Birnbaum and Couture[18] found that the gastrointestinal absorption of octachlorodibenzodioxin (OCDD) in rats was very limited, ranging from 2 to 15% of the administered dose. Lower doses (50 μg/kg) in an *o*-dichlorobenzene:corn oil (1:1) vehicle were found to give the best oral bioavailability for this extremely insoluble compound.

The data above indicate that gastrointestinal absorption of TCDD and related compounds is variable, incomplete, and congener specific. More soluble congeners, such as 2,3,7,8-TCDF, are absorbed almost completely, while the extremely insoluble OCDD is absorbed very poorly. In some cases, absorption has been found to be dose dependent, with increased absorption occurring at

lower doses (2,3,7,8-TBDD, OCDD). The limited database also suggests that there are no major interspecies differences in the gastrointestinal absorption of these compounds.

Conditions Affecting Bioavailability Following Oral Exposure Oral exposure of humans to TCDD and related compounds usually occurs as a complex mixture of these contaminants in food, soil, dust, water, or other mixtures that would be expected to alter absorption.

The influence of dose and vehicle or adsorbent on gastrointestinal absorption has been investigated in rats by Poiger and Schlatter,[19] using hepatic concentrations 24 hours after dosing as an indicator of bioavailability. Administration of TCDD in an aqueous suspension of soil resulted in a decrease in the hepatic levels of TCDD compared with hepatic levels resulting from administration of TCDD in 50% ethanol. The extent of the decrease was directly proportional to the length of time the TCDD had been in contact with the soil. When TCDD was mixed in an aqueous suspension of activated carbon, absorption was almost totally eliminated ($< 0.07\%$ of the dose in hepatic tissues).

Since TCDD in the environment is likely to be absorbed to soil,[20] Lucier et al.[21] compared the oral bioavailability of TCDD from contaminated soil to that from TCDD administered in corn oil in rats and guinea pigs. As indicated by biological effects and the amount of TCDD in the liver, the intestinal absorption from Times Beach and Minker Stout, Missouri soil was about 50% less than from corn oil. Shu et al.[22] reported an oral bioavailability of about 43% in the rat dosed with three environmentally contaminated soil samples from Times Beach, Missouri. This figure did not change significantly over a 500-fold dose range of 2 to 1450 ng TCDD/kg body weight for soil contaminated with about 2, 30, or 600 ppb of TCDD. In studies of other soil types, Umbreit et al.[23,24] estimated an oral bioavailability in the rat of 0.5% for soil at a New Jersey manufacturing site and 21% for a Newark salvage yard. These results indicate that bioavailability of TCDD from soil varies between sites and that TCDD content alone may not be indicative of potential human hazard from contaminated environmental materials. Although these data indicate that substantial absorption occurs from contaminated soil, soil type and duration of contact may substantially affect the absorption of TCDD from soils obtained from different contaminated sites.

Schlummer et al.[25] used a mass balance approach to assess the gastrointestinal absorption of PCDDs, PCDFs, PCBs, and hexachlorobenzene (HCB) from food in seven persons, 24 to 81 years of age, with different contaminant body burdens (*body burden* refers to the amount of the chemical present in the entire body; Table 6.1). The net absorption is calculated as the difference between contaminant input with food and contaminant output with feces, normalized to the contaminant intake. Positive values in Table 6.1 indicate net absorption, and negative values indicate net excretion, with absorption or excretion expressed as a percentage of daily intake. Three types of net absorp-

tion were observed in this study: (1) nearly complete net absorption (e.g., PCBs 28, 52, 77, 101, 126; 2,3,7,8-TCDF; and 1,2,3,7,8-penta-CDF), (2) incomplete net absorption (e.g., PCBs 105, 138, 153, 180, 202; TCDD; and 1,2,3,7,8-penta-CDD in the younger subjects), and (3) net excretion excretion to a greater extent than ingestion (e.g., 1,2,3,6,7,8-hexa-CDD and OCDD). In the case of the coplanar PCBs, 77 and 126, the congener-specific levels in blood lipids of the subjects (given in parentheses) were very low and absorption was nearly complete (90% or greater for PCB 126 in all but two subjects). In the 76- and 81-year-old subjects, PCB 126 was found at higher levels in the blood lipids, and the estimated net absorption of this congener was reduced to 77 and 53%, respectively. When PCB 126 (3,3′,4,4′,5), with a TEF (toxicity equivalency factor) of 0.1, is included with the PCDDs and PCDFs in the TEQ (dioxin toxic equivalents) calculation, the TEQ balance was dominated by this congener, resulting in a maximum net TEQ absorption of 80% and a net TEQ absorption in all but the oldest subject. Net excretion or limited absorption was observed for PCBs 138, 153, and 180 in the three older subjects, who had the highest levels of these congeners in their blood lipids. Thus, the gastrointestinal absorption or excretion of PCDDs, PCDFs, and PCBs from food in humans is not only congener dependent but is related directly to the concentration of a given PCDD, PCDF, or PCB in blood, or the congener-specific body burden. In most cases of background dietary exposures to PCDDs, PCDFs, or PCBs, the blood level or body burden of these congeners increases with the person's age, which often results in reduced gastrointestinal absorption of these compounds.

Table 6.1 illustrates that compounds showing nearly complete net absorption had very low or nondetectable levels in the serum lipids, and for other congeners, there was a trend toward decreasing net absorption/increasing net excretion with increasing congener concentration in serum lipids. Together, the data support the passive diffusion model for gastrointestinal absorption, where the concentration of the contaminant in the blood is the major factor determining absorption. However, the relatively high absorption levels of many congeners could not be explained on the basis of diffusive gradients estimated from the difference between the lipid-based food and serum concentrations, since the lipid-based food levels were always lower, favoring net excretion. Schlummer et al.[25] propose a "fat-flush" theory, which hypothesizes that the fat compartment of the absorbing tissue (gut wall) expands due to the uptake of dietary fat, resulting in a decrease in the lipid-based concentration of PCDDs, PCDFs, and PCBs in the gut wall below that of the food, thus facilitating absorption. Therefore, as food passes through the duodenum and the jejunum, PCDDs, PCDFs, and PCBs experience a diffusion gradient and net absorption as a result of the fat flush. As the gut contents reach the colon, the fat flush has subsided and the PCDDs, PCDFs, and PCBs have a diffusive gradient favoring net excretion. Thus, the fat flush theory supports the hypothesis that absorption and excretion of PCDDs, PCDFs, and PCBs are distinct processes occurring at different locations in the digestive tract. In addition, the results suggest that

TABLE 6.1 Net Gastrointestinal Absorption or Excretion of PCDDs, PCDFs, and PCBs in Humans and Dependence on Congener-Specific Blood Lipid Levels[a]

	Gender (Age in Years)						
	F (24)	M (25)	M (28)	M (36)	M (53)	F (76)	F (81)
2,3,7,8-TCDF[b]	> 78	> 72	69	> 81	> 50	> 78	87
	(< 1.16)	(< 1.08)	(< 1.89)	(< 1.19)	(< 0.78)	(2.43)	(< 1.03)
1,2,3,7,8-penta-CDF	> 81	nd	> 84	> 80	> 65	> 75	75
	(< 1.00)	(< 0.55)	(< 1.63)	(< 1.26)	(0.79)	(1.28)	(< 0.73)
2,3,4,7,8-penta-CDF	60	24	41	67	−41	−11	−106
	(13.5)	(23)	(27)	(13.2)	(46)	(37)	(89)
1,2,3,4,7,8-hexa-CDF	46	29	36	64	−5	15	−85
	(5.0)	(7.5)	(8.9)	(6.4)	(11.4)	(13.4)	(22)
1,2,3,6,7,8-hexa-CDF	31	13	52	56	−25	−10	−130
	(5.2)	(6.9)	(7.7)	(6.4)	(10.7)	(13.9)	(21)
2,3,4,6,7,8-hexa-CDF	64	42	68	66	32	59	< 11
	(< 3.0)	(< 5.0)	(4.4)	(< 3.8)	(4.7)	(6.8)	(6.5)
1,2,3,4,6,7,8-hepta-CDF	−5	21	55	−3	−101	38	−45
	(11.4)	(9.3)	(13.1)	(16.6)	(11.4)	(12.7)	(14.5)
1,2,3,4,7,8,9-hepta-CDF	nd	nd	nd	> 68	nd	> 98	nd
	(< 0.47)	(1.8)	(1.13)	(0.96)	(< 0.76)	(< 0.35)	(< 0.45)
Octa-CDF	−35	31	55	−59	−124	32	46
	(< 3.62)	(< 6.32)	(8.2)	(< 4.55)	(< 4.62)	(6.00)	(< 3.23)
2,3,7,8-TCDD[b]	38	42	62	55	−74	−68	−98
	(2.60)	(2.80)	(3.10)	(2.80)	(4.80)	(8.50)	(11.3)
1,2,3,7,8-penta-CDD	43	17	8	48	−82	−17	−117
	(7.2)	(9.4)	(10.8)	(7.1)	(16.1)	(15.5)	(24)

1,2,3,4,7,8-hexa-CDD	27	16	22	40	−79	−63	−171
	(4.50)	(5.5)	(6.6)	(5.5)	(12.6)	(14.9)	(21)
1,2,3,6,7,8-hexa-CDD	−47	−37	−35	−50	−338	−117	−381
	(28)	(23)	(36)	(33)	(69)	(44)	(81)
1,2,3,7,8,9-hexa-CDD	−67	−10	24	39	−150	−130	−300
	(7.2)	(3.8)	(5.7)	(4.2)	(7.7)	(11.6)	(16.8)
1,2,3,4,6,7,9-hepta-CDD	25	9	52	1	24	64	64
	(< 3.1)	(< 2.2)	(< 4.0)	(< 3.0)	(< 3.3)	nd	nd
1,2,3,4,6,7,8-hepta-CDD	−132	16	38	−18	−69	−173	−361
	(63)	(34)	(44)	(49)	(59)	(123)	(133)
Octa-CDD	−339	−39	−48	−197	−129	−459	−289
	(490)	(200)	(450)	(310)	(240)	(600)	(810)
HCB[c]	70	77	80	82	1	−56	−210
	(79)	(71)	(82)	(65)	(230)	(680)	(1420)
PCB 28[c]	65	84	85	87	64	89	84
	(5.0)	(2.8)	(1.9)	(1.9)	(4.5)	(7.8)	(6.6)
PCB 52	73	82	90	89	69	93	92
	(1.52)	(1.09)	(0.89)	(< 0.46)	(0.75)	(1.84)	(< 0.69)
PCB 77	> 91	83	> 90	> 90	> 82	> 93	92
	(nd)	(nd)	(nd)	(nd)	(0.007)	(0.064)	(< 0.02)
PCB 101	56	81	91	90	48	92	82
	(1.50)	(1.11)	(1.22)	(0.78)	(1.38)	(2.3)	(1.43)
PCB 105	78	87	99	90	63	3	61
	(2.2)	(1.21)	(1.65)	(0.86)	(2.9)	(3.2)	(5.7)
PCB 126	90	93	95	96	92	77	53
	(0.066)	(0.068)	(0.042)	(0.029)	(0.082)	(0.174)	(0.39)

(Continued)

TABLE 6.1 *(Continued)*

	Gender (Age in Years)						
	F (24)	M (25)	M (28)	M (36)	M (53)	F (76)	F (81)
PCB 138	80	72	70	87	6	33	6
	(55)	(63)	(131)	(50)	(174)	(133)	(270)
PCB 153	74	60	65	85	−54	31	−42
	(89)	(135)	(230)	(84)	(410)	(250)	(600)
PCB 180	83	70	59	82	−41	34	−75
	(51)	(115)	(171)	(67)	(330)	(175)	(380)
PCB 202	51	36	2	19	−324	−63	−123
	(0.69)	(0.97)	(2.3)	(1.59)	(3.4)	(1.53)	(3.3)

Source: Data from Ref. 25.

[a]Net absorption is calculated as the difference between contaminant input with food and contaminant output with feces, normalized to the contaminant intake and is expressed as a *percentage of the daily intake*. Positive values indicate net absorption, and negative values indicate net excretion with absorption or excretion expressed as a percentage of daily intake. Congener-specific levels in blood lipids are given in parentheses. nd, Not determined due to detection problems. <, values did not exceed three times blank values.

[b]PCDD/F blood levels: picograms per gram of blood lipids, shown in parentheses.

[c]HCB and PCB blood levels: nanograms per gram of blood lipids, shown in parentheses.

the ingestion of highly contaminated food should result in nearly complete absorption, due to the high diffusion gradient associated with high levels of PCDDs, PCDFs, and PCBs in the gut contents.

Because PCDDs, PCDFs, and PCBs are present in human milk, McLachlan[26] investigated the net absorption of these compounds in a nursing infant. The contaminant input, through the ingestion of mother's milk, and the contaminant output in the feces were measured to estimate the digestive tract absorption of these compounds. For almost all congeners, more than 90% of the ingested compound was absorbed, indicating that the common assumption of 100% absorption of PCDDs, PCFFs, and PCBs in nursing infants is reasonable. Dahl et al.[27] provide further evidence of this as they report over 95% absorption in postpartem infants (1, 2, 3 months) in Sweden. Abraham et al.[28] assessed the oral intake and fecal excretion of PCDDs and PCDFs in two breast-fed and one formula-fed infant at 1 and 5 months of age. The breast-fed infants had significantly more exposure to PCDDs and PCDFs, with more than 90% of the TCDD, 2,3,4,7,8-penta-CDF, 1,2,3,7,8-penta-CDD, and 1,2,3,6,7,8-hexa-CDD (> 93% of TEQs) being absorbed from mother's milk. Less complete bioavailability of higher PCDDs was observed, with 62 to 88% of 1,2,3,4,6,7,8-hepta-CDD and 16 to 75% of OCDD absorbed from mother's milk (Abraham et al.[28]). Furst et al.,[29] Hong et al.,[30] Schecter et al.,[31] and Georgii et al.[32] provide further evidence for the presence of PCDDs, PCDFs, and PCBs in human milk. This important route for excretion and exposure is discussed later in the chapter.

6.2.2 Absorption Following Dermal Exposure

Brewster et al.[33] examined the dermal absorption of TCDD and three PCDFs in male Fischer 344 rats (10 weeks old; 200 to 250 g) at 3 days after a single exposure using acetone as a vehicle. At an exposure of 0.1 μmol/kg, the absorption of TCDF (49% of administered dose) was greater than that of 2,3,4,7,8-penta-CDF (34%), 1,2,3,7,8-penta-CDF (25%), and TCDD (18%). For each compound, the relative absorption (percentage of administered dose) decreased with increasing dose, while the absolute absorption (μg/kg) increased nonlinearly with dose. Results also suggest that the majority of the compound remaining at the skin exposure site was associated with the epidermis and did not penetrate through to the dermis. In a subsequent study, Banks and Birnbaum[34] examined the rate of absorption of TCDD over 120 h after the dermal application of 200 pmol (1 nmol/kg) to male Fischer 344 rats. The absorption kinetics appeared to be first order, with an absorption rate constant of 0.005 h^{-1}. First-order kinetics indicates that there is a constant rate of absorption (% dose absorbed/time). A first-order kinetic model can be described by the equation $S = S_0 e^{-kt}$, where S is the amount of compound at any time t, S_0 the initial amount administered, and k the rate constant. This equation can be rearranged, solving for the half-time ($t_{1/2}$), the time it takes one-half of the compound to be absorbed: $t_{1/2} = \ln 2/k = 0.693/k$. With a rate constant of

0.005 h^{-1}, one-half of the dose of TCDD is absorbed in 139 h. Together, these results on dermal absorption indicate that at lower doses ($\leq$ 0.1 μmol/kg), a greater percent of this administered dose of TCDD and three PCDFs was absorbed. Nonetheless, the rate of absorption of TCDD is still very slow (rate constant of 0.005 h^{-1}), even following dermal application in acetone at a dose of 200 pmol (1 nmol/kg).

Rahman et al.[35] and Gallo et al.[36] compared the in vitro permeation of TCDD through hairless mouse and human skin. In both species, the amount of TCDD permeated increased with the dose, but the percent of the dose permeated decreased with increasing dose. The permeability coefficient of TCDD in human skin was about one order of magnitude lower than that in mouse skin. The hairless mouse skin does not appear to be a suitable model for the permeation of TCDD through human skin since the viable tissues were the major barrier to TCDD permeation in hairless mouse skin, whereas the stratum corneum layer provided the greater resistance in human skin. A significant increase in TCDD permeation through human skin was observed when the skin was damaged by tape stripping, which removed the stratum corneum layer. Gallo et al.[36] suggested that washing and/or tape stripping of the exposed area might remove most of the TCDD and reduce the potential for systemic exposure and toxicity since most of the TCDD remained within the horny stratum corneum layer of human skin even at 24 h following exposure. Weber et al.[37] also investigated the penetration of TCDD into human cadaver skin at concentrations of 65 to 6.5 ng/cm^2. This study also found that the stratum corneum acted as a protective barrier, as its removal increased the amount of TCDD absorbed into layers of the skin. With intact skin and acetone as the vehicle, the rate of penetration into the dermis and epidermis ranged from 6 to 170 pg/h per square centimeter, while penetration into the dermis and epidermis ranged from 100 to 800 pg/h per square centimeter. With mineral oil as the vehicle, there was about a 5- to 10-fold reduction in the rate of penetration of TCDD into the intact skin.

Conditions Effecting Bioavailability Following Dermal Exposure Dermal exposure of humans to TCDD and related compounds usually occurs as a complex mixture of these contaminants in soil, oils, or other mixtures that would be expected to alter absorption. Poiger and Schlatter[19] presented evidence that the presence of soil or lipophilic agents dramatically reduces dermal absorption of TCDD compared to absorption of pure compound dissolved in solvents. In a control experiment, 26 ng of TCDD in 50 μL of methanol was administered to the skin of rats, and 24 h later the liver contained 14.8 $\pm$ 2.6% of the dose. Dermal application of TCDD to rats in Vaseline (a lipophilic ointment) or polyethylene glycol (hydrophilic) reduced the percentage of the dose in hepatic tissue to 1.4 and 9.3%, respectively, but had no observable effect on the dose of TCDD required to induce skin lesions (ca. 1 μg/ear) in the rabbit ear assay. Application of TCDD in a soil–water paste decreased hepatic TCDD to about 2% of the administered dose and increased the amount

required to produce skin lesions to 2 to 3 μg in rabbits. Application in an activated carbon–water paste essentially eliminated absorption, as measured by percent of dose in the liver, and increased the amount of TCDD required to produce skin lesions to about 160 μg. These results suggest that the dermal absorption and acnegenic potency of TCDD depend on the formulation (vehicle or adsorbent) containing the toxin.

Shu et al.[38] investigated the dermal absorption of soil-bound TCDD in rats. The authors observed that the degree of uptake does not appear to be influenced significantly by the concentration of TCDD in soil, by the presence of crankcase oil as cocontaminants or by environmentally versus laboratory contaminated soil.

A major limitation of the studies above is uncertainty regarding the extrapolation of dermal absorption data on these compounds from the rat to the human. The in vitro uptake of TCDD has been investigated in hairless mouse and human skin.[35,36] In vitro dermal uptake of TCDD from laboratory-contaminated soil found that aging of soils (up to 4 weeks) and the presence of additives (2,3,5-trichlorophenol and motor oil) in the soil did not have any significant effect on dermal uptake.[36] Since most of the TCDD remained in the stratum corneum layer of human skin, the permeation of TCDD was significantly lower in human than in hairless mouse skin.

6.2.3 Absorption Following Inhalation Exposure

The use of incineration as a means of solid and hazardous waste management results in the emission of contaminated particles that may contain TCDD and related compounds into the environment. Thus, significant exposure to TCDD and related compounds may result from inhalation of contaminated fly ash, dust, and soil. In an attempt to address the bioavailability and potential health implications of inhaling contaminated particles, Nessel et al.[39] examined the potential for transpulmonary absorption of TCDD after intratracheal instillation of the compound administered to female Sprague–Dawley rats either in a corn oil vehicle or as a laboratory-prepared contaminant of gallium oxide particles. Several biomarkers of systemic absorption were measured, including the dose-dependent effects of TCDD on hepatic microsomal cytochrome P450 (CYP) content, CYP1-mediated aryl hydrocarbon hydroxylase (AHH) activity, and liver histopathology. Significant dose-related effects were observed at an exposure of ≥ 0.55 μg TCDD/kg. The authors found that enzyme induction was slightly higher when animals received TCDD in corn oil than when animals received TCDD-contaminated particles, and was comparable to enzyme induction after oral exposure. Similar bioavailability was also observed following inhalation exposure to TCDD when bound to gallium oxide particles and when bound to soil.[40]

The transpulmonary absorption of TCDD was assessed in male Fischer 344 rats following intratracheal instillation of a 1 nmol/kg dose in Emulphor: ethanol: water (1:1:3).[42] Transpulmonary absorption was 95%, suggesting

that there was almost complete absorption of TCDD by inhalation under these conditions. Similar results were also observed for the transpulmonary absorption of TBDD under similar exposure conditions.[42] Comparative studies show further the importance of transpulmonary absorption for TCDD.[41] Tissue distributions were measured 3 days after administration via different routes. Comparisons show that the percentage of dose distributed to the liver after intratracheal (itr) injection is similar to that after intravenous administration (iv) (33% for itr, 37% for iv). Also, both the iv and itr routes show a preference for greater sequestration in the liver over fat compared to the oral (po) route. These results suggest that the transpulmonary absorption of TCDD and 2,3,7,8-TBDD was similar to that observed following oral exposure.

6.3 DISTRIBUTION

6.3.1 Distribution in Blood and Lymph

Once a compound is absorbed, its distribution is regulated initially by its binding to components in blood and its ability to diffuse through blood vessels and tissue membranes. Lakshmanan et al.[43] investigated the absorption and distribution of TCDD in thoracic duct-cannulated rats. Their results suggest that following gastrointestinal absorption, TCDD is absorbed primarily by the lymphatic route and is transported predominantly by chylomicrons, which are small lipid droplets about 1 μm in diameter. Ninety percent of the TCDD in lymph was associated with the chylomicron fraction. The plasma disappearance of TCDD-labeled chylomicrons followed first-order decay kinetics, with 67% of the compound leaving the blood compartment very rapidly ($t_{1/2} = 0.81$ min), whereas the remainder of the TCDD had a $t_{1/2}$ of 30 min. TCDD was then found to distribute primarily to the adipose tissue and the liver.

In human blood less than 10% of TCDD was associated with red blood cells, indicating that most of this compound is bound to serum lipids and lipoproteins.[44] In vitro studies of TCDD in human whole blood found about 80% of the compound associated with the lipoprotein fraction, 15% associated with protein (primarily human serum albumin), and 5% associated with cellular components.[45] Theoretical and limited experimental data also suggest that TCDD and related compounds may be associated with plasma prealbumin.[46,47] The distribution of [^{3}H]TCDD among lipoprotein fractions from three fasting, normolipemic donors indicated a greater percentage associated with low-density lipoprotein (LDL) ($55.3 \pm 9.03\%$ SD) than with very low density lipoprotein (VLDL) ($17.4 \pm 9.07\%$ SD) or high-density lipoprotein (HDL) ($27.3 \pm 10.08\%$ SD). The distribution of TCDD among the lipoprotein fractions was similar to that reported earlier by Marinovich et al.[48] When the binding of TCDD was calculated per mole of lipoprotein, it was suggested that the maximal binding capacity was exerted by VLDL, followed by LDL and

HDL.[48] The results also suggest that variations in the amounts of each lipoprotein class may alter the distribution of TCDD among lipoproteins in a given subject. Significant species differences also exist; in the case of the rat, which has markedly lower plasma lipids compared to humans, TCDD was distributed almost equally among the lipoprotein fractions.[48]

Congener-specific differences have also been observed for in vivo binding of the 2,3,7,8-substituted PCDDs and PCDFs to different serum fractions in the blood.[44] Binding to the lipoproteins gradually decreased with increasing chlorine content, with about 75% of TCDD bound to lipoproteins, while approximately 45% of OCDD was bound to this fraction. In contrast, binding to other proteins increased with chlorine content from approximately 20% for TCDD to 50% for OCDD. Considerably less of the PCDDs and PCDFs was bound to the chylomicrons in serum, with less than 10% bound to this serum fraction.[44] In general, these in vivo results indicate that in serum, the higher chlorinated congeners do not partition according to the lipid content of the fractions. Thus, upon absorption, TCDD and probably related compounds are bound to chylomicrons, lipoproteins, and other serum proteins that assist in distributing these uncharged, lipophilic compounds throughout the vascular system. These compounds then partition from blood components into cellular membranes and tissues, probably largely by passive diffusion. In addition, cellular uptake may be facilitated partly through the cell membrane LDL receptor,[49] the hepatic receptor for albumin,[50] and/or other systems.

6.3.2 Tissue Distribution in Laboratory Animals

Once absorbed into blood, TCDD and related compounds readily distribute to all organs. Abraham et al.[70] assessed the tissue distribution of TCDD in female Wistar rats at 7 days following a single subcutaneous dose of 3 μg/kg. The range of TCDD concentrations was 29.2 to 31.0, 3.7 to 4.1, 0.9 to 1.1, 0.76 to 0.96, 0.60 to 1.05, 0.64 to 0.68, 0.32 to 0.33, 0.27 to 0.29, 0.16 to 0.18, 0.08 to 0.12, and 0.07 to 0.09 ng/g for the liver, adipose tissue, adrenals, ovaries, thymus, skin, lung, kidney, serum, muscle, and brain, respectively. Tissue distribution within the first hour after exposure parallels blood levels and reflects physiological parameters such as blood flow to a given tissue and relative tissue size. For example, high initial concentrations of TCDD and 1,2,3,7,8-penta-CDF were observed in highly perfused tissue such as the adrenal glands during the 24-h period after a single exposure.[10,51,52] A high percentage of the dose of 2,3,7,8-TCDF and 1,2,3,7,8-penta-CDF was also found in muscle within the first hour after intravenous exposure, due to the large volume of this tissue.[4,13,52] Nevertheless, within several hours the liver, adipose tissue, and skin become the primary sites of disposition, when expressed as a percent of administered dose per gram tissue and percent of dose per organ. Liver, adipose tissue, skin, and thyroid were the only tissues to show an increase in the concentration of TCDD during the initial 4 days after a single intraperitoneal

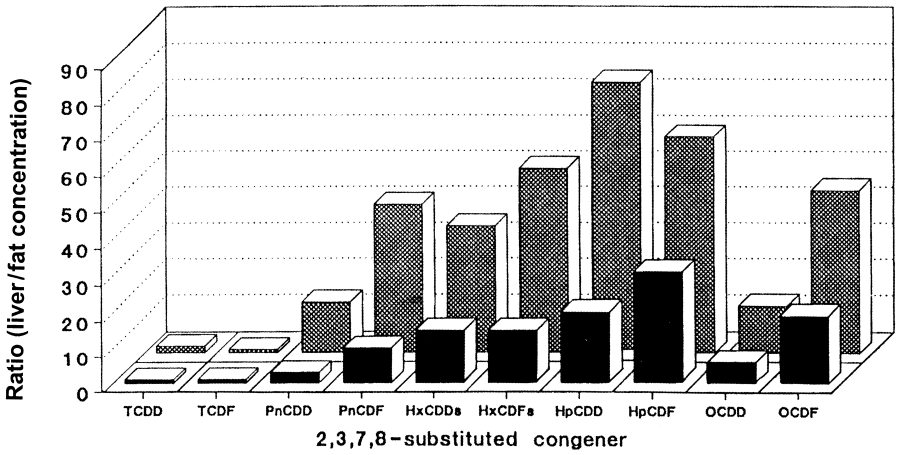

Figure 6.1 Liver/adipose tissue concentration ratios for 2,3,7,8-substituted PCDDs and PCDFs in marmoset monkeys (solid bars) and rats (shaded bars). The ratios were obtained from tissue concentrations on a wet weight basis. (Data from Refs. 53 and 54.)

exposure of rats.[51] In this study, a similar general pattern of disposition was observed in Han/Wistar and Long–Evans rats, which are, respectively, most resistant and susceptible to the acute toxicity of TCDD.[51]

While the liver and adipose tissue contain the highest concentrations of TCDD and 2,3,7,8-TCDF, there are some cogener-specific differences in the relative tissue distribution of related compounds. Figure 6.1 illustrates the liver/adipose tissue concentration ratios for 2,3,7,8-substituted PCDDs and PCDFs on a wet weight basis in marmoset monkeys and rats.[53,54] Monkey and rat data were obtained 7 days following subcutaneous administration of a complex mixture of PCDDs and PCDFs in toluene:DMSO (1:2). Monkeys received a total dose of 27,800 ng/kg (464 ng I-TEQ/kg), while rats received a total dose of 23,222 ng/kg (388 ng I-TEQ/kg). For most of the 2,3,7,8-substituted congeners, the highest concentrations on a wet weight basis were detected in hepatic and adipose tissue, with correspondingly lower values detected in kidney, brain, lung, heart, thymus, and testes. The hepatic and adipose tissue concentrations were similar for TCDD and 2,3,7,8-TCDF. However, with increasing chlorination the relative disposition to the liver markedly increases. Cytochrome P450 1A2 (CYP1A2) acts as a binding protein and contributes to the hepatic sequestration of these compounds.[55–58] The preferential hepatic accumulation of 2,3,7,8-substituted congeners was more pronounced in the rat, with the hepatic concentration of hepta-CDD (heptachlorodibenzodioxin) being approximately 80-fold greater than that in adipose tissue. Thus, there are differences in the disposition of PCDDs and PCDFs to liver and adipose tissue. Therefore, adipose tissue and/or serum concentrations of PCDDs and PCDFs may not reflect the concentrations of specific congeners in target tissues, such as the liver.

6.3.3 Tissue Distribution in Humans

Poiger and Schlatter[11] estimated that about 90% of the body burden of TCDD was sequestered in the fat after a volunteer ingested [^{3}H]TCDD in corn oil at a dose of 1.14 ng/kg. During this 135-day study, elevated radioactivity was detected in the blood only during the first 2 days after treatment. The data would be consistent with the high bioconcentration potential of TCDD in humans, as calculated by Geyer et al.[59] from daily intake assumptions, levels in human adipose tissue, and pharmacokinetic models. Geyer et al.[59] estimated a bioconcentration factor (BCF) of between 104 and 206 for TCDD in human adipose tissue.

Patterson et al.[60] developed a high-resolution gas chromatographic/high-resolution mass spectrometric analysis for TCDD in human serum. At the time of this publication in 1987, the arithmetic mean of the individual human serum samples was 47.9 parts per quadrillion (ppq) on a whole weight basis and 7.6 ppt on a lipid weight basis. Paired human serum and adipose tissue levels of TCDD have been compared by Patterson et al.,[61] Kahn et al.,[62] and Schecter et al.[63] All three groups reported a high correlation between adipose tissue and serum TCDD levels when the samples were adjusted for total lipid content. Furthermore, their correlation was observed over a concentration range of almost three orders of magnitude.[61] This correlation indicates that serum TCDD is a valid estimate of the TCDD concentration in adipose tissue.

Schecter et al.[63] investigated the partitioning of 2,3,7,8-substituted PCDDs and PCDFs between adipose tissue and plasma lipid content in 20 Massachusetts Vietnam veterans. The distribution ratio between plasma lipid and adipose tissue increased with chlorine substitution on the PCDDs and PCDFs. While 2,3,7,8-substituted TCDD, TCDF, penta-CDD, penta-CDF, hexa-CDD, and hexa-CDF had a plasma lipid/adipose tissue ratio of about 1.0, OCDD had a ratio of about 2.0. On the other hand, whole blood PCDDs and PCDFs seem to be found at the same concentrations as in adipose tissue, on a lipid basis.[64] Schecter et al.[65,66] also reported the mean PCDD and PCDF levels on a wet weight basis in human autopsy tissue samples from two patients from the United States. In general, tissues contain higher levels of the higher chlorinated congeners, and tissues with a greater lipid content contain higher levels of PCDDs and PCDFs.

The disposition of 2,3,7,8-substituted PCDDs and PCDFs in human liver and adipose tissue was assessed in a study of 28 people from the Munich area.[67,68] Table 6.2 summarizes these results, which are expressed on both a lipid and a wet weight basis. The concentrations of PCDDs and PCDFs in adipose tissue and liver are not the same when calculated on a lipid basis. This is in contrast to the high correlation that was reported between adipose tissue and serum TCDD levels when expressed on a lipid weight basis.[61–63] Furthermore, the liver/adipose tissue ratio increased with the higher chlorinated PCDDs and PCDFs. The congener specific hepatic deposition is also similar to that observed in rats and marmoset monkeys exposed to a complex mixture

TABLE 6.2 2,3,7,8-Substituted PCDDs and PCDFs in Human Liver and Adipose Tissue[a]

	Tissue Concentration on a Lipid Basis (ppt)			Tissue Concentration on a Wet Weight Basis (ppt)	
Congener	Fat	Liver	Liver/Fat	Liver[b]	Liver/Fat
TCDD	8.0	16.4	2.05	1.1	0.14
Penta-CDD	16.4	20.1	1.22	1.4	0.09
Hexa-CDD	94.7	166.8	1.76	11.7	0.12
Hepta-CDD	106.7	1002.4	9.39	70.2	0.66
Octa-CDD	373.2	4416.2	11.83	309.1	0.83
TCDF	2.5	5.5	2.20	0.4	0.15
Penta-CDF	35.2	173.7	4.93	12.2	0.35
Hexa-CDF	41.5	389.5	9.38	27.3	0.66
Hepta-CDF	14.2	218.9	15.42	15.3	1.08
Octa-CDF	4.0	29.7	7.43	2.1	0.52

Source: Data from Refs. 67 and 68.

[a] Values are the mean of 28 people from the Munich area.

[b] Estimated from the % fat in the liver ($7.02 \pm 5.33\%$, mean $\pm$ SD).

of PCDDs and PCDFs (Figure 6.1). Therefore, it is important to consider congener- and tissue-specific differences in disposition of PCDDs and PCDFs when blood levels are used to estimate tissue levels or body burdens.

Schecter et al.[69] assessed the disposition of PCDDs, PCDFs, and coplanar PCBs in the blood, milk, adipose tissue, placenta, and cord blood from five U.S. women. When expressed on a pg/g lipid basis, the mean total TEQs were 11.6, 12.1, 10.5, 5.8, 10.0, and 10.2 in adipose tissue, predelivery blood, placenta, cord blood, postpartum blood, and breast milk, respectively. The results suggest that PCDDs, PCDFs, and PCBs, when expressed as total TEQs on a lipid basis, partition to a similar extent between these tissues. Whereas 2,3,4,7,8-penta-CDF and PCB 126 were lower in cord blood than other tissues, the levels of TCDD were similar in these tissues.

6.3.4 Time-Dependent Tissue Distribution

TCDD and related compounds exhibit congener-specific disposition, which depends on tissue, species, and time after a given exposure. In general, these compounds are cleared rapidly from the blood and distributed to liver, muscle, skin, adipose tissue, and other tissues within the first hour(s) after exposure. This is followed by redistribution primarily to the liver and adipose tissue, which exhibit increasing tissue concentrations over several days after exposure. Elimination from tissues then occurs at rates that are congener-, tissue-, and species-specific. Thus, the ratio of the concentration of TCDD and related compounds in different tissues (i.e., liver/adipose) may not remain constant

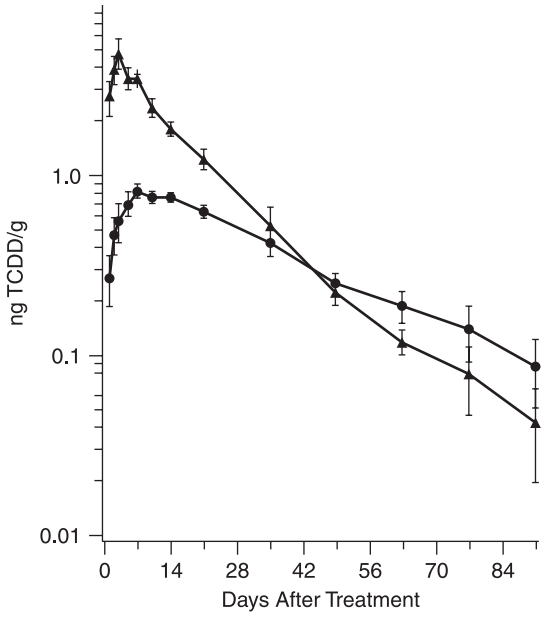

Figure 6.2 Time course of the concentration of [^{14}C]TCDD in rat liver (triangles) and adipose tissue (circles) after a single subcutaneous injection of 300 ng TCDD/kg body weight to female rats (mean $\pm$ SD). (Adapted from Ref. 70.)

over an extended period after a single exposure. Abraham et al.[70] examined the concentrations of TCDD in liver and adipose tissue of female Wistar rats over a 91-day period after a single subcutaneous exposure at a dose of 300 ng/kg body weight (Figure 6.2). The maximum concentration of TCDD in the liver and adipose tissue was reached at 3 and 7 days after exposure, respectively, with 26.5% of the dose administered in the whole liver at day 7. The liver/adipose tissue concentration ratio does not remain constant over time since the concentration of TCDD decreases more rapidly in the liver than in the adipose tissue. For example, the liver/adipose tissue concentration ratio for TCDD on a wet weight basis was 10.3 at 1 day after exposure and 0.5 at 91 days after exposure. The decrease in the TCDD concentration in adipose tissue is a linear function in the semilogarithmic plot of log concentration versus time, which indicates apparent first-order elimination kinetics with a half-life of 24.5 days in rats. First-order kinetics indicates that there is a constant rate of elimination (% dose excreted/time). As with absorption, the first-order kinetic model can be described by the equation $S = S_0 e^{-kt}$, where S is the amount of TCDD at any time t, S_0 the initial amount administered, and k the rate constant for elimination. Liver tissue exhibits a biphasic (two-component) exponential decay pattern with a half-life of 11.5 days for the first component (days 10 to 49) and a half-life of 16.9 days for the second component (days 49 to 91). TCDD is more persistent in the adipose tissue than in the liver. This is in contrast to the

mouse, where liver and adipose tissue have similar half-lives.[71] TCDD is exceptionally persistent in the adipose tissue of the rhesus monkey, with a half-life approximately 10- to 40-fold greater than that observed in the rat and mouse.[72] Thus, the relative persistence of TCDD is tissue specific and exhibits marked interspecies variability.

Most experimental tissue distribution and elimination data are obtained after exposure to a single congener, while real-world exposure to TCDD and related compounds occurs as a complex mixture of congeners. Neubert et al.[53] examined the persistence of various PCDDs and PCDFs in hepatic and adipose tissue of male and female marmoset monkeys. Animals received a single subcutaneous exposure to a defined PCDD/PCDF mixture (total dose of 27,800 ng/kg body weight), which contained 120 ng TCDD/kg body weight. Using the now somewhat dated I-TE (international TCDD toxic equivalence) factors,[73,74] the total administered dose corresponded to 464 ng I-TE/kg body weight. The concentrations of specific congeners in liver and adipose tissue were measured at 1, 6, 16, or 28 weeks after exposure, and elimination constants and half-lives were estimated assuming first-order kinetics (Table 6.3). The data in Table 6.3 were determined from pregnant and nonpregnant female and male marmosets since no obvious differences in tissue concentrations were observed among these groups. All 2,3,7,8-substituted PCDDs and PCDFs were consistently more persistent in the adipose tissue of marmoset monkeys. In general, the persistence in adipose tissue was from about 1.3- to 2.0-fold greater than that in liver, with the exception of 1,2,3,4,7,8-/1,2,3,4,7,9-hexa-CDF, hepta-CDFs, and OCDF, which were more than threefold more persistent in adipose tissue. For the latter congeners and OCDD, there was marked variance in half-life values, which may be due to delayed and incomplete absorption of the exceptionally persistent congeners and the relatively short (28 weeks) period of investigation.

The exposure of marmoset monkeys to a complex mixture of PCDDs and PCDFs included exposure to both 2,3,7,8- and non-2,3,7,8-substituted congeners.[53] One week after exposure to this complex mixture, the non-2,3,7,8-substituted PCDDs and PCDFs were present in liver and adipose tissue in relatively minor quantities compared with 2,3,7,8-substituted congeners; however, non-2,3,7,8-substituted compounds represented a considerable percent of the exposure mixture. In this study, none of the non-2,3,7,8-substituted TCDDs, penta-CDDs, TCDFs, or penta-CDFs could be detected in the liver by gas chromatography/mass spectroscopy. Some of the hexa and hepta congeners were detected in adipose tissue and liver, but after 1 week, the total amount in the liver was more than 5% of the dose administered only in the case of 1,2,4,6,8,9-hexa-CDF. Similar results were obtained in rats after exposure to a defined, complex mixture of PCDDs and PCDFs.[54] Additional short-term studies in rats provide evidence that the low tissue concentration of non-2,3,7,8-substituted congeners, measured 1 week after exposure, was the result of rapid elimination, since these congeners were detected at higher levels in the liver 13 to 14 h after exposure.[54] These results in monkeys and rats are compatible with

TABLE 6.3 Elimination Constants and Half-Lives of Various 2,3,7,8-Substituted PCDDs and PCDFs in Hepatic and Adipose Tissue of Marmoset Monkeys[a]

	Hepatic Tissue			Adipose Tissue		
Congener	K_e (week^{-1})	Half-Life (weeks)	95% Conf. Interval (weeks)	K_e (week^{-1})	Half-Life (weeks)	95% Conf. Interval (weeks)
2,3,7,8-TCDD[b]	0.0841 ± 0.0109	8.3	6.6–11.1	0.0658 ± 0.0072	10.5	8.7–13.4
1,2,3,7,8-penta-CDD[b]	0.0649 ± 0.0101	10.7	8.2–15.4	0.0490 ± 0.0057	14.2	11.5–18.3
1,2,3,4,7,8-hexa-CDD	0.0702 ± 0.0059	9.9	8.4–11.8	0.0411 ± 0.0083	16.9	12.1–27.9
1,2,3,6,7,8-hexa-CDD	0.0558 ± 0.0046	12.4	10.7–14.9	0.0373 ± 0.0073	18.6	13.4–30.2
1,2,3,7,8,9-hexa-CDD	0.0767 ± 0.0078	9.0	7.5–11.3	0.0525 ± 0.0089	13.2	9.9–19.7
1,2,3,4,6,7,8-hepta-CDD	0.0518 ± 0.0081	13.4	10.2–19.3	0.0372 ± 0.0060	18.6	14.2–27.2
Octa-CDD	0.0089 ± 0.0084	78	27–∞[c]	0.0122 ± 0.0093	101	20–∞[c]
2,3,7,8-TCDF	0.8012 ± 0.0549	< 0.87[d]	< 1.00	0.4986 ± 0.0829	1.39	1.05–2.06
1,2,3,7,8-/1,2,3,4,8-penta-CDF	0.7476 ± 0.0294	0.93	0.86–1.00	0.4735 ± 0.0408	1.46	1.25–1.76
2,3,4,7,8-penta-CDF	0.0786 ± 0.0048	8.8	7.9–10.0	0.0563 ± 0.0059	12.3	10.2–15.5
1,2,3,4,7,8-/1,2,3,4,7,9-hexa-CDF	0.0307 ± 0.0039	23	18–30	0.0103 ± 0.0074	68	28–∞[c]
1,2,3,6,7,8-hexa-CDF	0.0486 ± 0.0037	14.3	12.4–16.7	0.0290 ± 0.0091	24	15–62
1,2,3,7,8,9-hexa-CDF	0.0848 ± 0.0057	8.2	7.2–9.4	Not analyzed[e]	na	na
2,3,4,6,7,8-hexa-CDF	0.0373 ± 0.0057	18.6	14.3–26.5	0.0182 ± 0.0082	38	20–327
1,2,3,4,6,7,8-hepta-CDF	0.0186 ± 0.0072	37	21–152	−0.0140 ± 0.0137	∞[c]	54–∞[c]
1,2,3,4,7,8,9-hepta-CDF	0.0088 ± 0.0127	79	20–∞[c]	0.0011 ± 0.0112	660	30–∞[c]
Octa-CDF	0.0040 ± 0.0096	174	30–∞[c]	−0.0042 ± 0.0148	∞[c]	28–∞[c]

Source: Data from Ref. 53.

[a]Animals were treated subcutaneously with a single dose of a defined PCDD\PCDF mixture, and the tissues were analyzed at different times following treatment. Half-lives were calculated from tissue concentrations of the 2,3,7,8-substituted congeners in hepatic and adipose tissue. Values are given as elimination rate constant K^e, including estimated SD, and half-life, including 95% confidence intervals. na, not applicable.

[b]Calculated from the time period: > 6 weeks after injection.

[c]Calculated half-life is apparently infinite. Data for octa-CDD and octa-CDF are unreliable due to delayed absorption.

[d]Not detected in hepatic tissue 6 weeks after treatment; limits of detection used for calculation.

[e]Due to interference.

data from analysis of human tissue samples and milk in which the non-2,3,7,8-substituted congeners have also not been shown to be present in significant concentrations compared with the 2,3,7,8-substituted congeners.[67,75–78]

6.3.5 Dose-Dependent Tissue Distribution

Several studies suggest that the tissue distribution of TCDD and possibly related compounds is disproportional relative to dose. Abraham et al.[70] investigated the distribution of TCDD in liver and adipose tissue of rats 7 days after a single subcutaneous exposure to TCDD at doses of 1 to 3000 ng/kg body weight. Greater than 97% of the TCDD administered was absorbed at all doses, with the exception of the 3000 ng/kg group, where 84% of the dose was absorbed. Figure 6.3 illustrates the dose-dependent disposition of TCDD in liver and adipose tissue (% dose/g) 7 days after exposure. A sharp increase in TCDD concentration in liver was observed at exposure levels above 10 ng/kg body weight. Disposition in the liver increased from about 11% of the administered dose at an exposure level of 1 to 10 ng/kg body weight to about 37% of the dose at an exposure level of 300 ng/kg body weight. The increase in

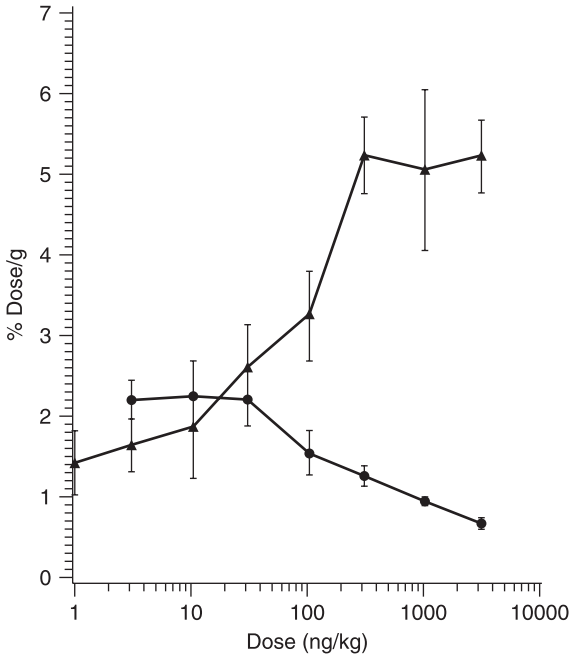

Figure 6.3 Dose-dependent tissue disposition of TCDD in female rats illustrated as the percentage of the administered dose of [^{14}C]TCDD/g liver (triangles) and adipose tissue (circles) at 7 days after a single subcutaneous exposure at doses ranging from 1 to 3000 ng/kg. (Values from animals that were treated with 3000 ng TCDD/kg body weight were corrected for 84% absorption.) (Adapted from Ref. 70.)

distribution to the liver was accompanied by a dose-related decrease in the concentration of TCDD in the adipose tissue. As a result, the liver/adipose tissue concentration ratio for TCDD increased with increasing doses, starting at an exposure level of 30 ng/kg body weight. Thus, the tissue-specific disposition of TCDD is regulated by a complex relationship, which includes species, time after a given exposure, and dose.

Other studies on the tissue disposition of TCDD and related compounds report similar dose-dependent behavior with disproportionally greater concentrations in the liver at high doses compared with low doses.[55,79–83] Chronic studies also support disproportional dose-dependent alterations in the tissue distribution of these compounds. Kociba et al.[84,85] found that female rats maintained on a daily dietary TCDD intake of 100 ng/kg per day for 2 years had an average TCDD content of 8100 ppt in fat and 24,000 ppt in the liver on a wet weight basis. Rats given 10 ng/kg per day had an average of 1700 ppt TCDD in the fat and 5100 ppt in the liver. For both of these exposures the liver/adipose tissue concentration ratio of TCDD was about 3. At the lowest dose level of 1 ng/kg per day, both fat and liver contained an average of 540 ppt TCDD. Kociba et al.[86] presented evidence that steady state had been reached after about 13 weeks of feeding of TCDD.

Other studies do not support the disproportional dose-dependent tissue distribution of TCDD and related compounds described above.[8] Tritscher et al.[87] also reported a direct dose-dependent hepatic disposition of TCDD in female Sprague–Dawley rats exposed biweekly to TCDD for 30 weeks at doses equivalent to 3.5, 10.7, 35.7, and 125 ng/kg per day. A linear relationship between administered dose and the concentration in the liver was observed over the dose range used in this chronic exposure study.

The dose-dependent tissue distribution of TCDD and related compounds is a critical factor that must be considered in estimating the concentration of these compounds in human tissues after chronic low-level exposure. This is particularly important since the general human population is exposed to much smaller daily doses than those used in experimental disposition studies.[7] Furst et al.[7] estimated human exposure to TCDD to be 0.3 pg/kg per day, while Schecter et al.[88] estimated the average daily human exposure to TEQs in the U.S. diet to be from 0.3 to 3.0 pg/kg body weight. Related at least partly to the long half-life of TCDD in humans, however, this exposure results in concentrations of 3 to 6 pg/g in human adipose tissue.[64] Similar levels of TCDD in adipose tissue (14 pg/g) were observed in rats 7 days after subcutaneous exposure to 3 ng/kg body weight.[70] Human data on the liver/adipose tissue concentration ratio of TCDD and related compounds are limited but suggest that the ratio may vary by at least an order of magnitude between individuals. Leung et al.[89] observed a geometric mean adipose tissue TCDD concentration of 7.78 ppt in 26 individuals and a concentration in liver at about one-tenth of that in adipose tissue on a whole weight basis. When measured on a total lipid basis, the concentrations of TCDD in both tissues were approximately the same. In a related study of 28 people from the Munich area, Thoma et al.[68] reported a liver/fat

ratio for TCDD of 2.05 when the concentration was expressed on a lipid weight basis (Table 6.2). Considerable variability between individuals was observed in this study, with TCDD concentrations ranging from 2.6 to 18 ppt in adipose tissue and 1.0 to 88.9 ppt in liver on a lipid weight basis. Considerable variability in PCDD and PCDF concentrations in liver and adipose tissues was also observed between individual marmoset monkeys,[53] suggesting that individual variability may also contribute to the difficulty in assigning a constant liver/adipose tissue ratio for PCDDs and PCDFs in humans and nonhuman primates.

6.3.6 Potential Mechanisms for the Disproportional Dose-Dependent Tissue Distribution

The observation that exposure to higher doses of TCDD and related compounds results in a disproportionally greater hepatic concentration of these compounds may be explained by a hepatic-binding protein that is induced by TCDD and other dioxinlike compounds that bind to the Ah receptor. The studies of Voorman and Aust[56,57] and Poland et al.[55,58] provide evidence that this binding species is cytochrome P450lA2 (CYP1A2).

Santostefano et al.[90] assessed the subcellular and tissue specific disposition of TCDD in rats and mice. TCDD was equally distributed between the hepatic P9 (mitochondrial, lysosomal, and nuclear) and S9 (cytosol and microsomal) fractions, with the microsomal fraction retaining the TCDD present in the S9 fraction. In contrast, TCDD was retained in the P9 fractions of lung and liver at all doses tested. The lack of pulmonary or renal sequestration coupled with the lack of localization of TCDD to pulmonary and renal microsomes supports the role of CYP1A2 as a hepatic microsomal binding protein involved in the hepatic sequestration of TCDD. Recently, Santostefano et al.[91] assessed the intralobular hepatic distribution of TCDD in rats and observed that centrilobular hepatocytes had a 2.7- to 4.5-fold higher concentration of TCDD than periportal hepatocytes. The enhanced centrilobular distribution of TCDD was associated with elevated CYP1A2-mediated MROD (methoxyresorufin *O*-deethylase) activity and CYP1A2 mRNA in centrilobular hepatocytes.

Diliberto et al.[92] used transgenic mice lacking the CYP1A2 gene to study the influence of CYP1A2 in the hepatic sequestration and distribution of TCDD, 2,3,4,7,8-penta-CDF, and PCB 153 (2,2′,4,4′,5,5′-hexa-CB), a nondioxinlike PCB. The liver/fat concentration ratios of these compounds in the parental lineage (C57BL6N and 129/Sv) were approximately 3.6, 18, and 0.07, respectively, indicating a high degree of hepatic sequestration for TCDD and 2,3,4,7,8-penta-CDF. Under identical exposure conditions, the 1A2 knockout mice had liver/fat concentration ratios of 0.17, 0.34, and 0.10, respectively. Thus, in the absence of the CYP1A2 gene, mice exhibited no hepatic sequestration of these compounds. This study and a related study in CYP1A2 knockout mice by Diliberto et al.[93] provides direct confirmation of the hypothesis that CYP1A2 is the dioxin-inducible hepatic binding protein

responsible for the hepatic sequestration of TCDD and related compounds, such as 2,3,4,7,8-penta-CDF.

The structure–activity relationship for the disposition and hepatic sequestration of CDDs, CDFs, and PCBs was investigated by DeVito et al.[94] Female B6C3F1 mice were treated per os (by mouth) for 13 weeks with different doses of TCDD, 1,2,3,7,8-penta-CDD, 2,3,7,8-TBDD, 2,3,7,8-TCDF, 1,2,3,7,8-penta-CDF, 2,3,4,7,8-penta-CDF, OCDF, PCB 126 (3,3′,4,4′,5-penta-CB), PCB 169 (3,3′,4,4′,5,5′-hexa-CB), PCB 105 (2,3,3′,4,4′-penta-CB), PCB 118 (2,3′,4,4′,5-penta-CB), and PCB 156 (2,3,3′,4,4′,5-hexa-CB). All of these compounds, with the exception of the mono-ortho PCBs (105, 118, 156) exhibited dose-dependent increases in the liver/fat concentration ratio. 4-Penta-CDF, penta-CDD, OCDF, TCDF, and PCB 126 were sequestered in hepatic tissue to a greater extent than was TCDD. Together, the results support the presence of an inducible protein (CYP1A2) and the congener-specific binding of some dioxinlike compounds to this hepatic sequestration protein.

6.4 METABOLISM AND EXCRETION

The metabolism of PCDDs and related compounds has been reviewed by Hu and Bunce.[2] There is evidence that a wide range of mammalian and aquatic species are capable of biotransforming TCDD to polar metabolites.[10,95–100] Although metabolites of TCDD have not been identified directly in humans, human fecal data from a self-dosing experiment suggest that humans can metabolize TCDD.[101]

Investigations of TCDD in rats, mice, guinea pigs, and hamsters found that more than 90% of the radiolabeled material excreted in urine and bile represented polar metabolites. Similar results were also observed for other congeners, with the exception of OCDD, although studies were often limited to the rat. OCDD is apparently not metabolized by the rat or is metabolized to a very minimal extent.[18] For all the congeners, essentially all the PCDD- and PCDF-derived radioactivity in liver, adipose tissue, and other tissues represented parent compound, suggesting that the metabolites of these compounds were readily excreted. Thus, with the exception of OCDD, the metabolism of TCDD and related compounds is required for urinary and biliary elimination and therefore plays a major role in regulating the rate of excretion of these compounds.

6.4.1 Structure of Metabolites

Sawahata et al.[102] investigated the in vitro metabolism of TCDD in isolated rat hepatocytes. The major product was deconjugated with β-glucuronidase, derivatized with diazomethane and separated into two compounds by high-performance liquid chromatgraphy. These metabolites were subsequently identified as 1-hydroxy-2,3,7,8-TCDD and 8-hydroxy-2,3,7-trichlorodibenzo-*p*-

dioxin. Poiger et al.[99] identified six metabolites in the bile of dogs that were given a lethal dose of [^{3}H]TCDD. The major metabolite was 1,3,7,8-tetrachloro-2-hydroxydibenzo-*p*-dioxin; however, 3,7,8-trichloro-3-hydroxydibenzo-*p*-dioxin and 1,2-dichloro-4,5-hydroxybenzene were identified as minor metabolites. The structures of the three remaining metabolites were not determined; however, two appeared to be trichlorohydroxydibenzo-*p*-dioxins and the third was apparently a chlorinated 2-hydroxydiphenyl ether. Poiger and Buser[103] reported differences in the relative amounts of various TCDD metabolites in dog and rat bile. Trichlorodihydroxydibenzo-*p*-dioxin and tetrachlorodihydroxydiphenyl ether appear to be major metabolites in rat bile. Furthermore, conjugates, presumably glucuronides, were formed in the rat but not in the dog. The investigators also observed a generally higher rate for the metabolism of TCDD in the dog.

6.4.2 Toxicity of Metabolites

The discussion above indicates that the metabolism of TCDD and related compounds is required for urinary and biliary elimination and thus plays a major role in regulating the rate of excretion of these compounds. At present, metabolism is also generally considered a detoxification process.[1–5,104,105]

Structure–activity studies of TCDD and related compounds support the widely accepted principle that the parent compound is the active species. The relative lack of activity of readily excreted monohydroxylated metabolites of TCDD[104,105] and 3,3′,4,4′-TCB[106,107] suggests that metabolism is a detoxification process necessary for the biliary and urinary excretion of these compounds. This concept has also been generally applied to TCDD-related compounds, although data are lacking on the structure and toxicity of metabolites of other PCDDs, PBDDs, PCDFs, PBDFs, PCBs, and PBBs.

Data on the metabolism of TCDD suggests that reactive epoxide intermediates may be formed. Poland and Glover[108] have investigated the in vivo binding of [1,6-^{3}H]TCDD-derived radioactivity to rat hepatic macromolecules and found maximum levels equivalent to 60 pmol of nucleotide in RNA and 6 pmol TCDD/mol of nucleotide in DNA. This corresponds to one TCDD-DNA adduct for each 35 cells. These investigators suggest that it is unlikely that TCDD-induced oncogenesis is through a mechanism of covalent binding to DNA and somatic mutation. Further studies of TCDD and related compounds are needed to confirm these results and assess the relationship between covalent binding and the short- and long-term toxicity of these compounds.

It is possible that low levels of unextractable and/or unidentified metabolites may contribute to one or more of the toxic responses of TCDD and related compounds. Further studies on the nature of the biotransformation products of these compounds will help to address this uncertainty. A possible explanation for the highly selective retention of the hydroxylated PCBs (OH-PCBs) in blood may be their structural resemblance with thyroxin, a thyroid hormone. Both rats and mice metabolize PCB 77 by CYP1A to the 1,2-shift metabolite, 4-OH-3,5,3′,4′-PCB, 5-OH-3,3′,4,4′-PCB, and 6-OH-3,3′,4,4′-PCB.[109,110]

Only the 4-OH metabolite was selectively retained, with blood containing 4-OH-3,5,3′,4′-PCB at a concentration 15 times higher than the parent compound, 5 days after oral exposure to PCB 77 in mice.[111] The selective accumulation of 4-OH-3,5,3′,4′-PCB in late gestational rat fetuses following maternal exposure to PCB 77 was also observed and probably is the result of transplacental transport, since fetal liver microsomes did not metabolize PCB 77.[109] This metabolite was found to be bound to a thyroxin-transporting protein (transthyretin) in the blood.[112] Competitive binding studies of OH-PCBs relative to thyroxine (T4) and computer modeling showed that OH-PCBs with the substituents in the meta or para positions were much more effective competitors for T4 than if the substituents were bound in an ortho position.[113]

6.4.3 Autoinduction of Metabolism

Accurate rate constants for metabolism are important in developing pharmacokinetic models that describe the disposition of TCDD and related compounds. Metabolism plays a major role in regulating the excretion and relative persistence of these compounds, since metabolism is required for urinary and biliary excretion. Although the relative rate of metabolism of TCDD and related compounds can be estimated from tissue and excretion half-life data, other factors, such as relative body composition, hepatic and extrahepatic binding proteins, and direct intestinal elimination of the parent compound, can also regulate the excretion of TCDD and related compounds. Therefore, in vivo disposition data provide only a limited approximation of the relative rate of metabolism of a specific congener in a given species. In vivo disposition data were also often obtained at exposures of TCDD and related compounds that were associated with induction of CYPlA1, CYPlA2, CYP1B1, and other potentially adverse responses that could alter metabolism and disposition. Autoinduction may occur where exposure to TCDD and related compounds increases the levels of these dioxin-inducible CYPs, which in turn could increase the rate of metabolism of TCDD and related compounds. Therefore, it may not be appropriate to extrapolate these data directly to predict the pharmacokinetics at low levels of exposure. Low-dose extrapolations can be assisted by assessments of the potential for autoinduction of metabolism, which may occur at exposures of TCDD and related compounds that are associated with induction of CYP1A1, CYP1A2, and CYP1B1. Characterization of the dose-dependent disposition of TCDD and related compounds is particularly important in the extrapolation of high-exposure animal data to low-exposure human data.

The excretion of metabolites of TCDD and related compounds into bile represents a direct means for estimating the rate of metabolism, since biliary elimination depends on metabolism and is the major route for excretion of these compounds. The small increase in metabolism and biliary excretion of TCDD in TCDD-pretreated rats observed by Poiger and Buser[103] and the negative results of Kedderis et al.[80] and Curtis et al.[81] suggest that autoinduction of TCDD metabolism and biliary excretion in the rat may not occur, or occurs to an extent that is not biologically relevant.

Limited data suggest that autoinduction of metabolism and biliary excretion does occur for PCDFs, in contrast to PCDDs. Pretreatment of rats with 2,3,7,8-TCDF (1.0 μmol/kg, 3 days earlier) significantly increased the biliary excretion of a subsequent dose of [^{14}C]2,3,7,8-TCDF.[114] The naive rats excreted 5.7 ± 2.4% of the dose over the initial 8 h, while the pretreated rats excreted 13.2 ± 3.2% of the [^{14}C]2,3,7,8-TCDF. Similarly, pretreatment of rats with 2,3,4,7,8-penta-CDF (500 μg/kg, per os, 3 days earlier) resulted in a two-fold increase in the biliary elimination of a subsequent dose of [^{14}C]2,3,4,7,8-penta-CDF.[16] These results suggest that pretreatment with 2,3,7,8-TCDF and 2,3,4,7,8-penta-CDF induces the metabolism of these congeners.

Isolated hepatocytes in suspension culture have been used as an in vitro system for studying the autoinduction of metabolism of TCDD and related compounds. In vitro results at a high substrate concentration (2.2 μ*M*) indicate that TCDD can induce its own rate of metabolism in the rat and hamster.[115] In contrast, TCDD was not able to induce its own rate of metabolism in guinea pig and mouse hepatocytes.[83,116] The kinetics of TCDD metabolism was also investigated in isolated rat hepatocytes incubated with [^{3}H]TCDD at concentrations of 0.01, 0.1, and 1.0 μ*M*.[117] Lower TCDD concentrations in the media result in concentrations in hepatocytes which are more similar to the levels in the liver after in vivo exposure. For example, the concentration of TCDD in hepatocytes incubated at 0.01 μ*M* are similar to hepatic levels after in vivo exposure of rats at a dose of about 10 μg/kg. At 0.01 and 0.1 μ*M*, the rate of metabolism of [^{3}H]TCDD was similar in hepatocytes isolated from control and TCDD pretreated rats, while at 1.0 μ*M*, [^{3}H]TCDD metabolism was greater in hepatocytes isolated from TCDD pretreated rats. The results indicate that TCDD can induce its own rate of metabolism in the rat, but only at high hepatic concentrations, which are generally not attained after in vivo exposure. Therefore, in vitro studies of the hepatic metabolism of TCDD (at 0.01 and 0.1 μ*M*) are consistent with the lack of autoinduction of TCDD metabolism and biliary excretion observed in vivo in the rat.[80,81]

The metabolism of [^{3}H]2,3,7,8-TCDF was also investigated in isolated rat hepatocytes incubated at concentrations of 0.01, 0.1, and 1.0 μ*M*.[117] At all concentrations, hepatocytes from TCDD pretreated rats metabolized 2,3,7,8-TCDF at a rate 4- to 25-fold greater than that observed in hepatocytes from control rats. Results indicate that 2,3,7,8-TCDF is metabolized in rat liver by the TCDD-inducible enzyme, cytochrome P450lA1.[118] These in vitro results support the in vivo autoinduction of 2,3,7,8-TCDF metabolism and biliary elimination observed in the rat.[114] The results also suggest that 2,3,7,8-TCDF will be far more persistent following exposures at low doses which do not significantly induce CYP1A1.

6.4.4 Excretion in Animals

Data regarding the excretion of TCDD and related compounds after exposure to a single radiolabeled congener support the assumption of a first-order elimi-

nation process consisting of one or more components. TCDD was excreted slowly from all species tested, with half-lives ranging from 11 days in the hamster to 7.2 to 8.7 years in humans. TCDD is exceptionally persistent in humans relative to other animal models.

Studies in the rat, guinea pig, hamster, and mouse have found that essentially all of the TCDD-derived radioactivity excreted in the urine and bile corresponds to metabolites of TCDD. The apparent absence of TCDD metabolites in liver and fat suggests that once formed, the metabolites of TCDD are excreted readily. Thus, urinary and biliary elimination of TCDD depends on metabolism of the toxin. The more limited data for other compounds also suggest that this relationship may be true for 1,2,3,7,8-penta-CDD, 2,3,7,8-TBDD, 2,3,7,8-TCDF, 1,2,3,7,8-penta-CDF, 2,3,4,7,8-penta-CDF, and 3,3′,4,4′-TCB.

Although urine and bile appear to be free of unmetabolized TCDD, TCDD and its metabolites are excreted in the feces of guinea pigs, rats, mice, and hamsters treated with [^{3}H]- and/or [^{14}C]TCDD.[1,10,97,98] The daily presence of unchanged TCDD in feces and its absence in bile suggests that direct intestinal elimination may be the source of the fecal excretion of TCDD. Lactation, direct intestinal elimination, and perhaps sebum may serve as routes for excretion of TCDD, which do not depend on metabolism of the toxin. These data suggest that the in vivo half-life for elimination of TCDD and related compounds only provides an approximation of the rate of metabolism of these compounds in a given animal.

The rate of excretion of TCDD and related compounds is species and congener specific. TCDD is most persistent in human and nonhuman primates. In the hamster, the least sensitive species to the acute toxicity of TCDD, the mean $t_{1/2}$ was 10.8 days,[10,119] and in the guinea pig, the most sensitive species to the acute toxicity of TCDD, the mean $t_{1/2}$ was 94 days.[97] 2,3,7,8-TCDF was also most persistent in the guinea pig, with a $t_{1/2}$ of 20 to 40 days.[14,15] Furthermore, results indicate that the relatively limited ability of the guinea pig to metabolize TCDD and 2,3,7,8-TCDF may contribute to the greater persistence and greater acute toxicity of these congeners in the guinea pig.

The tissue distribution, metabolism, and excretion of TCDD were also investigated in Han/Wistar and Long–Evans rats, which were, respectively, most resistant ($LD_{50} > 3000$ μg/kg) and most susceptible ($LD_{50} \sim 10$ μg/kg) to the acute toxicity of TCDD.[51] The results suggest that the metabolism and disposition of TCDD do not have a major role in explaining the strain differences in toxicity.

6.4.5 Excretion in Humans

Poiger and Schlatter[11] investigated the excretion of TCDD in a 42-year-old man (92 kg) after ingesting 105 ng [^{3}H]TCDD in 6 mL of corn oil. The half-life for elimination was estimated to be 2120 days, based on fecal excretion over a 125-day period following the single exposure (Table 6.4). The concentration of [^{3}H]TCDD-derived radioactivity was also measured in adipose tissue in the

TABLE 6.4 Half-Life Estimates for TCDD and Related Compounds in Humans

Chemical	Exposure Incident	Number of Individuals	Sample	Time Period between First and Last Analysis	Number of Time Points	Half-Life (yr)	Ref.
PCDDs							
2,3,7,8-TCDD	Male volunteer	1	Fecal excretion	125 days	28	5.8	11
	Male volunteer	1	Adipose tissue	6 yr	5	9.7	120
	Ranch Hand Vietnam veterans	36	Serum	5 yr	2	7.1[b]	121
	Ranch Hand Vietnam veterans	337	Serum	5 yr	2	11.3[c]	122
	Ranch Hand Vietnam veterans	213	Serum	10 yr	3	8.7	123
	Ranch Hand Vietnam veterans	97	Serum	15 yr	4	7.6	124
	German herbicide plant workers	48	Serum	6.3 yr	2–3	7.2	125
	Seveso, Italy	27	Serum	15.9 yr	2	8.2	126
1,2,3,7,8-penta-CDD	German herbicide plant workers	40	Serum	6.3 yr	2–3	15.7	125
1,2,3,4,7,8-hexa-CDD	German herbicide plant workers	41	Serum	6.3 yr	2–3	8.4	125
1,2,3,6,7,8-hexa-CDD	German herbicide plant workers	40	Serum	6.3 yr	2–3	13.1	125
1,2,3,7,8,9-hexa-CDD	German herbicide plant workers	39	Serum	6.3 yr	2–3	4.9	125
1,2,3,4,6,7,8-hepta-CDD	German herbicide plant workers	26	Serum	6.3 yr	2–3	3.7	125
Octa-CDD	German herbicide plant workers	32	Serum	6.3 yr	2–3	6.7	125
1,2,3,6,7,8-hexa-CDD	Technical pentachlorophenol in wood of home	1	Adipose tissue	28 mo	2	3.5	132
1,2,3,4,6,7,8-hepta-CDD	Technical pentachlorophenol in wood of home	1	Adipose tissue	28 mo	2	3.2	132
Octa-CDD	Technical pentachlorophenol in wood of home	1	Adipose tissue	28 mo	2	5.7	132

PCDFs							
2,3,4,7,8-penta-CDF	German herbicide plant workers	5	Serum	6.3 yr	2–3	19.6	125
1,2,3,4,7,8-hexa-CDF	German herbicide plant workers	42	Serum	6.3 yr	2–3	6.2	125
1,2,3,6,7,8-hexa-CDF	German herbicide plant workers	31	Serum	6.3 yr	2–3	6.0	125
2,3,4,6,7,8-hexa-CDF	German herbicide plant workers	6	Serum	6.3 yr	2–3	5.8	125
1,2,3,4,6,7,8-hepta-CDF	German herbicide plant workers	22	Serum	6.3 yr	2–3	3.0	125
1,2,3,4,7,8,9-hepta-CDF	German herbicide plant workers	6	Serum	6.3 yr	2–3	3.2	125
2,3,4,7,8-penta-CDF	Binghamton, New York, state office building	1	Adipose tissue	Initial 43 mo	4	4.7	130
			Blood	Final 29 mo	4	7.2	
			Combined	Total 6 yr	7	4.5	
1,2,3,4,7,8-hexa-CDF	Binghamton, New York, state office building	1	Adipose tissue	Initial 43 mo	4	2.9	130
			Blood	Final 29 mo	4	4.4	
			Combined	Total 6 yr	7	4.0	
1,2,3,6,7,8-hexa-CDF	Binghamton, New York, state office building	1	Adipose tissue	Initial 43 mo	4	3.5	130
			Blood	Final 29 mo	4	4.3	
			Combined	Total 6 yr	7	4.9	
1,2,3,4,6,7,8-hepta-CDF	Binghamton, New York, state office building	1	Adipose tissue	Initial 43 mo	4	6.5	130
			Blood	Final 29 mo	4	4.1	
			Combined	Total 6 yr	7	6.8	
2,3,4,7,8-penta-CDF	Yucheng	4	Blood	Initial 2.9 yr	2	1.3	129
		3		Final 2.7 yr	2	2.9	
		2		Total 5.6 yr	3	1.7	
1,2,3,4,7,8-hexa-CDF	Yucheng	4	Blood	Initial 2.9 yr	2	2.1	129
		3		Final 2.7 yr	2	5.1	
		2		Total 5.6 yr	3	2.4	
1,2,3,4,6,7,8-hepta-CDF	Yucheng	4	Blood	Initial 2.9 yr	2	1.6	129
		3		Final 2.7 yr	2	6.1	
		2		Total 5.6 yr	3	2.4	

(Continued)

TABLE 6.4 *(Continued)*

Chemical	Exposure Incident	Number of Individuals	Sample	Time Period between First and Last Analysis	Number of Time Points	Half-Life (yr)	Ref.
2,3,4,7,8-penta-CDF 1,2,3,4,7,8-hexa-CDF 1,2,3,4,6,7,8-hepta-CDF	Yucheng	3	Blood	9 yr	5–6	2–3	131
2,3,4,7,8-penta-CDF 1,2,3,4,7,8-hexa-CDF	Yusho	9	Blood	7 yr	3–5	> 5	131
1,2,3,4,6,7,8-hepta-CDF	Technical pentachlorophenol in wood of home	1	Adipose tissue	28 mo	2	< 1.7	132
Octa-CDF	Technical pentachlorophenol in wood of home	1	Adipose tissue	28 mo	2	1.8	132
PCBs							
3,3′,4,4′,5-penta-CB	Yucheng	na	Blood	na	na	< 1	131
3,3′,4,4′,5,5′-hexa-CB	Yucheng	na	Blood	na	na	10	131

[a] na, Not applicable.

[b] 95% confidence interval about the median of 5.8–9.6 yr.

[c] 95% confidence interval about the median of 10.0–14.1 yr.

same person over a 6-year period following exposure. A more accurate estimate of a TCDD half-life of 9.7 years was calculated based on adipose tissue concentrations over a 6-year period.[120] Table 6.4 summarizes additional half-life estimates for TCDD and related compounds in humans, based on serum and/or adipose tissue concentrations at two or more time points.

The Air Force is currently conducting a prospective study of veterans of Operation Ranch Hand, the unit responsible for the aerial spraying of herbicides, contaminated with 2,3,7,8-TCDD, in Vietnam from 1962 to 1971. A subset of the Ranch Hand cohort has had a series of up to four serum TCDD analyses conducted to investigate the elimination of TCDD in humans. Initially, the half-life of TCDD in humans was estimated to be about 7 years on the basis of TCDD levels in serum samples taken in 1982 and 1987 from 36 of the Ranch Hand personnel who had TCDD levels $>$ 10 ppt in 1987.[121] Wolfe et al.[122] investigated the half-life of TCDD in an expanded cohort of 337 Air Force veterans of Operation Ranch Hand that also included the 36 subjects of the earlier half-life study by Pirkle et al.[121] Based on paired TCDD measurements from serum collected in 1982 and in 1987, the authors reported a mean predicted half-life of 11.6 years and a median observed half-life of 11.3 years with a nonparametric 95% confidence interval of 10.0 to 14.1 years. The authors also investigated how the TCDD half-life varied with percent body fat (PBF), relative changes in PBF from 1982 to 1987, and age. They found that the TCDD half-life increased significantly with a high PBF value, suggesting that persons with more body fat tend to eliminate TCDD more slowly. In contrast, increasing age was associated with a shorter half-life. The redistribution of fat stores from subcutaneous to abdominal areas with aging, resulting in greater mobilization of TCDD, could in part explain the shorter half-life observed in older veterans. An increase in PBF from 1982 to 1987 was also associated with a decrease in half-life, which can be explained by a dilution of the existing body burden of TCDD into the increasing adipose tissue mass.

More recently, Michalek et al.[123] estimated the half-life of TCDD in 213 veterans of Operation Ranch Hand based on TCDD serum analyses conducted in 1982, 1987, and 1992. Of the 278 subjects with complete data in all three years, 213 were included for analysis of half-life based on the criteria of TCDD levels greater than 22.3 ppt in 1982, greater than 14.9 ppt in 1987, and greater than 10 ppt in 1992. All TCDD levels were background corrected by subtracting 4 ppt, and the logarithm of the background-corrected levels were modeled as a linear function of time to estimate decay rates using first-order kinetics. Using the Toeplitz assumption, the unadjusted estimated decay rate is 0.0797 per year (95% CI of 0.0727 to 0.0868), giving an unadjusted half-life estimate of 8.7 years (95% CI of 8.0 to 9.5 years). The adjusted half-life was found to increase significantly with an increase in percent body fat in 1982, but the half-life did not vary with age or relative changes in percent body fat. Most recently, Michalek and Tripathi[124] estimated the half-life of TCDD in 97 veterans of Operation Ranch Hand based on TCDD serum analyses conducted in 1982, 1987, 1992, and 1997. Of the 244 subjects with complete data at all four time

points, only 97 were included for analysis of half-life based on the criteria of TCDD levels greater than 39.5 ppt in 1982, greater than 25.0 ppt in 1987, greater than 15.8 ppt in 1992, and greater than 10 ppt in 1997. With increasing time since the initial exposure to TCDD, a greater proportion of the population was excluded from the analysis, as more subjects approach background body burdens of TCDD. Using the methods of the previous report,[123] the unadjusted estimated elimination rate was 0.0915 per year (95% CI of 0.0844 to 0.0986) giving an unadjusted half-life estimate of 7.6 years (95% CI of 7.0 to 8.2 years). Due to the smaller sample size, the current elimination rate estimate based on four measurements per subject has less precision than the earlier estimate of Michalek et al.,[123] which was based on three measurements per subject. Once again, the elimination rate decreased slightly but significantly as percent body fat increased, supporting the hypothesis that individuals with more body fat tend to eliminate TCDD more slowly than do those with less body fat. Michalek and Tripathi[124] also reported no significant change in the elimination rate with age or with relative changes in percent body fat.

The half-life of TCDD has also been investigated in two additional populations. Flesch-Janys et al.[125] studied a group of 43 German herbicide plant workers that had initial TCDD serum levels from 15.6 to 300 ppt. A median half-life estimate of 7.2 years was reported for this occupational cohort, which received an initial exposure to TCDD similar to that of the Ranch Hand veterans. A similar half-life estimate of 8.2 years was reported in 27 victims of the accident in Seveso, Italy[126] (see Chapter 20 for a discussion of the Seveso accident). This cohort had a greater initial exposure, resulting in serum levels of 130 to 3830 ppt TCDD. This study also included the early and later portions of the TCDD decay curve since the initial blood sampling began immediately following exposure and continued for 15.9 years. Thus, based on results from the Ranch Hand, German, and Seveso studies, the estimated half-life of TCDD in humans is from 7.2 to 8.7 years (Table 6.4).

Half-life estimates for other PCDDs and PCDFs have been estimated to range from 0.8 to 19.6 years (Table 6.4). Some of the half-life values in Table 6.4 are rough estimates based on a small number of persons and analysis at as few as two time points. Phillips[127] discusses this issue. Estimates also assume a simple, single-compartment, first-order elimination process.

In the largest and most comprehensive study, Flesch-Janys et al.[125] investigated the elimination of 2,3,7,8-chlorine-substituted PCDDs and PCDFs in a cohort of workers from a herbicide-producing plant in Germany (summarized in Table 6.4). The study group consisted of 45 males and 3 females with a mean duration of employment of 13.1 years. Mean time between end of employment and first blood sample was 5.4 years (median 2 years), and mean time between first and last blood sample was 5.6 years (median 6.3 years). A total of 43 subjects with two serum samples and 5 subjects with three serum samples were included in the study. For each congener, only those subjects whose congener serum levels exceeded 95% of German background concentration were included in the analysis. The mean background concentration was also subtracted from every original measurement before analysis. For 2,3,7,8-TCDF,

1,2,3,7,8-penta-CDF, and octa-CDF, no half-life was estimated because no person in the study passed the inclusion criteria above. 2,3,7,8-TCDF and 1,2,3,7,8-penta-CDF are excreted in animals much more rapidly than other congeners,[5] suggesting that these congeners may also be excreted more rapidly in humans. Conversely, 2,3,4,7,8-penta-CDF is far more persistent in animal models than TCDD, which supports the estimated 19.6-year half-life of 2,3,4,7,8-penta-CDF and 7.2-year half-life of TCDD in humans. However, this estimate was based on only five subjects, which met the criteria for inclusion in the study. With the exception of 1,2,3,7,8-penta-CDD and 1,2,3,6,7,8-hexa-CDD, the median half-lives of the PCDDs are generally similar. The estimates for these two congeners may be somewhat unstable, due to variable individual rate constants for elimination and the fact that about 25% of the population showed no decrease in serum levels over the sampling period. Furthermore, the investigation found that increasing age and percent body fat were associated with increasing half-life for most congeners. Finally, it is important to note that the half-life data reflect only the elimination of PCDDs and PCDFs from blood lipid and for all congeners may not reflect elimination from different storage sites. In the case of TCDD, it can be assumed that the half-life estimate reflects elimination from the main storage site, since about 90% of the body burden is sequestered in fat and the blood fat/adipose tissue concentration is about 1.[5,61–63] Data are more limited on the relative amount of other congeners stored in adipose tissue in humans, and limited and somewhat conflicting data suggest that the blood fat/adipose tissue concentration ratio may increase up to a factor of 2 for octa-CDD.[63,128] Thus, some uncertainties remain regarding the extent that the observed decrease in serum levels of higher PCDDs and PCDFs reflects the elimination of these compounds from the body.

Ryan and Masuda[129] reported on their continuing investigation into the elimination of PCDFs in humans from the Yusho and Yucheng rice oil poisonings (see Chapters 21 and 22, respectively, for descriptions of Yusho and Yucheng poisonings). Yucheng patients had PCDF blood levels on a lipid basis of 1 to 50 μg/kg, while Yusho patients had levels of 0.1 to 5 μg/kg. In the Yucheng patients, half-lives for three PCDFs were 2 to 3 years. Elimination from Yusho patients was more variable and slower, with half-lives over 5 years (see Table 6.4), and in several cases, no measurable elimination occurred during the 7 years in which samples were available. The limited results suggest that clearance of these PCDFs in the human is biphasic, with faster elimination at higher exposure. Schecter et al.[130] and Ryan and Masuda[131] also reported longer half-life values for PCDFs in humans at later time points after exposure, when concentrations are closer to the background levels of people with no unusual exposure.

6.4.6 Fecal Excretion: Approaches to Enhance Elimination

While results from animal studies suggest that direct fecal excretion of unmetabolized PCDDs and PCDFs represents a significant mechanism for the elimination of these lipophilic compounds, human data have been limited until

recently. The mass balance study of Schlummer et al.[25] provided the experimental human data in support of the two-step model of PCDD and PCDF transfer in the gastrointestinal tract, where absorption and excretion are distinct processes occurring at the small and large intestine, respectively (see Section 6.2.1). Rohde et al.[133] conducted a digestive tract mass balance study of six German men (age 41 to 73 years) with occupational exposure to PCDDs and PCDFs. Blood lipid levels of the subjects in 1996 ranged from 84 to 505 pg/g lipid for TCDD and 270 to 640 pg/g lipid for TEQs, compared with background levels in unexposed persons of 5.2 and 32 pg/g lipid, respectively. The daily quantity of nonmetabolized 2,3,7,8-chlorine-substituted PCDDs and PCDFs excreted in the feces exceeded the daily uptake from food, indicating significant clearance across the gastrointestinal tract. The concentration of these compounds in feces was also found to be highly correlated with that in blood, demonstrating that the fecal PCDD and PCDF content was related directly to the body burden of these compounds. No significant clearance (excretion via feces at least fourfold greater than uptake by food) was observed for congeners, including 2,3,7,8-TCDF, 1,2,3,7,8-penta-CDF, 1,2,3,4,7,8,9-hepta-CDF, or octa-CDF, which were not markedly elevated in the serum lipids. Together, these results support the relationship that fecal excretion is regulated by the lipid-based blood concentration of these compounds. The half-lives in these subjects, due to fecal clearance of nonmetabolized congeners, were estimated from the excretion rate and current body burden and ranged from 10 years for octa-OCDD (OCDD) to 22 years for TCDD to 33 years for 2,3,4,7,8-penta-CDF. Congener-specific half-lives, similar to that reported by Flesch-Janys et al.,[125] were also calculated based on the decrease in serum lipid level of congeners between 1990–1992 and 1996. The fecal clearance of nonmetabolized PCDDs and PCDFs contributed on average from 37% (TCDD) to 90% (OCDD) of the total elimination. Thus, fecal clearance plays an important role in the overall elimination of most congeners, with the daily fecal excretion estimated to be equivalent to the amount of TEQ present in about 1.7 g of blood lipids.[133]

Since direct fecal excretion is a significant route for the excretion of nonmetabolized PCDDs and PCDFs,[25,133] two recent studies investigated whether Olestra, a nonabsorbable sucrose–polyester synthetic fat substitute, may enhance the elimination of these compounds in humans. Moser and McLachlan[134] compared the fecal excretion of PCDDs, PCDFs, PCBs, and HCB in three subjects, with background exposures, while eating an Olestra-free diet and while eating a diet supplemented with 25 g/day of Olestra. The fecal excretion while on the Olestra diet was 1.5- to 11-fold, higher depending on the congener. If fecal excretion is estimated to contribute 40% of the overall elimination of TCDD, and the Olestra diet enhanced fecal excretion 5.7-fold, the overall rate of elimination of TCDD would be more than doubled while on the Olestra diet. Geusau et al.[135] investigated the effect of an Olestra-supplemented diet on the excretion of TCDD in two patients with chloracne and very high serum TCDD levels of 144,000 and 26,000 pg/g lipid. A diet supplemented

with fat-free potato chips (33 to 66 g Olestra/day) enhanced the fecal excretion of TCDD by up to 8- to 10-fold. Results suggest that the increase in fecal excretion of TCDD was due mainly to an increase in the amount of fat (dietary fat plus olestra) excreted via the feces. The resulting elimination half-lives of TCDD due to fecal excretion were estimated to be 1.4 years in the more highly exposed patient and 1.9 years in the other person. However, half-lives of 200 and 230 days, respectively, were determined based on analysis of serum and adipose tissue TCDD levels over the 8-month observation period. The observed half-lives were far shorter than can be explained by enhanced fecal or other elimination mechanism. In addition to the enhanced fecal excretion with Olestra, the authors speculate that the high levels of TCDD may have also induced the metabolism of TCDD in these subjects, but no data are available to support this speculation.

In related studies, Morita et al.[136–139] investigated the role of dietary fiber or *Chlorella* in the fecal excretion of PCDDs and PCDFs in rats. Rice bran fibers enhanced the fecal excretion of PCDDs from 0.6 to 2.3 and of PCDFs from 0.5- to 10.4-fold above that of rats on a control diet.[138] *Chlorella* is a unicellular green algae, sold as a health food or health supplement. *Chlorella* in the diet of rats also enhanced the fecal excretion of PCDDs from 0.8 to 5.6 and PCDFs from 0.9- to 11.1-fold above that of rats on a control diet.[139] Dietary fiber, chlorophyll, and/or lipid in the *Chlorella* may be factors responsible for the enhanced fecal excretion of PCDDs and PCDFs observed in this study. Thus, fiber and/or *Chlorella* may be other dietary factors capable of increasing the fecal excretion of PCDDs and PCDFs.

6.4.7 Lactation

Due to the lipophilic nature of milk, lactation can provide a relatively efficient mechanism for decreasing the body burden of TCDD and related PCDDs and PCDFs in women. As discussed by Schecter and Gasiewicz[140,141] and Graham et al.,[142] the elimination of TCDD and related compounds through mother's milk can result in high exposure levels in the infant. Since both milk and the fatty tissues of fish are essentially providing an oily vehicle, it would be likely that these sources would provide TCDD and related compounds in a form that is readily bioavailable. The relatively high bioavailability of PCDDs and PCDFs from mother's milk in nursing infants was discussed in Section 6.2.1. Further discussion of lactation as a route for excretion of PCDDs and PCDFs in women and exposure in infants is given in Section 6.6.

6.5 PHYSIOLOGICALLY BASED PHARMACOKINETIC MODELS

Physiologically based pharmacokinetic (PB-PK) models have been developed for TCDD in C57BL/6J and DBA/2J mice,[143] rats,[144] and humans.[145] PB-PK models incorporate known or estimated anatomical, physiological, and phys-

icochemical parameters to describe quantitatively the disposition of a chemical in a given species. PB-PK models can assist in the extrapolation of high-to-low dose kinetics within a species, estimating exposures by different routes of administration, calculating effective doses, and extrapolating these values across species.[146]

Andersen et al.[147] described a receptor-mediated PB-PK model for the tissue distribution and enzyme-inducing properties of TCDD. The data used for this analysis were from two previously published studies with Wistar rats.[70,148] The model was used to examine the tissue disposition of TCDD and the induction of both a dioxin-binding protein (CYP1A2) and CYP1A1.

Kohn et al.[149] developed a mechanistic model of the effects of dioxin on gene expression in the rat liver (referred to as the NIEHS model). The model includes the tissue distribution of TCDD in the rat and its effect on concentrations of CYP1A1 and CYP1A2 as well as the effects of TCDD on the Ah, estrogen, and EGF receptors over a wide TCDD dose range. Experimental data from Tritscher et al.[87] and Sewall et al.[150] were incorporated into the NIEHS model. Female Sprague–Dawley rats were injected with an initiating dose of dimethylnitrosamine, and after 10 days, the rats were exposed biweekly to TCDD in corn oil by gavage at doses equivalent to 3.5 to 125 ng/kg per day for 30 weeks. The NIEHS model predicts a linear relationship between administered dose and the concentration in the liver over this dose range, which is in agreement with the data of Tritscher et al.[87] The biochemical response curves for all these proteins were hyperbolic, indicating a proportional relationship between target tissue dose and protein concentration at low administered doses of TCDD.

A fugacity-based PB-PK model for the elimination of TCDD from humans was developed by Kissel and Robarge.[145] Transport within the body was assumed to be perfusion limited. TCDD was assumed to be uniformly distributed within each tissue or fluid phase, and tissue levels were considered to be in equilibrium with exiting fluids (blood, bile, and urine). TCDD is poorly metabolized in humans, thus reducing the necessity of modeling the fate of metabolites. TCDD also seems to exhibit fugacity-based partitioning behavior between tissues in humans, as evidenced by relatively constant lipid-based tissue distribution,[89,151] although this is not the case in rodents.[82,143,144] With a daily human background intake of TCDD in North America of about 50 pg/day,[152] the steady-state adipose tissue concentration predicted by the model, assuming no metabolism, was 7.7 ppt. This is similar to the lipid-based blood tissue levels reported in the general population with no known unusual exposure. The model was also used to predict the elimination of TCDD from Ranch Hand Vietnam veterans. The model simulation assumed a background exposure of 50 pg/day and no metabolism. Under these conditions, apparent half-lives of 4.4, 5.2, 5.9, 7.2, 9.1, and 20 years were estimated for persons with adipose tissue concentrations of 100, 50, 30, 20, 15, and 10 ppt, respectively. The model predicted half-lives are similar to the experimental value of 7.1

years, based on analysis of TCDD in blood lipids of veterans with adipose burdens greater than 10 ppt[121] (see Table 6.4). The apparent half-lives derived from the model increased as the adipose tissue concentrations approached the steady-state level associated with background exposure. Ryan and Masuda[129] reported a similar relationship for PCDFs, with experimentally derived half-lives increasing in persons with lower body burdens of the compounds. Finally, the model was found to approximate the elimination of TCDD from one volunteer as reported by Poiger and Schlatter.[11] Taken together, the comparisons described above suggest that a fugacity-based PB-PK model for TCDD in humans can provide one method for describing the elimination of TCDD from humans.

PB-PK models are limited primarily by the availability of congener and species-specific data that accurately describe the dose- and time-dependent disposition of TCDD and related compounds. As additional data become available, particularly on the dose-dependent disposition of these compounds, more accurate models can be developed. In developing a suitable model in the human, it is also important to consider that the half-life estimate of 7.2 to 8.7 years for TCDD was based on the assumption of a single-compartment, first-order elimination process.[121] It is likely that the excretion of TCDD in humans is more complex, involving several-compartment, tissue-specific binding proteins and a continuous daily background exposure. Furthermore, changes in body weight and body composition should also be considered in developing PB-PK models for TCDD and related compounds in humans.

6.5.1 Estimating Daily Intake of TCDD

Because TCDD is highly lipophilic, it has been shown that a majority of the TCDD in any body tissue is stored in the fat.[153] As a first approximation, a one-compartment pharmacokinetic model with first-order elimination may be used to compute the daily intake of TCDD based on steady-state concentrations in the fat. The mass balance model is

$$V_F \frac{dC_F}{dt} = D - k_e A_F \tag{6.1}$$

where V_F is the volume of fat, C_F the concentration of TCDD in fat, D the daily dose (mass/time), k_e the first-order elimination constant (time^{-1}), and A_F the mass of TCDD in fat. Concentration C_F is given by

$$C_F = \frac{A_F}{V_F} \tag{6.2}$$

Then, at steady state ($dC_F/dt = 0$), daily dose exactly balances elimination:

$$D = k_e A_F = k_e V_F C_F \tag{6.3}$$

Note that k_e can be expressed in terms of half-life:

$$k_e = \ln \frac{2}{t_{1/2}} \tag{6.4}$$

Substituting (6.4) into (6.3), one obtains the following expression for daily dose in terms of fat concentration:

$$D = \ln \frac{2}{t_{1/2}} V_F C_F \tag{6.5}$$

It is important to recall the two assumptions implicit in the derivation of the foregoing formula for daily uptake. First, steady-state conditions are assumed. Given that the half-life of some of these compounds is long (e.g., for TCDD the half-life is 7.2 to 8.7 years), steady-state levels would be approached only if the level of exposure were constant for 15 to 30 years. Pinsky and Lorber[154] compiled data from several studies which indicate that environmental concentrations of TCDD and related compounds have been decreasing over the past 20 to 30 years. They use a single-compartment model similar to the one presented above, but with a time-varying exposure profile rather than the constant input. The profile was determined statistically based upon previously recorded environmental trends. Using the prior exposure knowledge, Pinsky and Lorber found that the pharmacokinetic model was able to better predict body burdens that have been recorded over time than the steady-state model did. By manipulating the non-steady-state model and comparing results to the steady-state approximation, it can be shown for a given body burden measurement, the steady-state approximation would result in an overestimate of daily intake. Using some estimates of the decreasing exposure function presented by Pinsky and Lorber,[154] it appears that the overestimate of daily intake could be 20% or more with the steady-state model.

Another assumption of the simple model presented in this section is that the elimination kinetics are assumed to be constant over the person's entire life. Because TCDD and related compounds are stored primarily in fat, sudden weight loss and lactation would result in alterations of the TCDD elimination rate. Again, it would be assumed that for calculation of daily intake due to background exposure, the body burden data from such people would be identified and calculations handled accordingly.

The equation below shows a sample calculation for TCDD using equation (6.5). A fat volume of 14 L was chosen, representing 20% of the body weight. Also, for the purposes of this example, 1 mL of tissue was assumed to be equivalent to 1 g. Table 6.5 shows the estimated daily intake of TCDD at sev-

TABLE 6.5 Estimated Daily Intake of TCDD at Several Conditions

Half-Life (yr)	Fat Volume (L)	Fat Concentration (ppt)	Calculated Daily Intake (pg/kg per day)
5.8	14.0	6.72	0.44
7.0	14.0	6.72	0.37
5.8	14.0	5.00	0.33
7.0	14.0	5.00	0.27
5.8	7.0	6.72	0.22
7.0	7.0	6.72	0.18
5.8	7.0	5.00	0.16
7.0	7.0	5.00	0.14

eral conditions. The range of daily intakes calculated are in agreement with those reported by Fürst et al.[7] and Schecter et al.[88]

$$D = \left(\ln \frac{2}{t_{1/2}}\right) V_F C_F \left(\frac{1}{70 \text{ kg}}\right)$$
$$= \left(\ln \frac{2}{5.8 \text{ years}}\right)\left(14 \text{ L} \times 1000 \frac{\text{mL}}{\text{L}}\right)\left(6.72 \frac{\text{pg}}{\text{mL}}\right)\left(\frac{1}{70 \text{ kg}}\right)\left(\frac{1 \text{ year}}{365 \text{ days}}\right)$$

Thomaseth and Salvan[155] developed a minimal PB-PK model for TCDD in humans and utilized this model to estimate occupational exposures to TCDD. The model was reduced to one-compartment for ease of solution and was based on the following assumptions: (1) dynamic equilibrium of TCDD concentration between different body lipid distribution volumes, (2) first-order elimination proportional to TCDD liver content, and (3) daily intake proportional to body weight. The best parameter estimates based on Ranch Hand data were obtained with log-transformed data under a mixed-effects model, with liver elimination $k_f = 0.022$ day^{-1} (95% CI = 0.02 to 0.024), and background imput = 0.125 pg/kg per day (95% CI = 0.071 to 0.179). The model accounts for changes in body mass index (BMI) over time, with higher BMI being related to a longer half-life for TCDD. The model was then used to estimate occupational exposure of 253 U.S. chemical plant workers for whom one measure of serum TCDD was available. The estimated exposure of the NIOSH (National Institute for Occupational Safety and Health) cohort was 233 pg/kg per day (95% CI = 192 to 273). This model is much more rigorous than the simple steady-state approximation, and if a PK model is to be used to attempt to estimate intake, this model would be preferable. However, solving this model for daily intake is more complex and requires some assumption of the pattern of exposure over time. Alternatively, one could estimate daily intake by direct exposure calculations, that is, by examining the interaction of humans with environmental media containing the highest concentrations of dioxins.

6.6 PHARMACOKINETICS IN SPECIAL POPULATIONS

6.6.1 Prenatal and Postnatal Exposure of Offspring during Pregnancy and Nursing

Placental transfer of TCDD in rats and mice is relatively limited, with a single oral maternal exposure on gestation day 11 of mice, resulting in about 0.03% of the dose delivered to each embryo.[156] In contrast, excretion into milk represents a major pathway for maternal elimination of TCDD and for subsequent exposure of pups. During the first two postnatal weeks, mouse pups were given doses of TCDD via the milk that were, on a body weight basis, similar to that administered prenatally to their mothers.[157]

The transfer of PCDDs and PCDFs through the placenta and via the milk was investigated in rats and a marmoset monkey.[158,159] All of the congeners in rat fetal and neonatal tissues were 2,3,7,8-substituted with the exception of 2,3,4,6,7-penta-CDF. TCDD and 1,2,3,7,8-penta-CDD were found at the highest concentration in the liver of the newborn marmoset (about 0.15% of dose/g). For all other congeners, the concentrations in the liver of the newborn were less than 10% of the corresponding concentrations in adults. In contrast to liver, concentrations of 2,3,7,8-substituted congeners in the adipose tissue of the newborn marmoset were at least 33% of the levels in adults, and in the case of OCDD and OCDF, levels were threefold higher in the newborn than in the adult. Concentrations of the PCDDs and PCDFs in the newborn marmoset were highest in the adipose tissue, followed by the skin and liver. Thus, the hepatic concentrations in the marmoset fetus may not be representative of the rate of placental transfer of PCDDs and PCDFs in this animal model. As expected from rodent studies, the transfer of PCDDs and PCDFs via mothers milk was considerable, resulting in hepatic concentrations of TCDD, 1,2,3,7,8-penta-CDD, and 1,2,3,6,7,8-hexa-CDD in the suckled infant marmoset (postnatal day 33) higher than those in the dam. Transfer of hepta-and octa-PCDDs and PCDFs to the suckled infant was rather low, only about 10% of the levels in the dam. The pre- and postnatal transfer of TCDD to the offspring of rhesus monkeys was investigated by Bowman et al.[72] At weaning (4 months), the offspring had a TCDD concentration in adipose tissue about fourfold greater than that in the mothers. The mothers excreted from 17 to 44% of their TCDD body burden by lactation. Following weaning, the decrease in TCDD levels in adipose tissue of young monkeys apparently followed first-order, single-component kinetics, with a half-life of about 181 days.[160] The corresponding half-life in adult rhesus monkeys was reported to range from 180 to 550 days.[72]

Several studies provide data in support of the transplacental transport of PCDDs and PCDFs to the human fetus. Kreuzer et al.[161] measured the concentration of PCDDs and PCDFs in the lipids of adipose tissue and liver of three stillborn infants and detected 16 of a possible 17 congeners, with the exception of 1,2,3,7,8,9-hexa-CDF. TEQ levels ranged from 6.2 to 10.8 pg/g lipid, and TCDD levels ranged from 0.8 to 2.1 pg/g lipid for human infants

who died at birth. These levels are similar to those reported in the lipid fractions of maternal tissues, suggesting that prenatal exposure to PCDDs and PCDFs reflects the levels present in maternal tissues. Similar findings were reported earlier by Schecter et al.,[162] who detected TCDD (1.3 to 4.3 pg/g lipid) in the liver of three stillborn infants. Thus, significant prenatal exposure to PCDDs and PCDFs occurs, with the concentration of these compounds in the lipids of the newborn infants generally reflecting that in maternal lipids.

A significant source of postnatal exposure of human infants to PCDDs and PCDFs is through the ingestion of human milk. Several investigators have quantified the levels of TCDD in human milk samples. Many of the milk samples were pooled.[163] Rappe[164] reported levels of 1 to 3 ppt TCDD in milk fat from five volunteers in West Germany, and in a later report, Rappe et al.[165] reported an average level of 0.6 ppt TCDD in milk fat from four volunteers in northern Sweden. Furst et al.[166] reported an average level of 9.7 ppt TCDD in milk fat from three people in the Netherlands and < 1.0 ppt TCDD in milk fat from two people in Yugoslavia. Nygren et al.[167] reported average levels of TCDD in human milk samples from four subjects in Sweden to be 0.6 pg/g in milk fat, and in five subjects from West Germany to be 1.9 pg/g in milk fat. Schecter[64] compared PCDD and PCDF levels in human milk, in terms of milk lipid dioxin TEQs, in a number of countries characterized by varying degrees of industrialization. The United States, Japan, Canada, and Germany had values of 20, 27, 26, and 27 ppt, respectively, while Thailand, Cambodia, and Siberia had values of 3, 3, and 12, respectively.

High levels of TCDD have been detected in the milk of mothers exposed to high levels of TCDD in the environment. Reggiani[168] reported levels between 2.3 and 28.0 ppt TCDD in whole milk from mothers in Seveso. In a very early study, Baughman[169] reported levels between 400 and 1450 ppt TCDD in milk lipid from mothers in South Vietnam. Reanalysis of these South Vietnamese samples, originally collected in 1973, found 77 to 230 ppt TCDD in milk lipid.[170] Human milk samples collected in South Vietnam in 1985–1988 had 2.9 to 11.0 ppt TCDD in milk lipid,[171] while North Vietnamese samples contained 2.1 ppt TCDD in milk lipid.

Furst et al.[172] examined the levels of PCDDs and PCDFs in human milk and the dependence of those levels on the period of lactation. The mean concentrations of PCDDs in human milk (on a fat basis) ranged from 195 ppt for OCDD to 2.9 ppt for TCDD, with the levels of the other congeners decreasing with decreasing chlorination. This is in contrast to the generally lower levels of PCDFs in human milk, which range from 25.1 ppt for 2,3,4,7,8-penta-CDF to 0.7 ppt for 1,2,3,7,8-penta-CDF. An evaluation of the PCDD and PCDF levels in relation to the number of breast-fed children found that the concentrations in milk generally decreased with the greater number of children. The PCDD and PCDF levels in milk from mothers nursing their second child are on average 20 to 30% lower than those for mothers breast-feeding their first child. PCDD and PCDF levels were also analyzed in one mother over a period of 1 year after delivery of her second baby to assess the effect of duration of lacta-

tion. After breast feeding for 1 year, the mother had PCDD and PCDF levels that were 30 to 50% of the starting concentration. Levels in milk fat (ppt) at 1, 5, and 52 weeks after delivery were 251, 132, and 119 for OCDD; 7.9, 5.9, and 1.4 for TCDD; and 33.1, 24.5, and 10 for 2,3,4,7,8-penta-CDF, respectively. The results suggest a more rapid mobilization of PCDDs and PCDFs and excretion into human milk during the first few weeks postpartum. Although further studies are necessary, the limited data suggest that there are time-dependent, isomer-specific differences in the excretion of PCDDs and PCDFs in human milk.

Schecter et al.[173] assess the decrease in the levels of PCDDs, PCDFs, PCBs, DDE, and HCB in the blood and milk lipid in a mother who nursed twins over a 38-month period. During the first 23 months of nursing, the PCDD and PCDF TEQ decreased 68% (15.7 to 5.0 ppt, lipid) for blood and decreased 77% (13.6 to 3.1 ppt, lipid) for breast milk. Thus, lactation results in a similar reduction in PCDD and PCDF concentrations in the lipid fractions of blood and milk. During the first 23 months of nursing, the PCB 126 (3,3′,4,4′,5-penta-CB) milk concentration also decreased 71% (21.0 to 6.1 ppt, lipid). The authors estimate that approximately 115 ng TEQ (PCDDs, PCDFs, coplanar PCBs) was ingested by each infant from breast feeding for this extended period of time.

Abraham et al.[28] investigated the intake, fecal excretion, and blood levels of PCDDs, PCDFs, and PCB 126 in two breast-fed and two formula-fed infants. At 1 month, the concentration of PCDDs and PCDFs in breast milk were 19.7 and 22.2 TEQ (pg/g lipid), while the formula diet contained only 0.38 TEQ (pg/g lipid). At the age of 11 months, the breast-fed infants' blood PCDD and PCDF concentrations were 29.2 and 37.5 TEQ (pg/g lipid), while the formula-fed infants' blood PCDD and PCDF concentrations were 2.4 and 2.6 TEQ (pg/g lipid). At this time, the mothers that breast-fed had blood PCDD and PCDF concentrations of 12.3 and 10.5 TEQ, while the mothers that formula-fed had blood levels of 16.9 and 13.8 TEQ (pg/g lipid). Since PCB 126 has a TEF of 0.1, it is also important to note that at 11 months, breast-fed infants also have much higher levels of PCB 126 (287 and 374 pg/g lipid) relative to formula-fed infants (24 and 18 pg/g lipid). PCB 126 levels in mothers that breast-fed were 105 and 86 relative to levels of 193 and 52 (pg/g lipid) in the mothers that formula-fed. Thus, when PCB 126 is included in the TEQ calculation, the breast-fed infants total TEQ blood concentration (body burden) is more than doubled from that estimated based on PCDDs and PCDFs alone. The results of this study provide direct, quantitative data showing that the body burden (blood level) of PCDDs , PCDFs, and PCB 126 is more than 10 times higher in 11-month-old breast-fed infants compared with levels measured in 11-month-old formula-fed infants.

Although data are more limited for the coplanar PCBs, 3,3′,4,4′-TCB, 3,3′,4,4′,5-penta-CB, and 3,3′,4,4′,5,5′-hexa-CB have been detected in human milk from Swedish mothers, at concentrations of 16 to 32, 72 to 184, and 46 to 129 ppt on a fat basis, respectively.[174] Therefore, lactation appears to be an

effective means for the excretion of coplanar PCBs from mothers and a major source of postnatal exposure of nursing infants. Since 3,3′,4,4′,5-penta-CB and other coplanar PCBs are present in human milk at concentrations up to 60-fold higher than TCDD, it is important to consider the relative toxic potency of these dioxinlike compounds and their potential health impact on nursing infants.

Kreuzer et al.[161] measured the levels of PCDDs and PCDFs in the lipids of adipose tissue and liver of 17 infants (0.43 to 44 weeks of age) who died of sudden infant death syndrome. As expected, the concentrations of these compounds in breast-fed infants were higher than those in non-breast-fed infants; however, the magnitude of this difference varied due to differences in the age of the subjects and the duration of breast feeding. The TEQ concentration in the livers of these subjects were slightly, but not significantly, higher than the respective levels measured in the adipose tissue lipids. The results also suggest that the higher chlorinated congeners accumulate preferentially in liver lipids, an observation made earlier for adults in a study by Thoma et al.[67,68] (see Table 6.2).

Kreuzer et al.[161] used data from the study above and other published results to validate a physiological toxicokinetic model they developed to describe the body burden of TCDD for the entire human lifetime and the influence of breast feeding on the body burden. The model includes gender and age-dependent changes in the following parameters: body weight, volumes of liver, adipose, and muscle tissue, food consumption, and excretion of feces. The model also assumes that TCDD exposure occurs primarily from the ingestion of contaminated food, that TCDD is distributed freely in lipids, and that it is excreted unchanged in the lipids of the feces as well as following hepatic metabolism. More complex biochemical processes such as protein binding, saturation of metabolism at high TCDD concentrations, and induction of metabolism are not part of this model, which considers factors more relevant for low-level or background human exposures. With the basic assumption of this single compartment model and the free distribution of TCDD in all body lipids, including the gastrointestinal tract, the half-life of the nonmetabolic elimination ($t_{1/2}$) is proportional to the ratio of volume of body lipids (V) to the mass of lipids in stool excreted per unit time (dFa/dt). During aging, V increases at least 40 times but dFa/dt only 1.7 times (from 3 g/day in infants to 5 g/day in adults). Consequently, the half-life of the nonmetabolic elimination ($t_{1/2}$) is calculated to be only 0.42 year in newborns and 9.5 years in 40-year-old adults. According to this model, most TCDD is eliminated as unchanged compound in children, with the role of metabolism-dependent elimination becoming more important with age. Thus, the half-life increases almost linearly from its starting value of about 4 months in newborns and reaches a value of 5 years at the age of 40. An age-dependent elimination of TCDD has also been reported experimentally in the rhesus monkey.[72,160] Furthermore, the model of Kreuzer et al.[161] predicts that the relatively high TCDD concentrations that might be reached after 6 months of nursing do not lead to an elevated lifetime body burden of TCDD.

In a related study, Patandin et al.[175] investigated dietary, including lactational, exposure to PCDDs, PCDFs, and PCBs from early childhood until the early reproductive age of 25 years, to assess exposure risk to the next generation. Based on the analysis of 83 milk samples, previously reported analysis of food products and food questionnaire data, the daily TEQ intake per kilogram of body weight is 50 times higher in breast-fed infants and three times higher in toddlers than in adults. Although exposures are relatively high in breast-fed infants, breast feeding for 6 months contributes only 12 and 14% to the respective body burdens of men and women at the age of 25 years. After weaning, dairy products, processed foods, and meat are major sources of exposure to these compounds. Thus, while breast-fed infants have higher body burdens of PCDDs, PCDFs, and coplanar PCBs than those of formula-fed infants, the impact of breast feeding on the body burdens of these compounds decreases as people approach adulthood.

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CHAPTER 7

Dose–Response Modeling for 2,3,7,8-Tetrachlorodibenzo-*p*-Dioxin

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7.1 INTRODUCTION

There is an increasing consensus in the scientific community that high-level exposure to dioxins pose human health risks. As discussed in other chapters in this book, dioxins induce a variety of toxic effects in experimental animals, ranging from biochemical alterations to adverse effects such as developmental, reproductive, dermal, and hepatic toxicities. In addition, there are numerous studies in experimental animals demonstrating that 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is a complete carcinogen (reviewed in Chapter 11). Several human cohorts demonstrate an association between TCDD exposure and increased cancer risk. While human epidemiological data are accumulating, they are derived mainly from occupationally exposed workers (reviewed in Chapter 18), who are, for the most part, adult males. In several of these cohorts, there is an increased risk for all cancer types as well as an increased risk for lung tumors. The human and animal data clearly indicate that exposure to high doses of dioxins can result in adverse health effects, both cancer and

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noncancer. In fact, the human and animal cancer data are compelling enough that IARC[1] and NIH[2] have independently categorized TCDD as a known human carcinogen. What is much less certain are the quantitative estimates of the potential human health risks associated with exposure to TCDD and other dioxins, particularly background exposures in the general population.

Estimating potential human health risks from dioxins requires an understanding of the dose–response relationships for the various biological effects of TCDD and related chemicals. These relationships can be quantified through either empirical or mechanistic modeling. In this chapter we describe the present understanding of the dose–response relationships for some of the many biological effects of TCDD in animals and humans. In the following sections, we describe briefly the complexities associated with the simple terms *dose* and *response*. We also discuss the various modeling approaches used to describe the dose–response relationships for TCDD and related chemicals. Finally, we discuss the results of the modeling exercise examining the dose–response relationships for the cancer and noncancer effects induced by TCDD in experimental animals and humans.

7.2 CHOICE OF DOSE METRIC

One of the main principles of toxicology is that the dose makes the poison. The simplicity of this principle can be misleading. Dose can be described through a variety of metrics. Dose is typically expressed in units of magnitude of exposure and the frequency over which the exposure applies. Examples of dose metrics frequently used are administered dose, blood concentrations, area under the blood concentration curve, lifetime average daily dose, and body burdens. The choice of dose metric used is dependent on the intended use of the dose metric. For example, when extrapolating dose across species, different chemicals may require different dose metrics, depending on the response of concern, the physical properties of the chemical, and pharmacokinetic and pharmacodynamic differences between animals and humans. Dose metric issues are not limited to toxicology. Dose metrics issues are also important for therapeutic agents. Area under the blood concentration versus time curve (AUC) is used to describe dose for short-acting pharmacological agents applied in short-term exposures.[3] If chronic treatment with a therapeutic agent is required, as is typical in many disease states, steady-state blood concentration of the therapeutic agent is used as the dose metric. Both metrics assume that blood concentrations are related to target tissue concentrations. The difference between the use of dose metrics in toxicology and pharmacology is that in pharmacology, the focus is mostly on human data. In contrast, toxicologist's predominately use experimental data from animals and extrapolate this information to humans. Such extrapolations often require different approaches to describe dose.

When determining which dose metric to use, it is important to understand the toxic response the metric is describing and whether the dose metric is

intended for use in species extrapolation. Different types of toxicity may be better explained by different dose metrics. Some toxic effects are reversible and short-lived, requiring peak concentrations in order to observe the effect, such as narcosis. Others, such as developmental toxicities, have specific windows of sensitivity and exposures before or after these windows do not result in toxic responses. Responses such as cancer may require prolonged periods of exposure to develop. Dioxins induce a number of different biological effects, ranging from biochemical alterations to cancer. It is unlikely that for dioxins a single dose metric would best describe the dose–response relationship for their diverse biological effects.

One of the simplest means of describing dose is administered dose. Administered dose is often described as mg/kg, mg/kg per day, or concentrations in food, water, or air. Although administered dose can be a useful metric, it has limitations. During exposure, a chemical is absorbed, distributed, metabolized, and eliminated, and these processes influence the target tissue concentrations. Species differences in these processes result in different target tissue concentrations in different species following exposure to the same administered dose. Thus, expressing an exposure based on administered dose may not result in equal tissue concentrations across species. To correct for these species differences, default risk assessment methods apply either allometric scaling or uncertainty factors when attempting extrapolations across species. For many chemicals, these scaling methods provide reasonable estimates of comparable dose across species. For example, Bachmann[4] and colleagues examined the pharmacokinetic parameters of half-life and volume of distribution for 100 chemicals that had experimental data in both humans and rats. Based on these data, it appears that allometric scaling of the half-life and volume of distribution in rats can be used to predict these parameters in humans. However, there were exceptions to this rule. When another 15 highly lipophilic chemicals were added to the analysis, these chemicals did not scale based solely on body weight.[5] The model fit best when assumptions of body fat composition for rats and humans were incorporated.[5] These analyses indicate that administered dose may be used as a dose metric for species extrapolation provided that appropriate allometric scaling is applied.

Using administered dose as a dose metric has additional uncertainties when route and duration of exposures are varied. The route and duration of exposure influence tissue concentrations of a chemical. For example, the time course and peak tissue concentrations for the distribution of a dose of TCDD are different when the dose is administered orally, dermally, or intratracheally.[6] In addition, the amount and rate of absorption of a chemical in an oil vehicle can be very different when the chemical is dissolved or suspended in an aqueous vehicle or bound to soil.[7] Using administered dose as the metric for exposures occurring through the same route, rate, and dosing vehicle within the same species is clearly appropriate. However, in cases where these parameters vary across dosing scenarios, there is increasing uncertainty in the extrapolation.

The biological effects of a chemical require sufficient tissue concentra-

tions over a specific time frame. For some effects, such as cancer, prolonged exposures are required, whereas for other effects, such as developmental toxicities, the window of responsiveness lasts from hours to days. The difference in time required for these diverse effects calls for a dose metric that accounts for both the mass of chemical and the time frame of exposure as well. One of the uncertainties in risk assessment is time scaling across species. The difficulty is that there does not appear to be a consistent method of time scaling between species for different biological processes. If we were to compare the length of time of several common biological phenomena in rats and humans, we would observe different ratios for different biological phenomena. For example, rats live approximately 2 to 2.5 years, whereas the average human life span is estimated at 70 years. Gestation period is approximately 21 days in rats and 270 days in humans. Puberty in rats occurs between postnatal days 29 and 45, whereas in humans puberty occurs between 8 and 14 years old. In contrast, some processes, such as diurnal cycles or cell replication time, have equivalent time spans between species. Because of these uncertainties in species extrapolation across time, risk assessors use average daily dose or tissue concentration as a default dose metric.

Another reason that average daily dose or tissue concentration is the default dose metric is to account for the differences in the exposure patterns between experimental animals and humans. In toxicity studies, exposures tend to be at constant concentrations for defined periods of time. In contrast, human exposures to environmental chemicals are often intermittent and variable in the dose level. Average daily administered dose or tissue concentration is used as the default to approximate the intermittent human exposures compared to the constant dose rates used in toxicity studies. Several authors have recently attempted to examine tissue concentration as a useful dose metric for a number of effects of TCDD. Using different dosing regimens and time course studies, the relationship between tissue concentration and response has been compared. Hurst et al.[8] have presented data demonstrating that fetal tissue concentrations on gestation day 16 reasonably predict the developmental reproductive effects of TCDD in rats independent of the dosing regimen. Diliberto et al. demonstrated that target tissue concentrations predict hepatic enzyme induction in mice receiving either a single acute administration of TCDD or subchronic exposures for 4, 8, or 13 weeks.[9] Similar relationships between tissue concentrations and enzyme induction were also observed in rats.[10] Although tissue concentrations of TCDD reasonably predict hepatic enzyme induction in rats, there was no relationship between TCDD tissue concentrations and cell replication.[10] These studies demonstrate that for some responses, target tissue concentrations are good predictors of response, whereas other responses require further study for a more complete understanding of their dose–response relationships.

In the empirical modeling exercises described in this chapter, steady-state body burden will be used as the dose metric. This metric is best used when comparing animal exposures that approach steady state to the long-term back-

ground human exposures. Application of this dose metric to single-dose exposures in experimental animals or to high-level human exposure following industrial accidents should be used with caution. The use of steady-state body burden as a dose metric for species comparisons was proposed for TCDD and related compounds by DeVito et al.[11] This dose metric was chosen based on mechanistic understanding and practicality. The distribution of TCDD and dioxins is similar across species.[7] With the exception of the liver, TCDD and dioxins tend to distribute based on lipid content in tissues. In liver, the dioxin concentrations dependent on liver lipid content and CYP1A2 concentrations. CYP1A2 is inducible by dioxins and binds TCDD and related chemicals.[12,13] Whereas the distribution is similar across species, the half-life of TCDD and related chemicals varies significantly between species. TCDD has a much longer half-life in humans (approximately 7 years) compared to mice and rats, which have half-lives of 10 to 25 days.[7] Typical default procedures for species extrapolations do not account adequately for these differences in half-life between humans and rodents. The use of body burden as a dose metric is used as a surrogate for tissue concentrations and as such provides a useful dose metric across species despite the large species differences in the half-life of TCDD.

Another reason for the use of body burden is the uncertainty in human exposures. Because of the long half-life in humans, TCDD tissue concentrations represent past and present exposures. There are considerable uncertainties in estimates of past exposures to TCDD and related chemicals. Methods to detect the low levels of dioxins present in food were not available until the late 1980s, and food basket surveys are even more recently published. Estimates of daily intakes prior to the 1990s are based on the relationship between age, body burdens, dioxin concentrations in core sediment samples, and estimates of the biological half-lives of dioxin and related chemicals.[14] Over the past several decades, human dietary exposures to dioxins peaked about the 1970s and have been dropping since. Thus, different-aged populations have had very different exposures. However, many of the estimates of human exposures are based on tissue concentrations of dioxins. Human serum and adipose tissue concentrations of dioxins are much better characterized than are estimates of daily intakes. In contrast to the human data, the dose and dose rate are controlled in experimental studies, but serum or tissue concentrations of dioxins are frequently not determined. Because of the large difference in half-life between species and the uncertainty in past and present human exposures, in order to compare exposures, the USEPA has chosen to use steady-state body burdens as the dose metric for species comparisons.[15] Furthermore, the use of body burden as a dose metric allows use of the best characterized human exposure data.

Under steady-state conditions, total body burdens (ng/kg) of TCDD can be calculated based on daily dose (ng/kg per day):

$$\text{body burden (ng/kg)} = \text{daily dose (ng/kg per day)} \times \text{half-life}/\ln(2) \times f \quad (7.1)$$

TABLE 7.1 Estimated Species-Specific Half-Lives Used for Converting between Daily Exposures and Steady-State Body Burdens

Species	Half-Life (days)
Wistar rats	22
All other rat strains	25
C57BL/6N mice	10
All other mouse strains	11
Human	2593

Source: Data for rats and mice from Ref. 7; data for humans from Ref. 83.

where f is the fraction of dose absorbed and is assumed to be 50% for absorption from food[16] and 100% for other routes. The half-life is the species-specific half-life of TCDD. Half-lives for converting between daily exposures and steady-state body burden are presented in Table 7.1.

Attempts at describing dose based on a mechanistic understanding of the toxicity of a chemical have been presented for numerous chemicals. These dose metrics are developed based on a sufficient understanding of the mode of action of the chemical as well as a broad understanding of the biology of the toxicological process. Mode-of-action-based dose metrics are frequently estimated using physiologically based pharmacokinetic (PBPK) models. Several groups of investigators have developed PBPK models that can estimate tissue concentrations of TCDD in rats and mice. Some of the PBPK models also describe the dose–response relationships for biochemical alterations such as the induction of hepatic CYP1A1 and CYP1A2 or decreases in hepatic EGF receptor. The use of PBPK models to develop mechanistic-based dose metrics may be closer to the ideal dose metric. However, in practice, mechanistic dose metrics are infrequently applied in risk assessments because the mechanistic understanding required to develop these dose metrics is not attained for the vast majority of environmental chemicals. In addition, there is often difficulty in validating a model. Although theoretically, these models may provide a more sound reasoning for a dose metric, it is difficult to demonstrate statistically that the risk estimates are better using these models. A more detailed discussion of these models is provided later in the chapter.

7.3 BIOLOGICAL RESPONSES TO TCDD

Most, if not all, of the effects of TCDD are initiated by its binding and activating the Ah receptor. The Ah receptor is a ligand-activated transcription factor and a member of the PAS superfamily.[17] The responses elicited by

TCDD cover a broad range of observations ranging from initial biochemical alterations to more complex responses such as immune and developmental alterations. Once TCDD binds and activates the Ah receptor, it translocates to the nucleus and binds to specific regions of DNA designated as either dioxin-response elements or xenobiotic-response elements. These elements are upstream from the transcription start region of specific genes. The binding of the Ah receptor to these regions results in the bending of the DNA and altering the transcription rate of the specific genes. CYP1A1 is the best-studied TCDD-inducible gene. It is thought that the response to TCDD can be seen as a continuum of initial biochemical responses leading to toxicological responses. However, the qualitative and quantitative linkages between the biochemical and toxicological responses to TCDD remain uncertain in most cases.

Understanding the dose–response relationship for the biochemical and toxicological endpoints may provide guidance for use in the low-dose extrapolations required for human health risk assessment. Caution must be used when comparing sensitivities across endpoints for several reasons. Some endpoints are clearly adverse; others are adaptive responses. Still other endpoints may be considered as part of a continuum of adaptive to adverse. Cancer is clearly an adverse response. Biochemical alterations are somewhat more difficult to define as adaptive or adverse. For example, TCDD induces UDPGT1*6.[18–20] UDPGT1*6 is a member of a family of proteins that glucuronidates thyroxin. The glucuronidation of thyroxin is a primary catabolism pathway in its elimination. Inducing UDPGT1*6 increases the elimination of thyroxin and decreases serum thyroxin concentrations.[19,20] Thyroxin is an important factor during the development of the central nervous system. Decreases in serum thyroxin results in neurological developmental deficits in humans.[21] Clearly, neurological developmental deficits are adverse. However, whether the decreases in thyroxin or the induction of UDPGT1*6 are adverse is less clear. At some point, induction of UDPGT1*6 leads to significant decreases in serum thyroxin sufficient to result in developmental deficits.[21] However, there may be levels of UDPGT1*6 induction that do not lead to decreases in serum thyroxin, or there may be decreases in serum thyroxin that do not result in developmental deficits. Whether these responses are considered adverse remains controversial.

Another difficulty in comparing endpoints is that they are expressed in different units. Some biochemical endpoints are expressed as an enzymatic activity (EROD induction), while others are expressed as a concentration (changes in tissue retinoid concentrations). Some toxic endpoints are also expressed as continuous data, such as porphyrin accumulation; others are expressed as quantal responses such as the incidence of tumors or birth defects. Not only are the units different between endpoints, but the magnitude of change from controls is also different between endpoints. Biochemical endpoints, such as CYP1A1 induction, have maximum increases of greater than 5000% of control values. Other continuous endpoints have much smaller maximums often less than a twofold increase. In contrast, some toxic effects, such as carcinogenesis

or teratogenesis, can attain maximum response levels between 10 and 100% incidence.

To compare across endpoints, these different units of response must be converted into comparable units. It has been proposed that comparisons of endpoints could be facilitated if the responses were adjusted by describing the response in terms of percent of maximum achievable response.[22,23] It should be noted that although this transformation of the data aids in comparing different responses for a single chemical, comparisons between chemicals could be problematic. For example, TCDD and related chemicals decrease serum thyroxin concentrations by approximately 50%, while the nondioxinlike PCBs decrease serum thyroxin by over 90%. Using the method of Murrell et al.[22] to define effective dose results in differences in the effect level for the different chemicals (i.e., the ED_{50} for TCDD would be a 25% decrease in thyroxin and a 50% decrease for the nondioxinlike PCBs). Alternative approaches for modeling noncancer effects assume that the critical effect level is either a percent of the control value or is a designated number of standard deviations from controls.[24] These methods, while allowing for comparisons between chemicals, does not necessarily allow for direct comparisons between endpoints.[22] In the present exercise, our objective is to compare across endpoints and not across chemicals. Therefore, the effective dose will be defined as a percent of the maximum achievable response.[22]

In the dose–response modeling presented in this chapter, responses are compared based on excess risk or response. A common metric for comparison is the effective dose, or ED_p, which is the exposure or dose resulting in an excess risk (p) in the studied population. Although effective dose reporting for the 2, 5, and 10% increased risks has been the suggested approach for toxicity studies in experimental animals, the latter two levels are higher than response levels typically observed in the epidemiological studies on the effects of TCDD. On the basis of this observation, the dose resulting in a 1% response above controls (ED_{01}) is presented. Use of the ED_{01} value also avoids large extrapolations in the experimental data since the estimated exposure is in or near the range of the exposures in the experimental studies being compared.[22,23]

Risks above and beyond the background risks encountered in the general environment can be presented through different measures. In the present study excess risk is defined as the effective dose for risk ($p \times 100\%$), satisfying the following relationship:

$$p = \frac{R(dp) - R(0)}{R(\infty) - R(0)} \tag{7.2}$$

where $R(dp)$ represents the response (either risk or other measure) at p at a given exposure or dose level d, and $R(\infty)$ is the maximum response possible [e.g., $R(\infty) = 1$ for quantal responses, such as cancer]. In this exercise, p is equal to 0.01.

7.4 DOSE–RESPONSE MODELING

A model can be considered an illustration of how something works. Models can be conceptual (verbal descriptions), biological (animal models of human diseases), physical (three-dimensional models of chemicals), or mathematical (PBPK models). In each of these categories, the level of sophistication can vary from relatively simple to highly complex. The purpose of these models is to assimilate the information on the process under consideration and to represent the information in either a qualitative or a quantitative manner. Once developed, these models can be used to test hypotheses. The development of a model can then be seen as an iterative process that involves testing the model with data, comparing these predictions to the data, and refining the model as required, followed by further testing and refinement. The confidence placed in the predictions of a model is determined by the quality of the data and information used in its development. In this context, mathematical models of dose–response are quantitative descriptions of a biological process with inputs and outputs that have real-world counterparts. The application of mathematical modeling in toxicology has taken two approaches; one is strictly empirical and the other is based on mode of action. In the simplest terms, these models use empirical approaches that describe the relationship between dose (input) and response (output). More complex mode-of-action-based models have more inputs (dose, organ size, blood flows, etc.) and outputs (tissue concentrations, enzyme induction, cancer risks, etc). The goal of these mathematical models is to reduce uncertainties by using as much data as possible and to identify the areas where data gaps exist. There is a significant amount of biological data available for describing the dose–response relationships for the biological effects of TCDD. These data should be included to the greatest extent possible in any modeling exercise, either empirical or mode of action based. There are several generally accepted concepts that may aid in determining the types of mathematical models that can describe the dose–response relationship for TCDD. First, TCDD is a member of a class of xenobiotics and natural products that alter gene expression and cellular growth and differentiation by binding to the Ah receptor. Second, the biology of receptor-mediated events is well studied. For example, Roth and Grunfield[25] state: "At very low concentrations of hormone receptor, occupancy occurs but may be trivial; i.e., the curve approaches 0% occupancy of receptors. But if there are 10,000 receptors per cell (a reasonable number for most systems), the absolute number of complexes formed is respectable even at low hormone concentrations. One advantage of this arrangement is that the system is more sensitive to changes in hormone concentration; at receptor occupancy (occupied receptors/total receptors) below 10%, the concentration of occupied receptors is linearly related to the concentration of hormone, whereas at occupancies of 10 to 90%, the concentration of HR is linear with log hormone concentration, a given increase in the concentration is more effective in generating occupied receptors at the lowest part of the curve than at the middle." These concepts of dose–response rela-

tionships for receptor-mediated events should be considered when applying mathematical models to the biological effects of TCDD.

While we understand the relationship between hormone concentration and receptor occupancy, the quantitative relationship between receptor occupancy and biological response remains less certain. Different responses induced by the same receptor may have very different dose–response relationships. For example, simple responses, such as CYP1A1 induction by TCDD, have a different receptor occupancy–response relationship than coordinated biological responses, such as the TCDD-induced wasting. Thus, before applying mathematical models to biological data, an understanding of the biology mediating the response is invaluable.

Dose–response modeling is becoming an increasingly important tool in toxicology and risk assessment. The quantitative description of the relationship between dose and response can aid in both generating and testing hypotheses. In addition to their use in risk assessment, these models can also aid in experimental design. Empirical and mode-of-action-based modeling approaches are described more fully in the following sections.

7.4.1 Empirical Modeling

In the initial studies of the biological effects of a chemical, individual cells of animals or humans are exposed to the chemical, and some response is measured. In such studies, a pattern may be observed between increasing exposure and increasing response. Empirical dose–response modeling attempts to describe this pattern using simple mathematical models. While these models focus on mathematical forms that can fit a broad spectrum of data, they generally have little or no direct linkage to the underlying biology of the response. However, empirical models should be interpreted based on the biological data available and may provide qualitative insights into the biological mechanisms responsible for the response observed.

Examples of empirical models include linear functions (such as those used in linear regression), log-linear models, Poisson regression (commonly used in epidemiology), and Hill models (commonly used to analyze ligand-receptor data). Empirical models have several advantages. Compared to mode-of-action-based models, empirical models are easier to apply. There are user-friendly software tools capable of fitting these models to dose–response data readily available. Empirical models also provide a formal framework for hypothesis testing and interpolation between data points. One disadvantage of empirical models is that they are incapable of quantitatively linking multiple data sets in a mechanistic fashion.

7.4.2 Empirical Modeling of the Noncancer Effects of TCDD in Experimental Animals

As part of the USEPA reassessment of the exposure and health effects of dioxin and related chemicals, dose–response modeling of the noncancer effects of

TCDD in experimental animals was performed.[15] Understanding the dose–response relationship for the biological effects of TCDD could be very informative when assessing the potential health risks associated with exposure to dioxins. For example, if the dose–response relationships were either predominately linear or threshold, these results would have different influences on the types of models used to estimate low-dose risks. More important, this modeling effort provided estimates of effective dose that could be used as points of departure for determining exposure levels with minimal risks, such as a reference dose (RfD) or a tolerable daily intake (TDI).

Risk assessments for noncancer health effects have used NOELs and LOELs historically as points of departure for determining exposures associated with minimal risks. NOELs and LOELs are derived from experimental data and represent exposures that result in either no observable effects (NOEL) or the lowest exposure tested that resulted in an observable effect (LOEL). One of the criticisms of using the NOEL or LOEL approaches is that these estimates are sensitive to the study design. That is, poorly designed studies can result in higher NOEL values and LOEL values compared to better designed studies. Crump proposed an alternative approach designated as the benchmark dose.[26] The benchmark dose is the 95% lower confidence limit on a dose that produces a predetermined response rate. Typically, for quantal data, response rates of 1, 5, or 10% [designated the effective dose $(ED)_{01}$, ED_{05}, and ED_{10} value, respectively] have been used. The empirical models and the methods used to analyze the noncancer dose–response effects are described fully in the USEPA dioxin reassessment. Briefly, these models and the statistical details used were similar to two previous analyses.[22,23] Two different models were fitted to continuous data. Decisions between the two models depended on the number of dose groups used and the overall quality of the data. The first choice was to use a Hill model of the form

$$R(d) = b + \frac{vd^n}{k^n + d^n} \tag{7.3}$$

where $R(d)$ is the response at dose d, b the background response, v the maximum change from background, k the dose producing a response half of v, and n the Hill coefficient, which describes the curvature of the dose response. The shape of the dose–response curve is important in risk assessment and in understanding the biology of the response. The Hill coefficient can be classified based on the resulting shape of the dose–response curve. The response is predicted to be approximately proportional to dose when n is near 1. Sigmoidal or thresholdlike dose–response curves arise when n is much larger than 1 ($n > 1.5$). When n is less than 1, the dose–response curve becomes supralinear. For these reasons, n will also be referred to as the *shape parameter*.

In these analyses, n was not allowed to vary below 1. When $n < 1$, the slope of the dose–response curve at dose = 0 becomes infinite. Estimates of the ED_{01} value become unstable when $n < 1$ because they are sensitive to the slope of the

dose–response curves at dose = 0. An infinite slope is not plausible biologically and is difficult to estimate accurately, due to the limited data available from a particular study. The effect of restricting n to values of 1 or greater is a potential bias toward higher-than-expected ED_{01} values.

When the data did not demonstrate a clear maximum, a power model was fit to the data according to the equation

$$R(d) = b + sd^n \tag{7.4}$$

where s is the scale parameter and describes the magnitude of the effect per unit dose (d) and b and n have descriptions similar to those in the Hill model. Because this model has no fixed maximum, it was used for data with either no experimentally evident maximal response or with few dose groups. However, the lack of a maximum results in a considerable problem in defining effective dose. Effective doses derived from the power function model should be compared cautiously to those derived from the Hill model.

Quantal data were modeled using the Weibull model according to the equation

$$R(d) = c + (1 - c)[1 - \exp(-ad^k)] \tag{7.5}$$

where $R(d)$ is the probability of response at dose d, c is the expected response in untreated animals ($0 \leq c \leq 1$), a is the magnitude of response per unit dose raised to the kth power ($a \geq 0$), and k is the shape parameter ($k \geq 1$). When k is large, the Weibull model predicts thresholdlike behavior. In addition, k was not allowed to be less than 1 to avoid instability in the analysis. The ED_{01} values from quantal data satisfy the excess risk relationship described in equation (7.2), where $R(\infty)$ is equal to 1 for quantal endpoints.

The data analyzed in this exercise are from the published peer-reviewed literature. Due to the vast number of studies on the biological effects of TCDD, several criteria were used for inclusion in the analysis. The study must examine the effects of TCDD in experimental animals and must have used at least three dose levels of TCDD and a control. The data must be presented in tabular form and include the mean, an estimate of the variance, and an estimate of the number of animals studied in each dose group. Data presented only in graphical forms were not used in the analysis because attempts to estimate the means and variances of these data accurately were unsuccessful. It should be noted that most data examining the effects of TCDD are presented in graphical form. Thus our analysis is only a limited sampling of all the literature available on the biochemical and toxicological effects of TCDD.

The U.S. Environmental Protection Agency (USEPA) Benchmark Dose Software (BMDS) version 1.2b was used for model fits, calculation of the ED_{01} value, and 95% lower bound on the estimated ED_{01} value. Qualitative assessment of the goodness of the model fit was determined as good (the model curve included nearly all of the data point means), marginal (the model curve was

within 1 standard deviation of the data point means), or poor (the model fit was not within 1 standard deviation of the means). For a more complete description of the methodology employed, the reader is encouraged to read Part II, Chapter 8, ''Dose–Response Modeling for TCDD'' in the USEPA Dioxin Reassessment.[15]

Based on the criteria above, 36 manuscripts were identified. From these publications, there were 284 endpoints for which dose–response data was acceptable for analysis. Good or marginal fits were attained for 242 of these data sets (approximately 200 continuous endpoints and approximately 30 quantal effects). Because of the large number of data sets analyzed, the data were divided into several categories based on exposure regimen and endpoint. Exposure categories were grouped as either single or multiple exposures. For simplicity, effects were categorized as biochemical, hepatic, immune, toxicity, tissue, or endocrine. Alterations in mRNA, protein, or enzyme activities were designated as biochemical. Hepatic changes included measures of hepatotoxicity, such as serum enzymes and histological effects. TCDD-induced alterations in lymphocyte phenotypes and functional assays, such as altered responses to antigen challenge, were included in the immune category. Tissue responses included changes in tissue and body weights. Developmental, reproductive, and tissue toxicities were classified as toxic responses. Finally, endocrine responses included the effects of TCDD on thyroxin, TSH, or retinoid tissue or serum concentrations.

Comparisons between studies can be problematic for several reasons. The studies included in this analysis examine a variety of endpoints under a number of different exposure regimens. For example, studies examining the effects of TCDD after a single exposure often determined the response at very different time periods after the initial exposure. It is likely that for some effects, particularly reversible effects such as enzyme induction, the effective dose is influenced by the length of time after the initial exposure the response is determined. In addition, some of these studies employed different routes of exposure or different dosing vehicles, which could result in different rates and magnitudes of absorption of TCDD. Because of these uncertainties, caution must be used when interpreting these data.

Differences in exposures were also observed between studies employing multiple-dose exposures. The length of the multiple-dose studies analyzed was typically 13 weeks or longer and approaches steady-state conditions. In these cases, average daily dose was estimated for each study by calculating the total dose administered to an animal over the course of the study and dividing by the length of the study in days and was used as the dose metric for initial comparative purposes. To compare across species, average steady-state body burden at the ED_{01} value was calculated. The use of average steady-state body burden as a dose metric allows for comparisons of dose across species with differing elimination rates of TCDD.

When attempting to analyze a large data set such as in this example, a number of uncertainties are introduced for data sets in which the model pro-

vides a marginal fit. For example, in some cases, U- or inverted U-shaped dose–response curves were observed, thus reducing the confidence in the estimates of the ED_{01} value. For some data sets the Hill model did not provide the best fit, and other models could provide a better fit to the data, resulting in a very different estimate of the ED_{01} value. In addition, the ED_{01} value and the 95% lower confidence interval (LED_{01}) differed sometimes by more than 10-fold, suggesting that little confidence can be placed in some ED_{01} values as a useful index of toxicity. In such cases, the LED_{01} value can be considered as a bound. To base overall conclusions on the strongest results, less emphasis was given to data sets that demonstrated such problems.

Multiple-Dose Studies There were 139 endpoints examined from multiple-dose studies and 108 had fits described as either good or marginal. The ED_{01} values from studies exposing animals to multiple doses of TCDD are highly variable across and within response categories (Figure 7.1). The median ED_{01} value for the biochemical responses (13 ng/kg) is lower than the median ED_{01} value of the other response categories. Hepatic and immune responses had median ED_{01} values greater than 200 ng/kg. ED_{01} values were less than 50 ng/

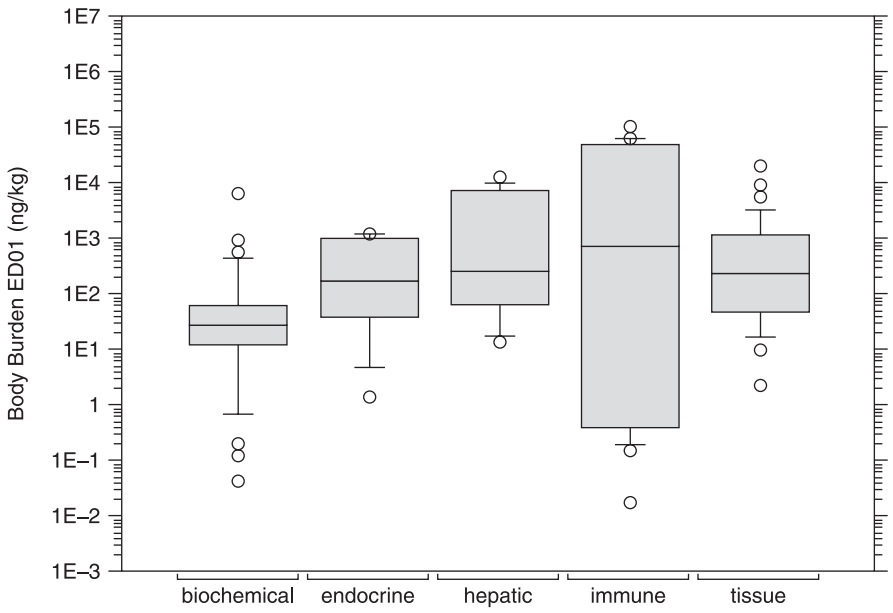

Figure 7.1 Distribution of ED_{01} values in multidose studies by response category. The distribution of individual body burden ED_{01} values is presented as box plots based on response categories. The boxed region contains values within the 25th to 75th percentiles of the sample distribution, with the median value (50th percentile) shown as a line within the boxed region. The error bars represent values within the 10th to 90th percentiles. Values above the 90th percentile and below the 10th percentile are shown as individual data points.

kg for 42 of the 108 data sets for which ED_{01} values were estimated. There were 11 data sets with ED_{01} values less than 5 ng/kg, six immune responses, three biochemical responses, and one each was an endocrine and tissue response. The immune responses should be viewed cautiously since the range of the ED_{01} values for immune responses was over six orders of magnitude. This large range decreases confidence in any particular immune ED_{01} value. Another limitation to this analysis is that few published studies examine male rats, male mice, or other species and that the dose–response information is predominately from female rats and mice. Because of these knowledge gaps as well as the large range of the ED_{01} values, extrapolation across gender and species should be done with caution.

The degree of confidence of the ED_{01} estimate can also be examined by the ratio of the ED_{01} value to the lowest dose used in the study from which it was derived. If the ratio is 1 or greater, the ED_{01} value is within the doses examined and suggests that the ED_{01} is a realistic value. The ED_{01} value can also be considered a reasonable estimate if the ratio of the ED_{01} value to the lowest dose examined is between 1 and 0.1. Caution should be taken when this ratio is less than 0.1, indicating that the estimate was more than an order of magnitude below the lowest dose used in the study. Of the 108 ED_{01} values examined, 47 had ED_{01}/lowest dose ratios of less than 1. However, of these 47, only 37 are less than one order of magnitude below the lowest dose used in the study. Hence, only approximately two-thirds of the ED_{01}s are within an order of magnitude of the lowest dose used in the study and can be considered reasonable estimates.

The shape parameter is also an important value estimated using these modeling approaches. In general, a shape parameter that is less than 1.5 indicates that the dose–response curve tends to be linear at low doses, and those with shape parameters greater than 1.5 tend to be thresholdlike. Forty-eight of the 108 endpoints for which an estimate was obtained had shape parameters less than 1.5, indicating linear dose–response relationships. Almost half of the biochemical and tissue responses demonstrated a linear dose–response relationship. However, the median shape parameter for the tissue responses is heavily influenced by the predominately linear shapes for alterations in thymic weight (10 of 11 dose–response curves for thymic weights had shape parameters of less than 1.5). In contrast, the immune function responses were predominately (81%) thresholdlike. Although there is some consistency in the shape parameters within some response categories, approximately half are linear and half are threshold. These data do not support a single-shaped dose–response curve for the effects of TCDD.

Single-Dose Studies: Adult Animals Ninety-eight data sets were analyzed that examined the effects of TCDD in adult rats and mice. Seventy-five of these data sets were assigned good or marginal fits. Both the Hill model (58 data sets) and the Weibull model (17 data sets) were applied to the single-dose studies. The median ED_{01} value was above 100 ng/kg for all response categories

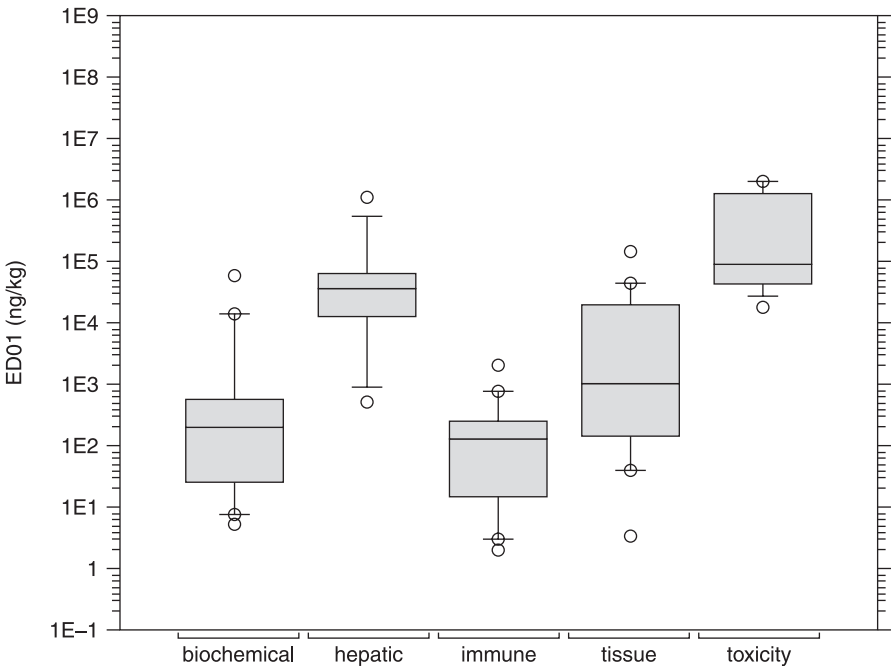

Figure 7.2 Distribution of ED_{01} values in single-dose adult studies by response category. See Figure 7.1 for details.

examined (Figure 7.2). Biochemical and immune responses had the lowest median ED_{01} estimates, 207 and 133 ng/kg, respectively. Hepatic and toxic responses had median ED_{01} values greater than 10,000 ng/kg. Similar to the multiple-dose studies, there is a large variability in the ED_{01} values within and between categories. The ED_{01} values generally varied approximately 1000-fold within each category. There were 14 data sets with ED_{01} values of less than 50 ng/kg. These responses were in the immune, tissue, and biochemical categories. Approximately one-third (21/74) of the ED_{01} estimates were below the lowest dose tested for the endpoints examined. Only 13 of 74 ED_{01} values were more than an order of magnitude lower that the lowest dose examined in the study.

A shape parameter of less than 1.5 was estimated in 30 of 75 endpoints examined. Both linear and thresholdlike dose–response relationships were observed in the biochemical, immune, and tissue response categories, and no consistent pattern emerged within these categories. Thresholdlike dose–response curves were observed for all endpoints in the toxicity category exhibited.

Single-Dose Studies: Developmental Studies A number of studies (90) examined the effects of TCDD during developmental exposures. Sixty of these data sets were assigned good or marginal fits. These data were analyzed sepa-

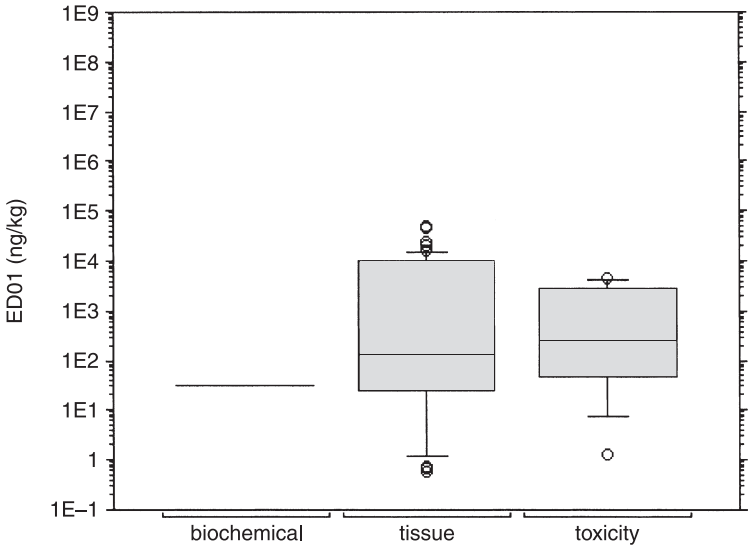

Figure 7.3 Distribution of ED_{01} values in single-dose developmental studies by response category. See Figure 7.1 for details.

rately from effects on adult animals. Similar to the adult single exposure, the developmental effects were categorized as biochemical, tissue, or toxic. Most of the developmental effects examined were considered tissue responses. Once again there was a large range of ED_{01} values spanning more than five orders of magnitude (Figure 7.3). The median values for all response categories were greater than 100 ng/kg, with an overall median of 139 ng/kg. Only 18 of the 60 ED_{01} values were less than 50 ng/kg, and 8 of these were less than 5 ng/kg. There were 38 data sets that had ED_{01} values lower than the lowest dose examined and approximately half (18) of these ED_{01} estimates were less than an order of magnitude below the lowest dose examined in the study. Only 18 of the 60 developmental endpoints analyzed had shape parameters of less than 1.5.

The developmental reproductive responses to TCDD observed in rats has raised considerable concern because of the low doses (50 to 100 ng/kg) at which these responses were observed. Developmental reproductive effects have been observed in rats, hamsters, and mice, although only the mouse and rat data have sufficient dose–response information that can be modeled.[27–31] Although some of the responses are observed in both rats and mice, there is a striking species difference in the ED_{01} values. The ED_{01} values for the reproductive developmental effects in mice are 10 to 1000 times higher than those determined in rats. There are also differences between ED_{01} estimates between studies examining the same species. For example, there is approximately an order-of-magnitude difference in estimates of the ED_{01} values between the

developmental reproductive toxicity studies of Gray et al.[30] and those observed in the studies of Mably et al.[27–29] Although both of these studies examined rats, they used different strains, and that may be one of the reasons for the differences between these studies. Although there is a qualitative consistency in the developmental reproductive toxicities of TCDD observed in rats, mice, and hamsters, there are considerable quantitative differences in the sensitivity to these effects between these species and possibly strains.

Summary of the Dose–Response Modeling Results for the Noncancer Effects of TCDD in Experimental Animals TCDD initiates a cascade of biochemical events through binding and activating the AhR that results in alterations in growth factors and hormones, their receptors, and proteins involved in regulating a variety of cellular functions, including the cell cycle and intermediary metabolism (see Chapters 12, 13, and 14). The alterations in these regulatory and homeostatic factors may mediate the toxicity of TCDD. The biochemical and toxicological effects of dioxins can be seen as a continuum that starts with biochemical changes, which leads to the toxicological responses. Based on the hypothesis above, the biochemical effects would occur at lower doses than the toxic effects. Thus, an understanding of the shape of the dose–response relationship for the biochemical effects may provide insight into the shape of the dose–response relationship for toxic responses at low doses.

The biochemical responses generally have lower ED_{01} estimates than those of the other response categories examined. Although these data are consistent with the hypothesis that the biochemical effects are precursors of the toxic effects, few of the biochemical responses examined are known to mediate the toxic responses. For example, while the CYP1A proteins are proposed as dose surrogates for the TCDD-induced carcinogenic effects,[32] the evidence directly supporting the role of these proteins in the pathway of carcinogenesis remains elusive.

The induction of thyroid tumors by TCDD is perhaps the most convincing example of the hypothesis that the biochemical responses are precursors to the toxic effects. TCDD decreases circulating thyroid hormones as a result of induction in hepatic glucuronosyltransferases (UGTs), which metabolize these hormones and increase their elimination. The decrease in serum thyroid hormones leads to increased serum thyroid-stimulating hormone (TSH). Increases in serum TSH lead to proliferative effects on the thyroid gland and prolonged stimulation of the gland by TSH leads to the induction of thyroid tumors. In the present analysis, two studies were examined which determined the dose–response relationship for alterations in serum thyroid hormones. Van Birgelen et al.[33] examined the effects on total and free plasma thyroxin concentrations and hepatic thyroxin glucuronidation (T4UGT) in female rats exposed for 90 days to a diet containing TCDD. The ED_{01} values for T4UGT, free plasma thyroxin, and total plasma thyroxin are 1.6, 4.9, and 33 ng/kg per day. The shapes of the dose–response curves were linear for T4UGT and free plasma

thyroxin and thresholdlike for total plasma thyroxin. Sewall et al.[34] exposed female Sprague–Dawley rats biweekly to TCDD for 30 weeks and determined the effects on UGT mRNA, serum total thyroxin, and serum TSH. All three responses had shape parameters greater than 1.5 and the ED_{01} values were 0.37, 1.3, and 26 ng/kg per day for UGT mRNA, total serum thyroxin, and serum TSH, respectively. In both studies, the ED_{01} for T4UGT is lower than effects on serum thyroxin concentrations, which is consistent with the hypothesis that TCDD decreases serum thyroid hormones by increased hepatic glucuronidation. In addition, the ED_{01} value for changes in serum thyroxin is lower than the ED_{01} value for changes in serum TSH, which is also consistent with the hypothesis. Interestingly, the ED_{01} for thyroid tumors in female rats is 33 ng/kg per day based on the effects observed from the National Toxicology Program.[35] These data support the hypothesis that biochemical responses have lower ED_{01} values than those of the toxic responses.

The data analyzed in this study were obtained from the published literature and used a variety of strains, species, genders, dosing regimens, and endpoints. One concern in interpretation of the ED_{01} values is the influence of study design on the estimates of these values. There are several examples demonstrating the influence of study design on estimates of the ED_{01} value. In single-dose studies, the time the endpoint is determined after the initial exposure influences both the ED_{01} value and the estimate of the shape parameter. Estimates of the ED_{01} value and shape parameters for hepatic ethoxyresorufin deethylase (EROD) activity in rats and mice 7 days after a single exposure demonstrate reasonable consistency. These ED_{01} values range from 16 to 84 ng/kg.[36–39] In addition, the shape parameters indicate a linear response in three out of four studies, with one demonstrating slight thresholdlike. These data indicate a consistency between rats and mice as well as between laboratories. However, Diliberto et al.[36] also examined the time course for induction of hepatic EROD activity at 7, 14, 21, and 35 days after a single exposure to TCDD. Both the ED_{01} value and the shape parameter increased with time after dosing. The ED_{01} value was 27 times greater at the 35-day time point than at the 7-day time point. The shape parameter increased from 1 at the 7-day time point to 6.5 at the 35-day time point. These increases are probably due to the decreasing tissue concentrations of TCDD and associated decreases in enzyme induction as TCDD is eliminated from the body over the course of the study.

Dose selection may also affect the results of the dose–response modeling. One of the lowest ED_{01} values determined is for hepatic EROD induction in female mice.[40] The ED_{01} value for hepatic EROD in the study by Vogel is 0.0094 ng/kg per day. The ED_{01} value was estimated for five other studies that exposed rats or mice to multiple doses of TCDD.[41–45] The ED_{01} values for the five other studies ranged from 0.4 to 3.2 ng/kg per day. The ED_{01} value from the Vogel et al. study is approximately 40 to 340 times lower.[40] The doses that Vogel et al.[40] used were approximately 100 times lower than those used in the

other five studies. It may be that the lower ED_{01} value in the Vogel study is due to the dose pattern and dose selection in this study compared to the other studies.

Species and strain selection have long been noted to influence the relative potency of a chemical. The developmental effects of TCDD have generated concern, particularly the developmental reproductive toxicities observed in rats, hamsters, and mice.[27–31] The studies in rats demonstrated decreased epididymal sperm counts on postnatal day 63.[28,30] However, the ED_{01} values vary over 200-fold (0.65 vs. 140 ng/kg) and the shape parameters indicate either a linear response or a thresholdlike response, depending on the study. Different strains of rats may account for the differences in the ED_{01} values and shape parameters between the data sets. In general, the responses observed in the Mably study were greater than those observed in the Gray et al. study, despite very similar doses and dosing regimens. Mably et al.[27] examined the effects of TCDD in the Holtzman rats, whereas Gray et al.[30] used Long–Evans rats. The data from Gray et al.[30] had ED_{01} values almost two orders of magnitude lower than those from Mably et al. and demonstrate highly nonlinear responses (shape parameters greater than 2 for all but 3 out of 32 responses examined). In contrast, the shape parameters indicate a more linear dose response relationship in Mably et al.[27] Theobald et al.[31] examined the reproductive developmental effects of TCDD in mice. The mice were more resistant to the effects of TCDD than were the rats and had ED_{01} values that were generally 10 to 100 times those of the Long–Evans rats.

An important finding in this analysis is that the biochemical effects tend to have lower ED_{01} values than those of more complex effects such as immunotoxicity or tissue weight loss. This finding is consistent with the hypothesis that the biochemical responses are precursors to the toxic responses of these chemicals. Another difference between the biochemical and toxicological responses is that the biochemical responses tend to have lower shape parameters. Thus, the dose–response relationships for the biochemical responses tend to be linear more often than the toxicological responses. Because of the limited dose–response data available for many of these analyses, caution must be taken when making some of these generalizations. For example, the decrease in thymus weight tends to have estimated shape parameters of 1.

The shape parameters have been used to categorize the data into linear and nonlinear dose–response relationships. There are some limitations to this categorization. Although the shape parameter describes the shape of the dose–response relationship in the experimental range, there is uncertainty in extrapolating this shape to lower dose levels.

Understanding the dose–response relationship for the biochemical and toxicological effects of TCDD is important to describe the potential human health effects of dioxins and related chemicals. The analysis demonstrates that a number of factors can influence the dose–response assessment. The design of the study can have strong influences on the estimates of the effective

dose and the shape of the dose–response curve. In addition, the choices of species and strain have considerable effect on the outcome of the dose–response analysis.

7.4.3 Empirical Modeling of the Carcinogenic Effects of TCDD in Experimental Animals

TCDD is carcinogenic in rats, mice, and hamsters. However, dose–response relationships are only available for several studies in mice and rats.[35,46] In these studies, three dose levels of TCDD and a control group were examined. Application of mathematical modeling to these data can be a powerful tool for combining mechanistic information and understanding the dose–response relationship for carcinogenesis. There are a variety of simple techniques for modeling chemical carcinogenesis.[47] These simple models can be improved by applying existing mechanism-based models of receptor-based effects of TCDD in concert with physiologically based pharmacokinetic (PBPK) models[48] and using these results in a multistage model of carcinogenesis.[88] Although both approaches have been attempted, in this section we deal with the more simple modeling exercise; the mechanism-based approaches are described later in the chapter.

There were dose-dependent increases in five tumor types in the 2-year feeding study of Kociba et al.,[46] and eight tumor types were increased in the 2-year gavage study conducted by the National Toxicology Program[35] in Osborne–Mendel rats and B6C3F1 mice. These data were modeled using a multistage model of carcinogenesis with up to two mutation stages affected by exposure.[47] Table 7.2 presents the results from this analysis. The ED_{01} values were calculated based on excess risk using equation (7.1). The lowest dose used in these experiments is approximately 1 ng/kg per day, and all but one of the estimated ED_{01} values are above this dose, indicating that they are within the experimental range. The one exception was liver cancer in female rats from the Kociba study, estimated at 0.77 ng/kg per day, which is very near the lowest dose used in this study. To compare across species, steady-state body burdens were estimated. Oral absorption of TCDD was assumed to be 50% for the Kociba et al. study (feed experiment) and 100% for the NTP study (gavage experiment).[49] The shapes of the dose–response curves were also determined, and these are presented in Table 7.2.

Similar to the noncancer dose–response modeling, the ED_{01} values for the carcinogenic affects range almost two orders of magnitude from 14 ng/kg for liver tumors in the Kociba study to 1190 ng/kg for the thyroid tumors in female rats from the NTP study. Eight of the 13 cancer dose–response curves were linear. However, because of the limited dose–response information available, it is unlikely that a linear or nonlinear model could be rejected statistically.[50] The results of the shape parameters should be viewed cautiously because of these limitations.

TABLE 7.2 Doses Yielding 1% Excess Risk (95% Lower Confidence Bound)[a]

		ED_{01}	
Tumor	Shape	Intake for 1% Excess Risk (ng/kg per day)	Steady-State Body Burden (ng/kg) at ED_{01}
Liver cancer in female rats (Kociba)	Linear	0.77 (0.57)	14 (10)
Liver adenomas and carcinomas in male mice (NTP)	Linear	1.3 (0.86)	20.6 (13.6)
Squamous cell carcinoma of the nasal turbinates or hard palate in female rats (Kociba)	Linear	5.0 (2.0)	90 (36)
Thyroid follicular cell adenoma in male rats (NTP)	Linear	4.0 (2.1)	144 (76)
Leukemias and lymphomas in female mice (NTP)	Linear	10.0 (5.4)	159 (86)
Liver adenomas and carcinomas in female mice (NTP)	Linear	15.1 (7.8)	239 (124)
Squamous cell carcinoma of the tongue in male rats (Kociba)	Linear	14.1 (5.9)	254 (106)
Liver adenomas and carcinomas in female rats (NTP)	Quadratic	13.0 (1.7)	469 (61)
Thyroid follicular cell adenomas and carcinomas in female mice (NTP)	Linear	30.1 (14.0)	478 (222)
Subcutaneous tissue sarcomas in female mice (NTP)	Linear–cubic	43.2 (14.1)	686 (224)
Squamous cell carcinoma of the lung in female rats (Kociba)	Cubic	40.4 (2.7)	730 (48)
Squamous cell carcinoma of the nasal turbinates or hard palate in male rats (Kociba)	Cubic	41.4 (1.2)	746 (22)
Thyroid follicular cell adenoma in female rats (NTP)	Cubic	33.0 (3.1)	1190 (112)

Source: Data from Ref. 47.

[a] Estimated by applying simple multistage models to 2-year animal carcinogenicity studies of TCDD.

Knowledge Gaps in Animal Cancer Dose–Response Modeling The dose–response data for cancer in animals following TCDD exposure are limited to three exposure groups. The application of nonlinear models to these data should be viewed with caution.[50] To differentiate between different dose–response curves, additional studies with more dose groups are needed. In addition, if biochemical and tissue response data were collected from these studies, mechanism-based cancer modeling could be facilitated.

In experimental animals most of the mechanistic-based research on the carcinogenicity of TCDD has focused on hepatocellular carcinomas. In the epidemiology studies, TCDD appears to induce tumors in multiple sites. Mechanistic research in experimental animals on other cancers, such as lung and thyroid, could aid in interpreting the human epidemiology data. Human exposure is to complex mixtures of PCDDs, PCDFs, and PCBs. These mixtures have not been tested sufficiently in experimental animals. Future studies involving mixtures of PCDDs, PCDFs, and PCBs may aid in understanding the potential human health risks associated with these exposures.

7.4.4 Empirical Dose–Response Modeling of Individual Human Data Sets

TCDD is carcinogenic in all species, strains, and genders of laboratory animals tested (mice, rats, and hamsters) (see Chapter 11). Tumors have been detected in a variety of tissues, including liver, lung, thyroid, skin, and other organs and tissues. The USEPA,[15] the U.S. Department of Health and Human Services,[2] and the International Agency for Research on Cancer (IARC)[1] all classify TCDD as a known human carcinogen. This classification is based on limited human epidemiological data, animal carcinogenicity testing and information on the mode of action, and the similarities of these pathways in humans and animals. Although this qualitative description is useful for hazard identification, risk assessments require more quantitative approaches. In the following section we describe the attempts at empirical modeling of both human and animal data to provide an understanding of the potential risks associated with exposure to TCDD.

There are a number of published reports examining the relationship between exposure to TCDD and adverse health effects in highly exposed populations. Generally, these data have limited dose–response information, limiting our ability to adequately describe the dose–response relationships in humans. As with any epidemiological study, there are a number of factors that are difficult to control adequately. There is always the possibility of disease misclassifications, and often the measurements of exposure are imprecise. The advantage of analyzing human data is that there are no assumptions concerning species extrapolations. For this reason, epidemiological data should be used maximally in understanding dose–response relationships. Several epidemiological studies of the effects of TCDD are available with varying degrees of utility for dose–response assessment. In the following section we describe dose–response analyses of these studies.

All Cancers Combined and Lung Cancer There are three studies of human occupational exposures that have sufficient dose–response information that are amenable for quantitative analysis. These are the NIOSH study,[51–53] the Hamburg cohort study,[54–57] and the BASF cohort study.[58]

NIOSH Study NIOSH conducted a cohort study of workers at 12 plants in the United States that produced TCDD-contaminated chemicals.[51] There were 5172 workers in this study, all of whom were males. Initial analysis of this population indicated that there was an increased mortality for total cancers and respiratory cancers for workers with more than 1 year of exposure and 20 years' latency since start of employment.

Steenland et al.[52] developed a job-exposure matrix for workers at eight of the 12 plants in the NIOSH study. These plants were chosen because they had sufficient information on work histories and TCDD levels on the job. In addition, these workers did not have exposure to pentachlorophenol. Steenland et al.[52] estimated TCDD exposure scores on a daily basis by multiplying TCDD concentrations in the industrial materials, the fraction of the workday spent working with these materials, and a qualitative degree-of-contact measure. Adding the exposure scores of each day then derived a cumulative TCDD exposure score. Workers were then divided into septile levels based on the exposure score. The data were also analyzed with or without a 15-year latency period. It should be noted that these exposure scores cannot be interpreted as a direct measure of TCDD exposure.

Standard mortality ratios (SMRs) were derived by septile for all cancers and for lung cancer. SMRs for all cancer with 0 or 15-year latencies and for lung cancer with no latency showed a statistically significant positive trend with exposure score. To compare the high-exposure groups to the low-exposure groups, a Cox regression analysis was applied to the data. When the data were analyzed assuming zero latency, there was no significant exposure–response relationship. When a 15-year latency was included in the analysis, Steenland et al. examined the exposure response relationship for all cancers, non-smoking-related cancers, lung cancer, and smoking-related cancers. Significant positive trends were observed for all cancers and non-smoking-related cancers with a 15-year latency. No significant effects were observed for lung cancers or smoking-related cancers when the cumulative exposures were used. When the logarithm of the cumulative exposure was used, significant trends were observed for lung cancer and smoking-related cancers.

Steenland et al.[53] further extended analysis of the NIOSH cohort to include estimated dioxin exposures in these workers. In 1988, serum lipid concentrations of TCDD were determined in 193 workers at one of the eight plants studied. Assuming first-order kinetics and a constant half-life of 8.7 years, serum TCDD concentrations were estimated at the time of last exposure. Using a first-order model for exposure between first exposure and last exposure, serum levels were regressed on the exposure scores. This formula was then used to estimate serum TCDD levels for all 3538 workers included in the study. Serum TCDD areas under the lipid-adjusted serum-level curves over time (AUC) were then estimated for the entire cohort.

The best-fitting model used a Cox regression with log(AUC) lagged by 15 years as a dose metric and had date of birth as a categorical variable. Based on this analysis, 1 pg/kg per day for 75 years is estimated to result in an excess

cancer risk for 0.0094 and 0.0080 for females. This assumes a background exposure of 0.5 pg TCDD/kg per day. Steenland also used the log of TEQ(AUC) as a dose metric, which resulted in a risk estimate of 0.0018 for males and 0.0015 for females from an intake of 10 pg TEQ/kg per day.

Steenland et al.[53] also used a piecewise linear model, which fit just as well as the model using log AUC. This analysis results in risk estimates for 1 pg TCDD/kg per day for 75 years at 0.0005 for males and 0.0004 for females. Risk estimates for 10 pg TEQ/kg per day were 0.0071 for males and 0.006 for females.

Hamburg Cohort Study In a series of papers,[54–57] a cohort consisting of 1189 men who worked in Hamburg, Germany at a herbicide plant were studied. Blood or adipose tissue TCDD concentrations were determined in 190 male workers in this cohort. A first-order kinetic model was used to estimate TCDD concentrations at the end of employment. Using a regression of the TCDD level on time worked in specific job areas compared to the estimated TCDD concentrations at the end of employment, the contribution of each job area to the TCDD exposure was estimated. Estimates of the TCDD concentrations (ng/kg body fat) at the end of employment for each member of the cohort were calculated using these regression results. Flesch-Janys et al.[55,56] then divided the cohort into the lower four quintiles and the ninth and tenth declass of the TCDD body burdens calculated. Relative risks for cancer mortality were estimated using a Cox regression. Relative risks were calculated using either the combined two lowest quintiles of the Hamburg cohort as an internal reference or a control group of gas workers as an external reference group. A significant positive trend between increasing TCDD concentrations and cancer mortality was observed using either reference group. Using national mortality data from the German Federal Office of Statistics and the method of Breslow and Day,[59] standard mortality ratios (SMRs) were calculated. Only in the tenth decile of TCDD concentrations were the SMRs elevated significantly compared to the two lowest quintiles from the cohort. When the gas workers were used as the comparison group, the SMRs were 129 or higher and were elevated significantly in three of the five exposure categories.

In a more recent analysis of this cohort, Flesch-Janys et al.[56] calculated time courses for TCDD blood lipid concentrations and used mortality up to 1992. The cohort was then divided into quartiles by integrated blood concentrations over time and SMRs were calculated. The SMR for all cancers combined was increased significantly for all workers combined (SMR 141, 95% CI = 117 to 168) and in the highest exposure quartile (SMR 173; 95% CI = 121 to 240). The SMRs increased in this analysis compared to the initial analysis of Manz et al.,[54] which used mortality data up to 1989. The SMR for lung cancer was significantly increased for all workers combined (SMR 151, 95% CI = 107 to 208), but not in any of the individual quartiles. There was a significant linear trend test on the SMRs by quartile for total cancer deaths ($p = 0.01$) but not for lung cancer deaths.

Becher et al.[57] examined the dose–response relationship of the Hamburg cohort for all cancers combined. Using the integrated blood concentrations of TCDD as calculated by Flesch-Janys et al.[56] and the SMRs for all cancers combined, a Cox regression model was fit to the data. Becher et al.[57] applied three different response models: a multiplicative model, an additive model, and a power model. The covariates used in the model were year of entry into employment, age at entry, duration of employment, and an exposure metric for β-hexachlorocyclohexane. Latency times of 0 and 10 years were also used in the models. Although the multiplicative model fit best, similar fits were observed with the other models, and Becher found no statistical reason to choose between them. Unit risk estimates for cancer death through age 70 assuming a daily exposure of 1 pg/kg per day of TCDD were calculated based on background German mortality rates. Unit risk estimates ranged from 0.0011 for females using the multiplicative model with a 10-year latency period to 0.0084 for males using the power model and no latency period. Becher et al.[57] also estimated the unit risk for lung cancer deaths using a Cox regression and the multiplicative model. This analysis resulted in similar unit cancer risks compared to all cancers combined.

BASF Cohort Study In 1953, there was an accidental release of TCDD at a BASF factory in Germany. Zober et al.[58] studied this cohort of 247 workers and found that the overall cancer mortality for all workers combined was not significantly increased. In this cohort there were 127 workers who developed either chloracne or erythema. In this group of workers, cancer mortalities were increased (SMR = 201; 90% CI = 122 to 315) in workers with a latency period of 20+ years. Zober et al.[58] also reported an increased cancer mortality in a subcohort of 153 workers who were considered likely to have been exposed to TCDD (SMR 198; 90% CI = 122 to 305).

Ott and Zober[60] extended their study of the BASF cohort using chloracne status and estimated TCDD concentrations at the time of exposure as dose metrics in 243 workers. A first-order pharmacokinetics model using a regression procedure estimated TCDD concentrations. Workers were then divided into three or four groups based on TCDD concentrations. There were no overall increases in total cancer mortality or respiratory system cancer. There was an increase in respiratory cancers in the highest of the three TCDD exposure groups (SMR 240, 95% CI = 100 to 500). A Cox proportional hazard model was used to analyze dose–response analysis and included calculated relative risks, with cigarette smoking, body mass index, exposure to asbestos, exposure to aromatic amines, age, and date of first exposure included as explanatory variables. There was a marginally significant dose effect related to total cancer deaths, but there was no effect on respiratory cancer deaths. The results of the study also suggest a trend for increasing total cancer deaths by TCDD concentrations in smokers and in all workers but not in nonsmokers or ex-smokers.

Other Studies A total of 1031 male workers at an herbicide factory in the Netherlands were exposed to dioxin.[61] Estimates of the peak TCDD concentration for all workers was calculated using a regression method.[61] The workers were then divided into exposure groups of low, medium, or high estimated peak TCDD concentrations. Estimates of the relative risk of mortality were calculated using the low-dose group as the reference group with adjustments for age, time of follow-up, and time since first exposure. Significant increases for the relative risks for total cancer deaths were observed for the medium [relative risk (RR) 1.9, 95% CI = 1.2 to 2.8] and high (RR 1.9, 95% CI = 1.3 to 2.8) exposure groups. It should be noted that there is no apparent dose trend. Insufficient information is provided in this study to estimate average body burden.

Three other studies were also not included in the present analysis. The Seveso, Italy cohort, which was exposed to a single episode of exposure to TCDD following an industrial accident (see Chapter 20), was not included due to limited exposure information. Studies are also available for the Yusho PCB and PCDF contaminated rice oil poisoning (see Chapter 21 for further discussion of Yusho incident). These studies are not included because this chapter focused primarily on the effects of TCDD and there was no TCDD in the contaminates reported. Finally, Collins et al.[62] examined cancer mortality in a subcohort of workers who developed chloracne following exposure to TCDD from an industrial accident in 1949. This plant was included in the NIOSH cohort, and thus this study was not included in the present analysis.

ED and Unit Risk Calculations In the USEPA dioxin reassessment, the ED_{01} values were estimated based on TCDD exposures in the NIOSH,[53] Hamburg,[57] and BASF[60] cohorts. In this analysis, life table data were obtained from the National Center for Health Statistics[63] for the years 1995–1997. Cancer deaths through age 75 attributed to TCDD exposure of 1 ng/kg body burden over background were estimated using the best-fit model to the individual data sets. The effective dose levels (ED_{01}, ED_{05}, and ED_{10}) were estimated by determining the dose resulting in the specified excess risk.

The ED_{01} and ED_{10} values (Table 7.3) are defined as exposures above background exposure that will produce either a 1 or 10% excess risk. The table also gives unit excess risks for exposures of 1 pg/kg per day intake above background. Only the NIOSH cohort provided enough information to estimate confidence bounds for the calculations. The Hamburg data had insufficient detail in the manuscript to estimate confidence limits on the risk estimates. In the BASF cohort, the lower confidence limit for the risk value in the Ott and Zober[60] model is zero (conditional risk ratio of 1.00). Thus the upper confidence limit on the ED values is infinite, and the lower confidence limit is zero. It should be noted that an unrealistically large fraction of the tumors were attributed to the background exposure based on the power model from the NIOSH cohort.[53]

TABLE 7.3 Total Cancer Risk from TCDD Exposure in Humans through Age 75[a]

Study	Model and Gender	ED_{10}	ED_{01}	Unit Excess Risk for 1 pg/kg per day Intake above Background
Steenland et al., 2001[53]	Power, male	500 (46.4, 2.91×10^7)	1.38 (0.71, 8.95)	0.0130 (0.0045, 0.0219)
	Power, female[b]	1315 (84.4, 4.5×10^8)	1.84 (0.92, 14.9)	0.0106 (0.0036, 0.0179)
	Piecewise linear, male	— (92.9, —[c])	18.6 (11.5, 48.3)	0.0010 (0.0004, 0.0017)
	Piecewise linear, female[d]	—[c] (108.9, —[c])	23.1 (14.3, 59.8)	0.00084 (0.00032, 0.0014)
Becher et al., 1998[57]	Power, male	120.3	5.971	0.0035
	Power, female[e]	170.9	7.580	0.0028
	Additive, male	192.8	18.22	0.0011
	Additive, female[f]	239.1	22.75	0.00088
	Multiplicative, male	258.9	32.16	0.00060
	Multiplicative, female[g]	304.4	39.82	0.00048
Ott and Zober, 1996[60]	Multiplicative, male	411.7 (201.9, ∞)	50.9 (25.0, ∞)	0.00038 (0, 0.00078)
	Multiplicative, female[h]	478.0 (234.4, ∞)	62.1 (30.5, ∞)	0.00031 (0, 0.00063)

[a] Dose is expressed as constant body burden in ng/kg not adjusted for lipid. Upper and lower 95% confidence limits (where available) are in parentheses after ED values.

[b] Relative risk RR proportional to $(AUC)^{0.097}$, with 15-year lag.

[c] When body burden exceeds 133 ng/kg, the AUC years exceed 40,000 ppt years and the model cannot achieve the prescribed risk level.

[d] Relative risk RR proportional to exp(0.000015 AUC). This is based on the linear function in the lower range of the piecewise linear model.

[e] Relative risk RR proportional to $(0.00017\ AUC + 1)^{0.326}$.

[f] Relative risk RR proportional to $1 + 0.000016$ AUC.

[g] Relative risk RR proportional to exp(0.00000869 AUC).

[h] Relative risk RR proportional to exp(0.0003522 × lipid concentration).

Noncancer Endpoints TCDD induces a number of noncancer effects in experimental animals. Several epidemiological studies have examined the relationships between TCDD exposure and noncancer effects of TCDD. There are a number of uncertainties in these analyses, and thus we have not examined the dose–response relationships for the noncancer effects of TCDD. For example, the incidence of cardiovascular disease is increased in the Hamburg cohort but

not in either the BASF or NIOSH cohorts. Because of the inconsistencies between studies, we did not examine this and other endpoints. Another problem with the noncancer effects is coexposure to other chemicals. A number of studies have examined the relationship between exposure to dioxinlike chemicals in developmental neurotoxic effects. These studies are confounded by coexposure to nondioxinlike PCBs. This coexposure complicates the dose–response analysis for TCDD. Future research efforts are necessary to better understand the role of TCDD and other dioxins in these effects.

Uncertainties in Estimates from Human Epidemiology As with any human study, a number of uncertainties associated with the risk estimates should be considered when interpreting these results. In these studies the estimate of dose is often imprecise. For example, in the NIOSH cohort TCDD concentrations were determined in 253 worker-years after the initial exposures. These workers were from 2 of the 12 plants studied and may not be fully representative of the entire cohort. In all three cohorts, TCDD concentrations were back-calculated to the time of exposure. These studies often used different estimates of the half-life of TCDD. A single compartment first-order elimination of TCDD was used in all these analyses. Although this provides a simple method for estimating body burdens at the time of last exposure, these models may be incorrect. Estimates of the half-life of TCDD vary by a factor of 2 in the peer-reviewed literature. There is also data that the elimination of TCDD is faster at higher body burdens. These assumptions clearly affect the exposure estimates.

Another assumption is that TCDD alone is responsible for the effects observed. In experimental studies, TCDD can be described as a tumor promoter, and the interaction between TCDD and cocarcinogens may be important for the carcinogenic effects of this chemical. Only in one of the cohorts examined is information provided for smoking history. In addition, these factories were producing chemicals in which TCDD was only a small contaminate (parts per million levels). These coexposures may also influence the effects of TCDD. In addition, the present analysis assumes that the effects are due solely to TCDD. Clearly, other dioxins were present in these workers as well as in the background comparison populations. Experimental data clearly demonstrate that these compounds induce TCDD-like effects and that the interactions of these chemicals is dose additive.

Conclusions for Human Cancer Dose–Response Modeling The epidemiological studies examined suggest that TCDD increases the overall risk of developing cancer, and lung cancer in particular. Other factors, such as smoking, may modify these risks. There are still a number of uncertainties that should be considered when interpreting these results. The studies examined are limited to exposures to adult males. Thus, risk estimates for fetal, infant, and childhood exposures are not assessible based on these studies. In rats, the female is more susceptible to the carcinogenic effects of TCDD. Because lim-

ited data are available for women, this is another uncertainty in the risk estimates presented. Future efforts should be made to better understand the health risks in these potentially susceptible populations. Despite these limitations, estimates based on the available human data suggest that the range of ED_{01} values is 1.4–62 ng/kg for all cancers combined.

7.5 MODE-OF-ACTION-BASED DOSE–RESPONSE MODELING

The goal of mode-of-action-based modeling is to express quantitatively the relationship between chemical exposure, target tissue dose, and the biochemical and physiological alterations leading to the toxic response. Mode-of-action modeling consists of a combination of PBPK models that estimate tissue dose and biochemical–tissue response models describing the relationship between tissue dose and response. Often, the distinction between tissue dose and response is maintained during the development of mode-of-action-based models. PBPK models provide information on the determinants of the pharmacokinetics of a chemical. In some cases, the PBPK models have been extended to generate predictions of the biochemical responses associated with the target tissue dose of the xenobiotic. The biochemical–tissue response models quantitatively describe the molecular steps leading to the observed responses. Few models have been developed to describe toxic responses. Most of these efforts have focused on carcinogenic effects of xenobiotics. In the following section we discuss PBPK and biochemical and tissue response models and how they can be used to represent the adverse effects of TCDD mathematically.

7.5.1 PBPK and Biochemical and Tissue Response Models

PBPK Model Structures and Model Development Pharmacokinetics encompasses the absorption of an administered chemical, its tissue distribution, metabolism, and elimination (ADME) from the body (see Chapter 6). The pharmacokinetic properties of a chemical depend on its physicochemical properties (e.g., tissue permeation constants, partition coefficients, and kinetic constants) and physiological parameters (e.g., organ volumes and blood flow rates). A PBPK model describes mathematically the relationship between physicochemical properties, physiological parameters, and ADME. These models describe the pharmacokinetics of a chemical by a series of mass-balance differential equations in which the concentration of a chemical in anatomically distinct regions of the body is represented by state variables. In addition, a PBPK model links these tissue "compartments" through physiologically realistic patterns of blood perfusion. Each tissue or blood compartment contains an equation with terms for input and loss of the chemical that describes the time course for the chemical concentrations. These and other equations make up the PBPK model and describe the structure and assumptions used in the model.

Evaluation of a PBPK model involves a comparison of the fit to the data and an understanding of the relationship of its structure to the underlying biology and the mathematical details linking the two.

The development and use of PBPK models in risk assessment was described by Clewell and Anderson.[64] PBPK models have been validated for numerous compounds in both animals and humans, and these models have been useful in risk assessments, particularly when describing cross-species dose extrapolations. PBPK models can also aid in extrapolating across structurally related chemicals since the structure of the models are the same or can be deduced for related compounds.

The validity of a PBPK model depends in part on how well it describes the basic biological interactions between the chemical and the organism. In the case of TCDD, a PBPK model must account for the interactions of TCDD with the Ah receptor. In addition, the model should account for the prolonged half-life of TCDD and the hepatic sequestration. Several research groups have developed PBPK models for TCDD. These models have provided insights into key determinants of TCDD disposition in animals. The development of the models are iterative processes resulting in more sophisticated models with each iteration. There are several levels of sophistication in PBPK models describing the dosimetry of TCDD. First are the traditional PBPK models by Leung et al.,[65–67] which include hepatic protein binding of TCDD. Further refinement of the model includes the use of diffusion-limited movement of TCDD using diffusion-limited modeling and induction of proteins through TCDD–AhR–DNA interactions.[68,69] Other levels of sophistication are represented by the models of Kohn et al.,[48] which include extensive hepatic biochemistry, and the models of Andersen et al.,[70,71] which describe the zonal induction of cytochromes P450 in the liver. More complex models have included coordinated responses of multiple organs[20] for hormonal interactions or the models of Roth et al.,[72] which focused on detailed descriptions of gastrointestinal uptake, lipoprotein transport, and mobilization of fat.

The models mentioned above are initial descriptions of dosimetry and biochemical response models. These models generally result in reasonable predictions of experimental data. However, there are subtle differences in the structures of these models, and these differences can have significant influence on the shape of the dose–response curve outside the experimental range. Because human health risk assessments are typically concerned with exposures below the experimental range, understanding the basic assumptions of the models and how they influence the predicted dose–response relationships is critical. Rather than examine all the models individually, we present some examples of how different assumptions influence the shape of the dose–response curves in the low-dose region.

The PBPK models for TCDD described above all accounted for an inducible binding protein in the liver. In recent iterations of these models, Hill kinetics are used to describe the rate-limiting step in the induction of CYP1A2 and 1A1 by TCDD. The Hill equation was originally derived to describe the

binary interactions between ligands and receptors or substrates and enzymes. The Hill model can be described as a very general kinetic model that allows for linear and nonlinear fits to the data below the maximal effective range. A number of investigators have applied the Hill model in their descriptions of ligand receptor interactions for soluble receptors such as the estrogen,[73] glucocorticoid,[74] and Ah[75] receptors. It should be noted that the Hill model is a general kinetic model and that when the Hill exponent is 1, it reduces to hyperbolic kinetics. Boeynaems and Dumont[76] provide a more complete discussion of kinetic models for ligand-receptor binding, including Hill kinetics.

Rather than examine all the models that employed the Hill equation individually, we focus on the work of Portier et al.[77] who developed a PBPK model to describe the relationship between hepatic TCDD concentrations and its effects on three hepatic proteins in female Sprague–Dawley rats. In this model, Portier et al. used the Hill equation to describe the rate-limiting step in the induction of CYP1A1 and CYP1A2 by TCDD. The reduction of EGF receptor was also modeled with Hill kinetics, assuming that the effect is mediated by a reduction in the expression of the receptor compared to control animals. For all three proteins, it was assumed that proteolysis followed Michaelis–Menton kinetics and was unaltered by TCDD. Two different models were used to fit the CYP1A1, CYP1A2, and EGF receptor data. These models differed only in the description of the mechanism of the basal rate of protein expression.

In the first model, designated the *independent model*, the TCDD-mediated alteration in protein expression is independent of the basal expression of these proteins. The second model, designated *additive*, assumes that the basal expression of these proteins is regulated by the Ah receptor, which is activated by an endogenous/dietary ligand. This ligand competes with TCDD for binding sites on the AhR. In the observable response range, there is virtually no difference in the ability to fit the data between the independent and additive models, going so far as to predict equivalent Hill coefficients for all three proteins.

Risk assessments frequently extrapolate below the observable experimental range. In these cases, the independent and additive models result in different dose–response relationships outside the experimental range. For example, in the low-dose range, the independent model demonstrates a thresholdlike dose response for CYP1A1. In contrast, the additive model indicates no thresholdlike response. Significant differences between the two models were also observed for CYP1A2 in the low-dose region. In contrast, these two models result in identical fits for both the low-dose region and in the observable range for the TCDD-mediated effects on the EGF receptor.

The analysis by Portier et al.[77] demonstrates that the assumptions used to describe the mechanism for the basal expression of TCDD-inducible proteins have dramatic effects on the shape of the dose–response curve in the low-dose range. The importance of these findings is that choosing either of these biochemical markers as dose surrogates results in risk estimates that could vary by

several orders of magnitude, depending on whether an independent or additive model was used. In reality, it is likely that the basal expression of these genes involves a combination of Ah-receptor dependent and independent pathways. For example, in AhR knockout mice, CYP1A2 is still expressed in hepatic tissue, but at lower concentrations than in the wild-type mice.[78] These data suggest that basal expression of CYP1A2 is mediated by both AhR-dependent and AhR-independent pathways. Future research efforts are required to better understand the mechanisms of basal gene expression for proposed dose surrogates prior to their use in risk assessments.

The model of Portier et al. assumes that the liver is a homogeneous tissue and that all cell types are equally sensitive to TCDD. Using antibody staining techniques, it was demonstrated that CYP1A2 and to a lesser extent CYP1A1 are expressed around the central vein of the liver in control animals.[79] Following exposure to TCDD, more cells around the central vein begin to express these proteins. As the dose increases, an increasing number of cells express these proteins until nearly all the hepatocytes express CYP1A1 and 1A2. This distinct division in observed regional induction within the liver might be due to differences in the sensitivity of individual hepatocytes to the effects of TCDD.

Andersen et al.[70,71] attempted to describe the regiospecific induction of proteins by TCDD by assuming that the observed distinct difference in protein expression suggested that individual hepatocytes were either noninduced or fully induced. Andersen et al. divided the liver into five concentric zones and varied the affinity of the liganded or activated AhR for the DREs by threefold between adjacent zones. Enzyme induction was modeled using Hill kinetics with a Hill exponent of 4. Qualitatively, the model reproduced the features of expanding zonal induction. Using parameters selected to yield a fit to tissue concentration time course data[37] and CYP1A1 mRNA data,[80] the model also produced a fit to P450 data comparable to that obtained with the homogeneous liver model of Kohn et al.[48] While both the zonal and homogeneous models provide similar fits in the experimental range, the zonal model predicts greater low-dose sublinearity than that of the comparable homogeneous model. Driving the low-dose nonlinearity of the zonal model is the predicted 81-fold difference in AhR–TCDD binding between periportal and centrilobular zones and the utilization of steep Hill kinetics.

The analysis of Portier et al.[77] and Andersen et al.[70,71] demonstrate that different assumptions used in the models result in very different predictions of the low-dose effects of TCDD. These models describe reasonably well the experimental data despite the significant differences in their structures and underlying assumptions. The different assumptions of some models are not readily reconcilable by our present understanding of the effects of TCDD. Clearly, future efforts are required that focus on determining which model structure more accurately reflects the biological effects of TCDD.

Modeling of Disposition of TCDD in Humans Although theoretically, one could convert a PBPK model for TCDD in rodents into one for humans simply

by substituting the equivalent human parameter values for the rodent values, there are a number of limitations to these attempts. A number of anatomical and physiological parameter estimates are available for humans. For some of these estimates, such as lung capacity and heart rate, we have an understanding of how age, gender, and body weight influence these values. In contrast, human-specific biochemical parameters such as TCDD metabolism, induction of CYP1A2, and other proteins are not available. Some parameters can be estimated based on in vitro studies of human tissues or purified preparations of human proteins. For example, human Ah receptor and CYP1A2 genes are cloned, and these could be used to estimate binding parameters of TCDD to these human proteins. Estimating parameters for tissue responsiveness to TCDD, such as CYP1A2 induction, requires tissue samples from a number of people exposed to various dioxin concentrations. Default methodologies, such as allometric scaling of the rodent values, are not likely to provide accurate estimates of the human values because protein expression does not appear to follow a simple scaling pattern for all proteins.

Alternatively, simple empirical models have been developed to account for the dose-dependent hepatic sequestration of dibenzofurans and other dioxinlike compounds.[81,82] This model has two primary parameters, $F_{\max}$ and K_d. $F_{\max}$ is described as the maximum proportion of the body burden sequestered in the liver. K_d is described as the half-saturation constant for the dose-dependent sequestration in the liver and is expressed in units of pg TEQ/kg. It should be noted that these parameters do not convey any specific information about the biology of TCDD. This model was fit to the Yusho and Yucheng food poisonings to describe the presumed dose-dependent hepatic sequestration of the PCDFs and resulted in reasonable fits to the data.

Pharmacokinetic Modeling Summary The first PBPK models for TCDD were described in the late 1980s. Over the past decade or more, these models have been refined through a series of iterations of development, experimental validation, and refinement. These iterations produce models of increasing biological complexity. The more complete models provide similar predictions of the disposition of TCDD in experimental animals. Further refinement of these models is not likely to have major impacts on their predictions in the experimental range.

Unfortunately, these models have rarely been applied in aiding analysis of the dose–response relationships for the effects of TCDD. Although there are numerous studies examining the effects of TCDD, differences in dosing regimens, such as route of exposure, exposure duration, and the length of time to necropsy after the last exposure, complicate the use of administered dose as a comparative dose metric between studies. Application of PBPK models to these data sets may provide a tool that would aid in the development of more scientifically based dose metrics.

Although these models have been applied to experimental animals, use of these models to assess human dose–response data has not been pursued. There

are several empirical models assessing the pharmacokinetics of TCDD in humans.[14,82,84] These models tend to be classical pharmacokinetic models with either one or several compartments. These empirical models have been used in human exposure assessments to back-extrapolate exposures based on a single measurement of serum, plasma, or blood concentrations of TCDD. The PBPK models for experimental animals can be readily scaled to humans. Although these models may have some uncertainties, it is unlikely that the use of these models would result in predictions that would be any less certain than the empirical models already in use.

In experimental animals, the PBPK models have been extended to include modeling of biochemical, tissue and/or toxicological response. These models provide adequate predictions of the experimental data. However, different model structures can produce divergent results outside the experimental data. Clearly, there is a need for more of a consensus on the underlying biology supporting particular model structures prior to their use in risk assessments. A better understanding of the mechanistic linkages between the biochemical effects of TCDD and its toxicological effects is needed.

7.5.2 Application of Models

One of the purposes of the PBPK and biochemical response models is to provide links between tissue dose, biochemical effects, and the toxic effects of TCDD. Although theoretically, these models can be applied to any effect, most of the work has focused on the carcinogenic effects of TCDD. Little attention has been given to the development and application of these models to the noncancer effects of TCDD. The focus of the mathematical and mechanistic modeling of the effects of TCDD has been on describing its hepatocarcinogenic effects. TCDD is described operationally as a tumor promoter. In tumor promotion models, TCDD increases the development of putatively preneoplastic altered hepatocellular foci (AHF). These are clusters of cells that exhibit altered expression of marker enzymes, such as placental glutathione-*s*-transferase (PGST), or γ-glutamyl transpeptidase (GGT). Mode-of-action-based modeling of the hepatocarcinogenic effects of TCDD may accomplished by incorporating linkages between the biochemical/tissue response models of TCDD to cell growth and mutations within the context of the quantitative dose–response models described above. In some cases, analysis of changes in hepatocyte replication has been used to estimate parameter values in some models.

Modeling Preneoplastic Lesions Mechanistic modeling of the carcinogenicity is based on a two-stage model of carcinogenesis. In this model, AHFs are treated as normal cells converted to initiated cells by a mutational event. Growth of the AHF cells and their numbers compared to normal cells is described relative to the birth and death rates of the respective cell populations. In these models, chemicals may affect the initiation stage (mutagens) and/or the birth and death rates (promoters) of normal and initiated cells. TCDD may or

may not affect the growth and/or mutational parameters directly. Three research groups have evaluated growth and development of AHFs using mode-of-action modeling. These groups used different mathematical approaches and assumptions of the phenotypic distribution of the AHFs as well as different linkages of the biological processes to the model parameters. Two groups assumed that there was a single initiated phenotype, while a third group assumed that there were multiple initiated phenotypes.

The liver tumor–promoting effects of TCDD have been studied extensively using several different experimental designs. In the tumor promotion models, animals are exposed to an initiator which is used to produce DNA damage. Typically, this is diethylnitrosamine (DEN). However, in these models, the mutation has to be "fixed". That is, the mutated cells need to replicate to ensure that the mutation is not repairable or causes cell death. Following the initiated step, the animals are exposed to the promoter, in this case TCDD, for a period of weeks to months. Pitot et al. examined the tumor-promoting effects of TCDD in rats in which a partial hepatectomy, where two-thirds of the liver was removed from the animals, was used to induce cell replication.[85] Following the partial hepatectomy, rats were exposed to a nonnecrogenic or non-hepatotoxic dose of DEN (30 mg/kg) 24 h later. In contrast, Maronpot et al. studied the tumor-promoting effects of TCDD using a dose of DEN that causes significant liver damage.[86] Both partial hepatectomy and liver damage cause liver cells to replicate. Comparison of these two methods results in differences in the background tumor rates and in the time course for tumor development following TCDD exposure. In addition to the differences in study design, Pitot[85] quantified three types of AHFs using the marker enzymes GGT, canalicular adenosine triphosphatase (ATP), and glucose-6-phosphatase (G6P), whereas Maronpot[86] used PGST only as a marker for AHFs. Buchmann et al.[87] also studied the tumor-promoting effects of TCDD using a slightly different study design compared to either the Pitot or Maronpot studies. Female Wistar rats were exposed to nonnecrogenic doses of DEN (10 mg/kg) for 5 days. This was followed by biweekly exposure to TCDD at the equivalent of 100 ng TCDD/kg per day.

Using a daily-administered TCDD dose as the dose metric, Portier et al.[88] applied methods developed previously[89] to estimate the parameters in the first half of a two-stage mathematical model of carcinogenesis from the initiation-promotion data.[86] The modeling results of Portier et al.[88] suggest that TCDD stimulates the production of PGST-positive AHF (a possible indication that TCDD has mutational effects) and that it promoted the growth of PGST AHF due either to increases in birth rate or decreases in death rate. Attempts to use the data on cell replication indices and liver weight did not appear to explain the mutational effect of TCDD. Based on the work of Kohn et al.,[48] Portier et al. suggested that the mutational effect of TCDD could be due to an increase in the metabolism of estrogens to catechol estrogens. The increases in catechol estrogens would lead to increases in oxygen free radicals and eventually, to mutations. The Portier model indicated an interaction between DEN and

TCDD, producing a TCDD dose-related formation of initiated cells throughout the study period. The best-fitting curves (using maximum likelihood methods) for the effects of TCDD on the birth and mutation rates reached saturation levels at doses below 3.5 ng/kg per day. Portier et al. applied the same model to the Pitot[85,90] data as a validation of the modeling results based on the Maronpot studies and found qualitatively similar results based on all four types of AHF from the two studies.

Moolgavkar et al.[91] modeled the hepatic tumor-promoting effects of TCDD by expanding on a previous mathematical model of tumor promotion.[87] In this model Moolgavkar et al. incorporated mathematical descriptions of cellular replication on the edge of the AHF. While the Moolgavkar et al. model was assessed based on a study using only one dose level of TCDD,[87] the results from their analysis were qualitatively similar to those of Portier et al.[88]

In the models of Portier et al. and Moolgavkar et al., it is assumed that all AHFs with the same phenotype have the same response to TCDD. In contrast, Conolly and Andersen[92] developed a model assuming that there are two types of cells initiated with the same phenotype. While the two cell types express the same biochemical marker, they have differential responses to the effects of TCDD. This hypothesis of hepatic tumor promotion was proposed initially by Jirtle et al.[93,94] and is described as the *negative selection model*. The assumption in this model is that the liver attempts to constrain proliferation following a promotional stimulus by generating mitoinhibitory signals. While some mutated cells respond to the mitoinhibition and do not grow out, the AHFs develop from cells that are insensitive to the mitoihibition and proliferate in response to the promotional stimulus. Thus, as the dose of TCDD increases, one group of initiated cells decreases in number and the other group increases in number and size. Because of this assumption, a U-shaped dose–response curve can result using the Conolly and Andersen model. In contrast, for the Portier et al. or Moolgavkar et al. model to produce U-shaped dose–response curves, the models would have to be modified by applying U-shaped parametric forms for the mutation rates or birth rates.

The Connolly and Andersen model was applied initially to the Pitot et al. data[85,90] to obtain parameter estimates for the birth and death rates of the two types of cells initiated. The Connolly and Andersen model used administered dose as the tissue dose metric. The two-cell model provides adequate fits to the data, and the estimates of the model parameters are biologically reasonable. Because the structure of the two-cell model is significantly different from the single-cell models of Portier et al. and Moolgavkar et al., comparison of the results of these analyses are difficult. For example, Conolly and Andersen estimate the birth and death rates independently. In contrast, Portier used a fixed death rate and Moolgavkar varied the death rate with the birth rate. Simultaneous estimates of the birth and death rates must be viewed cautiously, due to the limited data available to estimate these parameters. Recent data suggest that within PGST-positive AHF, TCDD does not influence the cell replication. However, in the PGST-positive AHF, there is a significant decrease in apopto-

sis.[94] Although these data are consistent with the negative selection hypothesis (the two-cell model), there is no information on the dose–response relationships for this effect.[95] The lack of dose–response information on apoptosis and cell replication make it impossible to estimate the birth and death rates simultaneously for the cell phenotypes initiated. Clearly, future research efforts are required to discriminate between the single-cell and two-cell hypotheses.

Estimation of Cancer Risks Portier et al.[88] combined a single initiated phenotype two-stage model of carcinogenesis with the biochemical response model[48] to estimate the dose–response data for liver tumors in female Sprague–Dawley rats.[46] In this model, CYP1A2 is used as the dose metric to describe the effects of TCDD on the initial mutation rate to the phenotype initiated. CYP1A2 was chosen as a dose metric because it is the major TCDD-inducible estradiol hydroxylase in the liver and the hypothesized role of estrogen metabolites in oxidative DNA damage.[96–100] Thus in this model, the initial mutation rate is proportional to the instantaneous concentration of CYP1A2, which was estimated using a biochemical response model.[48] In the simplest form of this model, all death rates and the second mutation rate to the malignant phenotype were held constant. The model adequately described the tumor data. However, the model overestimated the observed tumor response at the lowest dose examined. Based on this model, the dose–response curve was approximately linear and the ED_{01} value was estimated at a body burden of 2.7 ng/kg.

7.5.3 Mode-of-Action Modeling Knowledge and Data Gaps

There are a number of knowledge gaps in each of the models described. The biological structures of all the PBPK models described in this chapter are based on hypotheses about the mechanism of protein modulation by TCDD. At some point, however, each of these models incorporates curve fitting into the mathematical representations. For example, in the description of enzyme induction, ideally, one would have information on the rates of mRNA translation, mRNA transcription, and protein synthesis and degradation. In addition, it would be helpful to have information on the mRNA and protein concentrations. Typically, only mRNA, protein, and enzyme activity are available and frequently, not in the same study. Thus, at some point in the model, empirical approaches are used to describe induction. Despite the incorporation of empirical approaches in these models, the structure of the model is based on information on the anatomy, physiology, and qualitative effects of TCDD. In addition, these models reproduce data from studies not included in the development of the model, and often, these data are from experiments whose designs differ from those used in the model development. This can be considered a partial validation of these models.

A number of these models have different mathematical representations of the same physiological process and often provide comparable fits to the data. For example, the models of Kohn et al.[20,48] include descriptions of the TCDD

induction of the Ah receptor, binding to multiple DREs, and saturation kinetics for protein synthesis. The complexity of this sequence of events can lead potentially to nonlinearities for the overall response. However, the nonlinearities of the individual processes appears to compensate for each other, resulting in an approximate linear dose response in the low-dose region. In contrast, a regional induction model that describes the same series of steps as a single process using Hill kinetics predicts thresholdlike responses in the low-dose region.[70,71] It should be noted that both models provided adequate fits to a number of data sets in the experimental region. Thus, the choice of model structure can have pronounced effects on predicted low-dose behavior. Present data cannot resolve the discrepancies between these models.

Similar discrepancies were observed between models of TCDD-induced tumor promotion. That is, the hypothesis used to structure the model had significant impacts on the modeling results, despite the fact that these divergent models produced similar fits to the data in the experimental range. Presently, there are data supporting both model structures, but there is no conclusive evidence supporting one model over the other. The modeling efforts that describe the tumor-promoting effects of TCDD demonstrate the challenges present in the application of mathematical models to describe the complex behavior of biological systems.

7.6 DATA GAPS FOR DOSE–RESPONSE ASSESSMENT

In this chapter we describe quantitative descriptions of the dose–response relationships for biochemical and toxicological effects of TCDD. In the process of this exercise, a number of data or knowledge gaps were identified. Filling in these knowledge gaps would significantly improve our understanding of the dose–response relationship for TCDD and the risk assessment for this chemical. A summary of the more substantial data gaps is presented below.

There are both quantitative and qualitative similarities and differences in how a broad variety of species, including wildlife, laboratory animals, and humans, respond to TCDD. These are due to both pharmacokinetic and pharmacodynamic factors. Although the Ah receptor is necessary for TCDD to induce its biological effects, there are tissue- and species-specific biochemical responses that influence the action and function of this protein. For example, there are differences between Ah-receptor binding curves and the dose–response curves for biochemical and toxicological effects of TCDD. For these relationships to occur, there must be factors in addition to the Ah receptor which contribute to the biological responses to this chemical. Low-dose risk assessments would improve greatly if these factors were better understood.

There are a number of uncertainties in our understanding of the pharmacokinetics of TCDD and related chemicals in humans. In experimental animals, the disposition of TCDD is dose dependent. Humans are expected to have a similar dose-dependent tissue disposition. However, knowledge of the disposition of TCDD at or near background exposures of the general population is

limited. Although PBPK models can make predictions about tissue disposition at these low-level exposures, much of the data for which the models have been validated are at much higher exposures. Thus, the models would be making predictions outside the range for which they have been validated. In addition, there are no PBPK models for TCDD in a pregnant or developing animal. A major concern of the health effects of TCDD and other dioxins are their effects on the developing embryo and infant. Human data on these populations is rare. The lack of PBPK models in pregnant and developing animals limits their use in these potentially high-risk populations. There is also uncertainty about the half-life in humans and the population variability of the half-life. These uncertainties add to the difficulty in understanding the appropriate dose metric for species extrapolations. The present PBPK models for TCDD in experimental animals could aid in this problem if they were extrapolated to humans. Clearly, there would be uncertainty in these extrapolations; however, they may not be greater than the uncertainties associated with the current approaches.

In the present analysis, the ED_{01} values for some biochemical and toxicological effects of TCDD are at or near background human exposure. For example, based on the modeling of Portier and Kohn,[88] the ED_{01} value for liver tumors in rats is 2.7 ng TCDD/kg. Present human exposure is 5 ng TEQ/kg. In these animal models, more information is needed in the low-dose region. Like humans, experimental animals are exposed to low levels of dioxins in their diet. Typically, these exposures are discounted in experimental studies. More information on how background exposures alter the dose–response relationship in the low-dose region is required. This becomes increasingly important for effects with ED_{01} values at or near background human exposures.

Mathematical models, particularly mode-of-action models, can be used as hypothesis testing and generating. A number of quantitative mode-of-action models have been described in this chapter. These models all provide insight into the complex interrelationships of the molecular, biochemical, cellular, and physiological events resulting in a toxicological response to TCDD. Our confidence in a particular model and its predictions is based on the degree of scientific consensus about the mechanism or mode of action described by the model. One difficulty in applying these models for risk assessment is that there is a lack of consensus on the mechanism of action of TCDD for most endpoints. While there is a consensus that the biochemical alterations induced by TCDD lead to the toxic responses, there needs to be a better mechanistic linkage between these biochemical alterations and the toxic responses. Understanding these linkages could lead to improvements in our estimates of risk in the low-dose region.

7.7 SUMMARY

There are a number of lines of evidence that animals and humans may respond similarly to TCDD and related chemicals. There is significant structural and

functional homology of the Ah receptor found in experimental animals and that found in humans. A number of studies examining the effects of TCDD in human cells or tissues demonstrate remarkable concordance with the effects observed in cells or tissues derived from experimental animals. There are also similarities in some toxic responses. For example, the dose–response relationship for increases in cancer incidence in the epidemiological studies is qualitatively consistent with risk estimates based on the animal carcinogenicity data. The weight of the evidence supports the use of animal models as an appropriate basis for estimating human risk. A point of caution: There are notable differences in the pharmacokinetics and pharmacodynamics of TCDD between animal models and humans. Understanding and recognizing these differences is critical when estimating human risk based on animal data. Our level of confidence in estimates of risk depends on the accuracy of the description of the interspecies differences.

The experimental data on the molecular mechanism of action of TCDD is consistent with the hypothesis that binding of TCDD to the Ah receptor initiates a cascade of biochemical, cellular, and tissue responses that lead to the ultimate toxic effect in animals. Thus, when developing dose–response models, quantitative information on TCDD concentrations, Ah receptor occupancy, and biological response is crucial. It should be noted that multiple dose–response relationships for receptor-mediated biological responses are possible. As such, the dose–response relationships for the biochemical effects of TCDD may not be representative or predictive of the dose–response relationships for more complex responses, such as immune and developmental toxicities. The quantitative relationship between receptor occupancy and the biological effects of TCDD are strongly influenced by cell-specific factors as yet uncharacterized. The identification of these factors is one of the uncertainties in our understanding of the biological effects of TCDD.

One of the most important uncertainties in our understanding of the effects of TCDD is in our choice of dose metrics for species extrapolation. Although there are default procedures such as uncertainty factors and allometric scaling, the use of these methods for species extrapolations in risk assessments have proven unsatisfactory. An appropriate dose metric would incorporate both the magnitude and duration of exposure. In addition, the mechanistic relationship between this dose metric and the toxic response would be clearly defined. Because of the broad range of toxicities induced by TCDD, it is unlikely that a single-dose metric would be suitable for species extrapolation for all endpoints. In addition, widely different conclusions could be made based on the choice of dose metric. In the USEPA dioxin reassessment, steady-state body burden was suggested as an appropriate dose metric for species extrapolations. This metric was chosen, in part, because of the large difference in the half-life of TCDD between species and because target tissue concentrations should be equivalent between species when the dose is expressed as body burden. Clearly, body burden is not the ideal dose metric, and future research efforts should focus on developing more appropriate expressions of dose and exposure.

Risk assessments must also extrapolate across endpoints as well as species. Determining the critical effect as the basis of the risk assessment requires a method to compare across endpoints. Typically, this has been the NOEL or LOEL value from the toxicity studies. However, this method has some uncertainties. The ability to determine the NOEL or LOEL value depends, in part, on how precise the measurement of toxicity can be. In the present analysis, we have chosen to use the ED_{01} value. This is defined as the dose that produces 1% of the maximal response induced by TCDD. Here we have used empirical approaches to estimate both the ED_{01} value and the body burden that is associated with this effect level. Future efforts should apply existing PBPK models to estimate the tissue dose across experimental designs and between species. This approach may allow for a more accurate description of dose in place of steady-state body burdens.

The USEPA,[15] the National Institutes of Health,[2] and IARC[1] classify TCDD as a known human carcinogen. Experimental evidence demonstrates that TCDD is carcinogenic in all species and strains of laboratory animals tested. Although the classification of TCDD as a carcinogen is useful in hazard assessments, it is difficult to find epidemiological data that have sufficient dose–response information that would provide quantitative estimates of risk in these populations. There are considerable uncertainties in modeling the data from the epidemiological data available. Typically, these uncertainties include extrapolation of present TCDD exposures to past occupational exposures and the choice of type and shape of the dose–response model fit to the data. Often, a linear model is chosen because the limited number of exposure groups in the epidemiological studies do not provide enough information to fit more complex models. In the present analysis of dose–response relationships, approximately half of the cancer and noncancer effects of TCDD are linear in the experimental range and half are nonlinear. Thus, there is considerable uncertainty in the choice of a model given the limited epidemiological data sets available and the results of analysis of the experimental data.

Despite these limitations, it is possible to apply simple empirical models to the epidemiological data. Analysis of several of these epidemiological studies suggests that TCDD increases the risk for all cancers and lung cancers in adult males. Estimates of the ED_{01} values based on average excess body burdens of TCDD in these occupational cohorts range from 6 to 161 ng/kg. Using similar empirical approaches in analysis of the animal data results in a range of ED_{01} estimates of 3 to 1190 ng TCDD/kg steady-state body burdens. Lung cancer was the only tumor site that was increased in both the animal and human data. In humans the ED_{01} value ranged from 36 to 250 ng TCDD/kg, compared to the single estimate of the ED_{01} value in rats of 730 ng/kg. The similarities in the ED_{01} values between the rodent and human data increase our confidence that TCDD is a human carcinogen.

Our present understanding of the mode or mechanism by which TCDD induces its many biological effects is limited. There are a number of generalized theories of chemical carcinogenesis (i.e., tumor promotion vs. initiation), and

mechanistic models describing these processes have been developed. Application of these models to the biological effects of TCDD has provided insights into its mechanism or mode of action. In contrast, for a number of the noncancer effects of TCDD, our understanding of the mode of action is limited to the initial binding of TCDD to the Ah receptor. Because of the uncertainty of the mode or mechanism of action of TCDD, development of dose–response models for the noncancer effects is limited to enzyme induction and alterations of serum thyroid hormone concentrations.

Although the data and knowledge base is not amenable for mechanistic modeling of the noncancer effects, there are considerable data available for empirical analysis of these dose–response relationships. In this chapter we have described attempts at empirical modeling of the noncancer effects of TCDD. In this exercise, several difficulties and uncertainties were encountered that should be considered when comparing across endpoints and species. Estimates of the shape of the dose–response curves and the ED_{01} values are sensitive to the experimental design. Thus differences in the strain, gender, time of exposure, and the use of multiple or single exposures should be considered when comparing ED_{01} values or shape parameter estimates. In addition, comparisons across endpoints are also problematic and may be misleading. For example, a change of 1% in body weight is not nearly as adverse as a 1% increase in cancer risk. Confidence in the ED_{01} estimate is dependent on whether it is in the experimental exposure range or the change in the response is in the measurable range. Finally, because of the ubiquitous distribution of dioxins, even the control animals in the experimental studies have measurable dioxin body burdens. These exposures are not considered in the present dose–response analysis or in the vast majority of the experimental studies.

Despite these considerations, we observed several general trends. The biochemical responses tended to have lower ED_{01} values followed by hepatic responses, immune responses, and responses in tissue weight. Many of the shape parameters were consistent with linear dose–response relationships over the range of doses examined for a variety of endpoints. Although this does not imply that these curves would be linear outside the experimental range, the use of linear models in estimating risk should be given serious consideration. In all response categories, both linear and nonlinear dose–response curves were observed. However, the biochemical responses were more likely to have shape parameters consistent with linearity than were other response categories, such as tissue responses. Thus the data suggest that biochemical responses to TCDD are more likely to be linear within the experimental dose range and that more complex responses, including frank toxicity, are more likely to have a nonlinear shape. Thirteen cancer data sets were analyzed and the shapes were split between nonlinear (five endpoints) and linear (eight endpoints).

There were 50 data sets where the ED_{01} estimate was below 50 ng/kg, and 19 of these data sets had an ED_{01} value under 5 ng/kg. In addition, the ED_{01} value for several of the cancer endpoints was below 50 ng/kg. If we assume that the U.S. adult background body burden in the general population is about 5 ng

TCDD equivalents per kilogram of body weight, these results indicate that the margin of exposure is less than an order of magnitude for many toxic and biological responses.

The empirical models employed in this analysis have advantages and disadvantages. These models can be used to describe the pattern of response and a means of hypothesis testing and interpolation between data points. A major disadvantage is that they cannot provide a meaningful mechanistic and quantitative link between data sets. The mechanistic models are useful tools to describe these complex biological systems in a quantitative manner. Although these mechanistic models should be able to provide reliable extrapolations between species and dose levels, the uncertainty in the hypothesis underlying the model structure limits their use in risk assessments.

There are a number of PBPK models for TCDD that have been through varying rounds of refinement. Some of these models have very different structures. Despite these differences, they all provide reasonable predictions of the disposition and pharmacokinetics of TCDD in experimental animals. These models have provided a clearer understanding of the determinants of TCDD disposition in animals. Some of these models have been extended to describe biological responses from the molecular level to hepatic tumor promotion and carcinogenesis. All of these models provide reasonable predictions in the experimental range. However, because of the different hypotheses used to develop the models, there is a rapid divergence of predictions in the low-dose region. Although these models have potential for use in risk assessment, further research is needed to provide a better understanding of the biological effects of TCDD.

7.8 CONCLUSIONS

TCDD and related chemicals have been clearly identified as hazards. In order to estimate potential risks associated with exposure to these chemicals, we must better understand the shape of the dose–response functions in the observable range and what this implies about the dose–response relationships at the exposures of interest. In addition, we must also characterize the point of departure or critical dose level that we believe warrants concern. TCDD provides a difficult case study because of the multiplicity of responses and the complexity of its mechanism of action.

There is an unusually large database on the cancer and noncancer effects of TCDD in experimental animals compared to other chemicals. Empirical approaches to analyzing these data suggest that the biochemical responses tend toward linear dose–response relationships, and the toxicological effects tend to demonstrate more thresholdlike relationships. However, there is a considerable proportion of the toxicological effects that demonstrate linear dose–response relationships in the experimental range. The human dose–response data for toxicological effects is limited and does not provide clear evidence of threshold

or nonthreshold behavior. The results of mechanistic modeling of the dose–response relationships is highly dependent on the assumptions or hypotheses used in development of the models. The different models result in highly divergent risk estimates in the low-dose region, depending on the assumptions used, and as such their use in risk assessment is uncertain at this time. In light of these data, the choice of using either linear or nonlinear models for extrapolations in risk assessments remains uncertain.

In the present analysis we estimated the ED_{01} values for cancer and noncancer effects. Both human and animal cancer data were available for this analysis. Results from the human studies suggest a range of ED_{01} values from 6 to 161 ng/kg. Similar estimates of the ED_{01} values were obtained in empirical analysis of the animal data with ED_{01} values ranging from 14 to 1190 ng/kg. The only mechanistic model to estimate the ED_{01} values for cancer in experimental animals resulted in an ED_{01} value of 2.7 ng/kg. These results suggest that points of departure for cancer risk estimates may be within the range of background human exposures.

Estimates of the ED_{01} values for noncancer endpoints showed considerable variability and ranged over 10 orders of magnitude. While many of the noncancer responses had ED_{01} values significantly higher than the cancer ED_{01} values, a number of endpoints had ED_{01} values near background human exposures. In particular, the reproductive toxicities of TCDD in male and female rats had ED_{01} values similar to those for the cancer effects. In addition, many of the biochemical responses had ED_{01} values similar to the cancer ED_{01} estimates. Although the estimates of the ED_{01} values are highly variable, several conclusions can be made. The choice of a point of departure above 100 ng/kg would probably result in risk estimates greater than 1% for either cancer or noncancer effects. A point of departure below 1 ng/kg would be based on estimates that were well below the range of the experimental data and would more likely result in risk estimates below 1%. The weight of evidence from a number of different endpoints suggests that a point of departure between 10 and 50 ng/kg would be strongly supported by the experimental data. The use of these estimates suggests that the margin of exposure is approximately 2 to 10 for the background population.

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CHAPTER 8

Immunotoxicology of Dioxins and Related Chemicals

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8.1 INTRODUCTION

Landsteiner established early in the twentieth century that the immune system could specifically recognize and respond to small organic chemicals if these chemicals were first bound to larger proteins. Today, we recognize the clinical importance of such immune responses as the underlying cause of allergic reactions to certain drugs, such as penicillin, that occur when the chemical binds to self proteins in the body. Similarly, larger foreign chemicals, such as the proteins associated with parasites and molds, can provoke an immune response directly, leading to allergic reactions. Chemicals can also affect the immune system by altering its ability to respond to pathogenic agents. This aspect of chemical immunotoxicity was first recognized in the 1950s by the side effects produced by cytotoxic chemotherapeutic regimens that left cancer patient susceptible to unusual and life-threatening infections. Within a few years, a greater understanding of the basis for these side effects led to clinical applications of chemically induced immune suppression—to prevent rejection of tissue and organ transplants, to reduce overactive immune responses associated with allergy and asthma, and more recently, to treat autoimmune diseases such as multiple sclerosis and lupus. Today, the availability of a wide variety of immunosuppressive chemicals has revolutionized the field of organ transplantation and holds great promise for more effective treatment of allergic and autoimmune diseases in the future.

The early 1970s saw the emergence of the field of environmental toxicology, and along with it, new concerns regarding possible harmful effects of environmental chemicals on the immune system. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin

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(TCDD) was among the first environmentally relevant chemicals to be identified as a potent immunosuppressant, and research on its mechanisms of immunotoxicity has served as a prototype for the evolution of the discipline of immunotoxicology. During the past 25 years, the field of immunotoxicology has grown into a respected discipline of toxicology, and screening for effects of chemicals on the immune system is now required for premarket approval of many classes of new products, including drugs, pesticides, food additives, and other consumer products. In addition, research has continued to explore the characteristics and mechanisms of TCDD immunotoxicity. The goal of this chapter is to provide an overview of the current state of this research, focusing primarily on data published since 1994. The reader is referred to previous reviews for discussion of the older literature.[1,2]

8.2 BASICS OF THE IMMUNE SYSTEM

The immune system is a complex *recognition and response system* that has evolved as a means for the body to defend itself against invasion by disease-producing microorganisms (viruses, bacteria, and parasites). The immune system also plays a role in preventing the growth of certain types of cancers. In order to carry out this defense, an arsenal of sophisticated weaponry is utilized to attack and destroy the microbial invaders or transformed cancer cells. However, strict regulatory controls are also necessary to prevent inappropriate deployment of an immune response that could result in undesirable self-inflicted damage to the body. Thus, the immune system must not only be capable of identifying the enemy's cells as distinct from its own, but also be capable of sensing when to attack, when to marshal special forces, and when to retreat.

The cells that make up the immune system are derived from pluripotent stem cells in the bone marrow. As shown in the simplified diagram in Figure 8.1, these stem cells develop along two main lineages, entering either the lymphoid or myeloid precursor pool. Myeloid precursors differentiate into granulocytes, macrophages (MΦs), and dendritic cells (DCs), while lymphoid precursors differentiate into natural killer (NK) cells, B or T lymphocytes. The differentiation of T lymphocytes takes place in the thymus gland, where the cells undergo selection for antigen specificity and deletion if they recognize self-antigens. Prior to leaving the thymus and taking up residence in peripheral lymphoid organs, the T cells differentiate further into two distinct subpopulations of cells identified by their exclusive expression of CD4 or CD8 surface molecules. These T-cell subsets provide different functions during immune responses to pathogens.

When the body is first confronted with an infectious organism, a nonspecific *innate immune response* is initiated that involves phagocytic cells (MΦs, granulocytes), NK cells, and several soluble proteins and peptides that have antimicrobial actions. The resulting inflammatory response often leads to the inac-

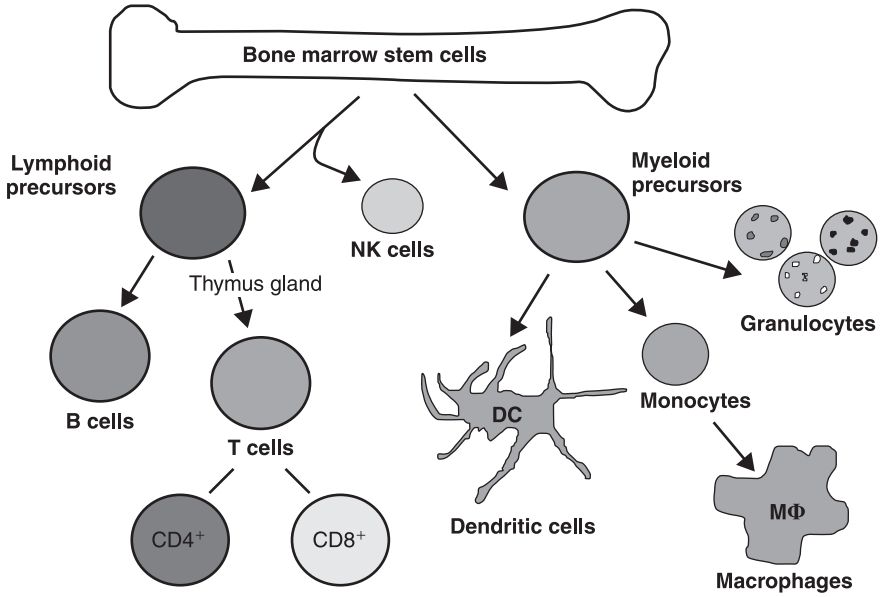

Figure 8.1 Hematopoietic stem cells in the bone marrow give rise to the cells that constitute the immune system.

tivation and clearance of the microbes. However, if these front-line defenses fail to clear the infection quickly, the *adaptive immune response*, which is mediated by lymphocytes, comes into play. This response takes longer to develop the first time that a particular pathogen is encountered, but the organism also develops a long-term immunity that can respond quickly and potently when the same pathogen is encountered again in the future.

The generation of an adaptive immune response takes place in the peripheral lymphoid organs, the spleen and lymph nodes. As shown in Figure 8.2, the response is initiated when DCs, located in tissues throughout the body, recognize and engulf infecting microorganisms. The microorganisms are taken up into acid vesicles where the proteins are processed into antigenic peptides and loaded onto major histocompatibility complex (MHC) class II molecules for display on the surface of the DCs. Antigenic peptides can also be generated within the proteosome of a cell and loaded onto MHC class I molecules for display on the cell surface. The antigen-bearing DCs then migrate into lymphoid tissue, where antigen-specific T cells will be encountered. $CD4^+$ T cells recognize antigenic peptide in association with the class II MHC molecules, whereas $CD8^+$ T cells recognize peptides in association with class I MHC molecules. The $CD4^+$ T cells will be activated to proliferate and differentiate into T "helper" cells. These cells will increase their surface expression of various adhesion and co-stimulatory molecules and secrete a variety of proteins called *cytokines* that promote the activation and differentiation of antigen-

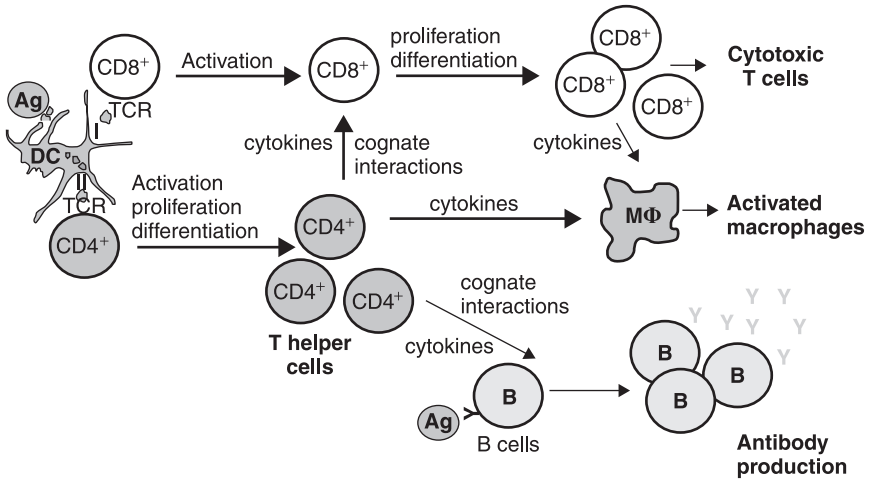

Figure 8.2 Many cellular interactions are involved in generating an adaptive immune response, leading to the generation of CTL activity, macrophage activation, and antibody production.

specific B cells and $CD8^+$ T cells through specific cytokine receptors. The antigen-bearing DCs are key to activating the adaptive immune response because they also express other cell surface molecules that provide co-stimulatory signals to the T cells that are essential for full activation. Inflammatory mediators produced by the innate immune response play an important role in the adaptive immune response by helping to induce co-stimulatory molecule expression on antigen-presenting cells (APCs). If T cells are presented with antigen in the absence of co-stimulation, the T cells become nonresponsive (anergic), and an immune response will not be generated.

Once $CD4^+$ T cells are activated, new molecules are expressed on their cell surface, allowing them to interact and promote the activation of B cells. They also produce a variety of cytokines that lead to the proliferation and differentiation of antigen-activated B cells. These cells produce and secrete antibodies that will neutralize, aggregate, lyse, or otherwise inactivate the antigen. Cytokines produced by $CD4^+$ T cells also induce antigen-activated $CD8^+$ T cells to proliferate and differentiate into cytotoxic T lymphocytes (CTL). These CTL recognize, bind, and kill virus-infected cells that express viral antigens on their surface. Cytokines produced by activated $CD4^+$ and $CD8^+$ T cells will also influence MΦ functions, leading to increased phagocytic capacity and increased microbiocidal activities.

Once the pathogen has been cleared from the body, downregulation of the immune response is a very important process that is necessary to prevent damage to normal tissues. Mechanisms such as the programmed cell death of activated DCs serve to terminate the signals that initiated and sustained the response. Increased production of cytokines that inhibit lymphocyte prolifera-

tion (e.g., transforming growth factor β or IL-10) and replacement of receptors for costimulation with surface molecules (e.g., CTLA4) that reduce the activity or induce apoptosis of the T cell also contribute to the decline in the response. Last, but certainly not least, as the primary response declines, some of the antigen-specific $CD4^+$ and $CD8^+$ T lymphocytes enter a quiescent state and remain in the body for a prolonged period of time. Upon reexposure to the same antigen, these ''memory'' cells will be able to respond more rapidly and more vigorously, thereby preventing infection. The phenomenon of memory cell generation is the basis for the efficacy of vaccinations.

8.3 APPROACHES TO IMMUNOTOXICITY ASSESSMENT

Hazard identification in relationship to the immune system presents some unique challenges when compared to other target organs. The fact that this target organ is located throughout the body and that the system must be challenged with antigen to fully appreciate the presence or absence of a hazard are two main distinctions. Hazard assessment is also complicated by the self-renewing nature of the immune system, with continual turnover and repopulation of cells in the periphery from the bone marrow and thymus. The fact that responses to different antigens may depend on different subsets of responding cells further complicates the process. Various screening methods to identify and characterize the immunotoxic hazard of chemicals have been recommended by several U.S. government agencies, including the Food and Drug Administration, USEPA, and National Toxicology Program. These protocols generally describe a tiered approach to testing the immune system using assays of increasing specificity as one moves through the tiers. The goal is to characterize the hazard in terms of types of immune responses affected by the xenobiotic, the dose–response for eliciting those effects, and identification of sensitive subpopulations as determined by age or genetics. The ultimate goal is to elucidate the mechanisms of action that underlie xenobiotic effects on immune function in order to strengthen the extrapolation of laboratory animal data for human health risk assessment.

8.4 OVERVIEW OF TCDD IMMUNOTOXICITY

After many years of research, it is clear that the immune system is one of the most sensitive targets for the toxic effects of TCDD and structurally similar halogenated aromatic hydrocarbons (HAHs), including polychlorinated biphenyls and dibenzofurans. In laboratory rodents, a single dose of TCDD in the low μg/kg range suppresses both antibody- and cell-mediated immune responses. In addition, animals exposed to TCDD show increased severity of disease symptoms and/or increased incidence of disease-induced mortality when challenged with a variety of infectious agents, indicating that the immune

system is compromised in a biologically significant manner. Importantly, the immunosuppressive effects of TCDD are dose-dependent and are observed at doses that are not overtly toxic to the animal.

The laboratory mouse has been the species of choice in the majority of the immunotoxicity studies on TCDD. Mice are the best characterized animal model for basic immunology research, and their wide use has led to the availability of essential immunological reagents and genetically defined strains that do not exist for other laboratory species. However, some interspecies variability in TCDD immunotoxicity has been noted, and the basis for such differences, particularly between rats and mice, is not entirely clear. Unfortunately, these differences contribute uncertainty to the risk assessment process of extrapolating data from animal studies to estimate the risk to humans from exposure to TCDD. Elucidation of the underlying mechanisms by which TCDD alters immune functions in various animal models will help to eliminate such uncertainties.

8.4.1 Dependence of TCDD Immunotoxicity on the Ah Receptor

One of the most important advances in the study of TCDD toxicity was the discovery of a genetic basis for the differences in sensitivity of different strains of mice. This genetic factor is the aryl hydrocarbon receptor (AhR) gene. The AhR is a cellular protein that exists in the cytoplasm in association with a number of other proteins, including a dimer of heat shock protein 90, a src protein kinase, and an immunophilin alternately named AIP, XAP2, or ARA9[3–5] (see Chapter 12). When TCDD binds to the AhR, the accessory proteins dissociate, allowing the AhR to move to the nucleus of the cell where it heterodimerizes with AhR-nuclear transport protein (ARNT). The AhR-ARNT nuclear complex then functions as a transcription factor, recognizing *dioxin response elements* (DREs) in the promoter region of various genes. Most, if not all, of the toxicity produced by TCDD is thought to result from altered gene transcription initiated through DRE binding. The best-characterized transcriptional response to TCDD-induced AhR activation is the increased expression of the genes for cytochrome P4501A1, 1A2, and 1B1, which are highly induced in liver and various other tissues following TCDD exposure. DRE binding sequences have also been identified in several other genes, but their direct induction by TCDD has not yet been demonstrated. In addition to gene induction, AhR activation has also been shown to suppress gene transcription through DRE binding by physically interfering with the binding of neighboring transcription factors. For example, this mechanism appears to explain the AhR-dependent inhibition of estrogen receptor–mediated induction of c-fos and cathepsin D following TCDD exposure.[6,7]

Studies in mice have shown that the immunotoxicity of TCDD and structurally related HAH is dependent on the activation of the AhR. In early studies, AhR involvement was deduced from structure–activity relationships using a variety of HAH congeners that differed in their affinity for binding to the AhR

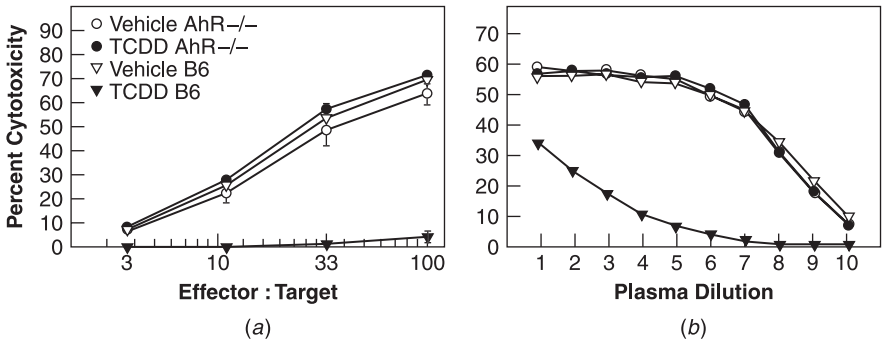

Figure 8.3 (*a*) The CTL response and (*b*) the alloantibody response in $AhR^{-/-}$ mice are not significantly suppressed by 15 μg TCDD/kg given orally one day prior to the injection of allogeneic P815 tumor cells. The same dose of TCDD highly suppressed these responses in $AhR^{+/+}$ mice. The generation of the immune responses was not altered by the absence of the AhR.

and their potency to suppress immune responses. Similarly, the greater sensitivity of certain mouse strains to TCDD-induced immune suppression directly correlated with the expression of high-affinity AhR (e.g., C57Bl/6 mice) compared to the reduced sensitivity of mice that expressed low-affinity AhR (e.g., DBA/2 mice). Recently, the generation of mutant mice in which the gene for the AhR has been disrupted has allowed direct testing of the hypothesis that the AhR is required for TCDD-induced immune suppression. The results of these studies showed that without functional AhR protein, C57Bl/6 mice exposed to TCDD generated normal immune responses to model antigens.[8] For example, as shown in Figure 8.3, when $AhR^{-/-}$ mice were injected with tumor cells derived from a genetically different strain of mouse, neither the CTL response nor the antibody response to the alloantigens expressed by the tumor cells was suppressed by exposure to TCDD. This same dose of TCDD was highly suppressive in normal $AhR^{+/+}$ C57Bl/6 mice. Interestingly, in the same studies, the $AhR^{-/-}$ mice mounted normal CTL and alloantibody responses, suggesting that the AhR itself is not essential for the development of a functional immune system. However, this conclusion must be tempered since only adult animals and only a few responses were examined. A recent study of bone marrow from $AhR^{+/+}$ and $AhR^{-/-}$ mice suggested that the AhR might play a role in the maturation of B cells.[8a]

One of the keys to understanding the mechanisms of TCDD immunotoxicity now appears to lie in determining what genes are regulated transcriptionally by AhR-ARNT and the role of such genes in the immune system. Although putative DREs have been found upstream of many genes with potential importance for immune function,[9] direct regulation by the AhR has been shown for only a few genes. Interestingly, the promoter region of the IL-2 gene was recently shown to contain DREs responsive to the AhR, and in vitro exposure of cells

from the thymus and spleen to TCDD resulted in increased production of IL-2.[10] IL-2 is a multifunctional cytokine that promotes T-cell growth as well as T-cell death.[11,12] Therefore, dysregulation of IL-2 production might play a role in suppression of T-cell responses following TCDD exposure. Two DRE-like sites within the Ig heavy chain 3′α promoter region have also been identified, and TCDD exposure induced the binding of the AhR nuclear complex to both sites.[13] Altered transcription of the 3′α promoter may play a role in the suppression of antibody production by TCDD. In other studies, TCDD was shown to inhibit the expression of the CD19 gene in a human B lymphocyte cell line.[14] CD19 is the hallmark differentiation antigen of the B-cell lineage and is expressed on all B cells except mature plasma cells. Expression of CD19 is required for optimal signaling through the B-cell antigen receptor complex, and $CD19^{-/-}$ B cells show a profound deficiency in response to antigenic stimulation.[15,16] The mechanism for TCDD's inhibitory effect on CD19 expression was postulated to be competition between the AhR and the B lineage specific activator protein (BSAP) for a common DNA binding site in the promoter region of the CD19 gene. Other DRE-regulated genes such as P4501A1 and 1B1 are not highly expressed in cells of the immune system and their induction has not been linked to TCDD's immunotoxicity. The recent emergence of commercial gene array technology should provide the means for rapid advances in this area of research.

In addition to a direct influence of TCDD on gene expression via DRE binding, other mechanisms by which AhR activation could alter immune cell functions have recently been described. For example, when the AhR becomes activated as a result of ligand binding, hsp90 and c-src proteins dissociate from the receptor. Enan and Matsumura[17] have presented evidence that AhR activation releases c-src in an active form that mediates tyrosine kinase activity which could disrupt normal intracellular signaling pathways. In addition, immunophilin-like proteins have been described that preferentially associate with the non-ligand-bound[4] or ligand-activated AhR.[3,18] A possible role for the immunophilin proteins is intriguing given that the potent immunosuppressive drugs cyclosporin A and FK506 exert their effects by forming complexes with immunophilins.[19] There are also reports that the ligand-bound AhR can associate physically with other transcription factors, such as the Rel A component of NFκB, to induce mutual functional modulation of gene expression controlled by these transcription factors.[20,20a] NFκB is widely expressed by cells of the immune system and has been shown to play important roles in both development and function. Similarly, physical association between the AhR and the retinoblastoma tumor suppressor protein, which controls cell cycle progression through G1, has been reported.[21] Thus, many pathways of cellular activation, proliferation, and survival have the potential to be influenced by AhR activation leading to alterations in the function of the immune system. Identifying and establishing the biologically significant pathways that are altered by AhR activation is a formidable, yet exciting prospect. Since activation of the AhR signaling pathway may induce a novel form of immune suppres-

sion, deducing this mechanism of action may provide new insights in basic immune regulation and may also reveal new possibilities for immunosuppressive therapies.

8.4.2 Indirect Mechanisms for TCDD Immunotoxicity

In order to function as a direct target of TCDD, a cell must express functional AhR. Myeloid cells including MΦs,[22,23] DCs (Ruby and Kerkvliet, unpublished data), and neutrophils[24] express detectable levels of AhR, whereas AhR expression in lymphocytes is very low.[22,23] However, B cells[25] and T cells[23] have been shown to increase their expression of the AhR upon immune activation, which may confer increased sensitivity to TCDD toxicity. If true, this may help to explain why responses of lymphocytes isolated from TCDD-exposed mice appear to respond normally to antigenic stimulation ex vivo while similar responses in vivo are suppressed.[26,27] If lymphocytes are removed from TCDD-treated animals prior to activation and upregulation of the AhR, lymphocytes may not retain sufficient concentrations of TCDD to suppress the response ex vivo, whereas surrounding tissue pools in vivo may provide ongoing exposure during the activation process. This bears special relevance to epidemiological studies, where the impact of TCDD exposure on the human immune system is usually evaluated by ex vivo testing of peripheral blood lymphocytes. The absence of changes in function of cells from TCDD-exposed persons[28–30] should be interpreted cautiously.

Although ex vivo testing for effects of TCDD on lymphocyte functions has not been fruitful, if TCDD alters lymphocyte functions directly via their AhR, this should be detectable by in vitro addition of TCDD to cells in culture. However, even using this approach, surprisingly few studies have documented direct effects of TCDD on lymphoid and/or myeloid cell functions in vitro. Although some direct effects of TCDD on B cells have been reported (see below), T cells appear to remain unaltered by direct in vitro exposure.[31] These observations led to the hypothesis that the immunotoxicity of TCDD seen in vivo might be induced indirectly through nonlymphoid tissues. Since the endocrine system produces several immunomodulatory hormones, it was considered a primary candidate. However, subsequent investigations into the role of glucocorticoids[26,32] or sex hormones[32] did not support their role as mediators of the immunotoxicity of AhR ligands, although castration of male mice provided partial restoration of the immune response following PCB exposure.[32] The immunosuppressive effects of TCDD were also not linked to excess production of arachidonic acid metabolites, including prostaglandin E_2,[33,34] or to oxidative stress[35] even though other studies have found that TCDD enhances the production of reactive oxygen species by MΦ[36] and other cell types.[37] Interestingly, it was recently shown that the human Cu/Zn superoxide dismutase gene is transcriptionally activated by DRE binding, suggesting that TCDD exposure, while inducing oxidative stress, may also accelerate neutralization of the superoxide anion, thereby reducing the oxidative damage.[38]

8.4.3 Effects of TCDD on B Cells

The suppression of the T helper cell-dependent antibody response to sheep red blood cell antigens is one of the most sensitive endpoints of TCDD immunotoxicity. A single dose of only 0.65 μg TCDD/kg body weight in C57Bl/6 mice suppresses the antibody response by 50% (reviewed in Ref. 39). Although the direct targets of TCDD that result in suppression of T-dependent antibody responses in vivo have not been fully resolved, alterations in B-cell function appear to play an important role. This conclusion is deduced from studies showing that antibody responses to antigens that do not depend on T cells are also suppressed by TCDD, albeit at higher dose levels, and that TCDD can directly alter B-cell responsiveness in vitro.

Kramer et al.[40] first reported that TCDD induced protein kinase activity directly and increased the intracellular calcium concentration in murine B cells in the absence of any other stimulus. At about the same time, Luster et al.[41] reported that TCDD selectively inhibited terminal B-cell differentiation into plasma cells in response to trinitrophenyl lipopolysaccharide (TNP-LPS) without altering early events in B-cell activation (measured as MHC class II and IL-2 receptor expression) or influencing B-cell proliferation. However, TCDD had to be present early in the culture in order to alter terminal differentiation, suggesting that an early event in B-cell activation was targeted by TCDD. Subsequent studies implicated increased tyrosine phosphorylation as an important early event.[42] However, other studies indicated that TCDD increased phosphorylation in activated, but not resting, B cells and that the effects of TCDD were similar in B cells from AhR-responsive and AhR-nonresponsive mice.[43] Interestingly, addition of IFNγ at the initiation of culture prevented TCDD-induced phosphorylation as well as the suppression of antibody production; however, the in vivo relevance of this finding has not been reported.

In other studies, TCDD inhibited B-cell proliferation that was triggered by treatment with LPS, surface immunoglobulin (Ig) cross-linking, or stimulation with phorbol 12-myristate 13-acetate (PMA)/ionomycin.[44,45] TCDD also suppressed IgM production induced by anti-IgM antibody plus lymphokines but not by activated T helper cells expressing CD40 ligand plus lymphokines. The selective effect of TCDD on surface Ig-cross-linked signals was correlated with TCDD-induced alterations in calcium homeostasis, seen as an early mobilization of calcium in resting B cells.[46] Taken together, these results indicate that the activation stimulus influences the effects of TCDD on B-cell responses and suggest that interference with specific signaling pathways used by different receptors is critical to TCDD's effects on B cells.

Although there have been conflicting data on the role of the AhR in B-cell dysfunction induced by TCDD, recent studies using two B-cell lines that differ in their expression of the AhR have linked the suppression of IgM production in B cells to activation of the AhR by TCDD. Using the CH12.LX B-cell line that constitutively expresses high levels of AhR, inhibition of μ-gene expression and IgM protein secretion by various dioxins was shown to follow a structure–

activity pattern predicted by AhR binding affinity.[47] Inhibition of IgM secretion was not observed in LPS-stimulated BCL-1 cells, a B-cell line that does not express the AhR.[13] As mentioned previously, DRE-mediated modulation of the Ig heavy chain 3′α promoter and of CD19 expression provide potential molecular mechanisms for TCDD's interference with B-cell responses.

8.4.4 Effects of TCDD on Antigen Presenting Cells

T-cell-dependent immune responses in vivo, including delayed and contact hypersensitivity responses and the generation of CTL, are highly sensitive to suppression following TCDD exposure (reviewed in Ref. 1). Because T cells recognize antigen only when it is presented by APCs and APCs must also provide appropriate and sufficient costimulation to induce T-cell activation, one potential pathway for TCDD to alter T-cell function is via the APCs. Although MΦs, activated B cells, and DCs are all capable of functioning as APCs, DCs represent the most potent APCs for initiating the responses of naive T cells.

Earlier studies have failed to show suppressive effects of TCDD on MΦ functions, including antigen presentation.[48–50] Thus, because of the unique role that DCs play in initiating primary immune responses, the influence of TCDD exposure on DC activity was examined. Interestingly, the results of these studies also failed to show suppression of APC function. To the contrary, exposure to TCDD resulted in enhanced expression of several accessory molecules on DCs that are known to be important in T-cell signaling, including CD86, CD40, CD54, and CD24, as well as MHC class II molecules.[51] These effects were dose-dependent, persisted for at least 14 days after exposure and did not occur in AhR-deficient mice. Consistent with enhanced co-stimulatory molecule expression, T cells cultured with DCs from TCDD-treated mice showed a greater proliferative response and increased production of several cytokines in vitro. Production of IL-12 by DCs from TCDD-treated mice was also enhanced. In addition, TCDD exposure did not alter the ability of DCs to internalize latex beads or to activate antigen-specific T cells in vitro, suggesting that uptake and processing of antigen is not impaired by TCDD[51a]. However, a potentially deleterious effect of TCDD on DCs was also noted—a significant and persistent decrease in the number of DCs recovered from the spleen as early as day 3 after TCDD exposure. Since activated DCs are programmed to undergo apoptosis, decreased numbers of DCs in TCDD-treated mice could reflect their inappropriate activation and subsequent deletion. More important, since the persistence of activated DCs has been shown to influence the strength and duration of an immune response,[52] a premature loss of DCs in TCDD-treated mice could result in insufficient contact time with T cells to sustain their full activation and differentiation. Although this remains to be tested, the ability of TCDD to activate rather than suppress DCs helps to explain why treatment of mice with an antibody to CD40, which was shown to increase co-stimulatory molecule expression on DCs and to enhance IL-12

production in TCDD-treated mice, failed to overcome suppression of the CTL response induced by TCDD exposure.[53]

8.4.5 Effect of TCDD on CD11b$^+$Gr-1$^+$ Cells

In recent years, Bronte and co-workers have characterized a population of myeloid CD11b$^+$Gr-1$^+$ suppressor cells (MSCs) that induce the death of CD8$^+$ T cells and lead to suppression of antiviral and antitumor immunity.[54–56] Interestingly, TCDD exposure increases the percentage and number of CD11b$^+$ cells in the spleens of mice challenged with P815 tumor cells that occurs in parallel with the suppression of CD8$^+$ CTL development.[57] Recent studies have addressed the hypothesis that the expanded population of CD11b$^+$ cells in TCDD-treated mice represent MSC and are responsible for suppressing the CTL response to the P815 tumor.[57a] The results of these studies showed that virtually all of the CD11b$^+$ cells in both vehicle- and TCDD-treated mice also expressed Gr-1. Furthermore, CD11b$^+$Gr-1$^+$ cells isolated from TCDD- but not vehicle-treated mice suppressed the development of CTL activity when co-cultured in vitro with spleen cells in mixed lymphocyte-P815 tumor cell culture (MLTC). This suppressive effect was found to require cell-to-cell contact since separation of the CD11b$^+$Gr-1$^+$ cells from the spleen cells with a semipermeable membrane prevented the suppression of CTL development. To provide a definitive link between these suppressor cells and TCDD-induced suppression of CTL activity in vivo, mice were treated with an antibody to Gr-1 during the allograft response that successfully eliminated the population of CD11b$^+$Gr-1$^+$ cells. Surprisingly, however, depletion of the CD11b$^+$Gr-1$^+$ cells from TCDD-treated mice failed to affect the suppression of the CTL response. These paradoxical results were potentially resolved by immunohistochemical examination of splenic tissue sections where the CD11b$^+$Gr-1$^+$ cells were found sequestered within the red pulp and physically separated from the T cells in the white pulp. This localization apparently precludes cell–cell contact between the CD11b$^+$Gr-1$^+$ cells and T cells shown to be required for inhibition of CTL development in vitro. Thus, although the increase in CD11b$^+$Gr-1$^+$ cells in TCDD-treated mice coincided temporally with the failure to develop a CTL response, an immunomodulatory role for the cells in CTL development could not be demonstrated.

Further studies to characterize the CD11b$^+$Gr-1$^+$ cells showed that up to 70% of circulating white blood cells expressed the CD11b$^+$Gr-1$^+$ phenotype in TCDD-treated mice on day 9 of the P815 response compared to around 25% in vehicle-treated controls.[57a] These cells were identified morphologically as neutrophils in the blood, whereas in spleen cell suspensions, mostly mature neutrophils as well as macrophages and immature cell types were observed. When the CD11b$^+$Gr-1$^+$ cells were isolated and stimulated with PMA in vitro, CD11b$^+$Gr-1$^+$ cells from TCDD-treated mice produced up to fivefold higher levels of superoxide when compared to vehicle-treated mice. Based on these

results, it suggests that the increase in $CD11b^+Gr\text{-}1^+$ cells may simply represent a compensatory response of the innate immune system resulting from an inability to clear the tumor in TCDD-treated mice. However, in contrast to cells from vehicle-treated mice, $CD11b^+Gr\text{-}1^+$ cells from TCDD-treated mice were unable to kill YAC-1 tumor cells in vitro, and treatment with PMA did not enhance their cytolytic activity, suggesting that TCDD alters the function of the cells. These findings are in general agreement with previous studies which showed that TCDD suppressed the antitumor activity of neutrophils elicited by casein injection.[24] However, in these studies TCDD did not alter superoxide or hydrogen peroxide release or degranulation, suggesting that the effects of TCDD on $CD11b^+Gr\text{-}1^+$ cells are stimulus-dependent.

8.4.6 Direct Effects of TCDD on T Cells

Over the years, many laboratories have been unsuccessful in demonstrating direct effects of TCDD on T cells in vitro.[26–31] However, the absence of in vitro toxicity alone does not exclude the T cell as a direct target of TCDD. For immunological studies in particular, it is possible that optimization of tissue culture conditions compensates for the defects induced by TCDD. Some possibilities include: optimization of antigen concentration alters co-stimulatory requirements of the T cells, mixing of cells in vitro eliminates need for T-cell migration, routine addition of antioxidants such as 2-mercaptoethanol compensates for oxidative stress induced by TCDD, and/or provision of unknown factors in the serum component of the media compensates for unknown defects. It is important to remember that although in vitro approaches are very useful for identifying potential mechanisms of action of chemicals, the results do not always reflect what is taking place in the intact animal. Thus, in the absence of convincing evidence for indirect effects of TCDD on T cells, the author's laboratory has continued to pursue studies to determine if TCDD directly targets T cells. Importantly, the recent availability of $AhR^{-/-}$ mice has provided a new opportunity to obtain definitive answers to the question.

To address the direct effects of TCDD on T cells, we incorporated the $AhR^{-/-}$ paradigm into a model of an acute graft versus host (GVH) response.[31a] In this model, T cells from C57Bl/6 (B6) mice (the *graft*) are injected into B6 × DBA/2 (D2) F_1 mice (the *host*). The B6 T cells recognize the genetic differences contributed by the D2 parent in the F_1 host, resulting in the generation of a $CD4^+$ T-cell-dependent anti-D2 $CD8^+$ CTL response that attacks host tissues.[58] By the second week, the GVH response is visually apparent as the host begins to lose body weight. CTL activity can be measured in vitro using ^{51}Cr-labeled P815 tumor cells that express D2 antigens. The main advantages of the GVH model are twofold: (1) graft T cells can be manipulated experimentally prior to transfer, and (2) the T-cell response can be tracked by flow cytometry after injection into the F_1 host. In our studies, by comparing the GVH-induced CTL response of T cells from $AhR^{-/-}$ and $AhR^{+/+}$ B6 mice, we

could assess directly whether or not the AhR expressed *in the T cells* is involved in the suppression of the CTL response by TCDD.

Preliminary studies established that the normal GVH-induced CTL response was highly sensitive to suppression by TCDD. Treatment of B6D2F1 host mice with 15 or 30 μg TCDD/kg 1 day prior to the injection of T cells from B6 mice profoundly suppressed the development of CTL activity. TCDD-treated mice were also protected from the GVH-induced body weight loss seen in control mice. The ability of TCDD to affect the GVH response of T cells from $AhR^{-/-}$ and $AhR^{+/+}$ B6 mice was then compared. As shown in Figure 8.4, T cells from $AhR^{-/-}$ mice generated a CTL response of comparable magnitude to T cells from $AhR^{+/+}$ mice, supporting previous data that the absence of

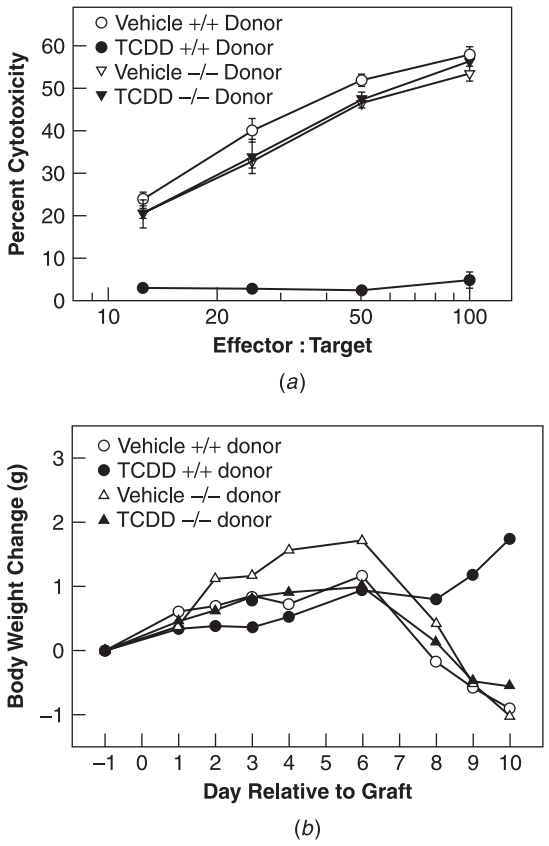

Figure 8.4 (*a*) CTL activity on day 10; (*b*) body weight change during GVH response. AhR expression is required in the T cells that respond in the GVH response in order for TCDD to suppress the response. B6D2F1 mice were treated with 0 or 15 μg TCDD/kg and injected 1 day later with T cells purified from $AhR^{+/+}$ or $AhR^{-/-}$ mice. Body weight was recorded daily and mice were killed on day 10 for assessment of CTL activity. Each point represents the mean of four mice.

the AhR does not alter the responsiveness of the T cells.[8] Amazingly, however, when host mice were treated with TCDD, the CTL response generated by $AhR^{-/-}$ T cells was completely resistant to TCDD, whereas the CTL response of $AhR^{+/+}$ T cells was profoundly suppressed (compare the closed symbols). The lack of suppression of the GVH response by TCDD when T cells lack the AhR was also reflected in the loss of body weight by the F_1 host mice (Figure 8.4*b*). These data clearly demonstrate, for the first time, that the AhR in T cells plays a critical role in the immunosuppressive effects of TCDD. With this insight, the extensive in vivo studies that have been carried out over the past decade to analyze T-cell responses in TCDD-treated mice can now be viewed as reflecting, at least in part, direct effects of TCDD on T cells.

8.4.7 Functional Effects of TCDD on T-Cell Responses In Vivo

P815 Tumor Allograft Model The effects of TCDD on T-cell functions in vivo have been extensively studied in the author's laboratory using the allogeneic P815 mastocytoma model in C57Bl/6 mice. The P815 tumor cells grow in the peritoneal cavity of the allogeneic recipient mice for about 8 days, by which time effective CTL and alloantibody responses develop to clear the tumor. The CTL response mediated by $CD8^+$ T cells and the alloantibody response mediated by B cells are dependent on $CD4^+$ T cells, and both responses are dose-dependently suppressed following TCDD exposure.[59] The suppression of CTL activity correlates with suppressed production of Th1-type cytokines (IL-2, IFNγ, TNF), while the production of Th2-type cytokines (IL-4, IL-6) is not altered by TCDD. This selective effect of TCDD on Th1-type cytokines was consistent with the selective suppression of IgM and IgG2a antibody production (promoted by IFNγ) while IgG1 (promoted by IL-4) was hardly affected. These results demonstrate that T cells play an important role in the suppressive effects of TCDD on antibody production.

Time-course studies showed that the initial production of IL-2, IFNγ, and TNFα was normal in TCDD-treated mice but then was abruptly terminated around day 5 or 6 after injection of the P815 tumor cells.[59] Since $CD8^+$ T cells were shown to be the source of the IL-2 and IFNγ at the measured time points, it suggests that initially, these cells were activated in TCDD-treated mice but then failed to proliferate and differentiate into CTL. The lack of CTL development did not appear to be due to the induction of cell death by TCDD since no increase in apoptotic or dead cells was seen by flow cytometric analysis.[60] $CD8^+$ cells expressing an activated CTL precursor phenotype ($CD44^{xhi}$, $CD28^{xhi}$, $CD54^{xhi}$, and $CD62^{lo}$) were suppressed as early as day 5 after P815 tumor injection,[61] suggesting that TCDD acted early to prevent their full activation. Interestingly, when P815 tumor cells were transfected to express high levels of the co-stimulatory molecule, CD86, the CTL response was not suppressed by TCDD.[57] These results suggested that direct provision of sufficient co-stimulation by the P815 tumor cells bypassed or compensated for the defect induced in $CD8^+$ T-cell activation by TCDD.

One of the signals for CTL development that is bypassed by CD86 transfection of the P815 tumor cells is the need for $CD4^+$ T cells.[57] The requirement for $CD4^+$ T cells is fulfilled within the first 4 days of the P815 response, since depletion of these cells after day 4 has no effect on the generation of the CTL response.[59] Interestingly, the time frame for $CD4^+$ T-cell help is identical to the time frame for effective TCDD-induced suppression of the CTL response. Thus, if TCDD exposure is delayed until after day 4, no suppression of the CTL response occurs.[59] These results suggest that the $CD4^+$ T cell may be the primary target of TCDD leading to suppression of the CTL response.

IL-2 production is one of the main consequences of CD86 co-stimulation and is also one of the main functions of $CD4^+$ T cells. Therefore, the ability of early IL-2 treatment to rescue the CTL response of TCDD-treated mice was examined. Unexpectedly, treatment of mice with IL-2 on days 1 to 3 after P815 sensitization (reflecting the time that $CD4^+$ T cells are required) not only failed to enhance the CTL response of TCDD-treated mice, but it suppressed the CTL response of vehicle-treated mice.[60] These results suggested that TCDD might enhance rather than suppress the production of IL-2 by $CD4^+$ T cells. Hypothetically, excess IL-2 production would then lead to deletion of the activated $CD4^+$ T cells by a well-characterized process known as activation-induced cell death.[11] The loss of helper signals from activated $CD4^+$ T cells would then prevent the continued expansion of the activated $CD8^+$ CTL precursor cells. Increased IL-2 production by T cells from TCDD-treated mice has been noted previously[59] and might result from direct induction of the IL-2 gene as recently described.[10] Unfortunately, this hypothesis has not been directly tested in the P815 tumor model due to the low frequency of antigen-activated $CD4^+$ T cells. However, in other studies using anti-CD3 antibody to activate T cells, TCDD has been shown to promote $CD4^+$ T-cell death by a process that may involve Fas-FasL signaling.[62–64] Interestingly, IL-2 has been shown to regulate several aspects of the Fas-triggered signaling pathway leading to T-cell death,[12] suggesting a potential link between the two phenomena in TCDD-treated mice.

DO11.10 Transgenic T-Cell Model Historically, the responses of antigen-specific $CD4^+$ T cells have been difficult to study in vivo due to the low frequency of such cells in the peripheral lymphocyte pool. However, the recent development of T-cell receptor (TCR)-transgenic mice, wherein essentially all of the T cells bear a common TCR for a specific antigenic peptide, allows this problem to be addressed. However, the transgenic T-cell response can be analyzed under more normal conditions by transferring a relatively low but detectable number of the cells to genetically identical (syngeneic) mice, where the cells home to the lymphoid tissues.[65] When the recipient mouse is challenged with the appropriate antigen, the antigen-specific T cells become activated, and their response can be followed using flow cytometry and a clonotype-specific antibody that identifies the transgenic T cells.

The effects of TCDD on the activation of antigen-specific $CD4^+$ T cells was investigated by Shepherd et al.[66] using the D011.10 ovalbumin (OVA) peptide-specific transgenic TCR model. When syngeneic BALB/c mice are injected with D011.10 T cells, the response of the CD4+ T cells expressing the transgenic receptor can be tracked using the clonotype-specific antibody, KJ1-26.[67] Interestingly, when mice were treated with TCDD prior to immunization with OVA, TCDD produced surprisingly few changes in the initial activation or proliferation of adoptively transferred, OVA-specific $CD4^+$ T cells. Peak numbers of KJ1-26^+ T cells were found in the spleen on day 3 after immunization and did not differ between vehicle- and TCDD-treated mice. In addition, the expression of several markers of activation (CD69, CD44, CD11a, CD62L, cell size) was similar between vehicle- and TCDD-treated mice. Although the antigen-specific T cells from TCDD-treated mice produced lower amounts of several cytokines (IL-2, IFNγ, IL-4, IL-10) on days 1 and 3 after immunization, the magnitude of the suppressive effects were relatively small and considered unlikely to account for the suppression of the antibody response. Taken together, the results indicated that TCDD had very little effect on the early stages of antigen-specific $CD4^+$ T-cell activation and proliferation.

Although activation of T cells is necessary for the initiation of an immune response, persistence of the activated T cells is also critical for the full development of effector activity. When $CD4^+$ D011.10 T cells were tracked beyond day 3, TCDD exposure resulted in significantly reduced numbers of OVA-specific T cells in the spleen on day 5 and day 7 after OVA challenge.[66] This finding suggested that TCDD might influence T-cell survival rather than activation. Subsequent studies using Annexin V and 7-AAD staining showed that TCDD significantly increased the percentage of apoptotic cells (Annexin V^+) as well as the percentage of dead cells (7-AAD^+) in the population of antigen-specific $CD4^+$ cells.[67a] In contrast, the survival of bystander $CD4^+$ cells was not affected by TCDD. These results provide direct evidence that TCDD selectively targets antigen-activated $CD4^+$ T cells to decrease their survival. Unfortunately, in the DO11.10 model, there was no evidence that TCDD increased IL-2 production to induce $CD4^+$ cell death, as postulated to occur in the P815 tumor model. Additional studies are under way to determine how TCDD alters the survival of the $CD4^+$ T cells.

Influenza A Virus Infection Model The suppression of host resistance to influenza virus infections is recognized as one of the most sensitive measures of TCDD immunotoxicity. In one study, mice exposed to as little as 10 ng TCDD/kg experienced increased mortality from influenza virus compared to vehicle-treated mice.[68] Although this profound sensitivity has not been corroborated in other laboratories, dose-dependent enhancement of influenza virus-induced mortality has been confirmed at higher doses of TCDD (10 to 15 μg/kg).[69,70] The immunological basis for the enhanced mortality has only recently been investigated.

Respiratory tract infection with influenza virus leads to the generation of

virus-specific CTL in the mediastinal lymph nodes (MLNs). There, virus-specific $CD8^+$ T cells undergo a $CD4^+$ T-cell-dependent activation, differentiation and clonal expansion into CTL. These CTL then migrate to the lung and mediate killing of virus-infected cells. When mice were treated with TCDD prior to virus infection, the development of virus-specific CTL activity and the associated production of IL-2 and IFNγ in the MLNs were suppressed.[70] In addition, fewer $CD8^+$ T cells were recruited to the lungs of TCDD-treated, virus-infected mice, and a reduced percentage of those cells expressed an effector phenotype ($CD8^+CD44^{hi}CD62L^{low}$).

Despite the suppression of the antiviral CTL response by TCDD, the overall cytolytic activity of lung lavage cells toward virus-infected fibroblasts in culture was unexpectedly normal in TCDD-treated mice.[71] Furthermore, IFNγ levels in the lung lavage fluid of mice treated with TCDD were enhanced 10-fold on days 7 and 8 after infection, while at the same time, IL-12 levels were significantly suppressed. Even more surprising was finding that the pulmonary virus burden was generally lower in mice exposed to TCDD on days 1 to 5 postinfection, and by day 9, no influenza virus was detected in lung homogenates from either vehicle- or TCDD-treated mice. These paradoxical results suggested that TCDD exposure might be enhancing the local, innate immune response to influenza A virus infection, perhaps by enhancing NK cells or granulocyte activity through the increased production of IFNγ. In fact, elevated neutrophil counts were found in the lung lavage of TCDD-treated mice on days 7 and 9 after virus infection; however, their cytolytic activity against virus-infected cells was not evaluated. Stimulation of NK activity by TCDD would be consistent with previous studies showing enhanced NK activity in mice treated subchronically with TCDD.[72] On the other hand, TCDD suppressed influenza virus-augmented NK activity in rats.[73]

The effects of TCDD on antibody production following influenza infection were also examined.[70] Plasma levels of IgM, IgG1, IgG2a, and IgG2b were approximately twofold lower in TCDD-treated mice on day 9 after infection compared to vehicle-treated mice. However, at the same time, the level of circulating IgA was increased approximately fourfold in TCDD-treated mice. Regulation of IgA production is not fully understood, although several cytokines, including IFNγ, have been associated with increased IgA production.[74] Thus, it is possible that increased IFNγ production in TCDD-treated influenza-infected mice underlies the increased IgA response. Alternatively, liver damage and decreased hepatic clearance of IgA could result in increased serum IgA levels, as noted previously in rats exposed to TCDD.[75]

Taken together, the data indicate that TCDD has complex effects on anti-influenza immune responses, suppressing some aspects while enhancing others. Given that the virus is effectively cleared from TCDD-treated mice despite the suppression of adaptive CTL and antibody responses, it suggests that the enhanced mortality following influenza virus infection may be due to an overly robust inflammatory response or to a direct effect of TCDD on virus replication.

Effects of TCDD on T Cell Development Thymic atrophy is one of the most extensively studied biological effects of TCDD on the immune system. However, despite the extensive database, the specific mechanisms by which TCDD induces thymic atrophy are still controversial. The controversy was initiated by a report demonstrating the induction of apoptosis (increased intracellular calcium, DNA fragmentation, decreased viability) in rat thymocytes treated in vitro with a high concentration of TCDD.[76] However, similar studies in mice failed to demonstrate similar effects.[77–79] Furthermore, overexpression of the antiapoptotic gene, *bcl-2*, in the thymus did not prevent TCDD-induced thymic atrophy in vivo[80] or in fetal thymic organ culture.[79] On the other hand, Kamath et al.[81] reported that in vivo TCDD exposure resulted in increased apoptosis of mouse thymocytes; however, the apoptotic phenotype determined by TUNEL assay was only apparent after an extended period of in vitro culture. Also, these findings were not corroborated in other laboratory studies.[79,82] Nonetheless, Fas/FasL interactions were proposed as a mechanism for increased apoptosis based on the finding that lpr and gld mice (defective in Fas and FasL expression, respectively) showed a modest decrease in sensitivity to TCDD-induced thymic atrophy.[50,83] An increased level of FasL mRNA was also found in thymic tissue taken from mice treated with TCDD.[83] However, since FasL is expressed in thymic stromal cells but not in thymic T cells,[84] the authors suggested that TCDD might induce FasL expression in thymic stromal cells, which would then trigger apoptosis in Fas-expressing thymocytes. This interpretation is consistent with in vitro studies showing that the thymic epithelium/stroma was a direct target of TCDD in vitro.[85,86] However, this conclusion appears to be incompatible with recent studies by Staples et al.,[82] who studied thymic atrophy in bone marrow chimeric mice that expressed AhR only in hematopoietic cells (e.g., thymocytes) or only in nonhematopoietic cells (e.g., stroma). The results of their studies showed that the AhR-dependent targets for TCDD-induced thymic atrophy were strictly in the hematopoietic compartment. TCDD-induced activation of nonhematopoietic cells in the stroma was not required for thymic atrophy. One possible explanation for these divergent results is that TCDD increases FasL expression in thymic DCs, which represent bone marrow-derived thymic stromal elements. Studies to address this possibility are in progress in their laboratory.

Alternatives to induction of apoptosis have also been proposed to explain thymic atrophy. Silverstone and his colleagues[78,87] have shown that thymic atrophy correlates with a TCDD-induced reduction in "prothymocyte" stem cells in the bone marrow and also with an inhibition of thymocyte maturation. Kremer et al.[86] demonstrated that the proliferation of thymocytes is impaired by TCDD. Further study will be needed to fully understand the process of thymic atrophy induced by TCDD.

Perinatal Effects of TCDD Perinatal exposure of the developing immune system to TCDD is recognized as the most sensitive method to induce

immunotoxicity, with T-cell-dependent responses primarily affected (reviewed in Ref. 1). The prenatal exposure effects of TCDD on T cells may result from altered thymic development, which has been demonstrated in mice at tissue concentrations of TCDD less than 200 fg/mg.[88] More recent work has shown that in utero exposure of rats to TCDD produces alterations in fetal and neonatal thymocyte subpopulations following a single dose of 1 to 3 μg/kg to the dam on day 14 of gestation.[89] One day after birth, thymic atrophy was not apparent in the pups but the percentage of $CD3^+CD4^-CD8^+$ thymocytes was increased. This skewing of thymocyte development toward a mature $CD8^+$ phenotype has also been reported in mice exposed prenatally or as adults to TCDD as well as in fetal thymic organ culture.[86] However, this T-cell skewing has not been shown to occur in peripheral lymphoid tissues,[90] and therefore its functional significance is unknown. Interestingly, Kronenberg et al.[90a] reported that treatment of $\beta 2$ microglobulin gene knockout mice with TCDD bypassed the need for MHC class I molecules for selection into the $CD4^-CD8^+$ cell pool. A transient upregulation of Notch gene expression by TCDD was suggested to explain these effects.

The effects of prenatal exposure to TCDD on immune responsiveness of mice were described several years ago.[90b] Recently, changes induced in the immune system of rats resulting from perinatal exposure to TCDD have been reported.[91] Exposure of the dams to 3 μg TCDD/kg suppressed the DTH response to bovine serum albumin (BSA) in both male and female rats at 14 to 17 weeks of age. The lymphoproliferative responses to T- and B-cell mitogens and the antibody response to sheep red blood cells were not affected in either gender except for a suppressed response to pokeweed mitogen in the females. In a second study, the differential effects of prenatal versus postnatal exposure to TCDD were examined.[91] Dams were exposed to vehicle or 3 μg TCDD/kg on day 14 of gestation. One day after birth, litters were cross-fostered to produce control, placental-only, lactational-only, and placental/lactational exposure groups. Changes in thymic phenotypes were assayed 1, 2, or 3 weeks postpartum, while the DTH response was assessed in 5-month-old males. Decreased percentages of thymic $CD3^+/CD4^-CD8^-$ cells and increased percentages of thymic $CD3^+/CD4^-CD8^+$ cells were seen through 3 weeks old in both genders after TCDD exposure. The severity of the effects was related to the route of exposure (i.e., placental/lactational > lactational > placental). At 5 months of age, the DTH response to BSA was suppressed only in the males receiving both placental and lactational exposure to TCDD. These results corroborate findings in mice that sufficient perinatal exposure to TCDD can result in persistent immune deficits into adulthood.

In other studies, Nohara et al.[92] documented the tissue dose of TCDD resulting from perinatal exposure of pregnant rats to 800 ng TCDD/kg on gestation day 15. On postnatal day 21, the thymus and spleen of the pups contained 102 and 62.7 pg TCDD/g tissue, respectively, and amounts decreased thereafter. Dose-dependent CYP1A1 mRNA expression was observed in the thymus following maternal exposure to 50 to 800 ng TCDD/kg on postnatal

day 5, reflecting activation of the AhR by TCDD. In contrast, CYP1A1 mRNA induction in the spleen was very weak. These levels of TCDD were not associated with reproducible changes in thymocyte or splenocyte populations except for a transient dose-dependent decrease in spleen cell numbers on postnatal day 49. Functional effects were not reported.

8.5 EPIDEMIOLOGY STUDIES OF TCDD IMMUNOTOXICITY

Existing epidemiological studies have not provided convincing evidence that TCDD and related chemicals cause immune dysfunction in humans. However, since human cells have been shown to express AhR, there is no plausible basis for hypothesizing that the immune system of humans is uniquely resistant to TCDD-induced immunotoxicity. Rather, it is likely that the level of exposure has not been sufficient to induce immune defects of sufficient magnitude to be detected by the clinical assays used. Also, as mentioned previously, ex vivo testing of lymphocyte function is not a sensitive approach for detecting TCDD immunotoxicity, even in mice that are highly sensitive to TCDD toxicity. Furthermore, TCDD exposure in the absence of specific antigen challenge has little effect on lymphocyte numbers or functions. Thus, it is not surprising that even persons exposed to relatively high concentrations of TCDD in the Seveso, Italy, accident (see Chapter 20) failed to show significant changes in blood cell counts, blood differentials, or lymphocyte proliferation assays, despite the presence of chloracne in several of the subjects.[93]

Since 1994, only a few new epidemiologic investigations of TCDD immunotoxicity have been published. Tonn et al.[94] examined 11 workers from a German factory that manufactured trichlorophenol. The subjects had elevated blood lipid concentrations of TCDD (43 to 873 pg/g compared to 4 pg/g found in controls), and some were afflicted with chloracne. The proliferative response of peripheral blood lymphocytes to IL-2 or to culture with allogeneic cells was reduced in the TCDD-exposed cohort; however, the change was significant only if one high responder was removed from the analysis. The workers were reported to be generally healthy, and with one exception, had no history of increased susceptibility to infections or cancer. Jung et al.[95] also examined German pesticide plant employees ($n = 192$), including a subgroup of 29 people with particularly high blood lipid concentrations of TCDD (33.6 to 2252 pg/g). The incidence of 14 types of infectious diseases was similar in exposed and nonexposed groups, as were serum antibody levels and blood cell counts. A small decrease in $CD8^+$ T cells was detected in the exposed population; however, mitogen-induced proliferation of blood cells was normal.

Geusau et al.[96] reported on the health status of two women, 30 and 27 years of age, with verified high-level TCDD intoxication. Patient 1, who had the highest TCDD level ever recorded in an individual (144,000 pg/g blood fat), developed severe generalized chloracne, whereas the second patient had only mild facial lesions despite heavy intoxication (26,000 pg/g blood fat). Patient

1 demonstrated a moderate elevation of blood lipids, leukocytosis, anemia, and secondary amenorrhoea. The laboratory parameters in patient 2 were all normal. Monitoring over a 2-year period revealed few clinical and biochemical health effects despite the high TCDD levels.

In contrast to adult exposure studies, Weisglas-Kuperus et al.[97] reported that perinatal exposure of nursing infants to background levels of PCBs and dioxins correlated with subtle changes in immune parameters and in the incidence of infectious and allergic disease. Perinatal exposure levels to PCBs correlated with an increased number of T lymphocytes and slightly lower antibody titers to mumps and measles at preschool age. PCB body burden at 42 months of age correlated with the likelihood of having recurrent ear infections or chickenpox but reduced allergic symptoms. These changes in disease susceptibility, including fewer allergic reactions, are consistent with suppression of immune functions by AhR-interacting dioxins and PCBs. These findings are also consistent with earlier laboratory studies establishing the heightened sensitivity of the developing immune system to these immunotoxicants. Interestingly, even though higher dioxin and PCB exposure was associated with breast feeding, the negative effect of exposure was counteracted by the positive effect of a longer duration of breast feeding. Additionally, as we have seen in animal models, TCDD influences infectious disease processes by mechanisms that do not involve immunosuppression which could also play a role in these findings.

8.6 SUMMARY

The immunotoxicity of TCDD has been studied extensively over the past 25 years. However, apart from the requisite role of the AhR and the identification of bone marrow-derived cells, including T cells and B cells, as critical AhR-expressing targets, the underlying biochemical mechanisms by which TCDD disrupts immunological functions remain uncertain. Many pathways of cellular activation, proliferation, and survival have the potential to be influenced by AhR activation, leading to alterations in the function of the immune system. Identifying and establishing the biologically significant pathways that are altered by AhR activation is a formidable yet exciting prospect. Gene array technology promises to provide exciting new insights into this question. Although much remains to be learned about how inappropriate cellular activation via the AhR induces immune suppression, deducing this mechanism of action and the signaling pathways involved should lead to new insights into basic mechanisms of immune regulation and potentially to new methods for treatment of immune-mediated diseases.

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CHAPTER 9

Developmental and Reproductive Toxicity of Dioxins and Related Chemicals

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9.1 BACKGROUND

9.1.1 General Concepts

The potential for dioxins and related compounds to cause reproductive and developmental toxicity has been recognized for many years. Recent laboratory studies have broadened our knowledge in this area and demonstrate that altered development is among the most sensitive endpoints of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) exposure in all vertebrate animals. In this chapter we review much of the literature on the developmental and reproductive toxicity of TCDD but do not intend to be exhaustive. Special emphasis is placed on findings that have been published since our last major reviews of this

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PCB designations: PCB 15, 4,4′-DCB; PCB 28, 2,4,4′-TCB; PCB 47, 2,2′,4,4′-TCB; PCB 52, 2,2′,5,5′-TCB; PCB 77, 3,3′,4,4′-TCB; PCB 80, 3,3′,5,5′-TCB; PCB 95, 2,2′,3,5′,6-PCB; PCB 118, 2,3′,4,4′,5-PCB; PCB 126, 3,3′,4,4′,5-PCB; PCB 128, 2,2′,3,3′,4,4′-HCB; PCB 133, 2,2′,3,3′,5,5′-HCB; PCB 136, 2,2′,3,3′,6,6′-HCB; PCB 153, 2,2′,4,4′,5,5′-HCB; PCB 155, 2,2′,4,4′,6,6′-HCB; PCB 156, 2,3,3′,4,4′,5-HCB; PCB 169, 3,3′,4,4′,5,5′-HCB.

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topic.[1,2] In addition, the data have been examined with a constant consideration for the aryl hydrocarbon receptor (AhR) model of TCDD action that appears to be applicable to most biological effects of TCDD and related chemicals. The AhR is present in the unfertilized oocyte, preimplantation embryo, and in many different organs of the developing fetus. New studies using transgenic AhR-null mice have demonstrated not only that the majority of TCDD endpoints evaluated thus far are AhR-mediated, but that a few developmental endpoints may be affected by TCDD in the absence of AhR. More extensive research needs to be done to verify that the latter TCDD effects are indeed AhR-independent, against the alternative that more than one AhR exists in mammals, as has been shown for fish. Nevertheless, the mechanisms by which in utero and lactational TCDD exposure causes adverse developmental and reproductive effects may potentially have AhR-independent, as well as AhR-dependent components. However, nearly all developmental endpoints of TCDD exposure that have been investigated thoroughly absolutely require AhR expression, and no known developmental or reproductive effect of TCDD exposure can be said to be totally independent of AhR. Therefore, phylogenetic conservation of AhR structure and function indicates that there is a potential for reproductive and developmental toxicity of TCDD and related chemicals in humans.

In this chapter we focus almost exclusively on laboratory mammals and to a far lesser extent on humans (see Chapter 16 for further discussion of the latter). The rapidly expanding database related to effects of TCDD-like AhR agonists on other vertebrate classes, such as fish and birds, has been mentioned only briefly (see Chapter 15 for a discussion of dioxin toxicity in fish). The vast majority of studies reviewed involve developmental or reproductive effects on six genera of laboratory mammals (mice, rats, hamsters, guinea pigs, rabbits, and monkeys) exposed only to TCDD, TCDF, or coplanar PCBs. However, a few studies of laboratory animals exposed to mixtures of halogenated aromatic hydrocarbons have been cited to make a particular point. Pregnant women have most commonly been exposed to mixtures of TCDD, TCDD-like PCB congeners, and related TCDD-like AhR agonists and a variety of other chemicals that are not AhR agonists. Effects of in utero and/or lactational exposure to these chemical mixtures on children are described for the Yusho and Yucheng incidents and the Seveso incident, which is typically evaluated as if it involved exposure primarily to TCDD (see Chapters 21, 22, and 20, respectively, for discussions of these incidents). However, there are very few cohorts of infants and children that have potentially not been affected by multiple chemical exposures. This makes it more difficult to ascribe effects in such children to TCDD-like AhR agonists and related chemicals.

9.1.2 Developmental Toxicity

The prenatal, neonatal, and weanling animal is highly sensitive to the adverse effects of TCDD. In utero and/or lactational exposure to single or combined

doses of TCDD that produce maternal body burdens in the ng/kg range result in a variety of developmental effects in mammalian offspring. Developmental and reproductive effects following exposure to TCDD and related chemicals have been observed in several representative species of three vertebrate classes: fish, birds, and mammals. In utero and/or lactational exposure to relatively low doses of TCDD results in reduced offspring viability, structural malformations, growth retardation, and functional alterations. Exposure to relatively large maternal doses of TCDD results in prenatal mortality in all species of laboratory animals examined in this chapter. However, the species variation in maternal doses of TCDD required to cause offspring mortality is less than the variation in LD_{50} values following adult exposure, indicating that offspring exposed in utero and via lactation are more similarly sensitive to the lethal effects of TCDD than those exposed in adulthood.

Nevertheless, TCDD-induced prenatal mortality is a relatively high dose endpoint, and unlike cleft palate and hydronephrosis in the mouse, it can be ameliorated by simultaneous exposure to antioxidants such as vitamin E. Therefore, it is possible that this and other high-dose endpoints, such as subcutaneous edema in the mouse and gastrointestinal hemorrhage in the rat, may result, in part, from oxidative stress in the dam. In contrast, other effects of TCDD which occur at lower maternal doses in susceptible species, such as cleft palate and hydronephrosis in the mouse and male reproductive system dysfunction and neurobehavioral alterations in rats and monkeys, respectively, appear to result from direct actions of TCDD exerted within the developing fetus or neonate. These effects are produced at TCDD exposure levels that result in no apparent maternal toxicity and probably involve AhR-mediated mechanisms that do not require oxidative stress in the mother.

The timing of chemical exposure during gestation, or shortly after birth, can be critically important, because the developing embryo, fetus, or neonate may be susceptible to toxic effects only during short time periods when particular developmental events are occurring. As summarized previously,[1] functional alterations in learning and sexual behavior (monkey and rat), and impaired growth, development, and function of the reproductive system (rat and hamster) occur at maternal TCDD doses that result in some of the lowest exposure levels tested in the offspring of these species. Some of the most striking findings regarding TCDD exposure during gestation and lactation relate to effects on the developing male reproductive system. Only a single low-level dose of TCDD to a pregnant rat or hamster is required to delay the onset of puberty, reduce sperm counts, decrease accessory sex gland weights, and/or alter sexual behavior in her male offspring. The most sensitive effects of TCDD on reproductive system development in female rats include a constellation of structural malformations of the external genitalia that appear to be rat-specific and are not as sensitive as the functional alterations in male rat offspring. In addition, perinatal TCDD exposure in female rat offspring increases the incidence of premature reproductive senescence, constant estrus, and cystic endometrial hyperplasia as well as altering mammary gland development and increasing

susceptibility to mammary tumor formation. Maternal TCDD dose–response studies have yet to be conducted for these functional female reproductive endpoints. When this is done, these endpoints may prove to be of similar sensitivity to TCDD as those observed in male rat offspring.

Neurobehavioral endpoints affected by TCDD, coplanar PCBs, and ortho-substituted PCBs have been studied more extensively in the rat and monkey than in the mouse. In the mouse, however, AhR and ARNT2 mRNA are expressed in the fetal central nervous system (CNS), and this probably will be the case in other mammalian species. Consistent with the observations in other species and the hypothesis that the developing CNS can respond to TCDD-like AhR agonists, in utero and lactational exposure to these chemicals has affected neurobehavioral development in human infants. However, human infants have almost always been exposed to complex chemical mixtures that include TCDD-like AhR agonists and other potentially toxic chemicals. Therefore, the effects observed might have been caused by exposure to PCB congeners contained in the mixtures that are not TCDD-like AhR agonists. In this context it is necessary to recognize that in utero and lactational exposure to TCDD and coplanar PCBs in rats can alter neurobehavioral development by producing effects that can be distinct from those produced by similar exposure to ortho-substituted PCBs. This suggests that the TCDD-like AhR agonists, that are contained in the mixtures to which human infants have been exposed may have played a role in producing the decreased optimality of neurobehavioral development observed in affected children.

Data on the developmental effects of TCDD-like compounds in children are limited to studies of cohorts that have been exposed in utero and via lactation to complex mixtures of these compounds. Although certain manifestations of developmental toxicity seen in laboratory mammals exposed to TCDD have been observed in children, there is little epidemiological evidence that makes a direct association between human exposure to TCDD-like AhR agonists and these effects. Effects of exposure to mixtures of PCB and CDF congeners in the Yusho and Yucheng poisoning episodes have been described collectively as an ectodermal dysplasia syndrome. This syndrome includes hyperpigmentation of the skin and mucous membranes, deformation of fingernails and toenails, conjunctivitis, gingival hyperplasia, and abnormalities of the teeth, which collectively can resemble effects of TCDD exposure in adult monkeys. Thus, it is possible to include adverse effects of AhR agonists on the developing CNS as a part of the ectodermal dysplasia syndrome, because the CNS is of ectodermal origin. Other developmental endpoints in humans include low birth weight, which has been associated with exposure to complex mixtures in cohorts of children in Japan, Taiwan, the Netherlands, and Michigan. Alterations in neurobehavioral development, thyroid function, and certain liver enzyme levels have been associated with exposure to near-background levels of TCDD-like CDD and CDF congeners in the Netherlands. Also in the Netherlands, environmental exposure to chemical mixtures that contain TCDD-like AhR agonists and non-AhR agonists has been associated with a higher incidence of

hypotonia in the neonate, a lower pyschomotor development index at 3 months, a less optimal neurological condition at 18 months, and lower cognitive scores at 42 months of age. Similarly, an increased level of hypotonia at birth has been found at the highest exposure level in a cohort of neonates in North Carolina, and some of these children were affected by hyporeflexia at birth and delayed motor development until 2 years of age. Adult men exposed prenatally to contaminated rice oil in Taiwan have been affected by decreased sperm motility, an increase in abnormal sperm morphology, and a decrease in sperm quality as determined by decreased performance in the hamster oocyte penetration test. It is possible that at least some of these developmental effects in human infants exposed to complex mixtures of PCBs, CDFs, and polychlorinated quarter-phenyls (PCQs) were caused by the combined exposure to those PCB and CDF congeners that are AhR agonists.

9.1.3 Reproductive Toxicity

The reproductive effects of AhR agonists following adult exposure in male and female laboratory mammals are summarized in the second major section of this chapter. TCDD and other AhR agonists administered to adult male animals can decrease testis and accessory sex organ weights, cause abnormal testicular morphology, decrease spermatogenesis, and reduce fertility. Some of these effects may be related to an androgenic deficiency caused by decreased steroidogenic responsiveness to LH stimulation and an increased pituitary responsiveness to feedback inhibition by androgens and estrogens. When administered to nonpregnant adult female animals, these compounds can reduce fertility, decrease litter size, cause estrus cycle abnormalities, and exert effects related to an inhibition of estrogen action. One effect of adult TCDD exposure in rhesus monkeys is an increase in the incidence and severity of endometriosis. Subsequent studies of exposed cohorts of women in Belgium and Israel found an association between exposure to TCDD-like AhR agonists and endometriosis. More recently, an altered sex ratio (decreased male births) has been discovered among babies born after the TCDD accident in Seveso. Interestingly, the altered sex ratio is associated with TCDD exposure in men before and during puberty. So far, evaluation of cohorts at other sites examined for this effect have not, generally, revealed a decreased proportion of male births. However, in these studies, fewer births may have been examined, the particular effect of prepubertal male exposure may not have been evaluated specifically, or individual exposure levels were not included in the analysis. The collective research results that are cited in this chapter demonstrate substantial evidence of reproductive toxicity following adult exposure to TCDD and other AhR agonists in male and female laboratory animals. Some of these endpoints may be applicable to effects that follow human exposures. However, female reproductive toxicity to this class of compounds in laboratory animals is described less completely because it has not been studied as extensively as that in males.

9.2 INTRODUCTION

9.2.1 TCDD-like Chemicals

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is one of 75 possible chlorinated dibenzo-*p*-dioxin (CDD) congeners, and in addition, there are 135 possible chlorinated dibenzofuran (CDF) congeners with a similar potential for toxicity. TCDD is one of the most potent of the CDDs, brominated dibenzo-*p*-dioxins (BDDs), CDFs, brominated dibenzofurans (BDFs), polychlorinated biphenyls (PCBs), and polybrominated biphenyls (PBBs) and as such, serves as the prototype congener for investigating the toxicity elicited by these classes of chemicals. For the slowly metabolized, bioaccumulative molecule TCDD, developmental and reproductive toxicity is generally believed to be caused by the parent compound. Its toxic potency is due to the number and position of chlorine substitutions in the dibenzo-*p*-dioxin ring system. CDD congeners with decreased lateral (2, 3, 7, and 8) or increased nonlateral chlorine and bromine substituents are less potent than TCDD[3]; however, most of these congeners will produce toxicity, and the pattern of responses within animals of the same species, strain, gender, and age will generally be similar to that of TCDD.[4,5] PCB congeners with zero or one ortho chlorine, two para chlorines, and at least two meta chlorines can assume a coplanar conformation sterically similar to TCDD and also produce a pattern of toxic responses in mammals similar to that of TCDD. In contrast, PCB congeners with two or more ortho chlorines cannot assume a coplanar conformation and do not resemble TCDD in signs of toxicity.[3,5]

An extensive database on the reproductive and developmental endpoints affected by TCDD-like congeners in fish, birds, and mammals has been reviewed previously.[1] A large amount of information about the effects of these chemicals on fish and bird reproduction and development has accumulated since that time. However, due to the considerable number of new studies that have become available on the developmental and reproductive toxicity of TCDD-like congeners in mammals, including humans, in this chapter we review only those studies that have been conducted in mammals. Developmental and reproductive toxicity to fish and birds is mentioned only briefly in the context of discussing certain evolutionary characteristics of this toxicity.

In mammals, CDD and CDF congeners chlorinated in the lateral positions, as compared with those lacking chlorines in the 2, 3, 7, and 8 positions, are preferentially bioaccumulated. This is of concern because combined effects of the lateral-substituted CDD, BDD, CDF, BDF, PCB, and PBB congeners acting through an aryl hydrocarbon receptor (AhR) mechanism have the potential of decreasing mammalian wildlife populations secondary to developmental and reproductive toxicity.[6] Gestational exposure to TCDD produces a characteristic pattern of fetotoxic responses in most laboratory mammals, consisting of thymic hypoplasia, subcutaneous edema, decreased fetal growth, and prenatal mortality.[1] Added to these common effects on development are

other effects of TCDD that are highly species-specific. Examples of the latter are cleft palate formation in the mouse, intestinal hemorrhage in the rat, and structural abnormalities of the external female genitalia in rats and hamsters.[7,8] In addition, PCB/CDF/CDD mixtures that contain both TCDD-like PCB congeners and non-TCDD-like PCB congeners have been implicated in causing developmental and reproductive toxicity to humans in the Yusho and Yucheng poisoning incidents in Japan and Taiwan[9–11] and in areas with high levels of fish consumption, such as Sweden and the Netherlands.[12,13] Therefore, exposure to TCDD-like halogenated aromatic hydrocarbons is a human health concern; however, the relative contributions of TCDD-like and non-TCDD-like components to the toxicity of mixtures of these chemicals are generally not known following human exposure to the mixtures.

The basis for most of this chapter is the new information that has been obtained in laboratory studies of mammalian species since 1993. We have also described the effects on reproduction and development that have been observed after accidental, environmental, and occupational exposure of human populations to mixtures of these chemicals. Laboratory animal studies, however, are given more weight than the human exposure studies, because unlike humans, laboratory mammals are typically exposed to known doses of single agents at experimentally selected times during gestation or soon after birth, whereas human cohorts are typically exposed to complex mixtures that contain TCDD-like congeners and other substances. In addition, laboratory animal studies are emphasized because they seem to provide the only basis for determining the mechanisms by which reproductive and developmental toxicity is produced. For example, transgenic mice have recently been used to determine whether or not developmental and reproductive toxicity of TCDD is AhR dependent.

9.2.2 AhR Evolutionary Characteristics

The AhR and aryl hydrocarbon nuclear translocator (ARNT) are members of a growing family of bHLH-PAS proteins that have been identified and characterized in invertebrates such as *Caenorhabditis elegans*, and in three classes of vertebrates: fish, birds, and mammals. The evolutionary characteristics of these and other members of the PAS family have recently been reviewed.[14–16] Chapter 14 gives a thorough analysis of AhR function by using a comparative/evolutionary biology approach. Briefly, the results indicate that the AhR is a phylogenetically ancient protein with a considerable degree of structural and functional homology between different vertebrate and even invertebrate classes. In fact, if conservative substitutions are included, the DNA base-pair sequence for the AhR from the killifish shares 78 to 80% similarity and 63 to 67% identity with that from mammals.[18] Within mammals, the highly conserved PAS-A repeat of the mouse AhR is 96% identical with that of the human, and the PAS-B repeat which has been shown to be part of the ligand binding domain in the mammalian AhR is 88% identical in the mouse and human.[14]

Functionally, the AhR is a ligand-activated transcription factor involved in the regulation of several genes, especially those for xenobiotic-metabolizing enzymes such as the cytochrome P450 1A and 1B forms. Evidence of cytochrome P4501A1 inducibilty has been obtained for bony fish and cartilaginous fish but not for jawless fish such as hagfish and lamprey.[15] This suggests that the most ancestral functions of the AhR may not have been related to cytochrome P4501A1 regulation. Interestingly, members of the bHLH-PAS family have been shown to play key roles in the adaptation to hypoxia and control of circadian rhythmicity in mammals,[19–21] and deletion of the AhR in the mouse can have adverse effects on development and reproduction.[22,23] Therefore, it is possible for the AhR to be involved in a number of fundamental biological functions in various species that are not related to cytochrome P450 induction.

9.2.3 AhR and ARNT Expression in Oocyte, Embryo, Fetus, and Placenta

In the mouse AhR mRNA and protein is expressed in unfertilized oocytes and preimplantation embryos, but by the time that the two-cell and four-cell stages are reached after fertilization, the AhR expression level is transiently diminished.[24,25] AhR expression is abundant from the eight-cell embryonic stage onward, where it probably results from zygotic gene expression rather than from maternal inheritance.[25] Similarly, in rabbit embryos on gestation day (GD) 4, the AhR is expressed only in trophoblast cells, not in embryoblast cells. However, by GD 6, embryoblast cells express this protein, and the increase in its expression is coincident in time with the degeneration of Rauber's trophoblast layer that occurs just prior to implantation.[24] Although the function of the AhR is unknown at these early time points, there is a more than 100-fold increase in the constitutive level of cytochrome P4501A1 (CYP1A1) mRNA expression 12 h after fertilization in the mouse zygote. Consistent with the decline in AhR protein expression already mentioned, the dramatic increase in CYP1A1 mRNA abundance is gone completely by the two-cell stage (GD 1.5) as well as in the blastocyst at GD 3.5.[26] It is postulated that this transient increase in CYP1A1 just after fertilization might ensure the destruction of an endogenous AhR ligand and prevent AhR-mediated gene transcription during this critical part of very early mammalian embryogenesis.[26]

After implantation, and particularly during organogenesis, spatial and temporal patterns of ARNT, ARNT2, and AhR mRNA expression occur in specific developing tissues and organs of the mouse embryo from GD 9.5 to GD 16.[27–29] On GD 9.5, ARNT mRNA is expressed strongly in the neuroepithelium of the brain and spinal cord, trigeminal ganglion, branchial arches 1 and 2, heart, hepatic primordia, and primitive gut. ARNT 2 message is also expressed in the neuroepithelium and in the remainder of the embryo, but at comparatively lower levels. In contrast to ARNT and ARNT2, AhR mRNA is not expressed significantly on GD 9.5, but by GD10, AhR mRNA is expressed

in the neuroepithelium of the developing brain, visceral arches, and heart. By GD 13.5 or 14, AhR mRNA is expressed abundantly in the primitive pituitary, palatal shelf, nasal septal cartilage, dorsal surface of the tongue, developing thymus, lung parenchyma, liver, developing gut mucosa, kidney, urogenital sinus, and tip of the genital tubercle. ARNT mRNA is expressed to a high extent in various cell types of endodermal and mesodermal origin, such as the lung and tongue muscle, and is barely above background in the developing nervous system. The tissue distribution of ARNT2 mRNA is the inverse of ARNT, being highest in the mantle layer of the spinal cord and brain and lowest in the endodermal and mesodermally derived tissues. The expression patterns observed at GD 13.5 or 14 continue to be found at GD 15 and 16, with the additional finding that ARNT2 is expressed clearly in neural crest derivatives like the dorsal root ganglia, adrenal medulla, and in developing tubules in the renal cortex. Thus, the expression of AhR, ARNT, and ARNT2 mRNAs is specific for cell type, organ/tissue, and developmental stage. Furthermore, immunohistochemical localization of AhR and ARNT protein correlated, in general, with in situ localization of AhR and ARNT mRNA expression at each gestational age.[27,28] That the AhR, ARNT, and ARNT2 expression levels vary with developmental stage suggests that the AhR pathway may exert important functions in normal development that can be affected adversely by the presence of TCDD-like xenobiotics.

9.2.4 AhR Signal Transduction and Mechanisms of Toxicity

The mechanism whereby TCDD-like congeners mediate toxicity to the developing embryo is thought to involve the AhR signal transduction cascade.[5] The current model of this pathway hypothesizes that ligands such as halogenated aromatics and biphenyls bind the cytosolic AhR, resulting in a protein capable of dimerizing with the nuclear AhR nuclear translocator (ARNT) protein. At least two forms of ARNT exist and have been designated as ARNT: the original form and the newly discovered ARNT2.[30] The AhR complex with ARNT or ARNT2 then associates with putative dioxin or xenobiotic response elements in the promoter regions of genes to stimulate or repress gene induction. The details of this cascade will not be discussed here but can be found in several excellent reviews[31–35] (see also Chapter 12). The linkage between the AhR signaling pathway and many of the adverse responses caused by exposure to TCDD and related chemicals has been established through analysis of mouse models that are defective in AhR protein expression.[36–41] However, while the AhR has been implicated in mediating the toxicity of TCDD-like chemicals, it remains to be established how the AhR signaling pathway actually produces the biological responses that have become the hallmark signs of dioxin toxicity. Fortunately, there are promising insights into the mechanism of action of AhR ligands at the molecular and cellular levels. First, since the AhR is a ligand-activated transcription factor, the induction or repression of specific genes are certain to be involved in the process of toxicity. At present, several novel gene

targets have been identified and characterized, but direct linkage to a specific toxic endpoint has not been established.[42–46] The use of microarray technology is certain to have a significant impact on the discovery of many novel gene targets; however, the identification of these genes must be viewed with caution, as each gene will need to be examined rigorously to determine whether it is truly a direct target of the AhR or a target of a gene affected by the AhR. Second, several studies have shown that ligand binding results in rapid and sustained degradation of the AhR protein both in vivo and in vitro.[47–52] Reductions in AhR protein appear to affect subsequent stimulation of AhR-mediated signaling[47–53] and also appear to affect growth in cell culture models.[54] In addition, mice with a gene-targeted deletion of the AhR exhibit a variety of growth defects, including immune system impairment,[36,37] reduced mammary gland development,[55] lower incidence of large interfrontal bones,[36,39,56] liver fibrosis,[36,37] adverse reproductive outcomes and increased pup mortality,[36,56] and an increase in primordial follicles in the ovary.[57,58] Taken together, these findings suggest that the AhR is involved in important aspects of growth and development and make it possible that unprogrammed reductions in AhR protein may disrupt endogenous signaling pathways that influence transcriptional events involved in growth and differentiation. Third, in the context of AhR signaling, effects on phosphorylation and the interaction with different signaling pathways through ARNT must also be considered.[59–65] Finally, several studies suggest that TCDD and related compounds might produce effects on growth factor pathways and cell cycle progression through changes in kinase activities that are independent of the AhR ARNT transcription complex.[66,67] Thus, it is clear that establishing the sequence of molecular and cellular events between binding of TCDD and related chemicals to the AhR and the subsequent expression of various signs of toxicity is one of the most challenging tasks to this field.

9.2.5 Developmental Toxicity in Fish and Birds

Developmental toxicity caused by TCDD-like AhR agonists occurs not only in mammals, as described extensively herein, but also in fish and birds.[1] Although the specific effects of TCDD and other AhR agonists on development in lower vertebrate species have not been described in this chapter, the mere occurrence of these effects is indicative of the large number of biological processes affected by AhR activation throughout the vertebrate phylum. In addition, the adverse effects of TCDD and related compounds on embryo development and viability in several different species of fish and birds clearly indicate that other classes of vertebrates, besides mammals, are also susceptible to alterations in AhR-dependent processes during the earliest stages of life.[1] The wide diversity of vertebrate species that are sensitive to TCDD-induced developmental toxicity (fish, birds, domestic animals, and laboratory mammals, including nonhuman primates) increases the likelihood that TCDD-like AhR agonists are capable of causing dose-related developmental toxicity in humans.

9.2.6 Cell Proliferation and Differentiation

AhR-mediated developmental toxicity can be produced by the ability of in utero TCDD exposure to cause striking alterations in cell proliferation and differentiation in the developing organism. This response can occur in the preimplantation embryo,[68] or at different stages of development in the fetus or neonate. Many of these developmental alterations become manifest in the epithelium of reproductive organs such as the prostate, mammary gland, and uterus, where epithelial growth and development of these organs is influenced by interactions with the underlying mesenchyme or stroma.[69] The ability of TCDD to inhibit estrogen-induced uterine epithelial proliferation in the mouse, for example, is dependent entirely on stromal AhR; AhR located in the uterine epithelium is not involved in this antiestrogenic effect of TCDD.[70] An AhR mechanism also is involved in the ability of TCDD to produce the structural malformations of cleft palate and hydronephrosis in mice.[1]

9.2.7 Lipid Peroxidation and TCDD Developmental Toxicity

It is likely that increases in lipid peroxidation following in utero TCDD exposure are mediated via TCDD interaction with the AhR to induce certain lipid-metabolizing enzymes. While increases in lipid peroxidation could result in toxicity, it is also true that lipid peroxidation is required for normal development. As stated previously, the functional significance of increases in AhR expression and cytochrome P450 activity that occur in the early embryo prior to implantation is not known. In this section we evaluate events in mammalian reproduction and development where the normal involvement of prostaglandin biosynthesis, a lipid peroxidation process, could potentially be modulated by the AhR. We then describe experiments that evaluate the possible role that abnormal lipid peroxidation may have in developmental or reproductive toxicity. In terms of mechanisms in this section we differentiate between high-dose effects of TCDD that may be due to increased lipid peroxidation associated with cytochrome P450 induction and low-dose effects that result from alterations in gene expression other than cytochrome P450. In the remainder of the chapter we focus on developmental endpoints that probably result from effects of low-dose TCDD exposure exerted directly within organs of the developing embryo or fetus and which are in all likelihood not secondary to increased xenobiotic metabolizing enzyme activity.

Lipid peroxidation in the arachidonic acid pathway is an essential part of early embryonic development. Reproduction in transgenic female mice that are deficient in cyclooxygenase 2 (prostaglandin H synthase-2, Cox-2), an enzyme necessary for the formation of prostaglandins, is affected by reduced levels of ovulation, failure of ova to be fertilized, inability of blastocysts to implant, and defective decidualization.[71] In the mouse, decidualization is a highly regulated process of differentiation that is accompanied by a rapid induction of uterine Cox-2.[71] Although the effects of decidualization on uterine AhR and ARNT

expression have not been studied in the mouse, this process in the rabbit is accompanied by a strong induction of the AhR and ARNT in maternal stromal cells at the site of implantation.[72] Since TCDD can induce the expression of Cox-2 in mouse hepatoma cells, by what appears to be an AhR-dependent mechanism,[73] it was suggested that AhR-mediated upregulation of Cox-2 might be involved in implantation of the rabbit blastocyst.[24] However, in transgenic mice that are deficient in AhR, embryo implantation occurs normally.[23] Therefore, it does not appear that the AhR pathway is necessary for the induction of Cox-2 during implantation. In addition, the ability of TCDD to inhibit ovulation[74] could not be caused by its ability to induce Cox-2 mRNA and protein expression, since an increase in the ovarian level of this enzyme is required for ovulation to occur.[75] That an AhR-mediated induction of Cox-2 is not required for ovulation is indicated further by the fact that transgenic AhR-null mice are more affected by difficulty in maintaining conceptuses during pregnancy than by reduced fertility.[23]

Although it does not appear that an AhR-mediated increase in arachidonic acid peroxidation via Cox-2 is necessary for ovulation and embryo implantation, it is possible that exogenous AhR ligands affect development adversely by increasing lipid peroxidation in the embryo, uterus, or placenta. Indeed, Cox-2, CYP1A1, and several other enzymes that are induced by TCDD can bioactivate xenobiotics oxidatively and cause the formation of free-radical intermediates and/or the subsequent formation of reactive oxygen species.[76] It is believed that these metabolic oxidation and lipid peroxidation pathways represent important bioactivation mechanisms by which exposure to certain xenobiotics can cause prenatal death and/or chemical teratogenesis.[76]

To test the influence of lipid peroxidation in TCDD-induced developmental toxicity, pregnant CF1 mice were administered 30 μg TCDD/kg or vehicle on GD 12, and placental and fetal tissues were examined 48 h later.[77] Fetal and placental nuclei contained a 1.8- and 2.3-fold greater incidence, respectively, of single-strand breaks in their DNA. Concomitant with this there were 1.5- and 1.9-fold greater incidences in lipid peroxidation products in fetal and placental tissues, respectively. In addition, TCDD administration resulted in increased amniotic fluid levels of malondialdehyde, formaldehyde, acetaldehyde, and acetone relative to control animals dosed with vehicle. As the formation of these substances is a measure of lipid peroxidation, these results suggest that reactive oxygen species formed by increased lipid peroxidation after TCDD exposure may be involved in TCDD-induced increases in prenatal death.[77] Consistent with this hypothesis, the administration of the antioxidants α-tocopherol (vitamin E) and ellagic acid to TCDD exposed C57BL/6J mouse dams significantly ameliorated the TCDD-induced increase in fetal death as well as the decreases in fetal and placental weight. Since vitamin E and ellagic acid reduced the production of superoxide anion, the level of lipid peroxidation, and the formation of single-strand DNA breaks, these results suggest that fetal death, as well as the fetal and placental weight reductions, may be due to oxidative damage caused by TCDD-induced enzyme induction.[78]

In contrast, vitamin E and ellagic acid had no significant effects on the incidence of TCDD-induced cleft palate and hydronephrosis. This indicates that the mechanisms by which TCDD causes prenatal mortality and structural malformations are different. It is likely that oxidative stress contributes to TCDD-induced fetal death but not to structural malformations. Although some effects of in utero and lactational TCDD exposure on development could potentially result from maternal toxicity, TCDD can also act independent of the dam to cause changes in developing tissues of the embryo or fetus. Indeed, as the rest of this chapter will demonstrate, TCDD and other AhR agonists cause structural malformations and/or functional alterations at doses that do not result in overt toxicity to the dam or fetus. This again indicates that mechanisms which play a role in large dose effects such as prenatal mortality may not be operative in causing low-dose effects such as structural malformations and postnatal functional alterations.

9.3 DEVELOPMENTAL TOXICITY

The manifestations of developmental toxicity from exposure to TCDD have been divided into three categories in order to fully describe the pattern of effects observed. These categories include death/growth/clinical signs, structural malformations, and functional alterations. Exposure-related effects on death/growth/clinical signs are indicated for laboratory mammals and humans, along with structure–activity results that are consistent with, but do not prove, an AhR-mediated mechanism. Structural malformations, such as cleft palate formation and hydronephrosis, are unique to mice. In other species, postnatal functional alterations, some of which may be irreversible, are the most sensitive adverse developmental effects of TCDD-like congeners. These include effects on the male and female reproductive systems of mice, rats, and hamsters, and neurobehavioral effects in mice, rats, and monkeys. Effects of TCDD exposure on developmental endpoints discussed in subsequent sections of this chapter are categorized in Table 9.1 with respect to the laboratory animal species that display these effects.

9.3.1 Death, Growth, and Clinical Signs

AhR-Null Mice and ARNT-Null Mice AhR-null (knock-out) mice have been developed to determine which adverse effects of TCDD exposure are AhR-mediated, and to identify effects on organ system development and function that are caused by absence of the AhR. Three lines of AhR-null mice have been generated using different targeting methods and they are on the following genetic backgrounds: C57BL/6N × Sv/129,[36] substrain of C57BL/6 × Sv/129,[37] and C57BL/6J × Sv/129.[38] AhR-null mice in all three lines are viable and offspring of both sexes are fertile and capable of reproduction. However, Abbott et al.[23] reported adverse reproductive outcomes, including deaths of

TABLE 9.1 Effects of In Utero and Lactational TCDD Exposure on Prenatal Mortality, Birth Weight, Structural Malformations, and Developmental Landmarks[a]

Endpoint	Mouse	Rat	Hamster	Rabbit	Guinea Pig	Monkey
Prenatal mortality	+	+	+	+	+	+
Decreased birth weight	+	+	+			+
Lipid peroxidation	+					
Cleft palate	+	+				
Hydronephrosis	+	+	+			
Extra ribs				+		
Gastrointestinal hemorrhage		+				
Accelerated eye opening	+	+				
Accelerated tooth eruption	+					

[a]References for these effects in the various species are given in the text and/or in Peterson et al.[1]
+, Effect is produced.

the females during pregnancy and lactation, small litter size at birth, poor survival of pups during the first 2 weeks after birth, and death of AhR-null pups after weaning in homozygous AhR-null female mice in the line of Fernandez-Salguero et al.[36] Since low survival of the weaned homozygous AhR-null pups was independent of genotype of the dam, it was probably not caused by maternal factors such as lactational insufficiency or aberrant maternal behaviors. However, the increased mortality of fetuses and pups prior to weaning could be due in part to impaired ability of the homozygous AhR-null female to support development of the fetuses, to survive pregnancy and lactation herself, and to rear pups until weaning.[23]

The profile of effects observed in the homozygous AhR-null offspring are dependent on the AhR-null line investigated and can consist of lesions in the immune system, skin, liver, heart, stomach, spleen, and uterus.[36,37,79,80] These findings suggest that the AhR signaling pathway plays an important physiological role in development and in maintaining homeostasis as offspring age. Transgenic mice with a null mutation in the ARNT gene have also been evaluated, and unlike their AhR counterparts, homozygous ARNT null embryos are not viable.[81,82] They are affected by neural tube closure defects, forebrain hypoplasia, delayed rotation of the embryo, placental hemorrhaging, visceral arch abnormalities, and death between GD 9.5 and 10.5. The primary cause of embryo mortality may be failure of the embryonic component of the placenta to vascularize and form the labyrinthine spongiotrophoblast, which is consistent with ARNT's role in hypoxic induction of angiogenesis.[82] Thus, the ARNT protein, unlike the AhR, plays an indispensable role during development that is essential for embryo survival.

Prenatal Mortality When exposed to TCDD during adulthood, laboratory mammals display wide differences in the LD_{50} of TCDD. It is interesting to

note, however, that when exposure occurs during prenatal development, the potency of TCDD tends to be more similar across species.[1] Thus, while the magnitude of the species differences in lethal potency of TCDD is affected by the timing of TCDD exposure during the life history of the animal, exposure to TCDD during pregnancy can cause prenatal mortality in the monkey, guinea pig, rabbit, rat, hamster, and mouse. The rank order of susceptibility to TCDD-induced prenatal mortality from most sensitive to least sensitive mammalian species would appear to be monkey = guinea pig > rabbit = rat = hamster > mouse.

Interestingly, it is possible that not all mechanisms of TCDD-induced prenatal mortality are AhR dependent. Peters et al.[39] administered a single maternal dose of 25 μg/kg of TCDD on GD 10 to AhR wild type or null female mice.[36] In the homozygous AhR-wild-type dams this dose of TCDD did not increase prenatal mortality. In contrast, prenatal mortality was increased in AhR-null dams, as there was resorption of a greater percentage of fetuses. Mimura et al.[38] also found that TCDD increased resorptions to a greater extent in AhR-null dams compared to AhR-wild type dams. These findings suggest that mechanisms that do not require the AhR may mediate, in part, the increase in prenatal mortality caused by TCDD.[39]

An important finding about predicting TCDD-induced prenatal mortality is that strain differences in lethal potency of TCDD when animals are exposed in adulthood does not predict strain differences in lethal potency of TCDD for the embryo/fetus. Certain rat strains display wide differences in sensitivity to lethality when TCDD is given in adulthood. The Long–Evans rat has a wild-type AhR, while the Han/Wistar rat contains a point mutation in its AhR gene that results in a splice variant AhR protein that binds TCDD with similar affinity.[83] Long–Evans and Han/Wistar rats are equally sensitive to hepatic CYP1A1 induction in response to TCDD, but the Han/Wistar strain is far less sensitive to TCDD-induced lethality than the Long–Evans strain when both strains are treated with TCDD in adulthood.[84,85] However, when these rat strains are exposed to TCDD during pregnancy, the maternal doses of TCDD administered on GDs 8 and 12 that cause fetal toxicity and lethality are similar.[86]

Influence of Maternal Toxicity In the mature female rabbit, AhR and ARNT are expressed in the nonpregnant and pseudopregnant uterus. In pregnant rabbits the AhR is expressed in the preimplantation uterus on GD 6. However, a stronger expression of AhR and ARNT mRNA occurs just after the attachment of the blastocyst on GD 7.[72] These gene products are observed in the luminal and glandular epithelium of the antimesometrial uterine compartment. In addition, AhR mRNA is also present in trophoblast cells. In contrast, AhR and ARNT expression is relatively lower in the luminal epithelium of the paraplacental and the mesometrial placental fold. Expression of the AhR and ARNT in the region of placentation is first observed in perivascular decidualized stromal cells on GD 9. By GD 12 the expression of these proteins

occurs in decidualized stromal cells of the placental bed. Within the placenta, however, the syncytiotrophoblast expresses only low levels of AhR and ARNT mRNA, with no detectable protein.[72] These expression patterns suggest functional roles for both AhR and ARNT in feto-maternal interactions in the rabbit. Therefore, the AhR pathway provides a receptor-mediated mechanism to produce carefully timed changes in gene expression that are necessary for normal development, and a mechanism for TCDD-like chemicals to cause adverse effects.

In the guinea pig, rabbit, rat, and mouse exposed to sufficiently high doses of TCDD, prenatal mortality is often associated with maternal toxicity that is not severe enough to result in maternal lethality.[1] In these cases maternal toxicity is indicated by decreased maternal weight gain and/or marked subcutaneous edema of the dam. In the hamster, where maternal toxicity is less severe, fetuses exhibit increases in neutrophilic metamyelocytes and bands, and increases in leukocyte number and bands are also found in maternal blood.[87] In the mouse, TCDD exposure causes rupture of the embryo–maternal vascular barrier, which results in hemorrhage of fetal blood into the maternal circulation.[88] Also, pregnant CF1 mice treated with 30 μg TCDD/kg on GD 12 exhibit increases in lipid peroxidation in placental and fetal tissues on GD 14.[77] These results suggest that there may be an association between the feto-lethal effect of TCDD and maternal toxicity in these species.

Despite this association between maternal and fetal overt toxicity in some laboratory mammals at high maternal doses of TCDD, prenatal and postnatal lethality can occur in the apparent absence of overt maternal toxicity at lower maternal doses. Olson and McGarrigle[87] reported prenatal death but no maternal toxicity in the hamster at 18 μg/kg TCDD, the highest dose used in their study. Similarly, studies in the rat demonstrate that both prenatal death[89] and postnatal death[7] can occur in response to TCDD exposure during gestation that does not result in overt maternal toxicity.

In *Rhesus* monkeys, fewer studies are available to make the association between prenatal mortality and maternal toxicity. Nevertheless, the results following dietary exposure to 25 ppt TCDD[90,91] and 50 ppt TCDD[92–95] before and during pregnancy suggest that TCDD-induced prenatal mortality can occur in monkeys in the absence of overt toxic effects in the mother. In other studies, a cumulative maternal dose of 1 μg TCDD/kg administered to monkeys during the first trimester resulted in a high incidence of prenatal mortality, with maternal toxicity occurring in some but not all of the mothers.[96,97] Thus, while some levels of TCDD exposure can result in prenatal mortality in monkeys even though overt toxicity seems absent in the mother, less attention has been given to female reproductive toxicity in general and to effects of maternal toxicity during pregnancy on fetal development in particular. Therefore, the influence of maternal toxicity on prenatal mortality has not been described adequately in monkeys.

Increased prenatal mortality was observed in affected Yusho and Yucheng women.[10,98–103] Since most women affected in these episodes exhibited chlor-

acne, Rogan[104] suggested that "exposure to amounts insufficient to produce some effect on the mother probably lessens the chance of fetopathy considerably." In support of this interpretation, overt signs of halogenated aromatic hydrocarbon toxicity were not observed in infants born to apparently unaffected mothers in the Seveso, Italy, and Times Beach, Missouri, TCDD incidents.[105,106] However, the interaction between maternal toxicity and overt toxicity in exposed human offspring does not yet seem to have been characterized adequately enough to be certain what relationship exits, if any. The possibility that paternal TCDD exposure could lead to prenatal mortality was examined in a cohort of men that worked in U.S. factories where Agent Orange was made. No association was found between paternal serum TCDD levels and spontaneous abortion.[107]

Of importance to this chapter, is the fact that structural malformations and functional alterations following in utero and lactational TCDD exposure can occur at relatively low maternal doses in laboratory animals, and perhaps in humans, that do not appear to cause maternal toxicity. In addition, there is some evidence that certain of these effects are due to a direct interaction of TCDD with organs of the developing fetus or neonate. Therefore, maternal toxicity seems involved less in causing structural malformations and functional alterations than in causing prenatal mortality.

Critical Periods of Developmental Exposure Mammalian pregnancies (including human) are characterized by critical periods or "windows" during which the embryo/fetus exhibits different susceptibilities and responses to chemical exposure. The susceptibility of any particular endpoint depends on the developmental state of that endpoint at the time of exposure. The embryo/fetus is changing constantly at all biological levels (e.g., cellular, tissue, organism) and the mechanisms of action, response, and recovery for a particular endpoint at the time of exposure are the determinants of whether or not a response to a given level of TCDD exposure will result in a developmental alteration.

The existence of a critical window for TCDD-induced lethality in the embryo/fetus is indicated by the ability of TCDD to cause prenatal mortality when administered at early times points during pregnancy but not at later times. For example, a single 24-μg TCDD/kg dose increases the incidence of prenatal mortality when administered to pregnant C57BL/6 mice on GD 6 but not when administered on GD 8, 10, 12, or 14.[108] Similarly, it was found that the largest increase in prenatal mortality occurred when a single dose of TCDD was given to the dam on GD 6 as compared to when TCDD was administered on any one of the GDs 7 to 15.[109] Thus, TCDD may be most effective at causing prenatal mortality early in gestation prior to the onset of organogenesis, but the window of susceptibility is not absolute, because some prenatal mortality can be induced even after GD 7.[109] Structural malformations, on the other hand, are characterized by the fact that the critical times extend into the period of organogenesis and have a definite end time. Cleft palate formation is

induced in the mouse when TCDD is administered to the dam on GDs 6 to 13 but not after the palatal shelves have fused.[108] Hydronephrosis, which has a broader window of susceptibility, can be induced by exposure to TCDD on GDs 6 to 18 and by exposure during the first few days of lactation.[108] After this time, however, hydronephrosis cannot be induced. Impaired development and function of certain male reproductive tract organs in rats, mice, and hamsters is caused by TCDD exposure late in gestation. Lactational exposure to TCDD is without effect for most of these endpoints.[89]

Structure–Activity Relationships The structure–activity relationship for developmental toxicity in laboratory mammals is generally similar to that for AhR binding. Gestational treatment of rats with CDD congeners that do not bind the AhR, 2-MCDD, 2,7-DCDD, 2,3-DCDD, or 1,2,3,4-TCDD, do not cause TCDD-like effects on development.[110] On the other hand, hexachlorodibenzo-*p*-dioxin, which has intrinsic AhR agonist activity, produces fetotoxic responses in rats that are essentially identical to those of TCDD.[111] Similarly, when administered to pregnant rhesus monkeys or CD-1 mice, PCB congeners that act by an AhR-mediated mechanism, 3,3′,4,4′-TCB (PCB 77) and 3,3′,4,4′,5,5′-HCB (PCB 169), cause the same type of developmental effects as TCDD. In contrast, 4,4′-DCB (PCB 15), 3,3′,5,5′-TCB (PCB 80), 2,2′,4,4′,5,5′-HCB (PCB 153), 2,2′,4,4′,6,6′-HCB (PCB 155), and 2,2′,3,3′,5,5′-HCB (PCB 133), which have essentially no or very weak affinity for the AhR, do not produce a TCDD-like pattern of prenatal toxicity in mice.[97,112–114] Thus, most structure–activity results for overt developmental effects of the halogenated aromatic hydrocarbons are consistent with an AhR-mediated mechanism. Nevertheless, one finding that stands out as being inconsistent is that 2,2′,3,3′,4,4′-HCB (PCB 128), which has very weak, if any, affinity for binding to the AhR, causes the same pattern of developmental effects in mice as TCDD.[112] However, a potential complication is that these effects may have resulted from contamination of the PCB 128 used in this study with a low concentration of a highly potent TCDD-like congener.

Humans In the Yusho and Yucheng poisoning episodes (see Chapters 21 and 22, respectively), developmental toxicity was reported in babies born to affected mothers who consumed rice oil contaminated with PCBs, CDFs, and polychlorinated quarterphenyls (PCQs).[9–11,101,115,116] Prenatal mortality and low birth weight suggestive of fetal growth retardation were observed in affected Yusho and Yucheng women.[10,98–103] In a follow-up of the Yucheng children at elementary school age, Guo et al.[117] reported decreased height and muscle development in children who were the first born to exposed women. A structural malformation, rocker bottom heel, was observed in Yusho infants, and other effects reported in Yusho and Yucheng include the formation of larger and wider fontanels, and abnormal lung auscultation.[98,101,102] The mechanisms by which these effects are produced are largely unknown. However, the decreased birth weight in infants born to exposed mothers 4 years after the ini-

tial Yucheng exposure incident is associated with decreased autophosphorylation of placental EGF receptors.[118]

Monkeys and humans exposed to TCDD-like AhR agonists in utero and/or via lactation display a clustering of effects in organs derived from ectoderm.[1] These effects include hyperpigmentation of the skin and mucous membranes, hyperpigmentation and deformation of fingernails and toenails, hypersecretion of the meibomian glands, conjunctivitis, gingival hyperplasia, presence of erupted teeth in newborns, altered eruption of permanent teeth, missing permanent teeth, and abnormally shaped tooth roots.[9–11,99,101–103,119–121] Consistent with this ectodermal displasia syndrome, some Yusho and Yucheng exposed infants displayed subcutaneous edema of the face and eyelids similar to that seen in monkeys exposed to TCDD in adulthood,[92,98,101,102,122] and a high frequency of hypomineralized dental defects has been reported after CDD/CDF exposure in Finland.[123] Effects associated with the ectodermal dysplasia syndrome such as hyperkeratinization of the skin[124] and accelerated tooth eruption[125] may involve changes in the level of EGF receptor expression.

The central nervous system (CNS), which is derived from ectoderm, is a site of action of TCDD following in utero and lactational exposure in laboratory animals. Neurobehavioral effects have been reported following transplacental and neonatal exposure to TCDD-like congeners in mice and to TCDD itself in rats and monkeys.[1] Similarly, there were effects of exposure in Yucheng children that were characterized as a delay in attaining developmental milestones. Some of these children were affected by neurobehavioral abnormalities, and there was a clinical impression of developmental delay or psychomotor delay, including impairment of intellectual development.[102] These results indicate that further research is needed to characterize the mechanisms by which TCDD-like AhR agonists affect the CNS, particularly since these children were coexposed to substances that are not AhR agonists which may have played a role in producing the effects observed.

Subsequent to the Yusho and Yucheng exposure incidents, other global regions have been found where children were exposed to elevated levels of TCDD-like chemicals in utero and via lactation. In northern Europe, a high dietary intake of fish contaminated with PCBs, CDFs, and CDDs by women in Sweden has been associated with an increased risk of low birth weight among their infants.[12] Similarly, in Dutch infants, high umbilical cord and maternal plasma PCB levels were associated with low birth weight. Infants with high cord plasma PCB levels (> 80 ng/L) weighed an average of 165 g less at birth than infants with low cord plasma PCB levels (< 20 ng/L).[13] In addition, umbilical cord and maternal plasma PCB levels were associated significantly with decreased growth of babies from birth up to 3 months of age, but this association was no longer detectable when the children reached 42 months of age.[13] Other effects of AhR agonist exposure in human infants include an association between total maternal dietary intake of these compounds and decreased thyroid hormone levels in breast-fed infants.[126] Also, an association between intrauterine and gestational exposure to near-background levels of

AhR agonists and increased plasma alanine amino transferase and aspartate aminotransferase levels has been reported for 11-month-old babies.[127]

In a Michigan cohort of infants born to mothers that consumed moderate amounts of contaminated Great Lakes fish, umbilical cord and serum PCB levels were predictive of lower birth weight, smaller head circumference, and shortened gestational age at birth.[128] The decrease in head circumference could apparently be accounted for by reductions in gestational age and birth weight. A similar conclusion was reached in another study which evaluated infants born to women occupationally exposed to PCBs during the manufacture of capacitors in upstate New York. In this study, also, a negative association between birth weight and PCB exposure was found that was reduced when gestational age was accounted for in the statistical analysis.[129] Taken together, these results indicate that decreased birth weight and head circumference due to PCB exposure is likely to be negligible except among already low-birth-weight or short-gestation infants.

While developmental toxicity was reported in babies born to affected mothers who consumed rice oil contaminated with PCBs, CDFs, and PCQs in the Yusho and Yucheng poisoning episodes,[9–11,101,115,116] and in babies born to mothers with a high level of dietary fish consumption,[12,13,128] it is difficult to determine the contribution of TCDD-like versus non-TCDD-like congeners, and the contribution of other factors to the fetal/neonatal toxicity. Nevertheless, high perinatal mortality was observed among hyperpigmented infants born to affected Yucheng women who themselves did not experience increased mortality.[10] Thus, in humans, as in laboratory mammals, the developing embryo/fetus appears to be more sensitive than the mother to mortality caused by TCDD-like AhR agonists.

Sex Ratio Female rats orally administered 2 and 8 μg TCDD/kg per day on GDs 6 to 15 delivered slightly more female offspring than male offspring.[130] However, this difference was not significant, and the possibility of adverse paternally mediated effects on reproduction in laboratory animals, including alterations in the sex ratio, have not been evaluated extensively. A similar effect of TCDD exposure, whereby male fetuses appear to be more susceptible than female fetuses to TCDD-induced prenatal mortality has been observed in human pregnancies in the most highly TCDD-contaminated area in the Seveso, Italy incident[131,132] (see Chapter 20 for a discussion of the Seveso incident). Thus, it seemed possible that in utero exposure to TCDD and other AhR agonists might alter the sex ratio in live born rat and human offspring. The ability of TCDD-like chemicals to alter the sex ratio in humans was examined subsequently in a population that had been exposed inadvertently to PCB- and CDF-contaminated rice oil.[133] Simultaneous exposure to coplanar PCBs, ortho-substituted PCBs, and CDFs during the Yucheng poisoning incident did not cause an excess of female births to male births, even though exposure levels were sufficient to produce signs of dioxin toxicity. Rogan et al.[133] concluded that sex ratio is unlikely to be a sensitive indicator of exposure to PCBs, CDFs,

and CDDs. Consistent with this finding, no trend toward a chemically induced change in sex ratio was found in Finland when births were examined from 1751 to 1997.[134]

Further results from Seveso have indicated that the effect of TCDD exposure on the sex ratio is exerted via paternal factors. An excess of girls were born to potentially exposed parents in the Seveso area between 1977 and 1996. When this was analyzed further, the probability of female births increased with increasing TCDD concentrations in serum samples from the fathers. Furthermore, men who were younger than 19 years of age at the time of the Seveso incident were more likely to sire female offspring than were men who were older at the time of the incident.[135] A subsequent report in a small Austrian cohort indicated that more girls than boys were sired by TCDD-exposed fathers who were less than 20 years old at the time of exposure; but the number of births was too small to reach the level of statistical significance. Nevertheless, there was no excess of female births sired by fathers that were older than 20 when exposed to TCDD.[136] This possibility that TCDD can cause paternally mediated alterations in sex ratio requires additional support for verification. The ovopathy concept has been suggested as a possible mechanism that could potentially explain the excess in female births.[137] However, the age of the father at the time of exposure appears to be an important factor that should be accounted for when data from additional human cohorts are analyzed.

9.3.2 Structural Malformations

Developmental effects consisting of cleft palate, hydronephrosis, and thymic hypoplasia are produced in mice following in utero exposure to halogenated dibenzo-*p*-dioxin, dibenzofuran, biphenyl, and naphthalene congeners, which bind stereospecifically to the AhR.[138–142] The oral surface of the palate in the mouse is characterized by eight or nine pairs of transverse ridges, rugae. TCDD and 3,3′,4,4′,5-PCB (PCB 126) produce palatal ruga anomalies in mice that have been associated with palatal cleft formation.[143] Hydronephrosis, on the other hand, is caused by hyperplasia of the ureteric lumenal epithelium, which results in ureteric obstruction. Both responses in the mouse can be induced at TCDD doses that are not otherwise overtly toxic[144]; however, cleft palate is less responsive than hydronephrosis, as the latter is induced in the absence of cleft palate.[108] Thymic hypoplasia is a fetal response to TCDD observed in virtually all laboratory mammalian species that have been tested.[145] Studies in humans have not identified an association between exposure to TCDD-like AhR agonists and structural malformations,[106,146–148] except that rocker bottom heel was observed in the Yusho cohort.[101]

The AhR is thought to mediate these structural malformations in laboratory mammals.[5] After GD 12 the AhR and its dimerization partner ARNT are expressed in the embryonic palate and developing urinary tract of the C57Bl/6 mouse fetus. Expression of AhR and ARNT mRNA increases significantly

during palatal shelf outgrowth from GD 12 to GD 14. While the increase in AhR expression was not affected by a maternal dose of 24 μg/kg of TCDD on GD 12, there was a decrease in the expression of ARNT.[149] Similarly, AhR protein levels in the mouse urinary tract increase from GD 12 to GD 14, regardless of exposure to 12 μg/kg of TCDD on GD 10, whereas the expression of ARNT protein on GD 14 is reduced by TCDD.[150] Thus, AhR and ARNT are expressed in the developing palate and urinary tract, and the opportunity exists for the AhR-ARNT complex to regulate gene expression in these developing tissues. It may be important for normal development that an appropriate relative expression of these genes is maintained and a decrease in the availability of ARNT may be a factor in the response of the embryonic palate and urinary tract to TCDD.

Cleft Palate The medial edge epithelium at the site of palate formation is an ectoderm that retains the ability to transform into mesenchymal cells. As the palate begins to form the apposing medial edge epithelia of the separate palatal shelves, each consists of an outer layer of periderm that overlays a strata of cuboidal-shaped basal cells. Palatal fusion is characterized by a sloughing of the outer periderm cells followed by the formation of junctions between the newly apposing basal epithelial cells. The midline seam between the developing palatal shelves then consists of the two layers of basal cells. Eventually, these basal cells will lose their epithelial characteristics and gain fibroblastlike features. Upon completion of the epithelial to mesenchyme transformation the apposing palatal shelves become fused so that a single continuous tissue is formed.[151,152] A palatal cleft can result from either a failure of the shelves to grow and come together or a failure of the apposing shelves to fuse.[153] TCDD-like chemicals are unusual inducers of cleft palate because the defect appears to result from a failure of shelf fusion rather than an inhibition of growth. GD 12 is a critical window for cleft palate induction. If in utero exposure of the mouse fetus to TCDD begins after this time, the incidence of cleft palate will decrease. No cleft palates can form when TCDD is administered on GD 14, since by this time the developing palatal shelves have completed fusion.[108]

Influence of Maternal Toxicity The possible influence of maternal toxicity on cleft palate formation was evaluated by performing reciprocal blastocyst transfer experiments using the high-affinity AhR NMRI and lower-affinity AhR DBA strains of mice.[154] After administration of 30 μg TCDD/kg or 8 mg 3,3′,4,4′-tetrachloroazoxybenzene (TCAOB)/kg to dams on GD 12, 75 to 100% of all NMRI fetuses developed cleft palates. This is true whether the fetuses remained within the uterus of their natural mother or were transferred into the uterus of a DBA dam. Under the same conditions, none of the 24 DBA fetuses transferred into an NMRI mother developed a cleft palate, even though 89% of their NMRI litter mates were affected. These results, along with the presence of AhRs in palatal shelves and responsiveness of palatal shelves in organ culture to TCDD, indicate that cleft palate formation in mice is due to a

direct effect of TCDD on the palatal shelf itself and is not secondary to maternal toxicity.

Evidence for an AhR Mechanism

RESISTANCE OF AhR-NULL MICE The AhR-null mouse line of Mimura et al.[38] is completely resistant to TCDD-induced cleft palate formation and the AhR-null line of Fernandez-Salguero et al.[36] is almost entirely resistant.[39] Taken together, these findings support the conclusion that the AhR plays a key role in TCDD-induced cleft palate formation. However, since 9% of the homozygous AhR-null fetuses of the Fernandez-Salguero et al.[36] transgenic line developed cleft palate when exposed to TCDD (compared to 0% of vehicle-exposed wild type and 0% of vehicle-exposed AhR-null fetuses) a TCDD-induced alteration in processes that do not require the AhR might also be involved. Further research is needed to explore this possibility.

STRUCTURE–ACTIVITY RELATIONSHIPS Of the halogenated aromatic hydrocarbons, TCDD has the greatest affinity for binding to the AhR, and it is the most potent teratogen in inbred mouse strains.[140–142,155] In addition, probit analysis of the dose–response curve for each congener, compared with those for each of the others, demonstrated that all dose–response curves were statistically parallel. This suggests that all the halogenated aromatic hydrocarbons evaluated produce cleft palate by a common mechanism. Therefore, relative potencies of the congeners are valid for any given incidence of cleft palate formation or hydronephrosis. The main finding, however, is that the rank-order potency of the various congeners for producing these teratogenic effects is generally similar to that for binding the AhR, with the notable exception that the apparent binding affinities of the BDFs have not yet been reported. Additional AhR ligands that cause cleft palate formation at maternally nontoxic doses include 3,3′,4,4′-tetrachloroazoxybenzene (TCAOB),[156] PCB 77,[114] PCB 169,[113] and a mixture that contained 1,2,3,4,6,7- and 2,3,4,5,6,7-hexabromonaphthalenes.[139]

Also consistent with the structure–activity relationship for binding to the AhR is the finding that a number of hexachlorobiphenyls do not induce cleft palate formation. These congeners either lack sufficient lateral substitution or are substituted in such a manner that they cannot achieve a planar conformation. Included in this category are the diortho and tetraortho chlorine-substituted PCBs: PCB 133, 2,2′,3,3′,6,6′-HCB (PCB 136), PCB 153, and PCB 155.[112] In addition, it is consistent with the structure–activity relationships that the mono-ortho chlorine-substituted 2,3,3′,4,4′,5-HCB (PCB 156) is a weak teratogen. Its potency relative to that of TCDD varies from 3×10^{-5} to 9×10^{-5} for cleft palate formation, aryl hydrocarbon hydroxylase (AhH) induction, and hydronephrosis.[157]

A result that would not be expected, according to the structure–activity relationships for binding to the AhR, is that the diortho chlorine-substituted

PCB 128 causes cleft palate formation and hydronephrosis in mice.[112] However, another diortho chlorine-substituted PCB congener, PCB 153, can also cause hydronephrosis and is a very weak inducer of the AhR-sensitive ethoxyresorufin-*O*-deethylase (EROD) activity.[158,159] It seemed consistent with the interpretation that PCB 153 is a partial AhR agonist, that it can competitively displace TCDD from the murine hepatic AhR, and that at large enough doses it can inhibit TCDD-induced cleft palate formation and immunotoxicity in C57BL/6 mice.[158,159] However, new information indicates that PCB 153 is a functional rather than a competitive antagonist of TCDD-induced immunotoxicity.[160] This suggests that the interaction between these two substances with respect to the inhibition of cleft palate formation may not be exerted at the level of AhR binding. Nevertheless, the ability of TCDD to cause cleft palate in a low percentage of AhR-null mice[39] indicates that a non-AhR-mediated component to this lesion exists, which could explain the effects of noncoplanar PCBs. Alternatively, it is possible that PCB 153 preparations used previously in laboratory studies could have been contaminated by a low level of a potent TCDD-like AhR agonist. Taken together, the data do not necessarily demonstrate that the results with PCB 153 and PCB 128 violate the expected structure–activity relationship for AhR-mediated toxicity.

Species and Strain Differences Differences exist between mouse strains with respect to susceptibility to cleft palate formation. Mouse strains that produce AhRs with relatively high affinity for TCDD respond to lower doses of TCDD than do mouse strains that produce relatively low affinity AhRs.[161,162] However, all strains, including those with AhRs of relatively low affinity, respond when exposed to sufficiently large doses of TCDD during the critical period of organogenesis.[142] Interestingly, the ability of TCDD to produce cleft palate is eliminated in transgenic mouse fetuses that do not express epidermal growth factor (EGF), whereas those that do not express transforming growth factor α (TGFα) are responsive to TCDD-induced cleft palate formation.[163]

Species other than the mouse develop cleft palate only at maternal doses that are fetotoxic and maternally toxic.[142,144] Although genetic differences between species might affect absorption, biotransformation, and/or elimination of TCDD by the maternal system and its absorption across the placenta, such species differences do not account for the lack of cleft palate formation in species other than mice.[142] Rather, the species differences appear to be caused by differences in the interaction between TCDD and the developing palatal shelves themselves. This is demonstrated by the occurrence of similar responses when palatal shelves from the rat, human, and mouse are exposed to TCDD in organ culture.[164–166] Under these conditions much higher concentrations of TCDD are required to elicit essentially the same palatal response in the rat and human that is seen in the mouse. In fact, the palatal shelves of the mouse are 200 times more sensitive to TCDD than those of the human. This suggests that human embryos have not been exposed to high enough concentrations of TCDD to

cause this effect.[167] Indeed, cleft palates have not been reported in human fetuses of mothers accidentally exposed to TCDD or mixtures of PCBs and CDFs.[11,146–148]

Biochemical and genetic differences between mouse and human palates that may explain their different sensitivities to cleft formation have been described. AhR concentrations in the mouse palate are 346 times greater than those in the human, and ARNT levels are also greater in mouse.[167] In addition, human and mouse palates cultured in vitro are dissimilar with respect to spatial and temporal patterns of EGF, EGF receptor, TGFα, and TGFβ3 mRNA expression. Since the proteins that are the translation products of these mRNAs are important for palate development, it has been suggested that species differences in the expression patterns of these genes could contribute to the lower sensitivity of human palates to TCDD compared to the mouse.[168]

In rats, as in humans, cleft palate is induced only at maternally toxic doses of TCDD-like AhR agonists. These doses are associated with a high incidence of fetal lethality, but even so, there can be differences between rat strains. Schwetz et al.[111] reported an increased incidence of cleft palate after maternal administration of 100 μg 1,2,3,6,7,8-HCDD/kg per day to Sprague–Dawley rats on GDs 6 to 15. Couture et al.[169] also observed an increased incidence of cleft palate formation after a single dose of 300 μg/kg of 2,3,4,7,8-PCDF in Fischer 344 rats. In Long–Evans rats administered 5 μg TCDD/kg on GD 8 there was a 71% incidence of cleft palate.[86] However, in Han/Wistar rats that have a mutated form of the AhR, exposure to 10 μg TCDD/kg on GD 8 failed to cause cleft palate formation.[86]

Cleft palate can also be produced in fetal hamsters following maternally toxic and fetotoxic doses of TCDD.[170] In monkeys, there are no corresponding soft tissue defects or clefts of the secondary palate, but bifid uvula[171] and bony defects in the hard palate[97] have been reported.

Hydronephrosis Hydronephrosis is one of the most sensitive developmental responses elicited by TCDD in mice. This effect of TCDD is characterized by hyperplasia of the ureteric luminal epithelium, resulting in a progressive obstruction of the ureter that occurs preferentially in the right kidney. The TCDD-induced kidney malformation occurs after the blockage of urine flow produces back pressure that damages the renal papilla.[172] Hydronephrosis can be accompanied by hydroureter and/or abnormal nephron development.[155,172–176] AhR and ARNT mRNA and protein are expressed in the fetal ureters and metanephric tubules of the mouse.[150]

When dissected on GD 12 from control embryos, isolated ureters exposed to 1×10^{-10} *M* TCDD in vitro display evidence of epithelial cell hyperplasia.[56] This, along with the expression of the AhR and ARNT, suggests that hydronephrosis is due to a direct effect of TCDD on the ureteric epithelium. Consistent with this interpretation, TCDD supports epithelial, but not mesenchymal, cell survival and stimulates epithelial cell proliferation and differentiation in

vitro.[177] Appropriate embryonic cell proliferation within the ureter is regulated by the actions of growth factors, including EGF.[56] In control ureteric epithelia, the expression of EGF receptors decreases with advancing development, whereas after TCDD exposure the rate of [^{3}H]thymidine incorporation into DNA and expression of EGF receptor does not decline. Therefore, in TCDD-treated mice there is a correlation between excessive proliferation of ureteric epithelial cells and an inappropriate expression of EGF receptors. Nevertheless, the induction of hydronephrosis by TCDD is enhanced in EGF-null fetuses compared to that in wild-type fetuses. This suggests that EGF (and TGFα) is not required for the induction of hydronephrosis by TCDD, even though it may influence the response.[177]

Other effects of TCDD on the developing kidney involve changes in the extracellular matrix and basal lamina.[172] In TCDD-exposed fetal kidneys, extracellular matrix fibers are of a diameter consistent with type III collagen in unexposed fetal kidneys. However, the abundance of type III collagen fibers is reduced by TCDD. This is important because these collagen fibers are associated with undifferentiated mesenchymal cells in the developing kidney. Similarly, the expression of fibronectin, which is also associated with undifferentiated mesenchymal cells, is decreased by TCDD exposure. In the glomerular basement membrane, the distribution of laminin and type IV collagen is altered by TCDD exposure. These changes in the glomerular basement membrane may affect the functional integrity of the filtration barrier and could exacerbate the hydronephrosis and hydroureter. The proteins within the extracellular matrix and basal lamina just described (collagen, fibronectin, and laminin) are markers of differentiation. Therefore, the effects of TCDD exposure on the expression of these proteins indicates that TCDD can alter mesenchymal and/or epithelial differentiation in the developing mouse kidney.

Evidence for an AhR Mechanism The effect of in utero TCDD exposure on the developing kidney is mediated by the AhR. This is indicated by the resistance of mouse strains lacking a functional AhR, including AhR-null transgenic mice to this effect of TCDD exposure. In addition, dose–response relationships for different PCB, CDF, and CDD congeners are consistent with the concept that the AhR mediates this response (reviewed in Ref. 1).

RESISTANCE OF AhR-NULL MICE Female transgenic mice that were heterozygous for the AhR-null mutant allele were mated to males of the same genotype and exposed during pregnancy to 40 μg TCDD/kg on GD 12.5.[38] Nearly all TCDD-exposed wild-type and heterozygous progeny developed hydronephrosis. In sharp contrast, there was no hydronephrosis in offspring from the same litters that were homozygous for the AhR-null mutation. Similarly, AhR-null mice generated by a different targeting method[36] were also completely resistant to TCDD-induced hydronephrosis.[39] These results demonstrate that this teratogenic response to TCDD is AhR-mediated. Since haplo insufficiency was observed for the cleft palate response but not for hydronephrosis, Mimura

et al.[38] suggest that the mechanisms by which the AhR mediates these two teratogenic effects of TCDD may be different.

STRUCTURE–ACTIVITY RELATIONSHIPS The rank order of potencies for various halogenated aromatic hydrocarbon congeners to cause hydronephrosis in mice is consistent with the structure–activity requirements for binding to the AhR.[140–142,155] The hydronephrosis produced by noncoplanar PCBs, mentioned previously, is morphologically different from that induced by TCDD (L. S. Birnbaum, personal communication). These data therefore provide further evidence that the AhR mediates the effects of these TCDD-like congeners on the developing mouse kidney.

Species and Strain Differences Mouse strains that produce an AhR with relatively high affinity for TCDD are more susceptible to hydronephrosis than mouse strains that produce a relatively low affinity AhR.[161,162] However, all strains, including those with an AhR of relatively low affinity, respond when exposed to sufficiently large doses of TCDD during the period immediately before and just after birth.[142] Transgenic mouse strains that lack either EGF, TGFα, or both are, if anything, even more susceptible to TCDD-induced hydronephrosis than are wild-type mice.[163]

Hydronephrosis is one of the most sensitive indicators of prenatal toxicity in hamsters.[87] Following 1.5 μg TCDD/kg administered to dams on GDs 7 and 9, the incidence of hydronephrosis in hamster fetuses was 11 and 4%, respectively. This is in contrast to an incidence of $< 1\%$ in control hamster fetuses. Thus, unlike cleft palate, hydronephrosis can be elicited in hamsters at TCDD doses that are neither fetotoxic nor maternally toxic.

In rats, the observed incidences of hydronephrosis after exposure to cumulative maternal doses < 2 μg TCDD/kg have not been significant.[173,178] Different rat strains are not equally susceptible to hydronephrosis. This is illustrated in the TCDD-resistant Han/Wistar and TCDD-sensitive Long–Evans rat strains by 1 and 10 μg TCDD/kg administered on GD 8, causing 3 and 12% hydronephrosis, respectively, in the Han/Wistar strain. In contrast, a 5 μg/kg dose of TCDD administered on the same day of gestation failed to cause hydronephrosis in the Long–Evans strain.[86] That the relative sensitivities of these two rat strains for cleft palate and hydronephrosis appear to be opposite suggests again that different mechanisms may be responsible for these two teratogenic effects.

Tooth Development Since mineralization defects observed in the first molars of human infants may have been caused by lactational exposure to CDDs and CDFs,[179] the effects of 1 μ*M* TCDD on primordial mandibular molar teeth from mouse embryos were studied in organ culture.[180] TCDD caused toxicity to odonotoblasts and ameloblasts, which led to a failure of dentin to undergo mineralization and to a lack of enamel deposition in culture. In addition, cuspal morphology was disrupted by TCDD exposure in the cultured teeth. Although a high concentration of TCDD was required to produce

these effects, it is possible that diffusion barriers in the teeth primordia may have resulted in TCDD concentrations at the cellular site of action that were much lower than those in the culture medium.[180] Exposure to EGF (10 μg/L) similarly retarded molar tooth development in cultured explants from wild-type embryos, and cultured primordial molar teeth from EGF receptor null embryos, were resistant to the effects of TCDD. In the presence of EGF and TCDD, the adverse effects of TCDD on mineralization and enamel deposition were largely, prevented but not completely.[180]

In utero and lactational exposure of male Holtzman rats to a maternal dose of 1 μg TCDD/kg on GD 15 tended to accelerate incisor eruption by about 1 day (9.9 days, control vs. 8.9 days, TCDD). Even though this effect was not significant,[181] when it is considered along with the results from the primordial tooth culture experiment, and the proposed association between accelerated tooth eruption and EGF receptor expression in mice, it is consistent with the idea that perinatal TCDD exposure may alter tooth development by interfering with EGF receptor signaling in vivo. The involvement of alterations in EGF receptor signaling in this effect would be consistent with aberrant tooth development being a part of the TCDD ectodermal displaysia syndrome. However, it is also possible that TCDD exposure may affect tooth development by perturbing other pathways, which either act in concert with or interfere with EGF receptor signaling and which probably involve additional mechanisms of cell and/or tissue interactions.

Effects of heavy lactational-only exposure to TCDD on tooth development were studied in Han/Wistar rats which are resistant to TCDD-induced lethality.[182] The offspring of dams administered large TCDD doses on postnatal day 1 and allowed to nurse their litters were affected by missing upper and/or lower third molars. Third molars were the only teeth still in the bud stage at the onset of TCDD exposure. However, defects in tooth development were found for the more advanced second molars even though these teeth were always present in TCDD exposed offspring. Incisor teeth, which erupt continuously in the rat, were affected by lactational exposure to large TCDD doses. Since early tooth development is controlled by inductive interactions between the epithelium and mesenchyme, these results exemplify the recurring theme that TCDD interferes with epithelial–mesencymal signaling during development.

Eye Opening In utero and lactational exposure to 1 μg TCDD/kg maternal body weight administered on GD 15 accelerated eye opening in the Holtzman rat.[181] Exposure to lower doses had no effect on eye opening in this rat strain. However, Gray et al.[183] found accelerated eye opening to be one of the most sensitive endpoints in the Long–Evans rat, occurring at 0.05 μg TCDD/kg maternal body weight administered on the same gestational day. In the ICR mouse exposure to 15, 30, or 60 μg/kg of TCDD on GD 14 accelerated eye opening in male pups at all dosage levels.[184] Interestingly, there was no effect on age to eye opening in female pups from the same TCDD-exposed mouse litters.

9.3.3 Functional Alterations

Male Reproductive System Testosterone and/or its active metabolite 5α-dihydrotestosterone (DHT) are essential prenatally and/or early postnatally for imprinting and development of accessory sex organs[185–187] and for initiation of spermatogenesis.[188] For example, exposure of the male rat fetus on GDs 14 to 16 to a 5α-reductase inhibitor, which inhibits conversion of testosterone to DHT, impairs development of the urethra and the urogenital sinus-derived accessory sex organs such as the prostate.[189] If perinatal imprinting fails to occur in either urogenital sinus- or Wolffian duct-derived accessory sex organs of the neonatal male rat, the male sex organs will not develop a normal trophic response to androgenic stimulation later in life and will not grow and develop normally as the animal matures. In addition, aromatization of testosterone to 17β-estradiol within the CNS is required perinatally for imprinting typical adult male patterns of reproductive behavior[190] and luteinizing hormone secretion.[191] Thus, normal development of male reproductive organs and imprinting of typical adult male sexual behavior patterns require that sufficient testosterone be secreted by the fetal and neonatal testis at critical times in early development before and shortly after birth.[192,193] Because TCDD exposure decreases plasma androgen concentrations in the adult male rat,[194] and TCDD is transferred from mother to young transplacentally and during lactation, alterations in a wide range of male reproductive system endpoints were evaluated in rats following in utero and lactational exposure to TCDD.[181,195,196] The effects of in utero and lactational TCDD exposure on the male reproductive system of mice, rats, and hamsters are summarized in Table 9.2.

The original Mably et al.[181,195,196] studies on impaired male reproductive system development caused by in utero and lactational exposure to TCDD have been expanded and further defined in subsequent studies using Holtzman, Long–Evans, Sprague–Dawley, and Wistar rats, Syrian hamsters, and mice. In the vast majority of these studies TCDD was used as the prototype AhR agonist. However, some studies used PCB 126, PCB 169, and 2,3,4,7,8-PCDF. In general, the collective findings have produced qualitatively similar results that define a significant effect of TCDD and related AhR agonists on the developing male reproductive system. The effects do not appear to result from reduced plasma androgen concentrations during the perinatal period as originally hypothesized by Mably et al.[181] and do not overlap completely with developmental effects of known antiandrogens.[197,198]

Androgenic Status To supplement the measurement of plasma androgen concentrations, the androgenic status of male rat offspring can be determined from the morphology and function of androgen-dependent organs. Anogenital distance (AGD), which is dependent on both circulating androgen concentrations and androgenic responsiveness,[199] was reduced in 1- and 4-day-old male pups by a single maternal TCDD dose as low as 0.16 μg/kg, even when slight decreases in body length were accounted for.[181] However, AGD was not

TABLE 9.2 Effects of In Utero and Lactational TCDD Exposure on the Male Reproductive System[a]

Endpoint	Mouse	Rat	Hamster
Decreased ventral prostate weight	+	+	+
Decreased dorsolateral prostate weight	+	+	—
Decreased coagulating gland weight	+	+	—
Decreased seminal vesicle weight	+	+	+
Decreased epididymal weight	+	+	+
Decreased testis weight	+	+	—
Decreased glans penis weight	—	+	—
Decreased fetal prostate epithelial bud formation	+	+	—
Delayed testis descent	0	+	—
Delayed preputial separation	0	+	+
Decreased daily sperm production	0	+	0
Decreased epididymal sperm numbers	+	+	+
Decreased ejaculated sperm numbers	—	+	+
Decreased fertility	—	+	—
Partial demasculinization of sexual behavior	—	+	+
Partial feminization of sexual behavior	—	+	—
Partial feminization of regulation of LH secretion	—	+	—

[a]References for these effects in the various rodent species are given in the text and/or in Peterson et al.[1] +, Effect is produced; 0, effect is not produced; —, effect not tested.

decreased in subsequent studies in Holtzman and Long–Evans rats exposed perinatally to TCDD when normalized to body weight[200,201] or crown-rump length.[202–204] Nor was relative AGD decreased in Long–Evans or Wistar rats exposed in utero and via lactation to TCDD or PCB 169.[198,205,206] Thus, these findings are consistent with the lack of effect of in utero and lactational TCDD exposure on plasma androgen concentrations and suggest that the androgenic status of male rat neonates is not affected by perinatal exposure to TCDD and PCB 169.

Two other external indicators of androgenic status are time to testis descent and time to preputial separation.[207,208] These occur in control rats between PNDs 20–23 and 42–45, respectively. Exposure to 0.16, 0.40, or 1.0 μg/kg of TCDD on GD 15 delayed testis descent in the Holtzman rat strain by 1.0 to 1.6 days.[181] However, this effect was significant in only two of four rat studies[181,202,203,205] and in the ICR mouse TCDD had no effect on the age at testis descent.[184] Puberty, assessed by age at preputial separation, was more reproducibly affected by TCDD across rat strains and species. It was delayed by as much as 3.6 days in Long–Evans rats exposed to 1.0 μg/kg TCDD on GD 15.[201,209] The effect was dose-related and significant at a maternal TCDD dose as low as 0.20 μg/kg.[209] Delays in age at preputial separation were also reported in Holtzman and Wistar rats and in the Syrian hamster following in utero and lactational exposure to TCDD.[201–205] The only species studied

where TCDD failed to delay the age at preputial separation was the ICR mouse.[184] The ability of TCDD-like AhR agonists to delay puberty in the Long–Evans rat was also observed following in utero and lactational exposure to 1.8 mg/kg of PCB 169 administered on GD 8.[198]

The spectrum of external effects caused by in utero and lactational exposure to TCDD and PCB 169 have been interpreted not to resemble those caused by antiandrogens such as flutamide.[198] This is evident in Holtzman and Long–Evans rats by perinatal exposure to TCDD or PCB 169 failing to affect external androgen-dependent tissues either by reducing relative anogenital distance or by inducing areolas, retained nipples, or hypospadias.[197,198,210] Flank gland development, an androgen-dependent process that occurs in young adult male hamsters, also was not affected by in utero and lactational exposure to TCDD.[201] However, other effects of in utero and lactational exposure to TCDD on the androgen-dependent endpoints of preputial separation, weight of the ventral prostate, seminal vesicle, glans penis, testis, and epididymis, daily sperm production, cauda epididymal sperm number, epididymal malformation, demasculinized and feminized sexual behavior, and feminized regulation of LH secretion resemble effects caused by antiandrogens.[197]

Prostate The fetal rat prostate begins to develop on GD 18.5 when solid cords of basal epithelial cells (prostatic buds) emerge from the urogenital sinus and invade the surrounding mesenchyme. By GD 20.5 this budding process, which TCDD partially blocks,[211] is complete. AhR and ARNT proteins are expressed at high levels in the rat urogenital sinus on GDs 16, 18, and 20 with mean concentrations of 600 fmol AhR and 140 fmol ARNT per milligram of total tissue lysate.[51] Since ARNT dimerizes with several members of the bHLH PAS family of transcription factors, it is significant that AhR protein levels in rat urogenital sinus tissue are approximately four times greater than ARNT. This raises the possibility that ligand-bound AhR might sequester ARNT and prevent it from participating in other developmentally essential protein–protein interactions. Whatever the mechanism, the hypothesis that AhR mediates effects of TCDD on prostate development is supported by the failure of in utero and lactational TCDD exposure to impair prostate development in AhR knockout mice but not in their wild-type littermates.[22] AhR and ARNT proteins also are expressed in human fetal, benign hyperplastic, and malignant prostate.[212] TCDD causes a dose-dependent inhibition of androgen-dependent transcriptional activity and prostate-specific antigen expression in LNCaP cells derived from human prostate.[213] Thus, like the rat and mouse prostate, the human prostate is capable of responding to TCDD.

In male Holtzman rat offspring exposed to TCDD in utero and via lactation, one of the most sensitive effects is a dose-related reduction in ventral prostate weight. The lowest TCDD dose administered to dams on GD 15, 0.064 μg/kg, caused an inhibition of prostate growth in male offspring on PND 32, and a maternal TCDD dose of 1.0 μg/kg caused a decrease in ventral prostate weight as late as PND 120.[181] In addition, weight of the ventral pros-

tate evaluated at various postnatal times is reduced by in utero and lactational TCDD exposure in Long–Evans and Sprague–Dawley rats[183,214] and in ICR and C57BL/6 mice.[22,184,215] Although a decrease in ventral prostate weight following in utero and lactational exposure to TCDD was not observed in Wistar rats; this may have been caused by the low level of TCDD exposure used in this study.[205] When the same investigators administered 10 μg/kg of PCB 126 on GD 15 to Wistar rats ventral prostate weights in 70- and 170-day-old offspring were reduced.[216] Administration of 1.8 mg/kg of PCB 169 on GD 8 also reduced ventral prostate weight in Long–Evans rats at 65, 260, and 600 days of age.[198] However, exposure to 100 μg/kg of PCB 77 on GD 15 had no effect on ventral prostate weight of Wistar rats at 70 and 170 days of age.[216] Taken together, these results demonstrate that in utero and lactational exposure to certain TCDD-like AhR agonists reduces ventral prostate weight in various strains of rats and mice. PCB 77 does not appear to share this effect with other AhR agonists in the rat. This may be due to a more rapid rate of metabolism and elimination of this PCB congener compared to TCDD and the other coplanar PCBs tested.

The ability of in utero and lactational TCDD exposure to decrease ventral prostate weight in the rat is greatest from the earliest age at which the organ can be weighed accurately until just after puberty (50 days of age). Thereafter, the magnitude of the weight reduction is attenuated progressively with advancing age, either completely or partially, depending on the dose of TCDD administered during pregnancy.[181] At minimally effective doses the reduction in ventral prostate weight is transient and not seen in adulthood. However, at maximally effective doses ventral prostate weight of adult males is reduced significantly. This has been demonstrated for: TCDD in Holtzman rats, PCB 169 in Long–Evans rats, PCB 126 in Wistar rats, and TCDD in ICR mice.[181,184,198,216] In utero and lactational exposure to TCDD also decreases the weight of the dorsolateral prostate and anterior prostate (coagulating gland) in the Holtzman rat, ICR mouse, and C57BL/6 mouse.[22,184,204,210,211,215] Thus, TCDD exposure is capable of interfering with ventral, dorsolateral, and/or anterior prostate growth and morphogenesis early in development. Depending on the dose administered during pregnancy, timing of the exposure, species or strain of animal, and lobe of the prostate, TCDD is capable of causing a prostate lesion that cannot be compensated for later in life. Besides size of the ventral prostate being smaller in adulthood, its responsiveness to testosterone stimulation in adulthood is also impaired by perinatal exposure to TCDD.[203]

In utero and lactational exposure to TCDD begins to impair rat prostate development during fetal life.[211] As fetal prostate development in the rat is initiated on GD 15, it was important to determine the concentration of TCDD that is present in the fetal urogenital tract shortly after this time. Administration of 1 μg/kg of TCDD on GD 8 to Long–Evans rats results in concentrations of TCDD in the urogenital tract of the fetus of 0.04 and 1.1 pg/g on

GDs 16 and 21, respectively.[217] This is significant because a similar dose of TCDD administered on either GD 8 or GD 15 causes a decrease in ventral prostate weight that lasts until shortly after the time of preputial separation.[198,200,201,218] Furthermore, 1 μg/kg of TCDD administered on GD 15 to Holtzman rats reduces the number of prostatic buds that emerge from the fetal urogenital sinus on GD 20 to form the various lobes of the prostate, and impairs prostate growth and development postnatally.[211] In addition, this same GD 15 dose of TCDD decreased cell proliferation in the ventral prostate of Holtzman rat neonates that were 1 day of age.[219] Subsequent analysis of the effects of TCDD on early postnatal development of the ventral prostate revealed that differentiation of both smooth muscle cells and luminal epithelial cells was delayed and striking alterations in the histology of the ventral prostate were apparent in male offspring at 32 days of age.[219] These alterations consisted of epithelial hyperplasia, decreased abundance of fully differentiated luminal epithelial cells, increased density of basal eithelial cells, altered spatial distribution of the androgen receptor, and increased thickness of the periductal smooth muscle sheath. Thus, the effects of in utero and lactational TCDD exposure on prostate growth and development are associated with impaired growth of the developing organ prenatally and neonatally, and by delayed and/or impaired differentiation postnatally that if the dose of TCDD is high enough, may be permanent.

The mechanism by which in utero and lactational exposure to TCDD impairs prostate growth and development is unknown. It cannot be explained in Holtzman rats by TCDD decreasing plasma androgen concentrations[201,204,220] or inhibiting the conversion of circulating androgens to DHT in the prostate.[204,221,222] Although this last conclusion is supported by an inability of TCDD to decrease rat ventral prostate type 2 5α-reductase enzyme activity or mRNA levels,[221,223] androgen metabolism has not been evaluated prior to birth. TCDD probably acts directly on the urogenital sinus from which the prostate develops and on the developing lobes of the prostate as they undergo differentiation. AhR and ARNT are expressed in both the rat urogenital sinus and the developing ventral and dorsolateral prostate[51,211]; and the infantile rat ventral prostate is responsive to in utero and lactational TCDD exposure in terms of CYP1A1 induction.[211] Also, various androgen-regulated mRNAs that code for prostatic secretory proteins, which are markers of luminal epithelial cell differentiation, show a developmental delay in their expression in response to perinatal TCDD exposure in the Holtzman rat.[219]

Essentially nothing is known about the long-term consequences of in utero and lactational exposure to AhR agonists on the prostate of laboratory rodent species during old age. The only study available found that the incidence of acute prostatitis in the dorsolateral prostate of 600-day-old Long–Evans rats was increased significantly by exposure to a single dose of 1.8 mg/kg of PCB 169 on GD 8.[198] Also 1 of 9 males displayed diffuse epithelial hypertrophy of the ventral prostate compared to 0 of 15 control males.[198]

Seminal Vesicle The rate of seminal vesicle growth is decreased in Holtzman, Sprague–Dawley, and Long–Evans rats, and in Syrian hamsters by in utero and lactational TCDD exposure.[89,181,201,214] The same effect has been observed in Long–Evans rats with PCB 169 and in Wistar rats with PCB 77.[198,216,224] However, seminal vesicle growth is not the most sensitive male reproductive endpoint affected. A multiple TCDD dosing regimen in Wistar rats decreased cauda epididymal sperm numbers and daily sperm production, and altered sperm morphology without affecting seminal vesicle weight.[205] Also, PCB 126 administered as a single dose of 10 μg/kg on GD 15 did not decrease seminal vesicle weight in the Wistar rat even though it did significantly decrease ventral prostate weight.[216] Similarly, in Holtzman rats and ICR mice sensitivity of the seminal vesicle to TCDD is not as great as that of the ventral prostate.[181,184,204] In contrast, these two accessory sex organs in Long–Evans rats seem equivalent in their sensitivity to TCDD administered on GD 15.[209]

The time course of the response of the prostate and seminal vesicle to in utero and lactational TCDD exposure in the Holtzman rat is very different.[204] The ventral and dorsolateral prostate respond with the greatest relative weight reduction early in development, and the magnitude of the response lessens with increasing age. Significant TCDD-induced decreases in seminal vesicle weight, on the other hand, are generally not detected until the peripubertal stage when androgen concentrations are rapidly increasing.[204] Consistent with the findings in Holtzman rats,[204] Long–Evans rats exposed on GD 15 to 1.0 μg/kg of TCDD and assessed on PNDs 15, 25, 32, 49, 63, and 120 did not exhibit a decrease in weight of the paired seminal vesicles and attached coagulating glands until PND 32.[225,226] Furthermore, in utero and lactational exposure to TCDD reduced the amount of secretory fluid contained in the seminal vesicles at this age, which contributed to their decreased weight.[225,226] Alterations have been found in the histology of the seminal vesicle at 32 days of age.[225,226] Compared to control animals where seminal vesicle epithelium displays extensive branching, TCDD exposure results in fewer and shorter epithelial branches. The epithelium of control rats is characterized by tall columnar cells, whereas that of TCDD-exposed rats contains smaller cells with a lower cytoplasmic volume/nuclear volume ratio.[225,226] Immunostaining for proliferating cell nuclear antigen (PCNA) in control seminal vesicles at 32 days of age is localized to undifferentiated basal cells, and no immunoreactivity has been observed in terminally differentiated luminal epithelial cells. In contrast, the undifferentiated seminal vesicles of TCDD-exposed rats at the same age exhibit PCNA immunoreactivity at both the basal and luminal surfaces of poorly branched glands.[225,226] Thus, in utero and lactational TCDD exposure interferes with epithelial proliferation and differentiation in the seminal vesicle as has been reported for the prostate.[211,225,226]

Testis Perinatal TCDD exposure in the Holtzman and Long–Evans rat causes a decrease in the daily sperm production of male offspring evaluated in adulthood.[196] In Holtzman rats spermatogenesis appears to be histologically

normal and there is no indication of any gross testicular lesion or degeneration of germ cells.[227] In Long–Evans rats there is generally no histological evidence for degeneration of the seminiferous tubules, Sertoli cell abnormalities, or retained spermatids in TCDD-exposed progeny.[209] On occasion, however, both Long–Evans rat progeny and Syrian hamster progeny of dams exposed to TCDD can exhibit severe atrophy of the seminiferous tubules that is associated with a marked loss of spermatogenic activity.[209]

In the male progeny of female Wistar rats exposed to a regimen of multiple TCDD doses administered prior to mating, during pregnancy, and throughout lactation; daily sperm production was decreased at all doses. Whereas pyknotic nuclei and luminal cell debris in some seminiferous tubuli were evident only at the highest TCDD exposure level,[205] in utero and lactational exposure to 1.0 μg/kg of TCDD on GD 15 caused no abnormal testicular histology in Sprague–Dawley rats evaluated at 62 days of age.[214]

Despite the lack of an effect on testicular histology, in utero and lactational TCDD exposure in Holtzman rats causes a dose-related decrease in daily sperm production when assessed at 49, 63, and 120 days of age.[196] Other studies have also reported significant decreases in the daily sperm production of Holtzman, Long–Evans, and Wistar rats,[89,209,216,228] but Sprague–Dawley rats,[214] ICR mice,[184] and Syrian hamsters[201] appear to be unaffected. In the three rat strains where decreased daily sperm production is observed, the response lessens in severity as the progeny age and in some cases returns to control levels.[196,201] The Long–Evans rat is an example of a rat strain where the decrease in daily sperm production caused by perinatal TCDD exposure is transient.[201,209] However, daily sperm production was decreased in the 170-day-old progeny of female Wistar rats exposed to TCDD prior to mating, during pregnancy, and throughout lactation. This indicates that the decrease in daily sperm production can be persistent under multiple dosing conditions.[216]

Effects on daily sperm production in Long–Evans and Wistar rat strains by certain non-ortho-substituted PCBs are variable. Administration of TCDD on GD 15 is more effective than administration on GD 8.[201,224] However, in utero and lactational exposure to PCB 169 on GD 8 reduced daily sperm production in Long–Evans rats,[198,224,229] whereas the exposure of Wistar rats to PCB 126 on GD 15 had no effect.[216] In contrast, in utero and lactational exposure to PCB 77 increased both testis size and daily sperm production in 65- and 140-day-old Wistar rats. It was hypothesized that the latter paradoxical effect may have been secondary to a non-AhR-mediated response to PCB 77, such as neonatal hypothyroidism.[216]

While severe undernutrition of rat pups and weanlings can adversely affect the male reproductive system and decrease spermatogenesis,[230–233] it is unlikely that this factor is involved in the reduced daily sperm production that follows in utero and lactational TCDD exposure in Holtzman rats.[196] At the two highest maternal TCDD doses, 0.40 and 1.0 μg/kg, feed consumption and body weight of male offspring were decreased up to 21%. However, there was essentially no effect on feed intake or body weight at the two lowest doses,

0.160 and 0.064 μg/kg, even though daily sperm production, cauda epididymal sperm numbers, and certain sex organ weights were reduced.

Because FSH and testosterone are essential for quantitatively normal spermatogenesis,[188] a potential explanation for the decrease in daily sperm production is a decrease in FSH and/or testosterone levels. In rats, the duration of spermatogenesis is 58 days,[234–236] so the decreases in plasma FSH concentrations in 32-day-old male offspring could contribute to the reductions in daily sperm production when the progeny were 49 and 63 days of age.[196] However, there was no effect on plasma FSH levels when the offspring were 49, 63, and 120 days old.[196] Therefore, it was concluded that reduced daily sperm production in 120-day-old rats, perinatally exposed to TCDD, is not due to decreases in plasma FSH levels during the period between PNDs 49 to 120.[196]

Epididymis In utero and lactational exposure to TCDD has been shown to reduce epididymal weight in Holtzman, Long–Evans, and Sprague–Dawley rat strains.[89,183,196,198,201,214] In these studies TCDD was administered on GD 15, except for the studies by Gray et al.,[201] where it was also administered on GD 8. In the Wistar rat, multiple TCDD doses administered prior to pregnancy and throughout lactation did not cause a reduction in the epididymal weight of the progeny.[205] Weight of the epididymis also was not reduced by in utero and lactational TCDD exposure in the ICR mouse,[184] but cauda epididymal weight was reduced by TCDD exposure on GD 11 in the Syrian hamster.[201] In rat strains that responded to perinatal TCDD exposure with reduced offspring epididymal weight, certain non-ortho-substituted PCB congeners had the same effect. Administration of PCB 169 on GD 8 decreased whole epididymal weight in Long–Evans rats, whereas weight of the epididymides in Wistar rats was either not affected or slightly reduced by PCB 77 or PCB 126 administered on GD 15.[198,216,224]

Compared to decreased testis weight, decreased epididymis and cauda epididymis weights in the Holtzman rat are more sensitive and persistent effects of in utero and lactational TCDD exposure. This is demonstrated by dose-related decreases in weight of the cauda epididymis occurring in Holtzman rats at 120 days of age and cauda epididymal weight being reduced significantly at this age by 0.064 μg TCDD/kg administered on GD 15.[196] The lowest dose of TCDD reported to decrease epididymal weight in other studies was 0.20 μg/kg in the Long–Evans rat[209] and the lowest dose tested in the Sprague–Dawley rat, 0.5 μg/kg.[214]

The reduction in epididymal weight following in utero and lactational exposure to 1 μg TCDD/kg maternal body weight on GD 15 could be permanent in certain rat strains. Significant decreases in whole epididymal or cauda epididymal weights have been observed in 120-day-old Holtzman rats and 240- to 330-day-old Long–Evans rats.[196,201] Since epididymal growth is androgen-dependent, a TCDD-induced androgenic deficiency and/or decrease in androgen responsiveness of the epididymis could account for the decreased size of the organ.[237,238] However, if such an antiandrogenic mechanism of

TCDD is involved, it does not appear to require decreases in plasma androgen concentrations or epididymal androgen receptor expression. This is because in utero and lactational exposure to TCDD has not been shown to reduce circulating androgen concentrations in Holtzman or Long–Evans rats at various stages of postnatal development.[201,204] Also, TCDD does not reduce androgen receptor concentrations in either the caput or corpus epididymis when measured in 240- to 330-day-old Long–Evans rat progeny exposed perinatally to TCDD.[201]

The highest dose of TCDD so far evaluated for effects on epididymal development, 2 μg/kg administered to Sprague–Dawley rats on GD 15,[214] induced a 27% incidence of malformations in the epididymis. These include the segmental absence of entire regions of the epididymis[214] and are similar to epididymal malformations reported for rats and mice exposed in utero to the antiandrogen flutamide.[239–241] The ability of TCDD to produce this lesion suggests that it may interfere with the testosterone-dependent differentiation of the Wolffian duct into the epididymis.[214]

Epididymal Sperm Numbers The epididymis has two functions. In the caput and corpus epididymis sperm mature gaining the capacity for motility and fertility, whereas in the cauda epididymis mature sperm are stored before ejaculation.[242] Mably et al.[196,243] found that motility and morphology of sperm taken from the cauda epididymis on PNDs 63 and 120 were unaffected by perinatal TCDD exposure. In contrast, exposure of Wistar rats to TCDD in a multiple-dosing regimen during mating, pregnancy, and lactation caused small but significant increases in the percent of abnormal sperm in 170-day-old male offspring.[205] However, in Wistar rats exposed in utero and via lactation to a single dose of PCB 77 or PCB 126 on GD 15, no effect on the percentage of abnormal sperm was found in 65- or 140-day-old male progeny.[216]

The most sensitive, robust, persistent, and reproducible effect of in utero and lactational exposure to TCDD on the male reproductive system of laboratory rodents is a decrease in cauda epididymal sperm numbers. This effect has been demonstrated for Holtzman, Long–Evans, Sprague–Dawley, and Wistar rats as well as ICR mice and Syrian hamsters.[184,196,201,205,209,214] The lowest dose of TCDD to produce this effect is 0.064 μg/kg administered on GD 15 to Holtzman rats.[196]

Following in utero and lactational exposure to a single dose of TCDD, there is a graded decline in sperm numbers as they travel from the testis through the caput, corpus, and cauda epididymis and are ejaculated.[183,201,228] Although this suggests that sperm transit rate through the epididymis may be increased by in utero and lactational TCDD exposure, three studies that have determined epididymal sperm transit rates by different methods have reached disparate conclusions. Sommer et al.[228] found no effect on epididymal sperm transit rate in Holtzman rats administered TCDD on GD 15 and ruled out the loss of sperm via spontaneous ejaculation or abnormal introduction of sperm into urine. In contrast, Sprague–Dawley rats similarly exposed to a single maternal

TCDD dose were affected by an increased epididymal sperm transit rate,[214] and Wistar rats exposed to multiple TCDD doses in utero and during lactation were affected by a decreased epididymal sperm transit rate.[205]

In association with the reduction in cauda epididymal sperm numbers, there is a distinct tendency for an increased incidence of a chronic inflammatory reaction in the epididymis of Long–Evans rats exposed in utero and via lactation to PCB 169[198] and in Holtzman rats exposed to TCDD (Sommer and Peterson, unpublished results). Furthermore, the decrease in cauda epididymal sperm numbers in adult hamsters following in utero and lactational exposure to TCDD is associated with an increased incidence of sperm granulomas in the epididymides and/or testes. This lesion is characterized by a nodular accumulation of fibrous connective tissue and mixed inflammatory cells surrounding degenerating sperm in the interstitium of the epididymides and testes. Taken together, these findings suggest that the reduction in cauda epididymal sperm numbers caused by in utero and lactational exposure to TCDD in the hamster are due in part to sperm resorption from the epididymis. Furthermore, since resorption of sperm in the epididymis is often associated with the accumulation of inflammatory cells in the organ, this effect might also occur in postpubertal Holtzman rats exposed to TCDD in utero and via lactation, because inflammatory cell infiltration is evident in the caput epididymis (Sommer and Peterson, unpublished results).

Ejaculated Sperm Numbers Although it has been assessed only in Long–Evans and Holtzman rats, and in the Syrian hamster, the effect on the male reproductive system which is detected at the lowest dose of TCDD administered during pregnancy is that which causes a decrease in ejaculated sperm numbers. The lowest single dose of TCDD known to cause this effect is 0.050 μg/kg administered on GD 15 in the Long–Evans rat, with ejaculated sperm numbers being assessed in adulthood.[183] In addition to the reduction in total number of sperm ejaculated during the mating period, there is a reduction in the number of sperm in copulatory plugs. The small reduction in sperm produced by the testis of Long–Evans rats, exposed perinatally to TCDD, is not sufficient to account for the larger reductions in cauda epididymal sperm numbers and ejaculated sperm numbers. Finally, there is no reduction in the number of copulatory plugs produced by TCDD-exposed males, indicating that copulation is not impaired.[201]

Male Reproductive Capability Male Holtzman rats born to dams given TCDD (0.064, 0.16, 0.40, or 1.0 μg/kg) or vehicle on GD 15 were mated with control virgin females when the males were 70 and 120 days of age.[196,243] The fertility index defined as the number of males impregnating females divided by the number of males mated was decreased by 11% and 22% at the two highest TCDD doses, respectively, in Holtzman rats. However, these decreases were not statistically significant, and at lower doses, the fertility index was not reduced. *Gestation index*, defined as the percentage of control dams mated with

TCDD-exposed males that delivered at least one live offspring, was also not affected by in utero and lactational TCDD exposure. In contrast, Gray et al.[201] reported that the Long–Evans strain is affected by fewer implants in females mated to GD 15 TCDD-exposed male offspring. Male Wistar rat offspring exposed to a multiple TCDD dosing regimen during mating, pregnancy, and lactation were able to impregnate unexposed female rats, resulting in viable fetuses, such that the mating, pregnancy, and fertility indices, as well as the gender ratio of the progeny were similar among control and TCDD treatment groups.[205] Similar to the results in Wistar rats, Mably et al.[196] reported that there was no effect on litter size, live birth index, or 21-day survival index for male Holtzman rat offspring that were mated to unexposed females.

Effects of in utero and lactational exposure to non-ortho-substituted PCB congeners on the reproductive capability of male rat progeny have been investigated. Exposure to 1.8 mg/kg of PCB 169 on GD 1 reduced the fertility of male Wistar rat progeny when they were mated with unexposed control females.[206] In contrast, treatment with either PCB 77 or PCB 126 on GD 15 had no effect on the outcomes when these PCB-exposed male progeny were mated with unexposed females and the number of implantations per litter, viable fetuses per litter, and percent resorptions were evaluated.[216]

Since rats produce and ejaculate 10 times more sperm than is necessary for normal fertility and litter size,[244,245] the absence of a reduction in fertility of male rats exposed perinatally to TCDD is not inconsistent with the substantial reductions in testicular spermatogenesis and epididymal sperm reserves. In contrast, reproductive efficiency in human males is very low, the number of sperm per ejaculate being close to that required for fertility.[246] Thus, measures of fertility using rats are not appropriate for low-dose extrapolation in humans.[247] A percentage reduction in daily sperm production in humans, similar in magnitude to that observed in rats,[196,243] would probably be associated with reduced fertility in men.

Humans There is a paucity of data from epidemiological studies pertaining to effects of TCDD exposure on human male reproductive tract organs. In the few studies that have been done, there is the possibility that the effects observed could be contributed to by concomitant exposure to other chemicals that are not TCDD-like AhR agonists. In the Yucheng incident that occurred in Taiwan in 1979 (see Chapter 22), it was found that boys born to women who had consumed PCB- and PCDF-contaminated rice oil during pregnancy did not experience a delay in either sexual maturation or testicular or scrotal development.[248] However, there was a decrease in penis size. Similarly, in male rat offspring exposed to TCDD in utero, both glans penis weight and diameter were decreased significantly, but glans penis length was not affected.[89] When 12 men who had been exposed to the contaminated rice oil prenatally were compared to 23 age-matched control men, semen volume and sperm counts were similar in the two cohorts. Nevertheless, sperm samples from the exposed men showed increased abnormal morphology, decreased sperm motility,

and poor performance in the hamster oocyte penetration test.[249] These results are comparable to those in rats, where in utero and lactational TCDD exposure reduced daily sperm production and increased the percentage of abnormal sperm.[205] It has been estimated that the lowest dose that caused these effects in the rat is associated with a tissue concentration as low as 2 pg TCDD/g.[250]

Possible Mechanisms for Male Reproductive Tract Effects In utero and lactational exposure to TCDD in the rat reduces accessory sex organ weights, delays preputial separation, and decreases daily sperm production by the testis, sperm storage in the cauda epididymis, and ejaculated sperm numbers. Most effects of in utero and lactational TCDD exposure on male reproductive system development are assumed to be AhR-mediated. Indeed, this is supported by studies in AhR knockout mice which have shown that the decreases in prostate and seminal vesicle weight are AhR dependent.[22] The effects of in utero and lactational TCDD exposure on male reproductive system development in the rat are consistent with decreased testicular androgen production and/or circulating androgen concentrations. However, circulating androgen levels and testosterone synthesis capacity in isolated testis preparations are not affected perinatally or at later times by in utero and lactational TCDD exposure.[181,201,204,220] It remains possible that developmental TCDD exposure could interfere with androgen action at the level of the androgen receptor. Although no effect of in utero and lactational exposure to TCDD on androgen receptor concentrations in the caput epididymis, cauda epididymis, ventral prostate, or seminal vesicle was found in 336- to 339-day-old Long–Evans rats,[201] alterations in the spatial distribution of androgen receptors occur in the ventral prostate of infantile and weanling Holtzman rats exposed perinatally to TCDD.[219] It is also possible that TCDD acts at a point beyond androgen receptor activation to interfere with prostate development, even in the presence of normal circulating androgen concentrations.[219] This is supported by the ability of in utero and lactational TCDD exposure to decrease androgen responsiveness in organ-cultured rat ventral prostate and dorsolateral prostate tissue, obtained from TCDD-exposed prepubertal male offspring.[221,222] Even though androgen receptor expression was decreased in the cultured ventral prostates, it is not known whether this effect fully accounts for the decrease in androgen responsiveness. Another possibility is that TCDD exposure interferes with mesenchymal/stromal–epithelial signaling in the prostate that is important for epithelial differentiation. However, the effects of in utero and lactational TCDD exposure on rat ventral prostate androgen responsiveness are exerted without an inhibition of DHT biosynthesis from steroid precursors in the organ postnatally.[221]

Just because development of androgen-dependent tissues such as the prostate, seminal vesicle, epididymis, and testis are affected by in utero and lactational TCDD exposure does not necessarily mean that an antiandrogenic action of TCDD is the only mechanism by which TCDD could disrupt their

development.[197,198] Impaired growth and development of these organs could arise by TCDD acting on multiple components of the endocrine axis to alter concentrations of other hormones, growth factors, and/or their receptors. Epidermal growth factor, prolactin, thyroid hormones, and growth hormones can influence development of certain of these organs, and their signaling pathways may be modulated by perinatal exposure to TCDD.[198] Also, an important finding is that the spectrum of TCDD effects on male reproductive system development and function does not quite match that which is produced by perinatal exposure to known antiandrogens, 5α-reductase inhibitors, or antiestrogens.[197,198] Therefore, it is possible that TCDD modulates cell proliferation and differentiation in these tissues by interfering with nonhormonal aspects of these processes. For example, prostatic budding and ductal morphogenesis are androgen-dependent, but they also involve important mesenchymal–epithelial interactions occurring downstream of androgen receptor action that might be modulated by TCDD.[197] An important observation is that ventral prostate epithelial cell differentiation in the rat is affected by a TCDD exposure-induced inhibition or delay in the development of luminal cells.[211] This effect may be the result of an alteration in the ability of the prostatic mesenchyme or stroma to support normal epithelial differentiation. Such an alteration in the cell type composition of the prostatic epithelium may underlie the postnatal inhibition of ventral prostate and dorsolateral prostate androgen responsiveness.

Female Reproductive System Effects of in utero and lactational exposure to TCDD on female reproductive system development have not been investigated as extensively as the male reproductive system. However, the results from several studies clearly show that effects of in utero and lactational TCDD exposure on reproductive system development, also occur in female offspring. Effects of such exposure to TCDD on the female reproductive system and mammary gland are shown in Table 9.3 (reviewed in Peterson et al.[1]).

Vaginal Thread Malformation One of the most sensitive effects of in utero and lactational exposure to TCDD on the female reproductive system is the occurrence of a vaginal thread malformation. It has been detected in Long–Evans and Holtzman rats but not in ICR mice or Syrian hamsters.[7,8,184,218,251,252] The lowest dose of TCDD to significantly increase the incidence of vaginal threads in female progeny is 0.20 μg/kg administered on GD 15 to Long–Evans rat dams.[218] The incidence of vaginal threads was greater in the Long–Evans rat when TCDD was administered on GD 15 compared to GD 8.[7] The incidence of vaginal threads in the Holtzman rat, on the other hand, was essentially the same when TCDD was administered on GDs 11, 15, or 18.[251] In utero and lactational exposure to other AhR agonists can also produce the vaginal thread in Long–Evans rats. PCB 169 administered on GD 8 at a dose of 1.8 mg/kg caused a significant increase in the percentage of female progeny displaying this effect.[198]

TABLE 9.3 Effects of In Utero and Lactational TCDD Exposure on the Female Reproductive System[a]

Endpoint	Mouse	Rat	Hamster
Vaginal thread malformation	0	+	0
Cleft phallus and mild hypospadias	0	+	+
Decreased number of ovarian follicles	—	+	—
Cystic follicles	—	+	—
Squamous hyperplasia of cervix	—	+	—
Hyperkeratosis of vagina	—	+	—
Increased estrous cycle abnormalities	—	+	+
Decreased fertility	—	+	+
Decreased fecundity	—	+	+
Premature reproductive senescence	—	+	—
Stunted mammary epithelial growth, fewer primary branches, and fewer terminal end buds	—	+	—
Failure of terminal end bud progression to more differentiated mammary structures	—	+	—
Increased susceptibility to DMBA-induced mammary tumors	—	+	—

[a]References for these effects in the various rodent species are given in the text and/or in Peterson et al.[1] +, Effect is produced; 0, effect is not produced; —, effect not tested.

The vaginal thread is manifested in pubertal rats as a persistent thread of mesenchymal tissue surrounded by keratinized epithelium that partially occludes the vaginal opening. Since the malformation can be identified in histological sections of the developing vagina in 2-day-old pups, it was hypothesized to be present at birth.[251] Accordingly, prenatal exposure to TCDD in Long–Evans rats causes the vaginal thread malformation, whereas postnatal-only exposure to TCDD does not.[218] The earliest time during fetal development that morphologic signs of the thread malformation can be detected is GD 19 in Holtzman rats[252] and GD 18 in Long–Evans rats.[253] At this time there is an increased thickness of mesenchymal tissue between the caudal Müllerian ducts. The presence of this mesenchymal tissue causes failure of the Müllerian ducts to fuse, a process that is normally completed prior to birth. TCDD was also found to block regression of the Wolffian ducts, which contributed to the changes in morphology of the vagina.[252] Therefore, the vaginal thread is produced by TCDD interfering with two critical morphogenetic events involved in the formation of the female reproductive tract: regression of the Wolffian ducts and fusion of the Müllerian ducts.[252,253]

The mechanisms by which TCDD produces these effects at the molecular level are unknown. TCDD modulates cellular responses to both hormones and growth factors, including androgens, estrogens, EGF, and TGF.[197,254,255] Developmental processes such as the timing of morphogenetic signals, and events such as cell proliferation, cell movement, receptor expression, apoptosis,

and terminal differentiation are tightly regulated by these and other hormones and growth factors. Thus, TCDD modulation or interference with the activity of hormones and/or growth factors in the female rat reproductive tract may play a role in causing the vaginal thread malformation.[252]

Cleft Phallus and Mild Hypospadias Other morphological effects of in utero and lactational exposure to TCDD on the female reproductive tract are cleft phallus and mild hypospadias. The hypospadias are considered mild because the urethral opening is always separate from the vaginal opening. These two types of malformations have been observed in rats and hamsters but not in mice.[7,8,184,218,251,252] The lowest dose of TCDD to significantly increase the incidence of cleft phallus and mild hypospadias is 0.8 and 0.2 μg/kg of TCDD, respectively, administered on GD 15 in Long–Evans rats.[218] The morphometric indices of mild hypospadias that were significantly affected by exposure to 0.2 μg/kg of TCDD on GD 15 were an increased length of the urethral slit, increased distance from the tip of the phallus to the urethral opening, and a decreased distance from the urethral to vaginal opening.[218] The incidence of cleft phallus was greater in female Long–Evans rat progeny administered TCDD on GD 15 compared to GD 8.[7] In Holtzman rats the incidence was greater when TCDD was administered on GD 11 compared to GD 15 or GD 18.[251] Other AhR agonists also produce cleft phallus and mild hypospadias in female Long–Evans rats. PCB 169 administered on GD 8 at a dose of 1.8 mg/kg caused a significant increase in the percentage of female progeny with cleft phallus.[198] It also caused female offspring to have a significantly longer urethral slit and a shorter distance between the urethral and vaginal openings indicative of mild hypospadias.[198]

These TCDD-induced malformations of the external genitalia in female rats and hamsters closely resemble the mild form of hypospadias caused by in utero exposure to diethylstilbestrol (DES) and other potent estrogens. In hamsters estradiol causes cleft phallus,[256] and in rats DES and the synthetic estrogen RU2858 produce a mild form of hypospadias.[257,258] This raises the possibility that TCDD, which is often characterized as being an antiestrogen, might cause these effects through an estrogenlike developmental action.[218] In this context, it is important to stress that the other type of malformation produced by in utero and lactational TCDD exposure in the female rodent, vaginal thread formation, is unique to TCDD-like AhR agonists. It is not known to be produced by any other class of chemical, including potent estrogens such as DES.

In addition to the external malformations of the female genitalia, Gray and Ostby[7] reported that in utero and lactational exposure to TCDD caused a significant reduction in ovary and brain weights in female rat offspring necropsied as adults. Hamster offspring display clefting of the phallus, mild hypospadias, and reduced ovarian weight, but do not form the vaginal thread.[7,8,218] Female ICR mouse offspring were not susceptible to either cleft phallus or vaginal thread malformations, nor were their ovary and brain weights reduced by perinatal TCDD exposure.[184]

Effects of AhR Agonists and AhR-Null Mutation on the Ovary Shiverick and Muther[259] reported that there was no change in circulating levels of estradiol in the rat after exposure to 1 μg/kg per day of TCDD on GDs 4 to 15. Similarly, Gray et al.[218] found no effect on serum estradiol levels after perinatal exposure to a single maternal dose of 1 μg TCDD/kg administered on GD 15 in the Long–Evans rat and evaluated on PNDs 21 and 28. In addition, these investigators found no effect on ovarian estradiol production when ovaries from vehicle- and TCDD-treated rats were removed on PNDs 21 and 28 and placed in organ culture for 3 h. However, other reports indicate that serum estradiol and ovarian secretion of estradiol in vitro are decreased by a similar TCDD exposure regimen in the Holtzman rat.[260,261] Histologic examination of ovaries from 21- to 22-day-old rats that were exposed in utero and via lactation to 1 μg TCDD/kg on GD 15 revealed decreases in number of ovarian follicles without alterations in ovarian size or apoptosis in the affected follicular regions.[262] Similarly, administration of 0.6 mg/kg of PCB 169 to rat dams on GD 1, combined with daily doses of 1 mg/kg of PCB 77 on GDs 2 to 18 caused a significant increase in cystic dilated ovarian follicles.[206]

Comparisons between fetal and neonatal wild-type and AhR-null mice indicate that the AhR may play a physiological role in the formation of primordial follicles and in the regulation of antral follicle numbers. In fetal mice there was no effect of the AhR-null mutation on the number of germ cells per ovary.[58] On PNDs 2 to 4, AhR-null mice had significantly more fully formed primordial follicles and fewer single germ cells than did wild-type mice. By PND 8 and on PNDs 32 to 35 there was no difference in the number of follicles, but by PND 53, AhR-null mice had significantly fewer antral follicles than did wild-type mice.[57,58] These results suggest that the AhR, perhaps activated by an as yet unknown endogenous ligand, may regulate the size of the oocyte reserve endowed at birth.[57]

Estrous Cyclicity and Reproductive Performance

RATS Long–Evans rats exposed to 1.0 μg/kg of TCDD on GD 8 are affected by a significantly increased incidence of constant estrus at 1 year of age accompanied by a significant reduction in fertility during a continuous breeding trial.[7] In contrast, TCDD exposure on GD 15 did not have the same effect on cyclicity, and the occurrence of constant estrus was not significantly different from that in controls. There also was no effect on female sexual behavior. Nevertheless, the number of mounts of control males and the latency to ejaculation were increased in matings with females exposed to TCDD on GD 15. This may have been due to TCDD-induced vaginal abnormalities interfering with normal copulation.

Gray and Ostby[7] compared the effect of exposure to 1.0 μg TCDD/kg on GD 15 in Long–Evans female offspring to that in female Holtzman offspring. There was a greater reduction in neonatal viability in Holtzman than in Long–Evans female offspring (50% vs. 11%, respectively) following TCDD exposure.

In the surviving Holtzman offspring, the morphological effects were similar to those in the Long–Evans offspring and included genital clefting and vaginal threads. Reproductive behavior was not assessed in female Holtzman offspring.

As Gray and Ostby[7] have noted, their observations are consistent with previous reports of infertility in female offspring after in utero exposure to TCDD[110] and are probably due to alterations in estrous cyclicity and ovarian function.[74] In utero and lactational exposure of Wistar rats to 0.5 μg/kg per day of TCDD administered on GDs 6 to 15 caused infertility in both genders.[110] Also in utero and lactational exposure to 1.8 mg/kg of PCB 169 on GD 1 decreased mating success and female fecundity in female Wistar rat progeny.[206]

HAMSTERS In utero and lactational TCDD exposure produced adverse effects in female hamsters that persisted into the F_2 generation. This occurred despite the F_1 generation being the only generation that was exposed in utero and via lactation to 2.0 μg TCDD/kg administered on GD 11.5; pregnant F_1 female offspring were not further dosed with TCDD.[8] In female F_2 progeny, vaginal opening was delayed and vaginal estrous cycles were altered. However, most TCDD-exposed females had regular 4-day behavioral estrous clycles. This suggests that in utero and lactational TCDD exposure did not cause a marked disruption in hypothalamic–pituitary–gonadal hormonal cyclicity. While F_1 TCDD-exposed females mated successfully with control males, 20% of them did not become pregnant and 38% of those that did become pregnant died near term. Both the number of implants in pregnant TCDD-exposed hamsters and the number of live born pups were reduced significantly. An important finding was that survival of F_2 generation offspring through weaning was impaired because the F_1 females had been exposed to TCDD in utero and via lactation.[8] The cause of death of the F_2 generation offspring has not been reported.

Histopathology of the Aging Female Reproductive Tract In utero and lactational exposure to TCDD affects histopathology of the female rat reproductive tract.[218] Cystic follicles with luteinization and sertoliform hyperplasia have been observed in the ovaries of 20-month-old female offspring exposed to TCDD. In addition, ovarian tumors were found in TCDD-exposed rats but not in vehicle-exposed controls. Diffuse squamous hyperplasia of the cervix and hyperkeratosis of the vagina have been seen in TCDD-exposed progeny but not in controls.

Mammary Gland The mammary gland of weanling rats and mice is a system of branching ducts that terminate in actively growing terminal end buds (TEBs). Elongation of the mammary ducts and penetration of the epithelium into the surrounding adipose stroma results from the rapid cellular proliferation of TEBs.[263] The density of TEBs (number of TEBs/mm^2) increases steadily after birth until it reaches a maximum in the rat on PND 21.[264] This is accompanied by a concomitant increase in the total area of the mammary

gland. After PND 21, numerous lateral buds develop along the growing ducts as further growth of the gland occurs. During this time septation and cleavage of the TEBs and lateral ducts result in the formation of three to five smaller buds per structure, the alveolar buds.[264] With the initiation of estrus cycling on PNDs, 35 to 42 alveoli form from the alveolar buds and increase progressively in number with each successive estrus cycle, resulting in the formation of lobules until cessation of mammary growth occurs. TEBs that do not differentiate in this manner regress into finger-shaped structures called *terminal ducts*.

AhR EXPRESSION AND EFFECTS OF AhR-NULL MUTATION Mammary glands from estrous cycling C57Bl/6J mice express high levels of AhR mRNA and protein.[55] Lower or undetectable levels of AhR mRNA and protein were found during late pregnancy and during mammary gland involution immediately after cessation of nursing. Transgenic female mice heterozygous for the AhR-null mutation were mated with transgenic heterozygous males. Comparative analysis of mammary gland development in 6- to 8-week-old female AhR-wild type and AhR-null littermates demonstrated that there was a 50% reduction in TEBs and an increase in terminal ducts in the AhR-null females. In most AhR-null females, ductal architecture, branching patterns, and overall organization of specific cell types in the mammary epithelium did not appear to be altered. However, a small percentage of mammary glands from AhR-null females exhibited little or no branching. These findings support the conclusion that AhR-dependent processes may play a role in TEB development.

MAMMARY DEVELOPMENT Pregnant Sprague–Dawley rats were orally administered 1 μg TCDD/kg or vehicle on GD 15.[265] Mammary development was evaluated in female offspring on PNDs 21 and 50. Body weight of the TCDD-exposed female offspring was reduced at both times. TCDD also delayed the time of vaginal opening and caused a disruption in the estrus cycle. However, uterus weight and mammary gland size were unaffected by in utero and lactational TCDD exposure. Nevertheless, the number of TEBs was increased in the mammary glands of TCDD-exposed female offspring on PND 50, and there was a corresponding decrease in the number of lobules. Cellular proliferation was not affected by TCDD exposure in terminal ducts at either time, but the results indicate that the differentiation pathway from TEBs to lobules was inhibited. A study in Long–Evans rats confirmed these findings and reported effects of in utero and lactational TCDD exposure on the mammary gland on PND 4.[266] At this time, development of the mammary gland epithelium was severely stunted and displayed a reduced number of primary branches. Unlike the results of Brown et al.[265] for PND 21, the later study found a decrease in the number of TEBs per mammary gland evaluated on PNDs 4, 33, and 37.[266] However, like the results of Brown et al.,[265] a retention of TEBs and failure to form more differentiated structures was found on PND 45. Interestingly, when evaluated after PND 90, TCDD-exposed mammary glands retain the ability to differentiate in response to estrogen.[267] Recombination experiments in which

mammary gland epithelium from TCDD-exposed donor rats was transplanted in the mammary fat pad of vehicle-exposed recipient rats, and vice versa, indicated that effects of perinatal TCDD exposure on either the mammary stroma or hormonal milieu may play a role in producing the toxicity.[266]

MAMMARY TUMOR FORMATION When the carcinogen 7,12-dimethylbenz[*a*]anthracene (DMBA) is administered to 50- to 60-day-old female rats that had been exposed to TCDD in utero and via lactation, the differentiation pathway of the mammary gland epithelium is disrupted.[264] Between 14 and 21 days post DMBA exposure, TEBs increase markedly in size and do not differentiate into lobules. Instead, these structures, termed *intraductal proliferations*, progress along an alternate pathway to form microtumors that have characteristics of rat mammary adenocarcinomas. In utero and lactational exposure to 1 μg TCDD/kg on GD 15 increased the number of TEBs in 50-day-old female offspring.[265] In addition, these glands were more susceptible to formation of DMBA-induced mammary tumors. Whereas neither epidemiological data nor occupational studies provide clear support for an association between TCDD exposure and occurrence of breast cancer in women, perinatal exposure to TCDD versus TCDD exposure after weaning can have opposite effects in laboratory animals. TCDD exposure after weaning decreased the incidence of DMBA-induced mammary tumors in female rats.[268] The latter effect was believed to be due to antiestrogenic properties of TCDD. The mechanism whereby in utero and lactational TCDD exposure alters TEB differentiation and promotes DMBA-induced mammary tumorigenesis is not known, but may be related to the retention of TEBs.

Central Nervous System Differentiated tissues derived from ectoderm such as the skin, conjunctiva, nails, and teeth, are affected by transplacental exposure to halogenated aromatic hydrocarbons in human infants. Therefore, the CNS, another highly differentiated tissue derived from ectoderm, should be a potential site of TCDD toxicity. In support of this assertion, AhR and ARNT are expressed in the CNS, and transplacental exposure of mice to PCB 77, rats to TCDD and various coplanar PCBs, monkeys to TCDD, and children to mixtures of PCBs, CDFs, and PCQs have resulted in neurodevelopmental toxicity. In rats and mice, effects have been described which suggest that in utero and lactational exposure to TCDD-like AhR agonists may alter some dopaminergic, cholinergic, serotonergic, and/or GABAergic pathways in the CNS. In addition, sexual differentiation of the CNS of adult male rats is irreversibly altered in a dose-related fashion by perinatal exposure to TCDD.[195,243] Thus, functional CNS alterations in multiple species, which may or may not be irreversible, can be affected by in utero and lactational exposure to halogenated aromatic hydrocarbons. However, the involvement of AhR-mediated mechanisms in producing these alterations has not been clearly demonstrated. The effects of in utero and lactational exposure of rats to TCDD, coplanar PCBs, and ortho-substituted PCBs on male sexual behavior, and various neuro-

TABLE 9.4 Effects of In Utero and Lactational Exposure to TCDD, Coplanar PCBs, or Ortho-Substituted PCBs on Sexual Behavior, Memory and Learning, Locomotor Activity, Temperature Regulation, and Night Vision in Rats[a]

Endpoint	TCDD	Coplanar PCBs	Ortho-Substituted PCBs
Demasculinization of sexual behavior in males	+	+[b]	—
Feminization of sexual behavior in males	+	—	—
Masculinization of saccharin taste preference in females	+	+	—
Improved working memory	+	+	0
Impaired working memory	0	+[c]	0
Delayed spatial alternation (T-maze)	0	0	+
Delayed spatial alternation (operant chamber)	—	0	—
Impaired visual discrimination learning	+	+	+
Prolonged haloperidol-induced catalepsy	—	+	0
Decreased dopamine synthesis in brain	—	0	+
Less responsive passive avoidance behavior	—	+	0/+
Increased locomotor activity	—	+	+
Spontaneous hypertensive-like hyperactivity	—	0	+
Altered body temperature regulation	+	—	—
Decreased night vision in male offspring	—	+	0

[a] References for these effects in the various rodent species are given in the text and/or in Peterson et al.[1] +, Effect is produced; 0, no effect was found; —, effect has not yet been determined in rats for TCDD.

[b] Fewer masculine sexual behavior endpoints are affected with coplanar PCBs than with TCDD.

[c] Results are dependent on the rat strain and/or testing paradigm used.

behavioral endpoints are summarized below and in Table 9.4. The purpose of including the ortho-substituted PCBs in Table 9.4 is so that apparent similarities and differences between the neurobehavioral effects of TCDD, coplanar PCBs, and ortho-substituted PCBs could be identified. Where differences do occur, however, it is usually the case that only a limited number of PCB congeners have been tested.

AhR and ARNT Expression AhR has been identified in the rat brain,[269] but an early study suggested that it may be associated with glial cells rather than neurons.[270] More recently, mRNAs for the AhR and ARNT have been localized to the same neuronal populations in the olfactory bulb, hippocampus, and the cerebral and cerebellar cortices by in situ hybridization using adult male rat brain.[271] Also in the rat, AhR, ARNT, and ARNT2 mRNAs are expressed in several hypothalamic and brain stem regions involved in the regulation of appetite and control of circadian rhythms. These include the arcuate nucleus, ventromedial hypothalamus, paraventricular nucleus, suprachiasmatic nucleus, nucleus of the solitary tract, and the dorsal and medial raphe

nuclei.[272] In addition, AhR and ARNT2 mRNAs have been identified in the developing neuroepithelium of the mouse fetus.[27] The highest concentrations of TCDD-derived ^{14}C radioactivity in the rat brain are found in the hypothalamus and pituitary, whereas much lower concentrations are found in the cerebral cortex and cerebellum.[273] This is consistent with the expression of detectable AhR and ARNT mRNA levels in the hypothalamus. However, in another study, AhR protein was not detected in whole rat or mouse brain but was detected in the cerebrum of the hamster, as well as the cerebrum and cerebellum of the guinea pig.[274] The significance of these findings is that TCDD and related AhR agonists could act directly via the AhR in some discrete neuronal populations of the brain. That AhR and ARNT mRNAs were found in several hypothalamic and brain stem regions that are involved in the regulation of appetite and circadian rhythms suggests an underlying basis for the disruption of these functions by TCDD. AhR, ARNT, and ARNT2 protein in distinct brain regions have not yet been fully characterized.

Sexual Differentiation in Rat Offspring In rats, the critical period of sexual differentiation extends from late fetal life through the first week of postnatal life.[192] In the absence of adequate circulating levels of testicular androgen during this time, adult males display high levels of feminine sexual behavior (e.g., lordosis), low levels of masculine sexual behavior, and a cyclic (i.e., feminine) pattern of LH secretion.[190,191] In contrast, perinatal androgen exposure will result in the masculinization of sexually dimorphic neural parameters, including reproductive behaviors, regulation of LH secretion, and several brain morphological indices.[275,276] The mechanism by which androgens cause sexual differentiation of the CNS in the rat is not completely understood. It appears that 17β-estradiol, formed by the aromatization of testosterone within the CNS, is one of the principal active steroids responsible for mediating sexual differentiation[277]; however, nonaromatized androgens are also involved. Effects of TCDD exposure on sexual behavior have been observed at low enough maternal doses so that undernutrition, due to a decrease in feed consumption by the dam, does not appear to be a factor.

DEMASCULINIZATION OF SEXUAL BEHAVIOR IN MALES Masculine sexual behavior in male rat offspring born to dams given graded doses of TCDD or vehicle on GD 15 was assessed at 60, 75, and 115 days of age.[195,243] Each male rat was placed in a cage with a receptive vehicle-exposed female and the first ejaculatory series and subsequent postejaculatory interval were recorded. The number of mounts and intromissions (mounts with vaginal penetration) before ejaculation was increased by a maternal TCDD dose of 1.0 μg/kg. The same males exhibited 12- and 11-fold increases in mount and intromission latencies, respectively, and a twofold increase in ejaculation latency. All latency effects were dose-related and significant at a maternal TCDD dose of 0.064 μg/kg (intromission latency) and 0.16 μg/kg (mount and ejaculation latencies). Copulatory rates (number of mounts + intromissions/time from first mount to

ejaculation) were decreased in a dose-related manner to less than 43% of the control rate.[195] The lowest maternal dose to produce a significant effect was 0.16 μg/kg. Postejaculatory intervals were increased 35% above the control interval, and a significant effect was observed at maternal doses of TCDD as low as 0.40 μg/kg.

The effect of in utero and lactational exposure to 1.0 μg/kg of TCDD administered on GD 8 or GD 15 on masculine sexual behavior was subsequently assessed in Long–Evans rats.[278] In males exposed to TCDD on GD 15, partial demasculinization of sexual behavior was evidenced by increases in total number of mounts prior to ejaculation, number of mounts with intromissions prior to ejaculation, number of mounts without intromissions prior to ejaculation, and latency prior to ejaculation.[201] Although the same profile of results was obtained in males exposed to TCDD on GD 8, the effects were not as great and were not significant.[201]

Masculine sexual behavior was also assessed in male Wistar rats exposed in utero and via lactation to TCDD administered to dams during mating, pregnancy, and lactation.[205] Mount latency and intromission latency were increased at two of three TCDD exposure levels. However, ejaculation latency, number of mounts with intromissions prior to ejaculation, and intromission frequency were not affected. Of these endpoints, only the number of mounts with intromissions prior to ejaculation was increased in Wistar rats exposed to PCB 126 on GD 15.[216]

Taken together, these results demonstrate in Holtzman, Long–Evans, and Wistar rats, that in utero and lactational exposure to TCDD affects some but not all endpoints and only partially demasculinizes sexual behavior in some strains. This response is therefore not as robust as other effects of TCDD on the male reproductive system. In addition, the degree to which the expression of masculine sexual behavior is affected depends on the species, AhR agonist, and dose administered. Male hamster progeny do not exhibit demasculinized sexual behavior following perinatal exposure to TCDD, making it difficult to extrapolate this response in the rat to other species.

FEMINIZATION OF SEXUAL BEHAVIOR IN MALES Male offspring of Holtzman rats administered graded doses of TCDD on GD 15 were castrated at 120 days of age.[195,243] Beginning at 160 days of age, these castrated males were injected weekly for 3 weeks with 17β-estradiol benzoate, followed 42 h later by progesterone. Four to 6 h after the progesterone injection in weeks 2 and 3, the male was placed in a cage with a sexually excited control stud male. Feminine sexual behavior, evaluated as the frequency of lordosis in response to being mounted by the stud male, was increased threefold, from 18% (control) to 54% by the highest maternal TCDD dose, 1.0 μg/kg. In addition, the lordosis intensity score was increased. Both effects on lordosis behavior in males were dose-related and significant at maternal TCDD doses as low as 0.16 μg/kg (increased lordotic frequency) and 0.40 μg/kg (increased lordotic intensity). Essentially the same results were found in a later study from the same laboratory.[203]

Together, they indicate that perinatal TCDD exposure can partially feminize sexual behavior in male rats. In a cross-fostering experiment it was found that this effect required lactational TCDD exposure, because when TCDD exposure was restricted to the in utero period there was no increase in lordotic behavior in male offspring.[89]

In contrast to three separate studies showing that in utero and lactational TCDD exposure increases the expression of feminine sexual behavior in male Holtzman rats,[89,195,203] this effect was not observed in male Long–Evans rats.[201] Lordotic behavior in adult male offspring was not increased after in utero and lactational exposure to a single maternal 1.0 μg TCDD/kg dose administered on either GD 8 or GD 15.[201] This lack of effect may be due to a rat strain difference in susceptibility to this type of alteration in behavior.

FEMINIZATION OF LH SECRETION REGULATION IN MALES The effect of perinatal TCDD exposure on the sexually dimorphic regulation of LH secretion by ovarian steroids was determined in male rat offspring at approximately 270 days of age.[195] Female rats ovariectomized as adults and primed with estrogen greatly increase their plasma LH concentrations when injected with progesterone, whereas similarly treated males fail to respond.[279] In contrast, progesterone causes significant increases in plasma LH concentrations in estrogen-primed male rats exposed in utero and via lactation to a maternal TCDD dose as low as 0.40 μg/kg, but progesterone has little effect in similarly primed control males. Thus, perinatal TCDD exposure increases pituitary and/or hypothalamic responsiveness of male rats to ovarian steroids in adulthood, indicating that regulation of LH secretion is permanently feminized.

MASCULINIZATION OF SACCHARIN PREFERENCE IN FEMALES Saccharin preference in the rat is a sexually dimorphic behavior that is hormonally organized during the period shortly before and just after birth.[280] Sodium saccharin can be used to assess the tendency of female rats to consume more of a sweet solution than do male rats. Female offspring of Sprague–Dawley rat dams that had been administered TCDD (0.025 or 1.0 μg/kg per day), PCB 77 (2 or 8 mg/kg per day), or PCB 126 (0.25 or 1.0 μg/kg per day) on GDs 10 to 16 consumed less saccharin and exhibited a reduced preference for this sweetener.[281] This result, which indicates that saccharin consumption is masculinized in female rats exposed to TCDD or coplanar PCBs during prenatal development, could be related to the antiestrogenic effect of these chemicals. Male offspring exposed to vehicle did not exhibit any preference for saccharin, and perinatal exposure to TCDD or coplanar PCBs did not change their saccharin consumption or saccharin preference.[281] Therefore, the effects of AhR agonists on saccharin consumption and preference are sex dependent.

BRAIN ESTROGEN RECEPTOR AND SEXUALLY DIMORPHIC NUCLEI In utero and lactational exposure to TCDD in the Holtzman rat partially demasculinizes and feminizes sexual behavior, possibly by causing incomplete sexual differen-

tiation of the CNS. To determine if TCDD exposure alters biochemical and morphological endpoints of sexual differentiation of the CNS in this rat strain, pregnant rats were administered a single maternal 0.7 μg TCDD/kg dose on GD 15. Estrogen receptor binding in specific brain nuclei that are dependent on hormone stimulation during the period of CNS sexual differentiation was evaluated in male and female offspring, and the volumes of certain sexually dimorphic brain nuclei were determined.[203] Estrogen receptor concentrations in the medial preoptic nucleus, ventrolateral aspect of the ventromedial nucleus, and periventricular preoptic area were higher in control females than in males, but in utero and lactational exposure to TCDD had no effect on estrogen receptor concentrations at these sites.[89] It also had no effect on estrogen receptor concentrations in other brain nuclei where there is known to be no gender difference in estrogen receptor concentrations. The volume of the sexually dimorphic nucleus of the preoptic area is normally greater in males, whereas that of the medial preoptic nucleus is greater in females. Perinatal TCDD exposure had no effect on the volume of either nucleus in male and female Holtzman rat offspring in adulthood.[89] Thus, in utero and lactational TCDD exposure is capable of partially demasculinizing and partially feminizing sexual behavior of male Holtzman rat progeny,[89,195,203] but these responses apparently are not associated with any effect on sexual differentiation of the estrogen receptor system in the brain or the volume of sexually dimorphic brain nuclei.[203,282]

POSSIBLE MECHANISMS FOR SEXUAL BEHAVIOR EFFECTS The most plausible explanation for the demasculinization of sexual behavior and the feminization of sexual behavior and LH secretion is that perinatal exposure to TCDD impairs sexual differentiation of the CNS. Neither undernutrition, altered locomotor activity, reduced sensitivity of the penis to sexual stimulation, nor modest reductions in adult plasma androgen concentrations of the male offspring can account for these effects.[195] On the other hand, exposure of the developing brain to testosterone, conversion of testosterone to 17β-estradiol within the brain, and events initiated by binding of 17β-estradiol to its receptor are all critical for sexual differentiation of the CNS and have the potential to be modulated by TCDD. If TCDD interferes with any of these processes during late gestation and/or early neonatal life, it could irreversibly demasculinize and feminize sexual behavior[283–285] and feminize the regulation of LH secretion[286,287] in male rats in adulthood. However, in utero and lactational exposure to TCDD does not alter either estrogen receptor concentrations in various brain nuclei or volumes of sexually differentiated brain nuclei of male and female Holtzman rat progeny at doses that affect the expression of masculine and feminine sexual behavior.[89] In humans, there is evidence that social factors account for much of the variation in sexually dimorphic behavior, but there is also evidence that prenatal androgenization influences both the sexual differentiation of such behavior and brain hypothalamic structure.[288–290] In utero and/or lactational exposure to TCDD could alter sexual behavior in species

other than the rat, including nonhuman primates[291–293] in which sexual differentiation is under androgenic control. However, this was not the case in male hamster progeny exposed in utero and via lactation to TCDD.[201]

Neurobehavior in Mice CD-1 mice exhibited neurobehavioral, neuropathological, and neurochemical alterations in adulthood after being exposed transplacentally to maternal doses of 32 mg PCB 77/kg administered on GDs 10 to 16.[294–296] The effects observed consisted of circling, head bobbing, hyperactivity, impaired forelimb grip strength, impaired ability to traverse a wire rod, impaired visual placement responding, and impaired learning of a one-way avoidance task. These effects were accompanied by neuropathological alterations in synapses of the nucleus accumbens which suggested interference with the development of dopaminergic systems.[294,295] In support of this suggestion, decreased dopamine levels and decreased dopamine receptor binding in the corpus striatum, both of which were associated with elevated levels of motor activity, have been found in adult mice transplacentally exposed to PCB 77.[296] Thus, transplacental PCB 77 exposure may permanently alter the development of striatal synapses in the mouse brain.

In NMRI mice exposed to a single oral dose of 0.41 or 41 mg PCB 77/kg on PND 10, muscarinic receptor concentrations in the hippocampus were decreased significantly at both dose levels on PND 17.[297] Similarly exposed mice evaluated at 4 months of age were significantly less active than controls at the onset of testing, but were more active than controls at the end of the test period.[298] Upon sacrifice after the activity testing was complete, a small but statistically significant increase in the muscarinic receptor concentration of the hippocampus was found in animals from the high-dose group. These results suggest that the neurochemical effects of PCB 77 in the mouse are complex because in utero and lactational exposure to this congener differs from postnatal-only exposure, and because cholinergeric as well as dopaminergic systems in the brain may be involved. Since these studies used PCB 77 rather than TCDD, it is possible that the effects observed may have been caused by a hydroxylated PCB 77 metabolite that is neurotoxic, but not by an AhR agonist. Therefore, further research is required to determine if PCB 77-induced neurotoxicity effects are relevant to TCDD-like developmental toxicity.

Neurobehavior in Rats A considerable number of neurobehavioral endpoints have been evaluated following perinatal exposure of rats to TCDD and coplanar PCBs that are AhR agonists, as well as to ortho-substituted PCBs that do not interact with the AhR. Interest in the latter arises from the fact that PCB mixtures to which children have been exposed in utero and via lactation contain coplanar and noncoplanar PCBs. Therefore, it is important from a mechanistic point of view to determine whether the effects of TCDD-like AhR agonists, which include the coplanar PCBs, can be distinguished from the effects of ortho-substituted PCBs, which, depending on their extent of ortho substitution, have either very weak or no AhR agonist activity.

Two hypotheses have been advanced: (1) TCDD and TCDD-like PCB congeners do not produce behavioral impairment at biologically relevant doses,[299,300] and (2) effects of TCDD and TCDD-like PCB congeners on learning and memory might be distinguishable from effects of ortho-substituted PCB congeners that are not AhR agonists.[301,302] However, these hypotheses have not yet been fully tested. To the extent that effects of individual ortho-substituted PCB congeners on neurobehavior may depend on reductions in thyroid hormone concentrations during the perinatal period,[303,304] it is important to note that TCDD and coplanar PCBs do not reduce thyroid hormone concentrations to as great an extent as the ortho-substituted PCBs.[305] Difficulty in resolving these hypotheses occurs because different laboratories, using the same testing methods, but sometimes different rat strains, have not always obtained similar results. In addition, different testing paradigms that appear, at least superficially, to test similar behaviors can arrive at discordant conclusions. Where the results of testing by different methods are not in agreement, the differences are not easy to resolve, in part because the relative sensitivities of the different measures are not always clear.

In utero and lactational exposure of rats to TCDD-like AhR agonists has affected endpoints that measure learning and memory, discrimination reversal learning, motivation to respond to incentives, transitional behavior, avoidance behavior, neurotransmitter function, and locomotor activity. In addition, perinatal exposure to AhR agonists has inhibited long-term potentiation (LTP) in the visual cortex but not in the hippocampus evaluated in vitro.[306,307] In some cases, effects observed with AhR agonists are similar to those of ortho-substituted PCB congeners.[301,308–310] Therefore, the available data obtained following in utero and lactational exposure of rats to these congeners tend to support the notion that TCDD and coplanar PCBs may affect neurobehavioral endpoints by a variety of mechanisms, only one of which is AhR-mediated.

SPATIAL LEARNING Female rats were administered PCB 77 (2 and 8 mg/kg per day), PCB 126 (0.25 and 1 μg/kg per day), and TCDD (0.25 and 1 μg/kg per day) by gavage on GDs 10 to 16.[301] Beginning on PND 80, radial arm maze behavior was evaluated in male and female offspring by using an eight-arm maze. While exposure to TCDD or these coplanar PCBs did not cause overt toxicity, the exposed offspring made fewer errors than controls. In contrast, exposure to the ortho-substituted PCBs—2,4,4′-TCB (PCB 28), 2,3,4,4′,5-PCB (PCB 118), and PCB 153—did not affect the number of errors.[308]

The ability of in utero and lactational TCDD exposure to reduce the number of errors made by male rat offspring in the radial arm maze test was confirmed, even at a reduced exposure level (0.1 μg/kg per day on GDs 10 to 16), but no significant decreases in the error rate were found in female rats.[310] However, further statistical analysis of the data suggested that the affected male rats were using a response strategy whereby they reduced their error rate simply by entering adjacent arms of the maze. This conclusion is supported by lack of an effect of TCDD exposure in the Morris water maze test, which does not

allow the rat to use a response strategy.[310] Nevertheless, the test for the response strategy of selecting adjacent radial arms was not significant in the original study at the higher level of TCDD exposure. This result suggests that working memory was improved by exposure to the 0.1 μg TCDD/kg per day maternal dosing regimen.[301] To test this further, similarly exposed male offspring were tested on a 12-arm maze with only eight arms baited. The results of this test indicated that the reduction in errors on the eight-arm maze was most likely not caused by any TCDD-induced improvement in spatial learning or memory.[311]

The low-dose TCDD exposure decreased latency in male rats in the radial arm maze test.[310] This reveals an apparent feminization of this parameter similar to that caused by the diortho-substituted congener PCB 153, which also decreased latency in male rats.[308] In addition, there may be other similarities between TCDD and at least some of the ortho-substituted PCBs, because when the radial arm maze was used to evaluate 2,2′,3,5′,6-PCB (PCB 95), an exposure-associated decrease in the error rate was observed in male rat offspring.[309] Similar to the original result with the larger exposure doses of TCDD,[301] the test for use of a response strategy was negative with PCB 95, but unlike the results with TCDD and PCB 153, there was no gender-related decrease in latency. In contrast to the decrease in error rate caused by perinatal exposure of Sprague–Dawley rats to PCB 77, PCB 95, PCB 126, and TCDD,[301] there was no effect of the coplanar PCB 77 or the diortho-substituted 2,2′,4,4′-TCB (PCB 47) on the error rate of male Wistar rats that were tested on an equivalent radial arm maze in a different laboratory.[312]

The male and female rat offspring that were exposed to the ortho-substituted PCBs (PCB 28, PCB 118, and PCB 153) and tested for working memory on the radial arm maze were subsequently evaluated for delayed spatial alternation on the T-maze beginning after PND 135.[308] Exposed females learned this task more slowly than exposed males, and all PCB congeners tested caused a decrease in the number of correct responses. There was no effect on the number of correct responses in exposed male offspring, but their latency to enter the maze was decreased compared to that of control male offspring. The latter effect again suggests a more femalelike pattern of response in male offspring exposed to the ortho-substituted PCBs, even though distinct gender differences still remained for the main response. Unlike the other ortho-substituted PCBs, PCB 95 did not affect the number of correct responses.[309] Therefore, not all ortho-chlorinated PCBs are equivalent, suggesting that metabolic differences or the existence of structural selectivity in the mechanism may have affected the responses to different PCBs.[308,309]

In contrast to several ortho-substituted PCBs, in utero and lactational exposure to TCDD or to the coplanar PCBs 77 and 126 produced no effect on the errors made by male or female offspring or in their latency in the T-maze test.[301] Another group also found that delayed spatial alternation was unaffected in both male and female rat offspring exposed to PCB 126, although these Long–Evans rats were tested in an operant chamber setting.[300] In this

case, even prolonged dietary exposure of female rats to PCB 126, which began 7 weeks before mating to an unexposed male and continued until the offspring were weaned, caused no treatment-related differences in performance.[300]

VISUAL DISCRIMINATION REVERSAL LEARNING Some effects were observed when the T-maze was used to assess visual discrimination reversal learning following in utero and lactational exposure of male and female rat offspring to maternal doses of 0.1 μg TCDD/kg per day on GDs 10 to 16.[310] This endpoint was tested by placing electric light stimuli onto the cross-arms of the same T-maze used to evaluate spatial discrimination reversal learning. At approximately 100 days of age, TCDD-exposed offspring performed similar to controls during the original learning phase of the trial but were slower to reach the testing criterion of 10 correct trials in a 12-trial session. This effect occurred equally in males and females and was most evident during the first and second reversal period. In the following reversal periods no further differences were evident between the TCDD and vehicle exposure groups. Similarly, PCB 118 and PCB 126 impaired visual discrimination learning in male rat offspring evaluated in an operant chamber.[313] However, these authors used more than one male offspring per litter, and they appear to have evaluated all rats from the same litter as if they were independent observations. Therefore, the results of this study appear to be uninterpretable on statistical grounds,[299,300] based on the criteria established by Holson and Pearce.[314]

One study using mixtures evaluated visual discrimination learning in the offspring of female rats fed a diet that contained Clophen A30 (32 mg total PCBs/kg diet) or a normal diet for 60 days prior to mating and through pregnancy.[315] After birth some offspring exposed to each diet were cross fostered to dams exposed to the other diet. When male and female offspring were evaluated at 120 to 180 days of age, there was no effect of PCB exposure during the acquisition phase of the paradigm (visual discrimination learning tested on a jumping stand). However, performance in all PCB-exposed groups was inferior during the retention phase, relative to their performance at the end of the acquisition phase. Since the effect was more pronounced in the prenatal only and prenatal and lactational exposure groups, compared to the lactational only exposure group, prenatal exposure to PCBs is required for visual discrimination learning to be impaired, at least when tested by this method.

ALTERED OPERANT RESPONDING FOR MOTOR REINFORCEMENT Female Holtzman rat offspring were exposed in utero and via lactation to various doses of TCDD that were maternally administered on GD 18. After PND 60, animals were trained to press a lever for brief opportunities to run on a running wheel.[316] Once they began to respond, the fixed ratio schedule of reinforcement was gradually increased so that it took increasingly more lever presses to activate wheel running time. Under each fixed ratio schedule of reinforcement, TCDD-exposed female offspring exhibited a dose-related reduction in the number of earned opportunities to run, lever response rate, and total number of revolu-

tions on the wheel. Bench mark dose (BMD) analysis of the data indicated that statistically significant deficits in performance occurred at dose levels similar to the current human body burden of TCDD equivalents, 6 to 8 ng/kg. At these doses estrous cyclicity was not affected, and overt toxicity was not observed in the offspring. However, their reduced motivation to respond to incentives is a significant manifestation of developmental toxicity that may extend to other motivational endpoints beyond this particular paradigm of wheel running. This decrease in motivational responsiveness represents the most sensitive effect of in utero and lactational TCDD exposure that has so far been found in the rat.

TRANSITIONAL BEHAVIOR Female Long–Evans rats were exposed to PCB 126 (0.25 and 1 μg/kg per day) via dietary supplementation which began 35 days prior to mating and continued through pregnancy and lactation.[302] After PND 400 transitional behavior was tested in male and female offspring by use of a concurrent random interval–random interval reinforcement schedule in an operant chamber. This test measures the rat's ability to adapt to changes under which it is rewarded for selecting the correct lever. However, if differences are found following in utero and lactational TCDD exposure, it is necessary to determine that the test rats do not differ from the controls in their perception of the reward. Offspring of both genders that were TCDD-exposed apportioned their responses less accurately than control offspring with respect to the concurrent random interval–random interval reinforcement pattern of scheduled reinforcements. That the exposed rats perceived the reward similar to the way it was perceived by the control rats was shown by testing the responses in a progressive ratio reinforcement schedule which resulted in no treatment-related differences in the relative strength of the reinforcing event.[302] In addition, there were no treatment-related differences when the same rats had been tested on PND 220 by using a multiple fixed interval–fixed ratio reinforcement schedule.[299] Therefore, the results obtained on the concurrent random interval–random interval schedule of reinforcement may indicate a selective effect of PCB exposure on adaptive ability in the offspring.[302]

BEHAVIORAL RESPONSES TO CNS DRUGS Haloperidol-induced catalepsy was evaluated in male Wistar and Long–Evans rat offspring exposed to the diortho-substituted PCB 47 (1 mg/kg per day) or the coplanar PCB 77 (1 mg/kg per day) from GDs 7 to 18.[312,317] At 100 and 180 days of age, catalepsy was induced in the male offspring by the dopaminergic antagonist haloperidol. Developmental exposure to PCB 77, but not to PCB 47, caused an increase in the time required for the affected rat to move its paw after the paw had been placed into certain positions by the experimenter. This result indicates an effect of PCB 77 on neuronal pathways where dopamine is the neurotransmitter.

The effects of similar exposure to PCB 77 on dopaminergic function have also been tested in Long–Evans rats by evaluating their ability to discriminate between the dopaminergic agonist apomorphine and saline.[318] As a positive control, the antithyroid drug propylthiouracil given to adult control animals

just prior to testing blocked their ability to discriminate between apomorphine and saline, whereas no similar inhibition of the ability to distinguish apomorphine was found in rats exposed perinatally to PCB 77. However, administration of buspirone to adult animals just prior to testing blocked the ability of vehicle-exposed rats to recognize apomorphine much more than it blocked this ability in the PCB 77-exposed offspring. As buspirone is a mixed serotonin receptor agonist and partial dopamine receptor antagonist, the authors suggested that perinatal exposure to PCB 77 may produce long-lasting effects on the interaction between dopaminergic and serotonergic processes in the CNS.[318]

Since PCB 77 was effective in prolonging haloperidol-induced catalepsy, whereas PCB 47 was not, it is interesting that perinatal exposure to coplanar PCBs and ortho-substituted PCBs also produce opposite effects on dopamine synthesis in the brain.[319,320] Perinatal exposure to ortho-substituted PCB congeners decrease dopamine synthesis in adulthood, whereas perinatal exposure to coplanar PCBs cause persistent elevations in brain dopamine and dopamine metabolite concentrations.[320] Therefore, perinatal exposure to coplanar and ortho-substituted PCBs can result in congener-specific effects on dopamine synthesis and function that may distinguish the different classes of congeners; however, only one PCB of each type was evaluated for dopamine function.

Female Long–Evans rats were exposed to PCB 126 (0.25 and 1.0 μg/kg per day) by dietary supplementation which began 35 days prior to mating and continued through pregnancy and nursing.[321] In control offspring evaluated on PND 12, all doses of chlordiazepoxide (CDP, 0, 3, 5, and 8 mg/kg) reduced performance in the CDP challenge test. This result suggested that the control offspring were affected by an increase in the visual threshold. In contrast, rats exposed in utero and via lactation to the low dose of PCB 126 were unaffected by CDP, whereas those exposed to the high dose exhibited less of a decrement in their performance than did the control offspring. Since additional test results demonstrated that PCB 126 did not cause deficits in attention, the altered performance of these rats after the administration of CDP suggests that perinatal exposure to PCB 126 may affect γ-aminobutyric acid (GABA)-mediated pathways in the CNS during development.[321]

PASSIVE AVOIDANCE BEHAVIOR Male Wistar rats were exposed to the coplanar PCB 77 or the diortho-substituted PCB 47 on GDs 7 to 18. Passive avoidance behavior was tested on a step-down platform when the rats were 220 days old.[312] The latency of male rat offspring to step onto a grid that had previously given them an electric shock was used to evaluate the effects of perinatal PCB exposure. Latency was decreased by both PCBs compared to control for up to 24 h after the initial shock. However, only the effect of PCB 77 was significant when evaluated at a single time (5 min, 4 h, and 24 h). A similar paradigm was used to evaluate the effects of perinatal exposure to PCB 77, PCB 47, and a combination of both PCBs in Long–Evans rats on PND 85.[317] Under these

conditions the most significant effect observed was a decreased latency in the PCB 77 and combined exposure groups at the 5-min timepoint. No significant effect was found when rats were exposed to PCB 47 only. These results suggest that differences in passive avoidance behavior may exist following perinatal exposure to nonortho- and ortho-substituted PCBs, but only one congener of each type has been tested.

OPEN-FIELD LOCOMOTOR ACTIVITY When open-field activity was tested in male offspring on PND 25, rats exposed to PCB 47 in utero and via lactation had a significantly higher activity level than did rats similarly exposed to PCB 77. However, there were no significant differences between the PCB-exposed groups and the unexposed control group.[312] When tested on PND 340, offspring exposed to PCB 47, PCB 77, and a combination of both PCBs were hyperactive compared to controls.[317]

Locomotor activity has also been tested in an operant chamber setting in rats exposed to PCBs only during lactation. Female DA/OLA/HSD rats were mated to male Lewis rats and the pregnant dams were administered vehicle, PCB 153 (5 mg/kg), or PCB 126 (2 μg/kg).[322] Pregnant rats were dosed every second day from PNDs 3 to 13. PCB-exposed, 112-day-old male rats were found to be hyperactive during both the fixed interval and extinction components of the reinforcement schedule. In addition, both congeners increased the activity level when exposure was limited to the postnatal period. The PCB 153-exposed offspring displayed a behavior pattern similar to that of spontaneous hypertensive rats, which are used as an animal model of attention-deficit hyperactivity disorder in children. With the results of only one congener of each type being tested, the spontaneous hypertensive behavior-like pattern of activity seems to be selective for the ortho-substituted PCB. In contrast to all results which show that in utero and/or lactational PCB exposure causes hyperactivity, perinatal exposure to PCB 95 caused hypoactivity in rat offspring.[309]

Neurobehavior in Monkeys Schantz and Bowman[90] and Bowman et al.[323] have conducted a series of studies on the long-term behavioral effects of perinatal TCDD exposure in monkeys. Because these were the first studies to evaluate the behavioral teratology of TCDD, monkeys exposed to TCDD in utero and via lactation were screened on a broad selection of behavioral tests at various stages of development.[323] At the doses studied (5 or 25 ppt in the maternal diet), TCDD did not affect reflex development, visual exploration, locomotor activity, or fine motor control in any consistent manner.[91] However, perinatal TCDD exposure did produce a specific, replicable deficit in cognitive function.[90] TCDD-exposed offspring were impaired on object learning but were unimpaired on spatial learning. Monkeys appear to be quite sensitive to this effect of TCDD exposure. Given that the half-life of TCDD in the monkey is approximately 400 days and that the mothers of the affected infants had been

administered 0.151 ng TCDD/kg per day for 16.2 months at the 5 ppt dose, it has been calculated that the maternal body burden reached a level of 42 ng TCDD/kg.[324]

TCDD exposure also produced changes in the social interactions of mother–infant dyads.[325] TCDD-exposed infants spent more time in close physical contact with their mothers. This pattern of effects was similar to that seen in lead-exposed infants and suggested that mothers were providing increased care to the TCDD-exposed infants.[325]

Neurobehavior in Humans The intellectual and behavioral development of Yucheng children transplacentally exposed to PCBs, CDFs, and PCQs was studied through 1985 by Rogan et al.[102] There was a developmental or psychomotor delay in 10% of Yucheng children compared with 3% of control children and of a speech problem in 7% of Yucheng children versus 3% of control children. Also Yucheng children scored lower than control children on three developmental and cognitive tests.[102] Neurobehavioral data on Yucheng children after 1985 shows that their intellectual development lags behind that of control children. In addition, they are rated by their parents and teachers as having a higher activity level; more health, habit, and behavioral problems; and a temperamental clustering closer to that of a "difficult child." It is concluded that in humans, transplacental exposure to halogenated aromatic hydrocarbons can affect CNS function postnatally. However, which PCB and CDF congeners, TCDD-like versus non-TCDD-like, are responsible for the neurotoxicity is uncertain.

Other effects of PCB exposure on neurodevelopment in human infants have recently been reviewed.[326] In Dutch children, exposure to PCBs, CDFs, and CDDs was associated with a higher incidence of hypotonia in the neonate, a lower psychomotor development index at 3 months of age, a less optimal neurological condition at 18 months of age, and lower cognitive scores at 42 months of age. Similarly, infants with the highest exposure levels in a North Carolina cohort showed hypotonia at birth. In addition, these infants were affected by hyporeflexia at birth, and delayed motor development up to 2 years of age but not when evaluated at a later age.[327,328]

Further research on the mechanisms of PCB-induced postnatal neurobehavioral and neurodevelopmental effects, dose–response relationships, and reversibility of the affected endpoints is needed to understand the role of TCDD-like PCB congeners versus non-TCDD-like PCB congeners in causing these effects in children. Mechanisms that respond to TCDD-like PCB congeners may not be involved, as three lightly chlorinated, ortho-substituted PCB congeners—PCB 28, PCB 47, and 2,2′,5,5′-TCB (PCB 52)—have been detected in monkey brain following dietary exposure to Aroclor 1016 and appear to be responsible for decreasing dopamine concentrations in the caudate, putamen, substantia nigra, and hypothalamus of these animals.[319] These noncoplanar PCB congeners are believed to cause this effect by acting through a mechanism that does not involve the AhR. On the other hand, results for

mice, rats, and monkeys suggest that in utero and lactational exposure to TCDD-like PCB congeners could be responsible for at least some, perhaps a unique subset, of the neurobehavioral effects reported in children.

Thermoregulation In adult rats TCDD-induced decreases in body temperature are associated with reduced serum thyroxin levels and a decrease in basal metabolism.[329,330] More recently, the offspring of rats exposed in utero and via lactation to a maternal diet that contained Aroclor 1254 were affected by decreased body core temperature, reduced metabolic rate, and marked reductions in serum thyroxin concentrations up to 14 days of age.[331] As part of a larger study male rats exposed to vehicle or 1 μg/kg of TCDD on GD 15 were castrated, for reasons unrelated to the study of thermoregulation, at approximately 8 months of age.[332] When evaluated at 15.4 to 17.7 months of age, TCDD-exposed animals exhibited significantly lower core body temperatures than controls when the ambient temperature was varied between 10 and 28°C. However, the metabolic rate was not affected by TCDD, indicating that the effector which regulates body core temperature during cold exposure was not impaired. In addition, in utero and lactational TCDD exposure had no effect on evaporative heat loss, or on skin blood flow when the rats were anesthetized so that this parameter could be measured. These results suggest that perinatal exposure to TCDD can decrease the core body temperature set point and cause a reduction in the regulated body temperature.

In a subsequent study, pregnant Long–Evans rats were administered a single maternal dose of vehicle or 1 μg TCDD/kg on GD 15, and their male offspring were implanted with transmitters to monitor core body temperature and motor activity.[333] At various ages the TCDD-exposed male offspring were affected by a nocturnal hypothermia that was accompanied by decreased motor activity. These effects were especially pronounced at 7 and 11 months of age, did not occur at 3 months of age, and were reduced at 16 months of age. In addition, TCDD-exposed animals exhibited a greater febrile response than that of vehicle-exposed control rats when challenged with a fever-inducing lipopolysaccharide. However, when 8-month-old rats were placed in a temperature gradient and allowed to select their own most favored ambient temperature, vehicle- and TCDD-exposed offspring selected the same ambient temperatures. This suggests that hypothalamic thermoregulatory centers are not altered permanently by TCDD in Long–Evans rats and that the effects on rats evaluated at 15 to 17 months of age were probably not caused by a change in body temperature set point.

When monitored by radiotelemetry, hamsters exposed to TCDD in utero and via lactation exhibit a persistent hypothermia despite normal metabolic responses to cold exposure.[334] In addition, there is no effect of TCDD exposure on the selection of an ambient temperature when hamsters are placed in a temperature gradient for 22 h. These results are important because the adult hamster has an unusually high resistence to the lethal and thyrotoxic effects of TCDD. However, the rat and hamster have approximately the same sensitivity

to perinatal TCDD-exposure-induced reproductive dysfunction and thermoregulatory dysfunction. The mechanisms for these responses have not yet been determined.

Night Vision Pregnant Long–Evans rats were exposed to the ortho-chlorinated PCB 47 and/or the coplanar PCB 77 on GDs 7 to 18. Daily doses of 1.5 mg PCB 47/kg per day, 1.5 mg PCB 77/kg per day, a combination of 1.0 mg PCB 47/kg per day + 0.5 mg PCB 77/kg per day, or an equivalent volume of vehicle were administered subcutaneously to each dam.[335] The effects of PCB exposure on visual processes were then assessed in male and female offspring at 200 days of age. The scotopic b-wave, maximum potential, and oscillatory potentials were recorded on the electroretinogram after the rats were adapted to the dark. Perinatal exposure to PCB 77 reduced the amplitudes of these potentials in female offspring in adulthood, but not in their male littermates. Exposure to PCB 47 alone was without effect; however, many of the decreases that resulted from PCB 77 appeared to be alleviated after simultaneous exposure to PCB 47. Although this suggests that functional antagonism between these ortho-substituted and coplanar PCBs can occur in the endpoints measured, it is also possible that this apparent antagonism resulted from the lower level of PCB 77 administered in the combination. These results indicate that in utero and lactational exposure to PCB 77, but not PCB 47 exposure, can produce long-lasting effects on night vision in female rat offspring.[335] Interestingly, the susceptibility to this effect was congener-specific, suggesting that the effect may be AhR-mediated. In addition, it was gender-dependent.

9.4 MALE REPRODUCTIVE TOXICITY

9.4.1 Reproductive Function and Fertility

When given to adult animals in doses sufficient to reduce feed intake and/or body weight, TCDD and related compounds decrease testis and accessory sex organ weights, cause abnormal testicular morphology, decrease spermatogenesis, and reduce fertility. Certain of these effects have been reported in rats, mice, guinea pigs, and monkeys treated with overtly toxic doses of TCDD, TCDD-like AhR agonists, or toxic fat that was discovered later to contain TCDD.[110,194,336–341] In the rat, a cumulative dose of 1 μg TCDD/kg per day administered 5 days a week for 13 weeks causes a 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, and similar effects have been found in other species.[336,337,339,340] This dosage regimen in the rat resulted in a TCDD body burden of approximately 20 μg/kg at the end of the dosing period,[342] and it significantly depressed feed consumption and body weight. A similar 13-week dosing study using adult male mice found that 3 and 30 mg of 3,3′,4,4′-

tetachloroazobenzene/kg per day caused reductions in epididymal sperm number.[343] In adult male Sprague–Dawley rats, a single dose of 25 μg TCDD/kg decreased epididymal sperm numbers, whereas testicular Leydig cell volume was decreased at 12.5 μg TCDD/kg. The decrease in Leydig cell volume is accounted for by a reduction in both the number and size of Leydig cells.[344] Daily sperm production was not affected even by 50 μg TCDD/kg.[345] Thus, the suppression of spermatogenesis and reduction in epididymal sperm number are not highly sensitive effects when AhR agonists are administered to sexually mature adult animals.

In contrast, daily sperm production assessed on PND 90 was decreased significantly in weanling Sprague–Dawley rats administered 1 μg TCDD/kg on PND 21.[346] In addition, testis histology revealed that 10 μg TCDD/kg caused a decrease in seminiferous tubule diameter compared to that in vehicle-dosed control rats. The spermatogonial population normally located in the basal area of the tubules was absent in the TCDD-treated rats. However, these effects on testis histology were not found at TCDD doses below 10 μg TCDD/kg. Motility studies were performed on epididymal sperm and dose-related decreases in sperm curvilinear velocity and beat cross frequency were found over the dose range from 0.1 to 5 μg TCDD/kg. Average path and straight-line velocity were decreased significantly at 5 μg TCDD/kg.[346]

Effects of TCDD administration to 21-day-old rats on epidermal growth factor receptor-, protein tyrosine kinase-, protein kinase A-, protein kinase C-, mitogen-activated protein 2 kinase-, and c-Src tyrosine kinase–mediated pathways in the testis were also examined.[346] Dose-related increases in the activity of c-Src kinase were found on PNDs 34 and 90 over the dose range from 0.1 to 5 μg TCDD/kg. In addition, the administration of multiple doses of the c-Src kinase inhibitor geldanamycin from PNDs 21 to 90 blocked the effects of TCDD on testis weight and daily sperm production. The authors concluded that these results provide evidence for the involvement of epidermal growth factor and c-Src kinase signaling pathways in the mechanism by which TCDD disrupts testicular development and function.

9.4.2 Alterations in Hormone Levels

Animals When TCDD is administered to adult male rats, the effects on male reproductive system endpoints are due in part to an androgenic deficiency. This deficiency is characterized in adult rats by decreased plasma testosterone and DHT concentrations, unaltered plasma LH concentrations, and unchanged plasma clearance of androgens and LH.[194,347–350] The ED_{50} of TCDD for producing this effect in adult male rats 7 days after dosing is 15 μg/kg,[194] and it can be detected within 1 day of treatment. The androgenic deficiency is believed to result from decreased testicular steroidogenic responsiveness to LH stimulation and increased pituitary responsiveness to feedback inhibition by androgens and estrogens.[347,350–353] Leydig cell volume was reduced significantly 4 weeks after a single dose of TCDD.[344] This effect was dose-related

and observed at the lowest dose, 12.5 μg TCDD/kg. It results from both a decreased number of Leydig cells and a reduced size of individual cells. It was subsequently established that these Leydig cell alterations could be prevented by the administration of human chorionic gonadotropin, an analog of LH.[354]

Humans The majority of data from epidemiological studies pertaining to effects of TCDD on the human male reproductive system have been collected from cohorts exposed as adults. Small decreases in serum testosterone concentration have been associated with adult occupational exposure to TCDD.[355] However, a clear association between serum TCDD concentrations and effect was most apparent in the data when differences in exposure patterns of individuals were considered by back-calculating serum TCDD concentrations to the time of exposure. When this was done, the lowest TCDD concentration in serum associated with decreased serum testosterone concentrations was 140 parts per trillion of TCDD.[324]

9.5 FEMALE REPRODUCTIVE TOXICITY

9.5.1 Reproductive Function and Fertility

TCDD and its approximate isostereomers affect female reproductive endpoints in a variety of animal studies.[1] Among the effects reported in monkeys, rats, and mice are reduced fertility, reduced litter size, structural malformations of the female gonads, and anovulatory menstrual/estrous cycles. In addition, TCDD exposure thwarts pregnancy maintenance and causes embryo/fetotoxicity. Some lines of AhR knockout mice exhibit difficulty in maintaining pregnancy and nursing offspring even in the absence of TCDD, suggesting that the AhR may play a role during pregnancy and/or in supporting lactation.

Monkeys Allen and colleagues evaluated the reproductive effects of dietary exposure to 50 or 500 ppt TCDD for approximately 9 months in rhesus monkeys.[92–95] In a series of studies, female monkeys exposed to 500 ppt showed obvious clinical signs of TCDD toxicity and lost weight throughout the period of evaluation. Following 7 months of exposure to 500 ppt TCDD, seven of the eight females were bred to unexposed males, but the remaining female showed such severe signs of toxicity that she was not bred, and five of the animals died within 1 year after exposure was initiated. Of the seven females evaluated for reproductive capability, only three were able to conceive and, of these, only one was able to carry her infant to term.[94] When females exposed to 50 ppt TCDD in the diet were bred at 7 months, two of eight females did not conceive and four of six that did conceive could not carry their pregnancies to term. As one monkey delivered a stillborn infant, only one conception resulted in a live birth.[95] Whereas the 500-ppt TCDD exposure level caused significant maternal toxicity, this was not the case at the lower dose. At 50 ppt TCDD the ability to

conceive and maintain pregnancy was reduced, but there was no apparent overt toxicity to the female.[93]

In a similar series of experiments, female rhesus monkeys were fed diets that contained 0, 5, and 25 ppt TCDD.[90,91] Reproductive function was not altered in the 5 ppt group, as seven of eight females mated to unexposed males after 7 months of dietary exposure to TCDD were able to conceive. Six of these females gave birth to viable infants at term and one gave birth to a stillborn infant. This was not significantly different from the control group fed a normal diet in which all seven monkeys gave birth to viable infants. At the 25-ppt dietary exposure level, however, only one of eight mated females gave birth to a viable infant. Since no serious health problems were exhibited by any females exposed to TCDD, the results in the 25- and 50-ppt groups show that TCDD exposure before and during pregnancy can cause fetal mortality in the monkey without producing overt toxic effects in the mother. These results with TCDD have been compared to a group of monkeys given dietary exposure to polybrominated biphenyls (PBBs, 0.3 ppm, Firemaster FF-1), in which seven of seven exposed females were able to conceive, five gave birth to live, normal infants, one aborted a mummified fetus, and one gave birth to a stillborn infant.[93]

McNulty[96] examined the effects of TCDD exposure during the first trimester of pregnancy (GDs 25 to 40) in the rhesus monkey. At a cumulative dose of 1 μg/kg given in nine divided doses, three of four pregnancies ended in abortion, two of which occurred in animals that displayed no overt maternal toxicity. At a cumulative dose of 0.2 μg/kg, one of four pregnancies ended in abortion, but this did not appear to be different from the control population. McNulty[96] also administered single 1-μg/kg doses of TCDD on GDs 25, 30, 35, or 40. The number of animals per group was limited to three, but it appeared that the most sensitive periods were the earlier periods, days 25 and 30, and that both maternal toxicity and fetotoxicity were reduced when TCDD was given on later gestational days. For all days at which a single 1-μg TCDD/kg dose was given (GDs 25, 30, 35, or 40), 10 of 12 pregnancies terminated in abortion. Thus, in 16 monkeys given 1 μg TCDD/kg in single or divided doses between GDs 25 and 40, only three normal births occurred.[96,97]

Rats The reproductive effects of exposure of male and female rats to relatively low levels of TCDD (0, 0.001, 0.01, and 0.1 μg/kg per day) were examined over three generations.[356] The results demonstrated exposure-related effects on fertility and litter size that were observed at 0.1 μg/kg per day in the F_0 generation and at 0.01 μg/kg per day in the F_1 and F_2 generations. Additionally, a 13-week exposure of nonpregnant female rats to 1 to 2 μg TCDD/kg per day resulted in anovulation and signs of ovarian dysfunction, as well as suppression of the estrous cycle.[337] The latter signs appeared to be reflected in the Murray et al.[356] study by an increased time between first cohabitation and delivery. However, at exposures of 0.001 to 0.01 μg/kg per day in an additional 2-year study, no effects on the female reproductive system were found.[357]

Effects of AhR Knockout on Female Reproduction Reproductive success is adversely affected in some AhR-null mouse lines (in the absence of TCDD exposure). AhR-null female mice[36] become pregnant at similar rates and implant similar numbers of embryos as control females. However, these AhR-null dams experience increased prenatal loss of conceptuses, difficulty in surviving the stress of lactation, and their pups show poor survival during lactation and shortly after weaning.[23] In contrast, offspring from a different AhR-null mouse line[37] exhibit low neonatal mortality. Possible reasons for this and other phenotypic differences between offspring of these two AhR-null mouse lines is unclear.[37]

9.5.2 Ovarian Function

The ability of TCDD to cause anovulation and reproductive cycle suppression in rats and monkeys was reviewed previously.[1] However, Li et al.[74,358] extended these findings. In the first study, adult female rats given a single oral dose of 10 μg TCDD/kg exhibited a 75% reduction in the number of ovulations per female and number of females ovulating.[74] In addition, the estrous cycle was altered by a significant increase in the time spent in diestrus with a corresponding decrease in proestrus and estrus. However, these findings were accompanied by overt toxicity to the females as evidenced by body weight loss. A subsequent TCDD dose response study in immature hypophysectomized chorionic gonadotropin-primed female rats showed that the inhibitory effects on ovulation were dose dependent but occurred only at exposure levels associated with significant body weight loss.[358] In rats that were hypophysecetomized on PND 23 and administered TCDD or vehicle on PND 26, hCG-stimulated ovulation was inhibited by TCDD.[359] However, in this model, there was no effect of TCDD on ovarian steroidogenesis in response to hCG. In addition, it appeared as though the TCDD-induced inhibition of ovulation was associated with a failure of the follicles to rupture in response to exogenously administered hCG. These results suggest that inhibition of ovulation in the hypophysectomized rat administered gonadotropins is due to effects of TCDD exerted directly on the ovarian follicles. In intact immature rats, TCDD delays ovulation that has been primed by exposure to equine chorionic gonadotropin.[360] This indicates that TCDD can disrupt the hypothalamus–pituitary–ovary axis during proestrus. Therefore, TCDD inhibits ovulation by both direct and indirect effects on the rat ovary.

9.5.3 Antiestrogenic Action

Estrogens are necessary for normal uterine development and for maintenance of the pregnant and nonpregnant adult uterus. The cyclic production of estrogens partially regulates the cyclic production of FSH and LH that results in the estrous or menstrual cycling of female mammals. Any effect that causes a decrease in circulating or target cell estrogen levels can alter hormonal balance

and action. Antiestrogenic effects of TCDD in female rats include a decrease in uterine weight, decrease in uterine peroxidase activity, and a decrease in the concentration of progesterone receptors in the uterus.[361] In addition, when TCDD and 17β-estradiol are coadministered to the same female rat, the antiestrogenic action of TCDD diminishes or prevents 17β-estradiol-induced increases in uterine weight, peroxidase activity, progesterone receptor concentration, and expression of EGF receptor mRNA.[361,362] Similarly, in mice TCDD administration decreases uterine weight and antagonizes the ability of 17β-estradiol to increase uterine weight.[363] This ability of TCDD to inhibit estrogen-induced uterine epithelial proliferation in the mouse was shown recently to depend entirely on stromal AhR; AhR located in the uterine epithelium was discovered not to be involved in this antiestrogenic effect of TCDD.[70] In cultured MCF-7 and T47D cell lines TCDD inhibits estrogen-induced alterations in cell proliferation, postconfluent focus production, and glucose–lactate conversion.[364] Estrogenic biochemical endpoints antagonized by TCDD in cultured MCF-7 cells include 17β-estradiol-induced increases in the expression of nuclear estrogen receptors, progesterone receptors, and cell cycle enzymes, and increases in the secretion of cathepsin D, pS2, and tissue plasminogen activator.[365–369] Interestingly, the interplay between the estrogen receptor and AhR pathways can be bidirectional. TCDD-induced induction of CYP1A1 is inhibited by 17β-estradiol in MCF-7 human breast cancer cells but not in Hep-3B human liver cells.[370]

Two prominent mechanisms that have been invoked to explain the ability of TCDD to antagonize estrogenic activity are a downregulation of estrogen receptors and increased metabolism of estrogen due to AhR-mediated enzyme induction within the target cell. However, some studies have not found a downregulation of estrogen receptors in MCF-7 cells exposed to TCDD,[371] whereas antiestrogenic responses can be induced by AhR agonists other than TCDD at concentrations that do not cause CYP1A1 induction.[364,372,373] Therefore, other mechanisms have been investigated. These include interaction of the AhR at DREs or other sites on DNA which overlap with the promoter regions of estrogen-regulated genes,[368,369,374] and competition between the AhR and estrogen receptor for coactivators such as SRC-1 and RIP 140.[368,375]

9.5.4 Endometriosis

Humans Endometriosis is characterized by endometrial cell growth outside the uterus and can be associated with infertility and pain. A critical review of the literature regarding the relationship between dioxinlike congeners and endometriosis has recently been published.[376] Of increasing interest is the initial report that women with endometriosis in Germany are more likely to have elevated concentrations of PCBs in their blood.[377] Although this report did not provide sufficient methodological detail [reviewed in Ref. 378], Koninckx and co-workers[379] reported that Belgium also has a high incidence of endometriosis and that TCDD concentrations in breast milk in Belgian women are among the

highest in the world. Similarly, a larger number of women in Israel with endometriosis were found to have measurable blood levels of TCDD compared to age-matched control women that had tubal infertility but no endometriosis.[380] More recently in Belgian women, high-serum TCDD-like toxic equivalent concentrations (TEQs) were associated with a greater risk for endometriosis,[381] but no association was found between endometriosis and total serum PCB concentrations in this study. This suggests that only TCDD-like PCBs may be capable of producing the response in women. However, data that might correlate an increased incidence of endometriosis with TCDD exposure at Seveso, where some women were heavily exposed, are currently being awaited.[382]

A recent study has demonstrated the occurrence of certain TCDD-induced biochemical changes that facilitate the ectopic growth of human endometrial tissue.[383] When the human tissue is exposed to TCDD in vitro and implanted into immunologically impaired nude mice, TCDD exposure inhibits the ability of progesterone to decrease the expression of matrix metalloproteinase enzymes. This effect, which is associated with a TCDD-induced decrease in the ability of human endometrial organ cultures to produce TGFβ_2, enhances ectopic growth of the endometrial lesions. These results begin to provide a biochemical basis for the ability of TCDD exposure to facilitate the expression of endometriosis in women, and they strengthen the association between elevated exposure to TCDD-like AhR agonists and the increased incidence and severity of this disease.

Monkeys An association between TCDD exposure and endometriosis has found some experimental support in studies using the rhesus monkey. However, the association between PCB exposure and endometriosis in monkeys is less clear. Rier and co-workers chronically exposed rhesus monkeys to TCDD in their diet for 4 years and then maintained the monkeys for an additional 10 years. These monkeys were then compared to similar unexposed animals in the same colony.[384,385] In monkeys exposed to dietary levels of 5 and 25 ppt TCDD, the incidence of endometriosis was 43 and 71%, respectively, whereas the incidence in control monkeys was 33%. Moreover, the severity of endometriosis was TCDD dose-dependent. Monkeys in the studies by Rier and co-workers appeared to be quite sensitive to TCDD-induced increases in the incidence and severity of endometriosis. It has been calculated that the female monkeys exposed to 5 ppt TCDD in the diet for 4 years had accumulated a TCDD body burden of 69 ng/kg.[324] However, another study found no association between the incidence and severity of endometriosis and exposure to Aroclor 1254 when rhesus monkeys were exposed for up to 6 years. Unlike the Rier studies, these monkeys were not held for evaluation a long time after exposure.[386] Interestingly, both the Rier and Arnold studies reported a similar high background incidence (33 to 37%) of endometriosis in unexposed monkeys. When taken together, the results of these studies indicate that it may take some time for a TCDD-induced increase in endometriosis to become manifest

above the background level, that sensitivity to halogen aromatic hydrocarbon-induced increases in endometriosis may be more readily detected when TCDD equivalent concentrations (TEQs) rather than total PCB concentrations are considered, and that the effect, if produced by PCBs at all in monkeys, could be PCB congener-specific. In this last sense, the results in monkeys correspond to those recently obtained by Pauwels et al.[381] in women, which also suggest that the effect on endometriosis is congener specific for those halogenated aromatic congeners with AhR agonist activity.

Rats and Mice An animal model has been developed in the rat and mouse to evaluate the effects of TCDD exposure on the development of endometriosis.[387,388] Although rodents do not develop endometriosis spontaneously, the surgical implantation of uterine tissue at ectopic sites in the abdominal cavity is a way of mimicking aspects of the disease. The formation of clear vesicles, fibrosis, inflammation, and adhesions are common to the disease in primates and to the rodent model of endometriosis.[388] Female rats and mice were administered 0, 3, or 10 μg TCDD/kg 3 weeks before, at the time of, and at 3, 6, and 9 weeks after surgery to induce endometriosis.[388] When evaluated at 3, 6, 9, and 12 weeks following surgery, dose-dependent increases in lesion diameter occur in both species if data from all time points are pooled. In addition, rats showed a decrease in body weight and ovarian weight at 9 and 12 weeks, accompanied by an increase in the time spent in vaginal estrus, and histology of the ovary at 12 weeks indicated ovulatory arrest. These effects on body weight and the ovary were not observed in the mouse, but the mouse was more susceptible to the TCDD-induced increase in lesion diameter than the rat at 9 and 12 weeks after the surgical implantation of tissue.[388] Mice were also susceptible to an enhancement of surgically induced endometriosis when they were exposed to TCDD in utero and via lactation beginning on GD 8.[389]

Additional studies done to assess the effects of TCDD exposure on surgically induced endometriosis in the mouse used a different model. Mice were first subjected to surgery to induce endometriosis, and then exposed chronically to daily doses of 0, 10, 50, or 100 ng TCDD/kg for 28 days. At 2 days after the last dose there was a dose-dependent decrease in lesion diameter.[390] In addition, uterine tissue implant survival and growth was decreased in ovariectomized mice and restored by estrogen replacement.[391] Exposure to TCDD inhibited the ability of estrogen replacement to promote implant survival and growth, suggesting that TCDD acted as an antiestrogenic compound. The authors attributed the differences between their results and those of Cummings' group to immune suppression which occurs when TCDD is administered prior to the surgical induction of endometriosis and facilitates implant growth; whereas the antiestrogenic effects of TCDD initially inhibit lesion growth when TCDD is administered after surgically induced endometriosis is established. Alternatively, they proposed that factors of ovarian origin other than estrogens, that are affected by the surgical procedure, may play a role in the establishment, maintenance, and growth of the uterine tissue implants. Despite these

suggestions, it seems possible that insufficient time is allowed when this model is used for the severity of surgically induced endometriosis to be increased by TCDD exposure subsequent to the initial inhibition. Similar to the mouse, the severity of surgically induced endometriosis is increased by TCDD in cynomologus monkeys 1 year after surgery.[392] This demonstrates that the increase in surgically induced endometriosis can be produced in more than one species and that it may take some time to develop. However, in agreement with the notion that the TCDD-induced increase in the incidence and severity of surgically established endometriosis may be related to an inhibition of immune function; such a relationship between TCDD-induced endometriosis and immune system dysfunction has been hypothesized for the increased severity of spontaneously occurring endometriosis in humans and monkeys.[393]

The potent TCDD-like AhR agonists TCDD and 2,3,4,7,8-pentachlorodibenzofuran increased lesion diameter in the mouse model. In addition, a similar effect that did not reach statistical significance with the number of animals evaluated was seen with the TCDD-like AhR agonist, PCB 126. In contrast, PCB 153 and 1,3,6,8-tetrachlorodibenzo-*p*-dioxin, which are not potent AhR agonists, did not alter lesion diameter or weight.[394] This is reminiscent of the results in humans and monkeys, in which the increases in endometriosis severity were correlated with serum TCDD toxic equivalent concentrations (TEQs) but not with total serum PCBs. Interestingly, the dose–response relationship for TCDD in the mouse model was U-shaped, with low doses promoting endometriosis and larger doses resulting in a decreased response.[388] This again suggests that either the antiestrogenic effects of TCDD, or some other effect on ovarian function may play a role, but when the TCDD is administered prior to the surgical induction of endometriosis, relatively large doses are required for the inhibition of lesion growth to occur.

9.5.5 Mammary Gland

Effects of Postnatal TCDD Exposure Oral administration of 2.5 μg TCDD/kg per day to female rats on PNDs 24, 26, 28, and 30 results in decreased cellular proliferation within the mammary gland and decreased mammary gland development.[395] While body weight was slightly but not significantly reduced 18 h after the last TCDD dose, the combined uterine–ovarian weights were less than half, and mammary gland size was only 61% that of vehicle-treated control rats. TCDD treatment caused a significant 59% reduction in the number of TEBs without significantly affecting the numbers of alveolar buds, lobules, and terminal ducts. Therefore, the postnatal TCDD-induced inhibition of mammary growth is accompanied by a selective size reduction within the most rapidly dividing portion of the mammary ducts, the TEBs. Although TCDD did not decrease the percentage of TEB cells that are proliferating (PCNA labeling index) or percentage of TEB cells in S-phase, it decreased these parameters in terminal ducts and lobules. Consistent with the decrease in the number of TEBs in TCDD-treated rats, the total numbers

of PCNA-labeled and S-phase cells were decreased (even though percentages were not) compared to the values obtained in vehicle-exposed control rats. In addition, TCDD exposure decreased the total numbers of PCNA-labeled and S-phase cells in terminal ducts and lobules. These results are consistent with the finding that early postnatal TCDD administration in the rat causes an inhibition of mammary epithelial cell proliferation, but the mechanism for this effect remains to be determined. It may be a consequence of the antiestrogenic properties of TCDD.[361,396]

Response of Mammary Organ Culture Interestingly, the effects of the AhR-null mutation on mammary gland development in vivo and direct AhR activation by TCDF in vitro turn out to be similar rather than opposite (see Section 9.3.3). Nulliparous C57Bl/6J mice were primed with 15 daily injections of estradiol and progesterone.[55] Mammary glands were removed 24 h after the last priming and cultured in the presence of 0.1% DMSO or 1 to 100 n*M* 2,3,7,8-TCDF for 5 days. Lobule size after culturing was suppressed by TCDF in a dose-related manner, such that lobules in mammary glands exposed to the largest dose of TCDF were less than half the size of vehicle-exposed lobules. The [^{3}H]thymidine labeling index was also reduced in the TCDF-exposed lobules. Therefore, the growth and development of TEBs into lobules appeared to be suppressed by TCDF. However, the effects of TCDF on TEB number after organ culture were not reported, so it is not known whether TCDF caused the expected decrease in number of TEBs. These results are consistent with the effects of TCDD on mammary growth and development in vivo in the rat. In addition, they suggest that TCDD-like AhR agonists can act directly on the mammary gland.

9.6 SENSITIVITY OF DEVELOPMENTAL AND REPRODUCTIVE ENDPOINTS

9.6.1 TCDD LOAELs

In utero and lactational exposure of laboratory animals to TCDD causes functional alterations, structural malformations, and mortality of offspring. Of these responses, functional alterations are the most sensitive. Table 9.5 shows, in rank order, the lowest observable adverse effect levels (LOAELs) of TCDD for developmental and reproductive endpoints in several laboratory animal species based on maternal TCDD body burden. Rats and rhesus monkeys are the most sensitive laboratory animals to the adverse developmental and reproductive effects of TCDD, followed by the guinea pig, hamster, and mouse. Rats are sensitive to both developmental and reproductive toxicity, where the most sensitive effect observed is a behavioral endpoint. Rhesus monkeys are sensitive both to developmental toxicity as indicated by the impairment in object learning and to reproductive toxicity as shown by the increased incidence and

TABLE 9.5 TCDD LOAELs for Developmental and Reproductive Endpoints

Endpoint	Species	Maternal TCDD Dose	Maternal TCDD Body Burden (ng/kg)	Refs.
Altered operant responding	Female rat	10 ng/kg on GD 18 (BMD, ED_{10})	10	316
Impaired object learning	Monkey	0.151 ng/kg per day × 16.2 months	42	95, 324
Accelerated eye opening	Male rat	50 ng/kg on GD 15	50	83
Decreased ejaculated sperm count	Male rat	50 ng/kg on GD 15	50	83
Decreased daily sperm production	Male rat	64 ng/kg on GD 15	64	196
Decreased epididymis weight	Male rat	64 ng/kg on GD 15	64	196
Decreased prostate weight	Male rat	64 ng/kg on GD 15	64	181
Demasculinized sexual behavior	Male rat	64 ng/kg on GD 15	64	195
Increased endometriosis	Female monkey	0.151 ng/kg per day × 4 years	69	324, 384
Feminized sexual behavior	Male rat	160 ng/kg on GD 15	160	195
Delayed preputial separation	Male rat	200 ng/kg on GD 15	200	183
Vaginal thread malformation	Female rat	200 ng/kg on GD 15	200	218
Hypospadias	Female rat	200 ng/kg on GD 15	200	218
Increased offspring mortality	Monkey	0.76 ng/kg per day × 4 years	345	324, 397
Decreased birth weight	Male rat	400 ng/kg on GD 15	400	181
Decreased testis weight	Male rat	400 ng/kg on GD 15	400	181
Altered working memory	Male rat	100 ng/kg per day on GDs 10–16	640	310
Cleft phallus	Female rat	800 ng/kg on GD 15	800	218
Premature reproductive senescence	Female rat	1000 ng/kg on GD 8	1000	7
Constant estrus	Female rat	1000 ng/kg on GD 8	1000	7
Cystic endometrial hyperplasia	Female rat	1000 ng/kg on GD 8	1000	7
Increased offspring mortality	Rat	1000 ng/kg on GD 15	1000	201
Increased prenatal mortality	Guinea pig	1500 ng/kg on GD 14	1500	87

TABLE 9.5 *(Continued)*

Endpoint	Species	Maternal TCDD Dose	Maternal TCDD Body Burden (ng/kg)	Refs.
Decreased ejaculated sperm counts	Male hamster	2000 ng/kg on GD 11	2000	8
Impaired reproductive function	Female hamster	2000 ng/kg on GD 11	2000	8
Hydronephrosis	Mouse	500 ng/kg per day on GDs 6–15	3800	398
Cleft palate	Mouse	1000 ng/kg per day on GDs 6–15	7700	174
Increased prenatal mortality	Mouse	24,000 ng/kg on GD 6	24,000	108

severity of endometriosis. These effects were observed in rhesus monkeys after prolonged exposure to TCDD that resulted in significant bioaccumulation of TCDD in the animal. The TCDD exposure regimen used for rats as well as for hamsters, guinea pigs, and mice was different than that for monkeys. Typically, it consisted of either a single dose or daily doses of TCDD being administered during pregnancy. Like the monkey studies, TCDD exposure of offspring was typically in utero and via lactation until weaning.

A cross-species comparison of TCDD LOAELs for developmental and reproductive endpoints, based on the maternal TCDD body burden in these species is shown in Table 9.5. TCDD LOAELs < 100 ng/kg were observed for two endpoints in rhesus monkeys, impaired object learning and increased endometriosis, and in rat offspring for male reproductive system developmental effects, including reduced sperm counts, decreased epididymis and prostate weights, and demasculinized sexual behavior. In addition, a BMD type of analysis resulted in an ED_{10} value of only 10 ng/kg for altered operant responding in female rats. TCDD LOAELs of 100 to 500 ng/kg were observed in male rat offspring for feminization of sexual behavior, delayed preputial separation (which is an index of the onset of puberty), decreased testis weight, and decreased birth weight, and in female rat offspring for hypospadias and for the vaginal thread malformation. The latter malformation is a unique effect of in utero TCDD exposure in female rat offspring. It has not been reported to occur in female offspring of other lab animal species or humans, and no structural malformations of the external genitalia have been found in TCDD-exposed male rat, hamster, or mouse offspring. In addition, the TCDD LOAEL for increased offspring mortality in the rhesus monkey, 345 ng/kg, is the lowest of all common laboratory animal species studied. TCDD LOAELs ranging from maternal body burdens of 500 to 1000 ng/kg have been reported

in rats for altered working memory in male offspring and for an increased incidence of cleft phallus, premature reproductive senescence, constant estrus, and cystic endometrial hyperplasia in female offspring. The TCDD LOAEL for increased offspring mortality in the rat, 1000 ng/kg, is three times higher than that for the monkey. TCDD LOAELs of 1000 to 2000 ng/kg have been observed for increased prenatal mortality in guinea pigs, reduced ejaculated sperm numbers in male hamster offspring, and impaired reproductive function in female hamster offspring. In contrast to the other species, where functional alterations have been assessed as a consequence of in utero and lactational TCDD exposure, in the mouse the historical focus has been primarily on structural malformations which are less sensitive endpoints. Accordingly, TCDD LOAELs $>$ 2000 ng/kg have been observed in the mouse for the classic TCDD teratogenic endpoints in this species, hydronephrosis and cleft palate. The TCDD LOAEL for increased prenatal mortality in the mouse is among the highest of the common laboratory animal species, 24,000 ng/kg.

9.6.2 Human Susceptibility

In a number of different exposure incidents, human fetuses and neonates have been exposed to PCBs, CDFs, and/or CDDs. Children have been affected by exposure level–related decreases in neurobehavioral development. However, due to the simultaneous exposure to multiple substances, these effects may not have necessarily been caused by TCDD-like AhR agonists, and no body burdens have been included for these effects in Table 9.5. However, the sensitivity of monkeys to at least one form of TCDD-induced developmental neurobehavioral toxicity suggests that TCDD-like AhR agonists in the mixtures to which children have been exposed either in utero or in utero and via lactation have the potential to play a role in causing the effects observed. Alterations in neurobehavioral development have been reported at lower TCDD body burdens in the monkey than in the rat, and this may be a function of differences in relative brain size and CNS complexity between the two species. Therefore, it is possible that neurobehavioral development in children may be adversely affected by maternal body burdens of TCDD and related AhR agonists that are not more than 10 to 100 times higher than the average background body burden of TEQs in adult humans in Western industrialized countries of about 6 to 8 ng TEQ/kg. The possibility also exists for the expression of other sensitive endpoints, such as reduced ejaculated sperm counts and endometriosis in humans as a consequence of exposure to TCDD and related chemcials. Since these TCDD endpoints have been observed in at least two laboratory animal species, people may be susceptible to these effects of TCDD.

9.6.3 Maternal and Fetal TCDD Body Burdens

Maternal and fetal body burdens associated with single doses of TCDD administered to pregnant Long–Evans rats on GD 15 are shown in Table 9.6.

TABLE 9.6 Relationship between Maternal and Fetal TCDD Body Burdens in Pregnant LE Rats[a]

Maternal TCDD Dose on GD 15 (ng/kg)	TCDD Body Burden (ng/kg)			
	GD 16		GD 21	
	Maternal	Fetal	Maternal	Fetal
50	31 ± 3	5 ± 1 (16%)[b]	27 ± 3	4 ± 1 (15%)[b]
200	97 ± 23	13 ± 4 (13%)	76 ± 17	15 ± 6 (20%)
800	523 ± 30	39 ± 5 (7%)	328 ± 59	32 ± 5 (10%)
1000	585 ± 98	56 ± 19 (10%)	431 ± 60	36 ± 9 (8%)

[a] Values are mean ± SE from Hurst et al.[399] rounded to the nearest 1 ng/kg.

[b] Fetal body burden as percent of maternal body burden on same GD.

The results indicate that increases in fetal body burdens on GDs 16 and 21 are directly proportional to increases in maternal body burdens on these days.[399] In addition, the fetal body burden when expressed as a percentage of the maternal body burden on each of these GDs, is dependent on the maternal body burden. At maternal TCDD body burdens less than about 100 ng/kg, the fetal body burden is 13 to 20% of the maternal body burden. On the other hand, at maternal TCDD body burdens greater than about 300 ng/kg the fetal body burden is only 7 to 10% of the maternal body burden on GDs 16 and 21. This distinction is important. It illustrates in the rat that the fetal body burden, when expressed as a percent of the maternal body burden, tends to be higher at low maternal TCDD body burdens. Whether this is the same for other species, including humans, remains to be determined.

The most sensitive adverse reproductive and developmental effect in Long–Evans (LE) rat offspring is a reduction in ejaculated sperm counts with a TCDD LOAEL of 50 ng/kg. This maternal body burden is associated with a fetal body burden of approximately 4 to 5 ng TCDD/kg, which is similar to the mean background body burden in adult humans in Western industrialized countries of 6 to 8 ng TEQ/kg. In addition, concentrations of TCDD found in the developing urogenital tract of the LE rat fetus on GD 21 were significantly greater than those found in the whole body of the fetus.[217] Thus, fetal urogenital tract tissues of the rat, which are among the most sensitive to adverse developmental effects of TCDD (Table 9.5), accumulate some of the highest TCDD tissue concentrations in the rat fetus.

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CHAPTER 10

Effects of Polychlorinated Biphenyls on Neuronal Signaling

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10.1 INTRODUCTION

The neurochemical and behavioral sequelae of developmental and adult exposure to polychlorinated biphenyls (PCBs) and dioxins was previously reviewed in 1994.[1] Unlike that review, in this chapter we focus on recent findings that PCBs, to a large extent, alter neurochemical function by influencing intracellular calcium concentrations ($[Ca^{2+}]_i$) and the consequences of these changes on neurotransmitter function, oxidative stress, and cell death. However, before beginning, there are two questions that should be addressed that will hopefully aid the reader in understanding both the choice of material to review and how these data fit into the larger context of the neurotoxicity of PCBs and related halogenated aromatic hydrocarbons (HAHs).

First, what important changes have transpired since the previous review was written? Although there have been numerous articles describing the behavioral consequences of developmental exposure to PCBs,[2,3] including the effects of exposure to individual PCB congeners;[4–6] the greatest progress (i.e., changes since the last review) has been made in understanding the potential mechanisms by which PCBs alter central nervous system (CNS) function. These more recent studies, using almost exclusively in vitro techniques, have demonstrated that (1) PCBs alter important neurotransmitters, including dopaminergic and cholinergic systems,[7–10] and (2) the majority of these effects are due to non-dioxin-like PCB congeners.[7,11–13] The need for understanding the mechanisms of action of these two major classes of PCB congeners on the CNS has become even more important since behavioral responses in offspring of rats developmentally exposed to either of these structurally disparate

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groups of HAHs differ—coplanar congeners decrease the number of errors in a maze task (noncoplanar congeners were without effect).[14] While in a T-maze delayed spatial alternation task, noncoplanar congeners increased the number of errors and coplanar congeners were without effect.[4,14]

Second, the question of why the majority of studies reviewed here focus on PCBs rather than dioxins is easier to answer than the previous question—there is simply a paucity of articles that have demonstrated that coplanar HAHs are active in vitro![15,16] Therefore, the consequences of exposure to PCBs are discussed with reference to dioxins and/or furans only when the available literature on these compounds either substantially adds to the knowledge of the consequences of exposure to noncoplanar PCB congeners or is in conflict with existing data.

10.2 IN VITRO VERSUS IN VIVO: THE QUESTION OF EXTRAPOLATION

The studies reviewed briefly have almost exclusively used in vitro techniques; thus, it is relevant, particularly for those readers who are most familiar with whole animal or epidemiological studies, to discuss some of the strengths and weaknesses associated with this technique. The greatest strength of in vitro techniques is that it allows the investigator to examine the actions of an agent in a much less complex system than is possible using in vivo procedures, thereby permitting the investigator to study toxicant actions in an isolated test system. In vitro techniques also allow for extensive control over variables that are difficult or impossible to control when using in vivo procedures (e.g., dose, timing, and duration of exposure to the active agent(s) or toxicant(s) of interest). In vitro techniques also allow the investigator (under circumstances where the preparation has minimal metabolic capacity) to examine the actions of the parent compound. As a consequence of this control, the investigator may have a greater opportunity to examine, and hopefully, begin to understand, a particular toxicological mechanism(s) of action of the parent compound.

The primary weakness of the in vitro technique is, not surprisingly, related to the extent to which the data can be extrapolated either to the whole animal or to the human, and in many cases, represents the "flip side" of the strengths described above. Thus, the ability to isolate and study simple systems in vitro often limits the ability to extrapolate these findings to the complex, interacting systems that exist in vivo. An example of this limited ability to extrapolate from in vitro to in vivo is the aforementioned lack of activity of coplanar HAHs in in vitro neuronal preparations and the neurochemical and behavioral data demonstrating the ability of these agents to alter CNS function when exposure occurs during development.[14,17] Often in vitro techniques use either continuous or primary cell cultures that limit the ability to investigate special windows of increased vulnerability, such as those seen during development. Furthermore, in many in vitro studies, the concentrations of the contaminants

are considerably higher than the toxicant body burdens seen either in animal studies or in the limited epidemiological studies that have determined contaminant body burdens. This discrepancy in doses often raises concerns (among those who are either epidemiologists or those who use in vitro techniques) that the findings may have little relevance in the "real" world.

10.3 PCBs ALTER INTRACELLULAR NEURONAL CALCIUM

10.3.1 Why Calcium?

One of the most active areas of PCB research since the previous review concerns the effects of noncoplanar PCB congeners on $[Ca^{2+}]_i$ in various neuronal preparations.[18–22] There are several reasons for this emphasis, related primarily to the key role that $[Ca^{2+}]_i$ plays in regulating neuronal function. First, transient elevations in $[Ca^{2+}]_i$, induced by depolarization, and involving entry of extracellular calcium, are necessary for the exocytotic release of the majority of neurotransmitters.[23] Thus, toxicant-induced changes in $[Ca^{2+}]_i$ may influence the exocytotic release of neurotransmitter(s) and the normal transfer of information between neurons. Second, alterations in $[Ca^{2+}]_i$ influence important second messenger systems, including cyclic adenosine monophosphate (AMP) and protein kinases,[24] involved either in additional transfer of information between neurons or in regulating cellular processes, including protein synthesis.[25,26] Third, particularly during development, alterations in calcium regulation influence neuronal growth and synaptogenesis.[27] Thus, a better understanding of the role that PCBs and related HAHs play in altering $[Ca^{2+}]_i$ will aid in determining some of the mechanisms by which these well-studied neurotoxicants influence neuronal function and ultimately behavior. Finally, although transient elevations in $[Ca^{2+}]_i$ are required for neurotransmitter release, prolonged elevations in $[Ca^{2+}]_i$ influence mitochondrial function and are linked with neuronal injury and the induction of processes that ultimately result in cell death.[28]

10.3.2 Noncoplanar PCB Congeners Elevate Intracellular Calcium by an IP_3 Mechanism

The early studies examining the consequences of PCBs on intracellular calcium were conducted by Kodavanti and his colleagues at the U.S. Environmental Protection Agency (USEPA). In the first of a series of papers,[18] these authors exposed cultured rat cerebellar granule cells, obtained from early postnatal rats, to one of two PCB congeners: a noncoplanar congener [2,2′-dichlorobiphenyl (2,2′-DCB)] and a coplanar congener (3,4,5,3′,4′-pentachlorobiphenyl) at concentrations ranging from 5 to 100 μM. These congeners were examined, to the best of my knowledge, because of prior research[7] demonstrating, respectively, their neurochemical activity, or lack thereof.

Changes in $[Ca^{2+}]_i$, calcium sequestration into mitochondria and microsomes and calcium extrusion from synaptosomes isolated from adult rat cerebellum were determined. 2,2′-DCB, at concentrations greater than 50 μ*M*, significantly elevated $[Ca^{2+}]_i$ while the coplanar congener was significantly less active. In addition, the authors demonstrated that 2,2′-DCB, but not 3,4,5,3′,4′-pentachlorobiphenyl, at concentrations greater than 5 μ*M*, decreased $^{45}Ca^{2+}$ uptake into cerebellar mitochondria and microsomes, providing a potential explanation for the elevations in $[Ca^{2+}]_i$ reported. The significance of these changes, given the high concentrations of 2,2′-DCB needed to alter $[Ca^{2+}]_i$, raise a number of questions that are addressed below.

The relationships of changes in $[Ca^{2+}]_i$ and other intracellular signaling molecules were next addressed by Kodavanti et al.,[11] who demonstrated that (1) both Aroclor mixtures (Aroclors 1016, 1254, and 1260) and many noncoplanar-substituted PCB congeners increased [^{3}H]phorbol ester ([^{3}H]PDBu) binding in rat cerebellar granule cells, a measure of activation and/or translocation of protein kinase C (PKC), and (2) this translocation was dependent on the presence of normal concentrations of extracellular calcium. The authors also determined a structure–activity relationship (SAR) for [^{3}H]PDBu binding in which only noncoplanar PCB congeners were active (Table 10.1). This SAR is remarkably similar to one first described by Shain et al. in 1991[7] based on reductions in pheochromocytoma (PC12) cell dopamine (DA) content (Table 10.1). The almost exact "overlay" between these two SARs, although based on different biochemical measures, suggests that alterations in either intracellular calcium or "downstream" events [e.g., altered phosphorylation of tyrosine hydroxylase (TH), the rate-limiting enzyme in the synthesis of DA], including alterations in second messenger systems, may influence cellular neurotransmitter content.

Shafer et al.[29] exposed cerebellar granule cells to 2,2′-DCB and measured inositol phosphate (IP_3) accumulation under both basal and carbachol-stimulated conditions. IP_3 was measured because stimulation of IP_3 receptors located on the endoplasmic reticulum releases calcium from intracellular stores.[30] 2,2′-DCB increased significantly both basal IP_3 accumulation and $[Ca^{2+}]_i$ that were dependent on extracellular calcium while inhibiting carbachol-stimulated IP_3 accumulation. The authors concluded that 2,2′-DCB-induced IP_3 accumulation was calcium dependent, but independent of PKC activation.

Mundy et al.[19] reviewed the findings above and concluded that (1) noncoplanar-substituted PCB congeners increased $[Ca^{2+}]_i$ in cerebellar granule cells, (2) the elevations were dependent on extracellular calcium, (3) these elevations were due to inhibition of sequestration of cytosolic calcium, and (4) the increased binding of IP_3 to receptors on cerebellar microsomes resulted in either enhanced release of microsomal calcium stores or further inhibition of cytosolic calcium sequestration.

More recently, Inglefield and Shafer[22,27] demonstrated that exposure of developing neocortical cells to Aroclor 1254 dose-dependently increased the

TABLE 10.1 Three Approaches to PCB SARs: EC_{50} Values for PCB Congeners Determined by PC12 Cell Cellular Dopamine Content,[a] Cerebellar Granule Cell [^{3}H]Phorbol Ester Binding,[b] and Brain or Muscle Sarcoplasmic/Endoplasmic Reticulum RyR/Ca^{2+}-Release Channel [^{3}H]Ryanodine Binding[c]

PCB Congener		EC_{50} (μM)			
				RyR/Ca^{2+}-Release Channel	
BZ No.	Structure	PC12 Cells	Cerebellar Granule Cells	Brain	Skeletal
4	2,2′	64	43	34.3	
11	3,3′	195	60		
14	3,5	> 201	74		
15	4,4	NEO[d]	NEO[e]		
28	2,4,4′	196	> 100		
47	2,4,2′,4′	115	89		
50	2,4,6,2′	71	41		
52	2,5,2′,5′	86	28	52.1	
54	2,6,2′,6′	NEO[d]	NEO[e]		
66	2,4,3′,4′	> 201		Inactive[f]	
70	2,5,3′,4′			166	
77	3,4,3′,4′	NEO[d]	NEO[e]		
82	2,3,4,2′,3′				1.2
88	2,3,4,6,2′			89.3	
95	2,3,6,2′,5′			17.1	0.33
103	2,4,6,2′,5′	157		50.8	
104	2,4,6,2′,6′	93	38	157	0.57
105	2,3,4,3′,4′		95		0.3
126	3,4,5,3′,4′	NEO[d]	NEO[e]	Inactive[f]	Inactive[g]
153	2,4,5,2′,4′,5′		> 100	178	

[a] Adapted from Ref. 7.

[b] Adapted from Ref. 11.

[c] Adapted from Refs. 12 and 13.

[d] No effect observed up to 200 μM.

[e] No effect observed up to 100 μM.

[f] Inactive up to 200 μM.

[g] Inactive up to 10 μM.

frequency of calcium oscillations that were dependent on entry of extracellular calcium, primarily via L-type voltage-sensitive calcium channels (VSCCs). Furthermore, the actions of Aroclor 1254 (20 μM) were blocked by both glutamate and γ-aminobutyric acid $(GABA)_A$ receptor antagonists, suggesting a role for these excitotoxicants (GABA is excitatory prior to 6 days in vitro) in regulating entry of extracellular calcium and influencing synaptic activity. Thus, as stated previously, PCBs may influence neuronal development by altering neuronal migration/survival, growth factor synthesis and release, and growth of developing neurons.[31–34]

10.3.3 Noncoplanar PCBs Alter Intracellular Calcium by Altering Ryanodine Receptor Activity

Pessah and colleagues[12,13] have also investigated the effects of PCBs on $[Ca^{2+}]_i$ and have also shown that only noncoplanar-substituted PCB congeners increase $[Ca^{2+}]_i$ in microsomes isolated from skeletal, cardiac, and brain tissue (Table 10.1). Their use of low concentrations of noncoplanar PCB congeners, combined with pharmacological manipulations (described below), allowed them to identify a putative molecular site of action responsible for the PCB-induced elevations in $[Ca^{2+}]_i$ [i.e., the ryanodine receptor (RyR)].

RyR is located on sarcoplasmic/endoplasmic reticulum (SR/ER) and three isoforms of the ryanodine-sensitive calcium-release channel complexes have been described: RyR1, RyR2, and RyR3. All isoforms are found in brain with RyR1 found in cerebellar Purkinje cells, RyR2 found in all brain regions, and RyR3 found in hippocampus, basal ganglia, and thalamus.[35–37] RyR/channel activation plays a key role in regulating $[Ca^{2+}]_i$.[38,39] Upon depolarization, VSCCs located on the plasma membrane open, permitting influx of extracellular calcium (present at concentrations approximately 1000-fold greater than found intracellularly in resting neurons).[40] These elevations in $[Ca^{2+}]_i$ increase the open probability of the RyR/channel, resulting in release of calcium from SR/ER stores, a phenomenon known as *calcium-induced calcium release*.[41]

Wong and Pessah[13] subsequently demonstrated that noncoplanar-substituted congeners dose-dependently induced both release and inhibited uptake of calcium from SR/ER vesicles. Because PCB-induced elevations in $[Ca^{2+}]_i$ were blocked by inhibitory concentrations of ryanodine, they concluded that the significant elevations in $[Ca^{2+}]_i$ are due to noncoplanar-substituted PCB congener activation of the RyR.

The authors further hypothesized that noncoplanar PCB congeners elevate $[Ca^{2+}]_i$ by an immunophilin-based mechanism. Support for this hypothesis was provided by Wong and Pessah,[42] who demonstrated that following exposure of SR isolated from skeletal muscle to noncoplanar-substituted PCBs, elevations in $[Ca^{2+}]_i$ were eliminated with either rapamycin or FK506. Because these compounds disassociate the immunophilin FKBP12 from the RyR, these results strongly implicate a FKBP12-immunophilin RyR/channel-based mechanism for the noncoplanar PCB-induced elevations in intracellular calcium.

Questions related to the ability to generalize these findings to the CNS were addressed by Wong et al.,[12] who demonstrated that exposure of microsomes isolated from cortex, hippocampus, and frontal cortex to the noncoplanar PCB congener number 95 (2,3,6,2′,5′-pentachlorobiphenyl) significantly elevated $[Ca^{2+}]_i$, with microsomes of hippocampal origin most sensitive. Similarly, Wong et al.[43] demonstrated that in vitro exposure to congener 95 depressed electrophysiological responses to stimulation in the rat hippocampus, providing further, albeit indirect evidence that this congener is likely to alter spatially mediated behavioral tasks.

What conclusions can be drawn from these studies that demonstrate that primarily noncoplanar-substituted PCB congeners elevate intracellular calcium? First, there are large differences in the concentrations of structurally similar PCB congeners needed to elicit significant changes in intracellular calcium between the two laboratories that have conducted the majority of these studies. The USEPA lab discerned differences in intracellular calcium following exposure of cerebellar granule cells to 2,2′-DCB at media concentrations greater than 50 μM, while changes in RyR binding and calcium release in isolated microsomes were seen by Pessah and colleagues at significantly lower concentrations (400 nM to 10 μM). It is conceivable that the differences in the concentrations needed to elicit an effect between these two laboratories reflect not only differences in potencies of the congeners selected for study, but also the preparations that were employed. However, Bemis and Seegal,[44] using a preparation similar to that employed by the USEPA group, detected significant elevations in intracellular calcium in cerebellar granule cells exposed to 2,2′-DCB at concentrations as low as 2.5 μM (Table 10.2). The reasons for the much higher concentrations of PCBs required to observe significant elevations in intracellular calcium by the USEPA group have not been determined.

Second, there are differences in the interpretation of the mechanisms responsible for the elevations in intracellular calcium. The USEPA group has suggested that noncoplanar PCB congeners, at concentrations equal to or greater than 50 μM, alter [^{3}H]PDBu binding, PKC activation and/or translocation and binding of IP_3 to its receptor. On the other hand, Pessah and colleagues present evidence that at nanomolar or low micromolar concentrations,

TABLE 10.2 Dose–Response Effects of 2,2′-Dichlorobiphenyl on Free Intracellular Calcium in Cerebellar Granule Cells Measured by Fluo-4 Florescence[a]

2,2′-DCB (μM)	Fluo-4 Florescence (Mean ± SEM)					
	< 1 min	2 min	5 min	10 min	15 min	30 min
Control	−0.26	−0.20	0.78	0.32	2.18	5.85
	±0.38	±0.53	±0.49	±0.62	±0.69	±1.13
2.5	3.24	1.39	1.99	0.94	1.64	4.13
	±0.57	±0.49	±0.34	±0.36	±0.39	±0.82
5.0	12.34	6.44	4.31	3.43	2.09	4.43
	±1.06	±0.62	±0.99	±0.75	±0.66	±0.82
10	31.65	20.27	11.84	7.49	8.68	9.91
	±2.50	±2.48	±1.67	±1.78	±1.99	±2.19
20	54.71	53.92	29.84	20.34	22.34	26.95
	±4.41	±5.05	±2.97	±2.27	±2.95	±3.93

[a]Fluo-4 data are expressed as the change in fluorescent intensity from a corrected baseline of zero. There was a significant, dose-dependent effect of 2,2′-dichlorobiphenyl on fluo-4 fluorescence ($F = 71.24$; df = 4,66; $p \leq 0.001$); $n = 21$ for the controls and 11–14 the individual doses.

noncoplanar PCB congeners increase the open probability of the RyR leading to a release of calcium from intracellular SR/ER stores.

10.3.4 Coplanar HAH Effects on Intra- and Extracellular Signaling

Findings from Legare et al.[16] and Hanneman et al.[15] provide an interesting exception to the vast majority of studies that have documented that only noncoplanar HAHs are active in vitro. In those studies, the authors demonstrated that 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) altered both hippocampal neuronal/glial gap junctional communication[16] and uptake of calcium into cultured hippocampal neurons,[15] suggesting the possibility that TCDD altered both intra- and intercellular communication.

However, given the exacting SARs for reductions in neurotransmitter function,[7] for alterations in neurotransmitter transporters,[45] and for alterations in intracellular calcium in other studies[11–13] (i.e., only noncoplanar PCB congeners are active), the results above are difficult to place in an existing framework. Nevertheless, if these results are replicated in other laboratories, they suggest that the mechanisms by which TCDD alters neuronal function differ from the mechanisms by which structurally similar coplanar PCB congeners alter function. This tentative conclusion is difficult to support, however, in light of the similar effects of developmental exposure to TCDD and coplanar PCB congeners on behavior.[14]

10.4 ARE IN VITRO CHANGES IN INTRACELLULAR CALCIUM SUFFICIENT TO PREDICT IN VIVO CHANGES IN CNS FUNCTION?

10.4.1 Alterations in RyR and Behavior

Little information exists on the relationships between PCB-induced changes in $[Ca^{2+}]_i$ and functional changes in the intact animal. In an attempt to address this problem, Schantz et al.[6] measured locomotor activity, spatial learning and memory, and RyR binding in rats developmentally exposed to the noncoplanar PCB congener 2,3,6,2′,5′-pentachlorobiphenyl. Compared to controls, PCB-exposed rats were hypoactive as adults and exhibited faster acquisition of working memory on a radial-arm maze task. Elevations in RyR binding were seen in cerebral cortex and cerebellum, while decreased binding was seen in the hippocampus. Although these results suggest involvement of the RyR in 2,3,6,2′,5′-pentachlorobiphenyl–induced behavioral changes, resolution of the role that these channels may play in altering behavior must await further experiments in which both RyR agonists and antagonists are used to either mimic or block the actions of PCBs. Furthermore, the binding studies were conducted in adult animals—examination of RyR function in adult animals may not reflect toxicant-induced changes in brain development that may occur at an earlier time. The authors appear to have considered this problem since they hypothesize that, because RyR/channels are found on growth cones of

developing neurons, developmental exposure to noncoplanar PCB congeners may alter the normal patterns of neurogenesis and synaptogenesis in brain, and thus induce deficits in CNS function that persist into adulthood. Thus, PCBs may alter RyR function during development (and induce behavioral changes evident in adulthood) but may not result in measurable (or interpretable) differences in RyR function when measured in adult animals. Therefore, a description of the developmental profile of RyR activation following in utero and lactational exposure to noncoplanar PCB congeners and its relationships to behavioral change would seem warranted. Nevertheless, RyR modification is likely to represent one of several mechanisms by which PCBs alter behavior—indeed, recent evidence strongly implicates RyR3 in the acquisition of spatial learning[46] and induction of hippocampal plasticity,[47] since RyR3 knockout mice exhibit deficits compared to wild-type control mice.

10.4.2 Changes in Neurotransmitter Function

Elevations in intracellular calcium induced by neuronal depolarization, due to entry of extracellular calcium via VSCCs and *N*-methyl-D-aspartate (NMDA) receptor channels, play a key role in the exocytotic release of neurotransmitters.[40] Indeed, the probability of vesicular release of neurotransmitter is highly correlated with localized concentrations of intracellular calcium.[40] Given that noncoplanar PCBs elevate intracellular calcium, elevations in extracellular DA seen in both striatal slices exposed to PCBs[48,49] (Table 10.3)

TABLE 10.3 Effects of 4-h Exposure to Aroclor Mixtures and Individual PCB Congeners on Striatal Tissue Punch and Media Dopamine (DA) Concentrations (Percent of Control Exposure)

	Tissue DA Concentration			Media DA Concentration		
Treatment	40 μ*M*	100 μ*M*	200 μ*M*	40 μ*M*	100 μ*M*	200 μ*M*
PCB congeners						
2,5,2′,5′	92	75	39	155	323	1330
2,2′	87	75	40	142	268	1416
2,4,2′,4′	89	62	34	160	632	1970
2,3,6,2′,5′	84	48	24	221	959	1660
2,4,4′	97	93	75	160	256	885
2,4,5,2′,4′,5′	92	83	78	151	274	241
3,4,3′,4′	90	95	87	100	108	131
2,6,2′,6′	93	95	95	108	117	119
3,4,5,3′,4′	102	96	99	112	114	107
	40 ppm	100 ppm	200 ppm	40 ppm	100 ppm	200 ppm
Aroclor mixtures						
1:1 1254:1260	78	70	43	242	480	1017

and in PC12 cells may, in part, be due to localized increases in intracellular calcium. Evidence suggesting that the PCB-induced release of DA may, in part, be due to activation of the RyR and the subsequent elevation in $[Ca^{2+}]_i$, is provided by Wan et al.,[50] who demonstrated that ryanodine increased release of DA from striatal slices obtained from adult rats.

In addition to alterations in RyR function, other mechanisms may contribute to the noncoplanar-induced elevations in basal release of DA. Recently, Mariussen et al.[51] have shown in vesicles isolated from adult rat striatal synaptosomes that noncoplanar PCB congeners inhibited vesicular monoamine transporter (VMAT2) uptake of DA, resulting in elevated cytosolic concentrations of neurotransmitter. Thus, our recent evidence of increased media concentrations of DA in striatal slices exposed ex vivo to PCBs[48,49] may in part be due to a PCB-induced VMAT2 inhibition of uptake of DA into synaptic vesicles, leading to short-term elevations in cytosolic DA and increased DA release. Similar effects (i.e., enhanced basal release of catecholamines; decreases in potassium-stimulated release of catecholamines, and reductions in cell catecholamine content) were observed by Messeri et al.[52] in bovine adrenal chromaffin cells exposed to the noncoplanar congener 2,4,2′,4′-tetrachlorobiphenyl for either 24 h or 5 days. These seemingly contradictory results—an increase in basal release of catecholamines accompanied by a decrease in potassium-stimulated release—are consistent with the previously described effects of noncoplanar PCB inhibition of DA uptake into VMAT2,[45] thus reducing the size (neurotransmitter content) of the vesicular pool released by high potassium-induced depolarization.

Thus, noncoplanar PCBs (1) inhibit neurotransmitter uptake into vesicles, resulting in an increase in cytosolic concentrations of neurotransmitter and enhanced basal release of cytosolic free DA into the extraneuronal space, as well as (2) decrease the neurotransmitter content of the vesicles evident following depolarization. We also suggest that VMAT2 inhibition of uptake of monoamine neurotransmitters should inhibit de novo synthesis of DA due to feedback inhibition of TH and/or activation of synthesis-modulating autoreceptors, which are sensitive to nanomolar concentrations of synaptic DA,[53] leading ultimately to reductions in cellular or tissue DA concentrations.

These in vitro data, demonstrating that PCBs enhance the release of neurotransmitter from DA terminals, have been supported in a recent series of experiments in which we determined extracellular brain concentrations of DA and its metabolites using in vivo microdialysis in adult rats exposed to Aroclor 1254 for 3 days, 1, 2, or 8 weeks.[54] Striatal extracellular concentrations of DA in PCB-exposed rats were increased significantly after 3 days compared to vehicle-exposed animals, but were reduced significantly at later times (Table 10.4). We suggest that the short-term elevations in extracellular DA concentrations may be due to VMAT2 inhibition, while the later reductions probably reflect inhibition of DA synthesis due either to feedback inhibition of TH or activation of synthesis-modulating autoreceptors.

In summary, the studies reviewed demonstrate that only noncoplanar PCBs

TABLE 10.4 Dopamine Concentrations in Baseline Striatal Microdialysates from Freely Moving Adult Rats Exposed to 25 mg/kg per day Aroclor 1254

Exposure Duration	Dopamine Concentration (ng/30 min collection; Mean ± SEM)[a]	
	Control	PCB
3 days	0.20 ± 0.02	0.31 ± 0.03*
1 week	0.14 ± 0.04	0.08 ± 0.001*
2 weeks	0.18 ± 0.06	0.05 ± 0.01^{-}
8 weeks	0.19 ± 0.04	0.07 ± 0.02*

[a]Data were statistically analyzed using two-way ANOVA with repeated measures; $*p \leq 0.05$, $-p \leq 0.1$; $n = 3$–5 animals per condition. Dopamine concentrations have been corrected for microdialysis probe efficiency.

significantly (1) elevate $[Ca^{2+}]_i$ in a variety of in vitro preparations,[12,19] and (2) enhance release of catecholamines from bovine adrenal chromaffin cells[52] and rat striatal slices.[49]

10.5 ADULT EXPOSURE TO PCBs: EFFECTS ON NEUROTRANSMITTERS, OXIDATIVE STRESS, AND NEURODEGENERATION

10.5.1 PCBs Are Not Just Developmental Neurotoxicants

Although there is considerably less interest in examining the consequences of adult exposure to PCBs, recent epidemiological evidence demonstrating that aging fish anglers (with elevated serum PCB levels) exhibit short-term memory deficits[55] has rekindled interest in earlier laboratory studies that have clearly demonstrated that PCBs alter adult CNS function. First, Seegal and colleagues have shown that exposure of both adult rodents[56] and nonhuman primates (NHPs)[57] to PCBs significantly reduces DA concentrations in the substantia nigra (SN), putamen, and caudate nucleus (Figure 10.1). That these neurochemical effects may have relevance to the occupational neurological effects of PCBs is shown by the fact that serum levels of PCBs, measured in NHPs immediately following PCB exposure, were nearly identical to those seen in capacitor workers several years after their exposure to PCBs ceased.[58] Most important, these decreases in central DA concentrations persisted well beyond the period of exposure to PCBs,[59] when brain and serum PCB levels were significantly reduced, suggesting that the reductions in central DA concentrations are long-lived, if not permanent.

Second, exposure of both adult NHPs and rats to PCBs significantly decreased the number of TH-immunoreactive cells in the SN,[60] suggesting

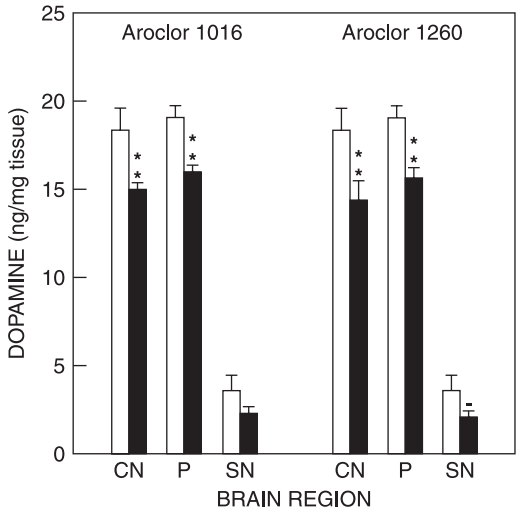

Figure 10.1 Regional brain dopamine concentrations in caudate nucleus (CN), putamen (P), and substantia nigra (SN) from control (open bars) and PCB-exposed (filled bars) adult nonhuman primates (*Macaca nemestrina*). PCB-exposed animals received 3.2 mg/kg per day of either Aroclor 1016 or 1260 for 20 weeks. Data include animals sacrificed immediately after exposure and animals sacrificed 24 or 44 weeks after exposure ceased. Data were analyzed statistically using one-way ANOVA; $^{**}p \leq 0.01$, $-p \leq 0.1$; $n = 3$–9 animals per group.

either a reduction in the expression of TH or a decrease in the number of neurons (Table 10.5). The latter results provide a potential mechanistic basis for the prolonged depression in basal ganglia DA concentrations we observed. The similarity of the changes in DA function, including reductions in basal ganglia concentrations of DA and the number of TH-containing neurons in the SN following adult exposure to PCBs, to those seen in Parkinson's disease (PD) provide support for our hypothesis that HAHs may play a role in the induction of this age-dependent neurodegenerative disease.

TABLE 10.5 Number of Tyrosine Hydroxylase-Positive (TH^+) Neurons in the Substantia Nigra of Rats and Nonhuman Primates Exposed Chronically to Polychlorinated Biphenyls

	TH Cell Count (Mean ± SEM)	
Treatment	Rat	Nonhuman Primate
Control	2,506 ± 271	38,000 ± 3,690
Aroclor 1254	1,345 ± 105	
Aroclor 1016		21,000 ± 450
Aroclor 1260		24,000 ± 1,350

10.5.2 PCBs Induce Oxidative Stress: Role of Intracellular and Mitochondrial Calcium

The mechanisms responsible for the loss of TH^+ neurons in NHPs or rodents following exposure to Aroclor mixtures are not fully understood but probably reflect neuronal degenerative processes that ultimately result in cell death. Because cell death, particularly in the nigroneostriatal system, is thought to involve increased oxidative stress,[61,62] a brief review of the relationships between contaminant-induced alterations in $[Ca^{2+}]_i$ and oxidative stress will aid in understanding how these contaminants may induce cell death.

First, do PCBs and related HAHs induce oxidative stress? Most appropriate for this discussion are the results of Hassoun et al.,[63] who demonstrated that exposure of female mice to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) at levels as low as 0.45 ng/kg per day for 13 weeks increased superoxide anion (SOA) production, lipid peroxidation, and DNA single-strand breaks in brain—effects similar to those seen in rat hepatic tissue following a single exposure to higher levels of TCDD.[64,65] Hassoun et al. suggested that these effects may be due to TCDD induction of either cytochrome P450 or aldehyde dehydrogenase activity—events also induced by exposure to PCBs.[66–68] However, only whole brain tissue was analyzed, thus limiting the ability to determine if regional brain differences exist in susceptibility to this environmental stressor. Furthermore, measures of oxidative stress were not examined in the presence of measurements of neurochemistry or behavior, further reducing the ability to determine the functional significance of these changes.

Induction of oxidative stress is not limited to TCDD or coplanar PCB congeners, which induce many of their toxic effects via the aryl hydrocarbon (Ah) receptor[69,70] (see also Chapter 12 for additional information). Ganey and co-workers[71,72] demonstrated that exposure of neutrophils to either Aroclor 1242 (which contains many lightly chlorinated noncoplanar congeners) or the noncoplanar congener 2,2′,4,4′-tetrachlorobiphenyl-inhibited Cu/Zn superoxide dismutase (SOD1) activity, resulting in enhanced SOA production at the expense of hydrogen peroxide formation. Although these studies reveal an important mechanism (i.e., SOD1 inhibition) by which PCBs may influence oxidative stress, there have been, to the best of my knowledge, no studies that have demonstrated a similar effect in nervous tissue.

Additional mechanisms by which PCBs induce oxidative stress were examined by Robertson and co-workers.[66,73,74] Both coplanar and noncoplanar PCB congeners, susceptible to in vivo metabolism to hydroxylated metabolites (i.e., biphenylols),[75] are metabolized to semiquinone intermediates that react with oxygen to form quinones and SOA.[66] In turn, the PCB quinones react with nitrogen and sulfur nucleophiles to form hydroquinone adducts that bind to DNA, induce strand breakage, deplete glutathione, and induce oxidative stress (see Chapter 17 for additional information). Although many of the reactions following metabolism of PCBs to biphenylols have been examined in vitro, these reactions are likely to also occur in vivo.

Second, what are the relationships between toxicant-induced elevations in oxidative stress and neuronal calcium homeostasis—many of which may be mediated by changes in nitric oxide?

Elevations in $[Ca^{2+}]_i$ (such as those induced by exposure of cells in culture to PCBs) induce nitric oxide (NO) production in neuronal cells,[76] increasing the likelihood of NO reacting with molecular oxygen or superoxide anions to form peroxynitrite, a highly reactive free radical species.[77] In turn, elevations in NO potently deenergize brain mitochondria,[78] contributing, either directly or as a consequence of subsequent elevations in $[Ca^{2+}]_i$, to mitochondrial dysfunction. Finally, increases in $[Ca^{2+}]_i$ release cytochrome *c* from mitochondria[28] and thus may contribute directly to induction of apoptosis.

Furthermore, alterations in NO influence both RyR and VSCCs that regulate $[Ca^{2+}]_i$. Thus, increased NO (or its reactive intermediates) interact with RyR,[79] with low concentrations of NO reducing the open probability of the RyR/channel and higher levels increasing the open-channel probability.[80] Similarly, Campbell et al.[81] demonstrated that NO-related species inhibit L-type calcium channels, a VSCC that plays a key role in regulating entry of extracellular calcium.[41] Although knowledge of the consequences of NO-induced inhibition of L-type calcium channels is incomplete, this inhibition is likely to influence RyR activity directly since entry of extracellular calcium via this channel enhances RyR/channel open probability (i.e., calcium-induced calcium release).

The rationale for positing a relationship between PCB-induced elevations in $[Ca^{2+}]_i$ and oxidative stress is more tenuous than the results described above since few studies have examined the consequences of PCB-induced alterations in $[Ca^{2+}]_i$. Nevertheless, we suggest that PCB-induced elevations in intracellular calcium are likely to initiate changes similar to those described, where intracellular calcium or NO concentrations were manipulated directly (i.e., enhanced NO production, altered mitochondrial energy production, increased mitochondrial calcium loading, release of mitochondrial cytochrome *c*,[28] and ultimately cell death).

What data support this tentative hypothesis? Maier et al.[82] demonstrated that noncoplanar PCB congeners reduced oligomycin-sensitive Mg-adenosine triphosphate(ATP)ase activity in brain mitochondria; reductions in energy metabolism lead to neuronal depolarization, activation of NMDA receptors, and further increases in $[Ca^{2+}]_i$.[61] Furthermore, Inglefield and Shafer[27] showed that noncoplanar PCBs increase both the frequency of calcium oscillations and basal $[Ca^{2+}]_i$ in developing neocortical cells and that these responses can be blocked by glutamate and $GABA_A$ receptor antagonists, demonstrating a role for excitatory amino acid neurotransmitters in PCB-induced elevations in $[Ca^{2+}]_i$. As mentioned previously, the consequences of these PCB-induced changes have not been adequately addressed: Whether these noncoplanar PCB–induced elevations in intracellular calcium mediate the observed increases in oxidative stress remains to be determined. Furthermore, PCBs inhibit free radical scavengers;[71,83,84] toxicant-induced increases in oxidative

stress may lead to an amplification of the cycle described above, resulting ultimately in opening of mitochondrial permeability transition pores, uncoupling of the mitochondrial respiratory chain, and cell death.

However, the role of $[Ca^{2+}]_i$ in coplanar HAH-induced oxidative stress is much less obvious since the vast majority of the in vitro studies demonstrate that only noncoplanar PCBs are active. Thus, in vitro detection of the putative mechanisms by which coplanar HAHs induce oxidative stress in vivo remains largely unknown. PCBs may also indirectly induce oxidative stress in brain by increasing concentrations of free catecholamines (i.e., those not stored in vesicles) by a mechanism involving both enzymatic and nonenzymatic catabolism of neurotransmitters, and in particular DA[85] and formation of reactive quinones and semiquinones, which contribute further to oxidative stress. These reactive quinones may also increase $[Ca^{2+}]_i$ by increasing the open probability of the RyR/channel since Feng et al.[86] demonstrated that quinones alter hyperreactive cysteines located in the lumen of the RyR, leading to an increase in $[Ca^{2+}]_i$. Thus, noncoplanar PCBs increase both $[Ca^{2+}]_i$ and free DA, both of which lead to enhanced oxidative stress and decreased cell viability. In turn, elevations in intracellular calcium activate a number of intracellular signals associated with cell death, including proteases, endonucleases, and phospholipase A and C.[87,88]

In addition to its recognized association with cell death, oxidative stress has also been linked to changes in neuronal plasticity that are reminiscent of those seen following developmental exposure to PCBs. Thus, bath application of hydrogen peroxide accelerated the decay of long-term potentiation (LTP) in CA1 of guinea pig hippocampus by enhancing free radical formation.[89] Similarly, exposure of hippocampal slices to low concentrations of hydrogen peroxide reduced slow-onset potentiation induced by exposure to carbachol.[90] On the other hand, Klan[91] and Klan et al.[92] present evidence that extracellular application of superoxide dismutase or catalase attenuates LTP induction in the CA1 area of hippocampus, suggesting that production of superoxide anion by the tetanic stimulation of hippocampus may be necessary for maintenance of LTP. It is tempting to suggest that PCB-induced increases in oxidative stress may contribute to the deficits in hippocampal[93] and cortical LTP seen following developmental exposure to PCBs.

10.5.3 Interactive Effects of PCBs with Other Environmental Contaminants

Despite the fact that human exposure to PCBs is due primarily to consumption of contaminated food products that contain many other known or suspected neurotoxicants, there is little information concerning potential interactions (e.g., synergism, antagonism) following coexposure of PCBs with other neurotoxicants. Seegal[8,44] examined the consequences of coexposure to PCBs (Aroclor 1254 or 2,2′-DCB) and methylmercury (MeHg) on DA function and changes in intracellular calcium. In the first experiment,[8] tissue and media

concentrations of DA were determined in striatal tissue from adult rats following ex vivo exposure to Aroclor 1254, MeHg, or the two contaminants in combination. Coexposure increased significantly the release of DA into media while significantly reducing tissue DA concentrations compared to effects seen following exposure to either contaminant alone. We suggest that the synergism between the two contaminants may be due partly to their ability to alter $[Ca^{2+}]_i$, which in turn influence the activity of TH[94]—the rate-limiting step in the synthesis of DA.[95] In the second experiment,[44] $[Ca^{2+}]_i$ were measured in cerebellar granule cells from early postweaning rats following exposure to 2,2′-DCB, MeHg, or the two contaminants in combination. The effects of coexposure were dependent on the absolute concentration of the binary pair of contaminants—low to moderate concentrations significantly elevated $[Ca^{2+}]_i$ compared to effects seen following exposure to either contaminant alone, while higher absolute concentrations led to antagonistic reductions in $[Ca^{2+}]_i$. The shift from synergism to antagonism may be due to MeHg interactions with reactive cysteines located in the lumen of the RyR and subsequent reductions in channel activity. Thus, PCBs may both enhance the open probability of the channel, as well as permit greater access of MeHg to the reactive cysteines located in the channel lumen.

These results demonstrate the need to examine for possible interactions between known and putative toxicants on CNS function to determine more appropriately the risks associated with exposure to complex environmental mixtures of contaminants. At present, there are insufficient data to predict whether particular contaminants will interact synergistically, resulting in greater risk to the individual, or interact in an antagonistic manner, perhaps reducing risk.

10.6 SUMMARY

In conclusion, despite evidence that environmental levels of PCBs, dioxins, and furans are decreasing, there continues to be considerable research efforts focused on understanding both the consequences of developmental exposure to these persistent organic pollutants and the mechanisms responsible for alterations in CNS function. Are these efforts misguided considering the ever-growing number of putative neurotoxicants that either already exist or are now being released into the environment? It is a difficult question to answer, due, in part, to the fact that an appropriate answer must be based on both economic (distribution of scarce research funds) and scientific criteria. Although scientists have little direct control over the first criterion, the studies that have been reviewed here suggest that additional research may provide answers to several important questions that have not yet been adequately resolved. These unanswered questions, in my estimation, include understanding (1) the risks associated with developmental environmental and adult occupational exposure to PCBs, (2) how PCBs and other HAHs interact with other environmental

neurotoxicants, and (3) the relationships between alterations in intracellular calcium and other signaling molecules and functional changes in the CNS following both developmental and adult exposure to PCBs.

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CHAPTER 11

Experimental Toxicology: Carcinogenesis

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11.1 INTRODUCTION

Understanding the potential for dioxins to influence human health requires basic understanding of the disciplines comprising the health sciences—physiology, pathology, biochemistry, and epidemiology, for example—which, at their union, form the field of toxicology. The various chapters of this book address specific topics evaluating the potential impact of dioxins on human health. Here, the experimental data on the carcinogenicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and selected dioxins is presented within a mechanistic framework to facilitate an understanding of the state of the science and its relationship to other responses and topics presented elsewhere in this book.

11.1.1 Value of Animal Studies in the Determination of Carcinogenic Potential

A carcinogenic response is the result of a complicated interplay of biological processes: pharmacokinetic, physiological, and biochemical, among others. Individually, these processes may be studied utilizing in vitro research tools, but the interplay and final outcome of exposure to chemical carcinogens can only be fully evaluated using suitable in vivo animal models. The use of animal models for studying carcinogenesis can be divided into two broad categories, long-term chronic bioassays and shorter-term mechanistic studies, each

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of which make important contributions to final assessment of carcinogenic potency in the human.

The most widely used paradigm for determining carcinogenic risk to humans is the 2-year rodent chronic bioassay.[1] Groups of 50 or more rats and/or mice are exposed to the maximum tolerated dose (MTD) of the test agent and several lower doses in the range $\frac{1}{2}$ to $\frac{1}{10}$ of the MTD. The 2-year chronic bioassay is designed to ascertain whether a chemical is capable of causing cancer under the experimental conditions of the study. The bioassay is an important qualitative test,[2] but in the absence of additional supporting data, does not provide sufficient information to allow high-confidence quantitative estimates of carcinogenic potency or risk to humans.[3] While successfully balancing the need to limit the probability of false negative results and limit the costs of testing, the assay has the additional limitation of not being able to discriminate between compounds that are genotoxic carcinogens (cause heritable DNA alterations) from those that are nongenotoxic. These determinations are made either by (1) accumulating biochemical (e.g., adduct formation) and cellular (e.g., growth dysregulation) evidence indicative of mode of action, or (2) functional tests, which are carried out within the framework of initiation-promotion studies in rodent liver or mouse skin. Initiation-promotion studies are based on the tenets of multistage carcinogenesis, a description of the process of tumor development that provides a framework into which experimental and epidemiological data can be integrated successfully.[4] Animal studies are also used successfully to generate mechanistic data, which is an important adjunct to cancer bioassays by virtue of its usefulness in evaluating the relevance to human risk assessment and in improving confidence in risk estimations.

11.1.2 Multistage Carcinogenesis: Framework for Understanding the Experimental Carcinogenesis of Dioxins

Neoplastic development occurs through a series of (multiple) steps or stages (Figure 11.1).[4] The multistage process of neoplastic development has been recognized in several organs in the human[5,6] and in two widely used model systems of chemical carcinogenesis, the mouse skin and rat liver.[7,8] At the genetic level, these steps or stages reflect the stochastic accumulation of heritable alterations in protooncogenes and tumor suppressor genes (TSGs).[9–11] Both types of alterations contribute to neoplastic conversion by disrupting biochemical processes involved in the control of normal cell growth and differentiation.[6]

Based on early work in experimental chemical carcinogenesis in mouse skin and rat liver,[7,12–15] the process of neoplastic development has been defined as comprising three stages: initiation, promotion, and progression (Figure 11.1). This basic construct, which defines the stages operationally, has been widely used and recapitulates faithfully the multistage process of neoplastic development in animals and humans.[16,17]

Each of the three stages of multistage carcinogenesis has unique character-

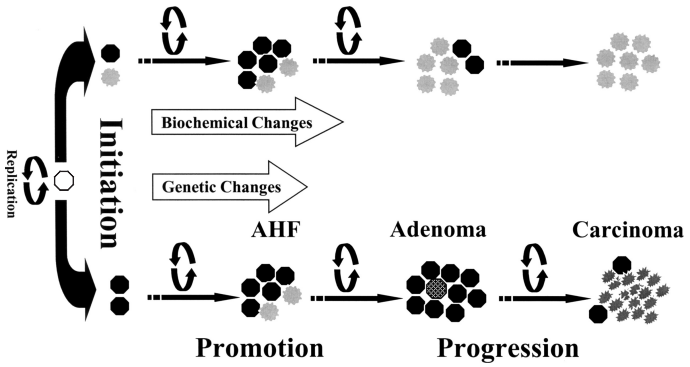

Figure 11.1 Integrated model of multistage hepatocarcinogenesis in the rat. ●, dead hepatoctyes; ●, clonal initiated hepatocytes; ●, cells in the early stage of progression; ✸, malignant hepatocyte; ⟲, cell division.

istics. The first stage, *initiation*, can be defined as the heritable genetic alteration of a cell. Initiation requires both alteration of the genome and cell replication to fix the alteration as a mutation. By definition, the mutation confers a growth advantage to the cell. The clonal expansion of these cells into benign lesions under the influence of endogenous or exogenous (e.g., promoting agents such as dioxins) is the second stage, termed *promotion*. At the cellular level, promoting agents reversibly alter the net growth rate of the clonally expanding (preneoplastic) initiated cells[18–20] or the normal cells[21] to produce a selective growth advantage for the initiated cells. The final stage, *progression*, begins when one or more initiated cell(s) acquire(s) the second genetic alteration required for malignant conversion. This represents an irreversible transition from preneoplastic to neoplastic growth.

The multistage description of carcinogenesis provides a useful framework in which to organize and interpret results from experimental carcinogenesis studies. The results of tumor bioassays for TCDD and selected dioxins, as well as whole animal mechanistic work, is presented with an emphasis on interpretation within the framework of multistage carcinogenesis. The evaluation of mechanisms of carcinogenesis is an important step in the overall evaluation of human carcinogenic risk. This is particularly important for dioxins, where there is considerable uncertainty regarding specific mechanisms of carcinogenesis, and the rodent–human concordance of dioxin-induced increases in the specific tumor sites that have been observed of rodent bioassay.[22,23] Moreover, TCDD has been classified as a known human carcinogen by both the International Agency for Research on Cancer (IARC) and the National Toxicology Program (NTP) of the U.S. Department of Human Health Services (DHHS). These determinations have been made through the evaluation of rodent bioassay data and mechanistic information in light of epidemiological data that by itself cannot fully support an unequivocal evaluation of human carcinogenicity.

11.2 CANCER BIOASSAY

11.2.1 Sites of Increased Tumor Incidence

Numerous studies conducted during the late 1970s and early 1980s were seminal in establishing the carcinogenicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD).[24–29] Since then there have been few new chronic animal bioassays examining the carcinogenicity of dioxinlike compounds. Numerous reviews summarizing the results of data from these chronic carcinogenicity studies have been conducted and the reader is encouraged to refer to these for more detailed information and conclusions on the respective studies.[30–33]

TCDD has been shown to be a potent carcinogen in these long-term animal cancer studies. Studies have been carried out in both sexes of rats (Table 11.1) and mice (Table 11.2), and also in male hamsters.[25,26,29,34] The most highly cited study is the Kociba study conducted by Dow Chemical.[25,35] This study, a chronic 2 year feeding study conducted on both male and female Sprague–Dawley rats, was the first study to analyze comprehensively the carcinogenicity of TCDD in multiple tissues and both genders (Table 11.1). Historically, many of the regulatory activities on assessing the health risk for human exposure to

TABLE 11.1 Rat Studies of Carcinogenicity of Dioxins and Dioxinlike Compounds

Strain	Gender	Duration	Doses	Regimen	Ref.
Sprague–Dawley	M	104	0, 1, 10, 100 ng TCDD/kg per day	Feed study	25
	F	104	0, 1, 10, 100 ng TCDD/kg per day		
Osborne–Mendel	M	104	0, 10, 50, 500 ng TCDD/kg per week	Twice a week, gavage	26
	F	104	0, 10, 50, 500 ng TCDD/kg per week		26
	M	104	1250, 2500, or 5000 ng hexa-CDD/kg per week	Twice a week, gavage	36
	F	104	1250, 2500, or 5000 ng hexa-CDD/kg per week	Twice a week, gavage	36
Sprague–Dawley	F	104	0, 3, 10, 22, 46, 100 ng TCDD/kg per day	Five times a week, gavage	40[a]
	F	104	0, 20, 44, 92, 200 ng penta-CDF/kg per day	Five times a week, gavage	40[a]
	F	104	0, 10, 30, 100, 175, 300, 550, 1000 ng PCB 126/kg per day	Five times a week, gavage	40[a]

[a] Ongoing study.

TABLE 11.2 Mouse Studies of Carcinogenicity of Dioxins and Dioxinlike Compounds

Strain	Gender	Duration	Doses	Regimen	Ref.
B6C3F1	M	104	0, 10, 50, 500 ng TCDD/kg per week	Twice a week, gavage	26
	F	104	0, 40, 200, 2000 ng TCDD/kg per week	Twice a week, gavage	26
Swiss/H/Riop	M	> 1 year	7, 700, 7000 ng TCDD/kg per week	Once a week for 52 weeks, gavage	28, 29
Swiss–Webster	M	99	0, 1 ng TCDD/animal	Three times a week, dermal	27
	F	104	0, 5 ng TCDD/animal	Three times a week, dermal	27
Tg.AC hemizygous transgenic	F	26	0, 5, 17, 36, 76, 121, 166, 355, 760 ng TCDD/kg	Three times a week, dermal	81
B6C3F1	M	104	1250, 2500, or 5000 ng HCDD/kg per week	Twice a week, gavage	36
	F	104	2500, 5000, or 10,000 μg HCDD/kg per week	Twice a week, gavage	36
Swiss–Webster	M	104	0, 5 ng HCDD/animal	Three times a week, dermal	171
	F	104	0, 5 ng HCDD/animal	Three times a week, dermal	171

TCDD have relied heavily on data from this study. The results of this study indicated that TCDD was a potent hepatocarcinogen in female but not male rats. In addition, statistically significant increases in tumors were observed at multiple sites, including the lung, adrenal cortex, nasal turbinates, and tongue. A summary of the sites where increases in the incidence of tumors were observed in the Kociba study[25] is presented in Table 11.3. Toth et al. showed further that TCDD is a potent hepatocarcinogen in male Swiss/H/Riop mice administered 700 ng TCDD/kg per week orally for a year.[29] In addition, the National Toxicology Program conducted a chronic 2-year gavage study of TCDD in both genders of Osborne–Mendel rats and B6C3F1 mice.[26] In addition to confirming the female-specific carcinogenicity of TCDD in female rat liver, this study showed that TCDD was a carcinogen in male and female mouse liver and identified the mouse thyroid gland as a tumor site for TCDD-

TABLE 11.3 Summary[a] of Sites of Increased Cancer Incidence in Chronic Studies of TCDD Carcinogenicity in Rodents

		Rat		Mouse		Hamster
Rodent	Organ	M	F	M	F	M
Rat and mouse	Liver		×	×	×	
	Thyroid	×			×	
	Lung		×	×		
	Subcutaneous fibrosarcoma		×		×	
Rat only	Adrenal cortex	×	×			
	Nasal turbinates/hard palate	×	×			
	Tongue	×				
Mouse only	Thymic lymphomas			×		
	lymphomas				×	
Hamster only	Facial skin-squamous cell carcinoma					×

[a] From Tables 11.1 and 11.2.

induced cancer. Together these studies form the basis for the conclusion that TCDD alone is able to induce tumors in multiple species and in both genders and causes tumors at sites distant from the site of administration.

In addition to TCDD, only two hexachlorodibenzo-*p*-dioxins have been tested for carcinogenicity in a chronic bioassay. The NCI/NTP tested a 1:2 mixture consisting of 1,2,3,6,7,8-hexa-CDD and 1,2,3,7,8,9-hexa-CDD in a design similar to that for the study of TCDD noted above.[36] Data from this study showed a pattern similar to that of TCDD in that the hexa-CDD mixture induced liver tumors in both male and female mice and in female rats. It is interesting to note that incidence of liver tumors exhibited a statistically significant dose–response "trend" in both the TCDD and HCDD NTP studies, although direct comparison of individual groups to untreated animals was not significant.

TCDD was tested in a long-term study in male golden Syrian hamsters.[34] Hamsters are the most resistant species in terms of acute TCDD toxicity. Within this long-term study, male golden Syrian hamsters were given two to six intraperitoneal or subcutaneous injections of TCDD over a 4-week period at doses of 0, 50, or 100 μg TCDD/kg, and the experiment terminated after 12 to 13 months.[34] Animals treated at the highest dose developed squamous cell carcinomas of the skin in the facial region, with the earliest lesions detected after 8 months. There was no indication that there was an increase in liver tumors.

A preliminary report also indicated that medaka (*Oryzias latipes*) immersed in TCDD-treated water (33.9 ppq TCDD) for 28 days, followed by immersion in clean water for up to 8 months, led to an increase in tumors at multiple sites, including gills, thyroid, and swimbladder.[37]

There are few carcinogenicity data on individual congeners of coplanar (dioxinlike) polychlorinated biphenyls (PCBs). However, laboratory studies

found statistically significant increased incidences of liver tumors in rats ingesting commercial PCB mixtures, including the American commercial PCB mixture Aroclor 1260 or the European PCB mixture Clophen A60. Significant increases in gastric cancer, leukemia, and lymphoma were found in rats ingesting Aroclor 1254. The Aroclor 1254 mixture contains the highest level of dioxinlike coplanar PCBs of these mixtures. Partial lifetime studies found precancerous liver lesions in rats and mice ingesting PCB mixtures of high or low chlorine content. More recent studies have compared the carcinogenicity of several Aroclor mixtures.[38] Of the Aroclors tested, exposure to Aroclors 1016, 1242, 1254, and 1260 resulted in an increased incidence of liver neoplasms in female rats. However, only Aroclor 1260 at high doses (100 ppm in feed) was carcinogenic in males. In addition, Aroclor, 1242, 1254, and 1260 induced the incidence of thyroid tumors in male rats. Analysis of liver levels of specific PCB congeners suggests that in males the induction of tumors is dependent on total PCB content, whereas in females it is dependent on the dioxinlike activity of the PCB mixture (as a result of accumulation of dioxinlike PCBs from the Aroclor mixture).[39]

With regard to studies of the carcinogenicity of other dioxinlike compounds, including PCBs, the National Toxicology Program is currently conducting 2-year carcinogenicity bioassays of TCDD, a polychlorinated dibenzofuran, several polychlorinated biphenyls, and mixtures of these compounds in female Sprague–Dawley rats.[40] These studies will serve to enhance our understanding of the carcinogenicity of dioxinlike compounds.

In summary, TCDD induces tumors in multiple sites in both male and female rodents and in multiple species. The main target organs where increased cancer incidence has been observed in both rats and mice are the liver, thyroid, lung, and skin. It is important to highlight that considering both species of all animal species tested, there are no specific consistent or ''hallmark'' target organs for TCDD-induced cancer. However, as shown in Table 11.3, the liver is clearly a common target site. Although it exhibits the highest concordance between gender and species, in the rat, TCDD is a female-specific liver carcinogen. At other sites there is gender concordance across species (skin); at others there is concordance within a species between genders (e.g., adrenal and nasal turbinates).

11.2.2 Sites of Reduced Spontaneous Tumor Incidence

Cancer Studies In addition to positive trends in tumor formation, several negative trends have been observed in laboratory animals chronically exposed to TCDD. In the Kociba study there was a significant reduction in the incidence of spontaneous benign tumors of the uterus, benign neoplasms of the mammary gland, mammary carcinoma and pituitary adenoma in female rats, and pheochromocytoma (tumor of the adrenal medulla or sympathetic paraganglia) of the adrenal gland and pancreatic adenoma in male rats.[25] There are essentially two mechanisms that are proposed to explain these negative trends in cancer incidence. First, the reductions in tumor incidence are secondary to

alterations in body weight as a result of TCDD exposure, and second, TCDD disrupts endocrine homeostasis, thereby reducing the incidence of hormone-dependent cancers such as mammary and uterine cancers.

Potential Mechanisms of Reduced Spontaneous Tumor Incidence

Reduced Body Weight Gain In a compiled analysis of multiple chronic 2-year bioassays for numerous chemicals tested by the National Toxicology Program, it was observed that there was a negative correlation between body weight and incidence of benign neoplasms of the mammary gland and anterior pituitary in male, but not female, Fisher F344/N rats.[41,42] Exposure to chemicals that resulted in a reduction of body weight gain by 10 to 20% was correlated with a decrease in incidence of both mammary tumors and anterior pituitary tumors in female rats. This phenomenon may be related to a homeostatic growth suppression in reproductive organs during periods of reduced nutritional status.

Chronic exposure to TCDD leads to a decrease in body weight gain in both male and female rats, without significant differences in food intake. The reduction in incidence of mammary and pituitary tumors in chronically treated animals is consistent with a reduction in body weight gain associated with chronic TCDD treatment. In addition, a statistically significant decrease in the incidence of acinar adenoma of the pancreas was observed in male rats exposed to 100 ng/kg per day.[25] Reduction in pancreatic adenoma has been observed in diet-restricted animals,[41] and consequently, the reduction in incidence of this lesion may also be due to TCDD effects on body weight. In contrast, the pheochromocytoma in male rats does not appear to be a consequence of changes with body weight, yet it exhibited a dose-dependent reduction in TCDD-exposed males. The mechanism for the reduction in pheochromocytoma is unknown.

Endocrine Homeostasis The decrease in classical hormone-dependent cancers in TCDD-treated rats may also be related to the ability of TCDD to alter estrogen metabolism and/or its ability to act, in some cases, as an antiestrogen. Increased estrogen metabolism may result from increased expression of CYP1A2, CYP1B1, UGT, and GST. Decreased estrogen action could result from effects on the estrogen receptor (ER) levels or ER transcriptional function. TCDD may also induce the inactivation of estrogen in target cells by metabolism by TCDD-inducible cytochromes CYP1A2 and CYP1B1, or conjugating enzymes such as UGT1/GST, without altering circulating estradiol concentrations.[43]

11.3 MODE OF ACTION

In contrast to complete mechanistic descriptions, a mode of action (MOA) more broadly characterizes the dominant biological activity of a chemical or

chemicals in terms of the obligatory steps leading to cancer. There are two principal MOAs by which carcinogens contribute to the development of tumors in vivo: genotoxic and nongenotoxic. Genotoxic carcinogens principally, if not exclusively, cause DNA lesions which result in the heritable accumulation of genetic alterations. Nongenotoxic carcinogens selectively increase the growth of genetically altered cells, chiefly through cytotoxic or mitogenic mechanisms.[44] MOA determinations (i.e., distinguishing between MOAs) influence the direction of mechanistic research and guide approaches to dose–response analysis and low-dose extrapolation. Therefore, the determination of a MOA is an important element in the characterization of the carcinogenic potential of a compound.

Numerous short- to medium-term two-stage models of chemical carcinogenesis suitable for distinguishing between and quantifying the effects of genotoxic and nongenotoxic compounds have be developed.[45–48] These protocols typically employ a single initiating dose of a genotoxic chemical, followed by enhancement of cell division [partial hepatectomy (PH) or cytotoxicity] to fix the genetic damage as a heritable mutation (initiation). Subsequently, animals are exposed chronically to a chemical (promoting agent) that causes clonal expansion of the genetically altered cells (promotion). Increases in the incidence of tumors or preneoplastic lesions are produced by carcinogenic agents administered at either stage. Promoting agents cause elevations in the incidence of these lesions when administered at appropriately high doses postinitiation, but not in the absence of initiation. This selectivity is central to the utility of these models to distinguish between chemicals that act by a predominately nongenotoxic or genotoxic mechanism. Results from these models for TCDD and related compounds are reviewed here since they provide substantial quantitative and mechanistic data characterizing TCDD as a nongenotoxic carcinogen.

11.3.1 Two-Stage Models of Liver Tumor Promotion

TCDD Following the 2-year chronic bioassay conducted by Kociba et al.,[25] which established the hepatocarcinogenicity of TCDD, Pitot and co-workers conducted an initiation promotion (IP) study that first demonstrated that TCDD was a potent liver tumor promotor.[49] Following initiation (DEN 10 mg/kg, partial hepatectomy), female rats received biweekly doses of TCDD (0.14 and 1.4 μg/kg) subcutaneously for 7 months. These doses were equivalent to the medium (10 ng/kg per day) and high doses (100 ng/kg per day) in the Kociba bioassay.[25] The incidence of hepatocellular carcinomas and altered hepatic foci (AHF) exhibiting altered expression of the hepatic marker proteins glucose-6-phosphatase, canalicular ATPase, and γ-glutamyl transpeptidase (GGT) was determined. Based on associations between the incidence of AHF and liver cancer in rodents, in addition to the understanding that tumors develop clonally—a clonal lineage exists between early single-cell lesions through to the fully malignant tumor—AHF are believed to be part of the biological continuum from initiated cells to carcinomas, and are therefore referred to as preneoplastic lesions.[8,48,50–52] Increased incidence of hep-

atocellular carcinomas and preneoplastic lesions were restricted to the TCDD treatment groups receiving initiating doses of DEN. Hepatocellular carcinomas were observed in the 100-ng TCDD/kg per day dose group, while increases in preneoplastic lesions occurred in both the 10-ng/kg per day (0.7% of the liver) and 100-ng/kg per day (43% of the liver) dose groups.

This first report was followed by other studies that confirmed and extended these results to include alternate doses and species, as well as the effects of exposure duration and schedule.[53–65] These initiation-promotion studies were particularly important because they demonstrated three important characteristics of TCDD-mediated tumor promotion which have implications for human risk assessment. The primary activity of TCDD is to expand previously existing populations of altered cells. The induction of significant numbers and volume fractions of placental glutathione-*S*-transferase-positive (PGST) AHF by TCDD was shown to require prior initiation with a genotoxic agent.[49,55,58–60,63] In the absence of initiation, a small number of AHF are observable, particularly after 60 weeks of promotion.[66] The origin, spontaneous or induced, of the altered cells is irrelevant to the tumor-promoting activity of TCDD, although the type of altered cell may affect the potency of TCDD.[49,63,67] Withdrawal experiments, which measured the number and volume fraction of AHF after nonexposure periods of varying length, demonstrated that the tumor-promoting effects of TCDD were reversible for TCDD-dependent lesions.[59,66] Furthermore, multiple-dose/exposure duration studies established that TCDD-dependent tumor promotion was a function of both dose and exposure duration.[49,55–57,59–63,66] Concentration–time relationships are commonly observed for toxicological endpoints.[68] At present, only a single study has provided characterization of dose–response on TCDD-mediated promotion of AHF at multiple time points.[63]

The prerequisite for initiation and potent induction of preneoplastic and neoplastic lesions provided strong evidence that TCDD is a potent tumor promoter in the liver, with a mechanism dominated by nongenotoxicity. This is consistent with the vast majority of in vitro genotoxicity tests, which are negative[69,70] for TCDD. In addition to providing consistent and substantial evidence indicating that TCDD acts predominately, if not exclusively, as a nongenotoxic carcinogen (promoting agent), the data from these models have been integrated into analytical or simulation models, which provide important insights into the underlying biology of TCDD-mediated hepatocarcinogenesis.

PCDD/F Tumor Promotion Studies While the majority of tumor promotion studies have been carried out with TCDD, other studies have also investigated the tumor promotion capability of other structurally related compounds, such as the polychlorinated dibenzodioxins and polychlorinated dibenzofurans. These data indicate that the capacity of these compounds to induce the development of preneoplastic foci in the liver exhibit a rank-order potency similar to that for the induction of CYP1A1 enzyme activity.[57,61] Studies also demonstrate that the potency of non-ortho-substituted (dioxinlike)

PCBs to induce the development of AHF exhibits a similar potency to that for induction of CYP1A1 activity.[65,71] Within the framework of the toxic equivalency factor (TEF) scheme (congeners are assigned a "toxic" potency value relative to the most potent compound, TCDD), which is used to assess mixtures of dioxinlike compounds, the database of information used in derivation of the WHO-TEFs indicates that the relative potencies for tumor promotion, where available, are similar to the current WHO-TEF values.[72] In addition, when mixtures of dioxins/furans have been analyzed, the effects on AHF development appear to be additive. These data suggest that tumor promotion by dioxins and dioxinlike PCBs probably act through similar mechanisms.

11.3.2 Two-Stage Models of Mouse Skin Tumor Promotion

Mouse TCDD Studies Tests of the tumor-initiating and tumor-promoting capacity of TCDD have also been conducted in two-stage (initiation-TCDD promotion) models of mouse skin tumorigenesis. These studies demonstrate that in mouse skin, TCDD is at least two orders of magnitude more potent a promoting agent than tetradecanoyl phorbol acetate (TPA), a well-characterized skin tumor promoter.[73] TCDD did not induce tumor promotion in early studies carried out in Swiss Webster and CD-1 mice, the classic skin tumor promotion models.[27,74] TCDD caused growth dysregulation in epithelial cells in mice carrying the hairless trait (hr), but not in wild-type or heterozygous mice[73,75] which established that TCDD was a potent skin tumor promoter in this genetically predisposed mouse strain.[73] Congeners that bind to the AhR (aryl hydrocarbon receptor) (2,3,7,8-tetrachlorodibenzofuran, 3,4,3′,4′,5′-hexabromobiphenyl) but not to non-AhR-binding congeners (2,7-dichlorodibenzo-*p*-dioxin, 2,4,5,2′,4′,5′-hexabromobiphenyl) were shown to cause skin tumor promotion, indicating that TCDD-mediated skin tumor promotion is AhR dependent.[73,76] A single study in the two-stage mouse skin model assigns only weak tumor-initiating activity to TCDD.[77] However, this conclusion is confounded by the lack of appropriate control animals. Given the absence of additional studies, there appears to be insufficient evidence that TCDD is a tumor initiator in the mouse skin (recently reviewed in Ref. 78).

Based on structure–activity studies and genetic studies, it appears that the skin tumor-promoting actions of TCDD are AhR dependent. The relative toxicity and tumor-promoting capacity of two polychlorinated furans (2,3,4,7,8-CDF and 1,2,3,4,7,8-CDF) investigated in hairless mice indicate that 2,3,4,7,8-CDF is 0.2 to 0.4 times as potent as TCDD and that 1,2,3,4,7,8-CDF is 0.08 to 0.16 times as potent as TCDD.[79]

Transgenic models for the classification of mechanism of action of carcinogens have been used to examine the mechanism of carcinogenicity of TCDD in mice.[80] These include the Tg.AC transgenic mouse, which harbors an activated mouse v-Ha-ras oncogene (an intermediate in growth factor signaling), and the p53 +/− transgenic mouse, which is heterozygous for the wild-type tumor suppressor p53. Dermal application of tumor promoters such as phorbol

esters results in the development of epidermal papillomas in the Tg.AC. Topical application of TCDD results in a significant increase in the incidence of squamous cell papillomas in both male and female Tg.AC mice,[80] supporting the conclusions that TCDD is not a tumor promoter. In contrast, treatment of p53 +/− mice with TCDD by gavage for 24 weeks did not result in any neoplastic lesions, supporting the conclusion that TCDD is not directly genotoxic. Subsequent studies showed that the induction of papillomas by dermal application of TCDD to the hemizygous Tg.AC mouse was dose-dependent.[81,82] In addition, the induction of skin papillomas in this model occurs when TCDD was given by oral administration. These data provide further support for the potent tumor-promoting action and weak initiating capacity of TCDD.

Two-Stage Models of Lung Tumor Promotion The positive trend for lung tumors observed in male mice in the NTP carcinogenesis bioassay of TCDD[26] was explored by Beebe and co-workers[83] to determine if the affect could be characterized as tumor promotion. Control or male Swiss mice initiated with *N*-nitrosodimethylamine (NDMA) were given single (1.6, 16, or 48 μg/kg intraperitoneally) or multiple (0.05 μg/kg per week, 20 weeks) doses of TCDD and sacrificed at 52 weeks of age. A 100% incidence rate of lung tumors in NDMA-initiated mice obscured any effect of TCDD on induction of tumor incidence. TCDD did, however, promote the development of lung tumors in these mice. Tumor multiplicity was significantly elevated in two NDMA/TCDD treatments groups (1.6 and 16 μg/kg) compared to NDMA-treated controls. A more comprehensive initiation-promotion study conducted in Sprague–Dawley rats, which have a much lower spontaneous incidence rate of lung tumors, demonstrated that TCDD alone can promote the development of bronchiolar hyperplasia (proliferation in number of cells) and AB metaplasia (abnormal transformation of cell type).[84] These lesions were reversible; incidence of these lesions returned to control levels following withdrawal of TCDD for 16 or 30 weeks. A mechanistically similar group of liver tumor promoters, PCBs, also promote the development of lung tumors in male Swiss mice following NDMA initiation.[85] The demonstration of promoting effects in rat and mice lung tumorigenesis provides additional support for the assertion that TCDD's dominant mode of action for carcinogenesis is via promotion.

11.4 MECHANISM OF ACTION

11.4.1 General Issues for Mechanism

Why are we interested in determining mechanism and mode of action for TCDD-mediated carcinogenesis? The human epidemiological data available provide only limited evidence of carcinogenicity in humans[31] and are not sufficient to provide high confidence estimates of potency and risks associated with exposure. Our dependency on data derived from animal studies for evaluating

the potency of TCDD and the risks associated with human exposure means that the results from animal studies must be extrapolated to humans. Specifically, qualitative and quantitative determinations of the relative sensitivity of humans and rodent test species must be made, to allow the extrapolation of rodent potency data to humans. In the absence of comparable test data in humans and rodents, equivalence of sensitivities across species is best evaluated by demonstrating species similarities in the underlying biological processes that contribute to the mechanism.[86,87] For example, in rodents, TCDD's biological effects are mediated by binding to the Ah receptor (AhR).[88–92] Binding is required for the tumor-promoting activities of TCDD.[89,91] Therefore, human sensitivity to the tumor-promoting effects of TCDD would then be a function of AhR binding. Differences in the expression, tissue distribution, binding characteristics, and signal-transducing potential of the AhR in rodents and humans could be compared to provide estimates of species sensitivity.

Risk assessment involves the extrapolation of high-dose incidence data acquired in test species to low doses in humans. The shape of the dose–response curve in this low-dose region cannot be determined empirically, due to statistical limitations imposed by the number of animals that can be practically utilized in bioassays.[93] However, extrapolation requires making assumptions regarding the shape of the dose–response curve in this region, which can take many forms, including linear, sublinear, and supralinear.[94] Since response is the net result of the interaction of the processes involved in the toxic response, determining the nature of these processes helps to inform the decision regarding the type of extrapolation (linear, nonlinear) that is taken. Biologically based dose–response (BBDR) models, which are quantitative representations of the mechanisms of action and associated pharmacokinetics, have been used successfully to demonstrate expected nonlinearities in low dose–response values.[95] Mechanistic information can be incorporated into BBDR models to improve confidence in dose–response assessments and determining the relevance of a compound to human health.

11.4.2 Liver Tumor Promotion

Two levels of responses with the potential to be informative exist regarding the mechanism of TCDD-mediated liver tumor promotion—those at the level of the cell and those at the molecular level—are discussed. Where reasonable, mechanistic inferences have been made in an effort to present the current state of understanding regarding potential mechanisms of action.

Cellular-Level Responses

Cell Replication, Apoptosis, and Tumor Promotion Three cellular-level processes govern the growth dynamics of preneoplastic (initiated cell) AHF lesions: cell division, death, and differentiation.[91,96] Cell death takes two forms, necrosis (pathologic death) and apoptosis (programmed cell death).[97]

Here we define differentiation broadly as loss of the cell phenotype initiated. The net growth rate of a population of tumor cells will then be the difference between the cell division rate and the combined loss of cells from differentiation, necrosis, and apoptosis. A balance between cell division and cell death, primarily apoptosis, is believed to maintain the steady-state size of the normal liver. Dysregulation of these processes leading to increases in cell division and/or reductions in apoptosis can result in net growth rates greater than those of normal cells and the expansion of a tumor. This is the principal effect of tumor promoters. These principles, with origins in basic tumor biology, guided the establishment of several growth dysregulation hypotheses regarding the mechanism of action of TCDD, which have been explored experimentally.

Mills and Andersen[98] hypothesized that TCDD establishes a mito-inhibitory environment in the rodent liver that prevents or reduces the division of normal hepatocytes. Altered hepatocytes that are refractive to this effect of TCDD expand to form AHF and eventually hepatic tumors under the influence of TCDD. Others have hypothesized that TCDD increases cell division rates in AHF and have examined this possibility by measuring cell division rates in AHF and normal cells.[20,99] The results of experimental work in the rodent liver, which differ in dose and exposure duration, are not consistent and do not substantiate or refute either hypothesis.

Using the initiation-promotion model, increases in non-focal-cell proliferation by TCDD have been reported in female rats after chronic exposure to a 100- to 125-ng/kg per day dose of TCDD, which is both tumorigenic and hepatotoxic,[25,100] for 30 weeks.[47,100,101] However, exposure to lower tumor-promoting doses of TCDD (0.1 to 10 ng/kg per day) and for shorter durations do not cause increases in non-focal-cell proliferation.[63,102] Others have also reported the lack of a consistent effect of TCDD on non-focal-cell proliferation.[47,103] Additionally, dose-dependent increases in focal cell proliferation have not been demonstrated.

Several investigators have observed TCDD-mediated reductions in non-focal-cell proliferation.[47,102] Teeguarden et al. observed statistically significant reductions in non-focal-cell proliferation in female Sprague–Dawley rats exposed to 0.1 or 1 ng TCDD/kg per day for 1 or 3 months but not for 6 months.[63] Furthermore, the effect was not dose dependent, decreases were not observed after exposure to 10 ng/kg per day. The hypothesis that TCDD establishes a mito-inhibitory environment in the liver is poorly supported by experimental work. The possibility remains that the methodology is not currently sensitive enough to observe mito-inhibition of normal hepatocytes, which have a low normal division rate (1.2×10^{-3} per cell per day).[104]

Increased division rates of hepatocytes within AHF have also been reported. Buchmann and co-workers exposed DEN-initiated (10 mg/kg per day, 5 days) female Wistar rats to 100 ng TCDD/kg per day (1.4 μg/kg subcutaneously, biweekly) for up to 17 weeks.[99] Cell division in AHF, as measured by 5-bromo-2′-deoxyuridine (BrdU) labeling, was statistically significantly higher

than non-focal-cell (presumably normal hepatocytes) labeling in TCDD-treated rats after 13 or 17 weeks of exposure. Exposure for 9 weeks or less did not result in elevated cell division in AHF. Although a dose–response value was not reported, and the elevated labeling indices were not consistent across the exposure period, this work does provide some support for the hypothesis that TCDD deregulates growth control within AHF, leading to elevated cell division and clonal expansion.

The hepatic tumor promoter phenobarbital has been shown to reduce focal cell apoptosis rats.[18] Stinchcombe[20] extended this work to TCDD in DEN-initiated (10 mg/kg in drinking water for 10 days) female Wistar rats exposed to 100 ng TCDD/kg per day (1.4 μg/kg subcutaneously, biweekly) for 115 days or given a single 1.4-μg/kg dose 3 days before sacrifice. This study demonstrated a significant reduction in apoptosis by TCDD. No changes in focal cell or non-focal-cell division were observed. The apoptotic index was unaltered in non-focal cells, but the mean value was 40% lower than controls in the single-dose group and 87% lower in the chronically treated group. Only the reduction in the chronic exposure group was statistically significant. In addition, others have shown that TCDD can inhibit ultraviolet-induced apoptosis.[105] This significant mechanistic work supports the hypothesis that TCDD may increase the net growth rate of intermediate cells through selective reductions in apoptosis. These observations should be confirmed and extended by determining if reductions in intermediate cell apoptosis are dependent on the presence of ovarian hormones in female rats, as tumor promotion appears to be. Substantiating this mechanism of action would provide specific targets for extending molecular-level mechanistic work in the rat. Elucidation of the molecular mechanisms underlying TCDD mediated effects on cell division and apoptosis is a significant area of mechanistic research (reviewed in Ref. 91).

Liver Weight Changes and Hypertrophy Hepatic hypertrophy is a common response to chemicals that induce drug-metabolizing enzymes, including promoting agents such as peroxisome proliferators, TCDD, and PCBs.[106] This AhR-dependent effect[107] has been reported consistently in several species and model systems.[57,79,108,109] Enlargement of the cytosolic compartment, specifically due to a large increase in the total amount of smooth endoplasmic reticulum, is primarily responsible for liver hypertrophy after treatment with PCBs[106] and is probably true for TCDD treatment as well. It has been suggested that liver hypertrophy is frequently associated with promoting activity of chemical. However, Teeguarden et al.[63] reported hepatic hypertrophy in female Sprague–Dawley rats after administration of 1 or 10 ng TCDD/kg per day for 1 month, but not 3 or 6 months. Significant promotion of AHF was observed throughout this time period, demonstrating a clear disassociation between promotion and hepatic hypertrophy. Further exploration of this cellular-level effect of TCDD in the liver is therefore unlikely to lead to additional insights into the mechanism of TCDD-mediated promotion.

Interaction with Ovarian Hormones The effect of ovarian hormones implied by the female-specific tumor response reported by Kociba[25] was explored in the two-stage model of hepatocarcinogenesis by Lucier.[101] Intact and ovariectomized Sprague–Dawley rats were initiated (DEN, 200 mg/kg intraperitoneally) and promoted with TCDD (100 ng/kg per day) for 30 weeks. These studies were then extended by Wyde and co-workers, who examined promotion after both 20 and 30 weeks, with and without chronic estradiol supplementation.[110,111] The major conclusions from these studies of similar design are that TCDD promoted the development (volume fraction) of γ-glutamyl transpeptidase (GGT)-positive AHF in intact but not ovariectomized rats. In addition, the promotion of GGT-positive AHF was estrogen-responsive. In contrast, TCDD induced the development of PGST-positive AHF in both intact and ovariectomized rats. These data indicate that the TCDD induction of GGT-positive but not PGST-positive foci are hormonally regulated. In addition, the duration of exposure-dependent induction of hepatocyte replication by TCDD was also ovarian hormone-dependent and estrogen-responsive.

Biochemical Responses

Cytochrome P450 Production of Promutagenic Estrogen Metabolites One mechanism that may contribute in part to the rat gender-specific liver tumorigenicity and ovarian hormone dependence for liver tumor promotion by TCDD is an indirect mechanism whereby TCDD induces the expression of cytochromes P450 that metabolize 17β-estradiol to catechol estrogens.[112,113] Catechol estrogens such as 2-hydroxyestradiol (2-OH-E2) and 4-hydroxyestradiol (4-OH-E2) can be converted to semiquinone intermediates that are able to undergo redox cycling between the semiquinone and quinone forms, resulting in the formation of superoxide anions and reactive singlet oxygen species. The increase in radical formation may subsequently lead to an increase in oxidative stress or DNA damage. Hepatic tumor-promoting doses of TCDD (ca. 10 to 100 ng/kg per day)[63] are within the same range (3.5 to 125 ng/kg per day)[114] as those that cause induction of hepatic CYP1A1 and 1A2 activity and, presumably, production of these estrogenic metabolites and reactive oxygen species. Induction of oxidative stress[115–117] and oxidative DNA damage[118] have been observed following exposure to TCDD. More recently, Wyde et al. showed that the induction of oxidative DNA damage by TCDD is female-specific, dependent on estrogen, and only observed following chronic but not acute exposure to TCDD.[119] Although these data indicate a role for indirect genotoxicity in the mechanism, the relative contribution of TCDD-mediated production of indirect genotoxicity by catechol estrogens in hepatic tumor promotion may be small in light of overwhelming evidence indicating that the primary effects of TCDD in the rodent liver is promotion of AHF and tumors, not genotoxicity.[63]

Disruption of Cell Signaling Pathways Liver growth and differentiation is under the control of networks of cell signaling pathways.[120,121] Direct or indirect disruption of these control processes can alter hepatic growth and or differentiation, resulting in neoplastic growth.[78,122–124] Several ligand-receptor cell signaling systems have been shown to be (TCDD does not bind to these receptor) affected indirectly by TCDD,[91,103] including those for epidermal growth factor receptor (EGFR) and the estrogen receptor (ER). The EGFR is a transmembrane ligand-activated tyrosine kinase present in hepatocytes.[103] The endogenous ligand of EGFR, epidermal growth factor, is a potent mitogen in the liver.[122,125] EGFR is downregulated in several experimental hepatocarcinogenesis protocols,[103] after administration of tumor-promoting doses of TCDD in initiated and noninitiated rats.[103] This effect is reported to be estrogen dependent[103] and may occur without increases in receptor activation or turnover.[126]

The tyrosine kinase c-SRC is an integral part of the signaling cascade for growth factors (e.g., platelet-derived growth factor) and hormones such as insulin, thyroid hormone, and estrogen,[127] and can phosphorylate the EGFR.[128] In several tissues, including mouse liver, c-SRC has been shown to be associated with the AhR. Binding of TCDD to AhR leads to the activation of c-SRC kinase activity in mouse liver tissue.[127] No mechanistic role for secondary activation of c-SRC in hepatic tumor promotion has been elucidated, but it is intriguing to consider that this kinase interacts with two hepatic receptor systems affected by administration of TCDD in rodents, EGFR and ER. Activation of this pathway is AhR-dependent and occurs in a dose range considered to be of toxicological significance.[127]

Demonstration of the potent antiestrogenic activity of TCDD and other AhR agonists in in vivo and in vitro systems[90,129,130] led to substantial research into potential mechanisms of action. Expression of estrogen-responsive genes is controlled by the concentrations of estrogen and the estrogen receptor to which it binds, and by binding of this complex to a positive regulatory domain, a promoter region, in proximity to the DNA sequence constituting the gene. TCDD does not bind to the estrogen receptor directly to inhibit estrogen action,[129] but has been shown to upregulate expression of cytochromes P450 capable of metabolizing estradiol, and to downregulate rat uterine estrogen receptor.[131] The TCDD:AhR complex is also capable of reducing expression of estrogen-responsive genes by binding to inhibitory dioxin-responsive elements (iDREs) within the promoter regions.[90] Crosstalk between AhR and the ER system has also been demonstrated.[132,133] Experimental data indicate multiple mechanisms by which TCDD, through the activated AhR, can interact and interfere with ER signaling pathways.

Although substantial evidence of the effects of TCDD on these and other cell signaling systems has accumulated,[91] some of which have a clear relationship to TCDD-mediated promotion (similar dose range, AhR dependence, estrogen dependency), a specific role for the alterations in these systems has not

been elucidated. The large number of cellular effects resulting from administration of a strong inducing agent with additional effects on several hormonal systems (estrogen/ER, glucocorticoids, thyroid-stimulating hormone) confound the association of single effects with tumor promotion.

Mathematical Models of Promotion Understanding the influence of TCDD on the three primary processes that determine the number and volume fraction of AHF in initiation-promotion experiments—mutation, cell division, and death (apoptosis)—is complicated by the lack of experimental data characterizing the variation of these parameters with time. Two approaches have been developed for evaluating these data within the framework of multistage carcinogenesis; both are based on cell growth dynamics and multistage concepts. One approach, developed by Conolly and Kimbell, simulates the growth of AHF,[134] while the other uses analytical and statistical methods to infer growth parameters from experimental data.[135–138] Both approaches fit cell mutation, cell division, and cell death (apoptosis) rates to determined AHF number and volume fraction data experimentally. These analyses have led to important insights that take the form of experimentally testable hypotheses. Independently, Moolgavkar and Portier used stochastic two-stage models to infer the effect of TCDD on initiation, and cell division and apoptosis (or differentiation rates) rates in AHF.[138,139] Fitting the model to the number and volume fraction of AHF in DEN-initiated, TCDD-treated female rats suggested that TCDD affected both apoptosis and division rates. Better fits to the number and volume fraction data were obtained when TCDD was assumed to increase the rate of initiation.[139] A similar analysis of a different data set also implied that TCDD modestly increases "mutation" rates,[138] solidifying this model-derived hypothesis. To the extent that the term *mutation* may imply genotoxicity, the overwhelming majority of in vitro and in vivo data indicate no direct genotoxic effect of TCDD. However, the increase mutation rate observed is consistent with the hypothesis that TCDD-mediated increases in the formation of weakly genotoxic estrogen metabolites or reactive oxygen species that could contribute to or result in indirect DNA damage and accumulation of DNA mutations.[113] Strict interpretation of this observation as evidence of direct genotoxicity of TCDD should be cautioned against. Alternatively, these results may also be interpreted to be the result of accelerated conversion of DEN-initiated cells to visible (marker enzyme positive/negative) AHF.[140] This initiation effect may also be the result of assuming a single TCDD-responsive phenotype of intermediate cells. Simulation models that assume two TCDD-responsive cell types, which is consistent with experimentally observed heterogeneity in AHF,[141] reproduce size and volume fraction data successfully without a TCDD affect on mutation rates.[142] Both the analytical and clonal growth models have supported the hypothesis that TCDD alters focal cell proliferation rates and apoptosis (in some cases) in a time-varying fashion. These models confirm the importance of continuing to measure changes in cell cycle kinetics (division/death) in normal and focal cells

during promotion[143] and that these approaches will eventually help elucidate the mechanism of TCDD tumor promotion.

11.4.3 Lungs and Respiratory System

Cell- and Organ-Level Responses In the chronic study of Kociba et al., a significant increase in the incidence of keratinizing squamous cell carcinoma of the lung was observed in female rats exposed to 100 ng/kg per day in the diet for 2 years.[25] However, significant increases in lung tumor in similarly treated male rats were not observed in this study. By contrast, in a 2-year gavage study conducted by the NTP, there was a significant increase in the trend of lung tumor incidence (adenoma or carcinoma) with increasing doses of TCDD (0 to 71 ng/kg per day) in male B6C3F1 mice but not in female mice.[26] However, the actual incidence of tumor formation at the highest dose of TCDD was not statistically significant.

Significant increases in incidence of stratified squamous carcinoma of hard palate or nasal turbinate were also observed following chronic exposure to TCDD at a dose of 100 ng/kg per day in both male and female Sprague–Dawley rats.[25] These data indicate that there is not a gender difference in the carcinogenicity of TCDD in rat nasal turbinates/hard palate as in lungs.

Biochemical Responses Fundamentally, the mechanism of carcinogenesis by TCDD in the lung may have similarities with the liver. The lung possesses significant levels of AhR, and expression of the AhR is localized to the bronchiolar Clara cells.[144] Cytochromes P450 CYP1A1 and CYP1B1 are induced in rat lung following exposure to AhR agonists and TCDD.[145,146] The AhR is present in the nasal turbinates, and this tissue is the only tissue besides the liver in the rat that has been shown to contain CYP1A2.[147,148] CYP1A2 was constitutive and inducible at levels similar to liver. The presence of CYP1A2 in this tissue may explain the high retention of TCDD, as CYP1A2 is believed to be a TCDD-binding protein.[149]

In female Sprague–Dawley rats exposed to an approximate daily dose of 125 ng TCDD/kg per day for 30 weeks, induction of CYP1A1 in the lung is localized to the Clara cells. This induction may be associated with a number of hyperplastic and metaplastic changes in the alveolar–bronchiolar region.[144] At higher doses of the AhR agonist 3-methylcholanthrene (25 mg/kg), induction of CYP1A1 is observed in Clara cells, endothelial cells, and alveolar type II pneumocytes.[150]

The AhR has been detected in human lung tissue.[151] Furthermore, a number of cytochromes P450 are inducible by TCDD in both normal[152] and malignant[153,154] human lung cells, indicating that a functional dioxin-inducible AhR-dependent pathway is likely to exist in humans in vivo. However, the linkage between activation of the AhR in the lung and the mechanism of neoplasia has not been elucidated.

11.4.4 Thyroid

Cell- and Organ-Level Responses TCDD causes a dose-related increase in thyroid follicular cell adenomas and carcinomas in rats and mice.[26] Increased thyroid follicular cell adenomas were observed in male Osborne–Mendel rats treated with doses as low as 1.4 ng TCDD/kg per day and in female B6C3F1 mice treated with 71 ng/kg per day. The development of thyroid tumors in the male mice occurred at 1.4 ng/kg per day, compared with doses of 7 and 71 ng/kg per day required for the development of liver tumors.

Biochemical Responses One hypothesis for the induction of thyroid tumors by TCDD involves the disruption of thyroid hormone homeostasis via the induction of the phase II enzymes UDP-glucuronosyltransferases (UGTs).[155] Thyroxine (T4) production and secretion is controlled by thyroid-stimulating hormone (TSH), which is under negative and positive regulation from the hypothalamus, pituitary, and thyroid by thyrotrophin-releasing hormone (TRH), TSH itself, T4, and triiodothyronine (T3). TCDD induces the synthesis of UDP-glucuronosyltransferase-1 (UGT1) mRNA by an AhR-dependent transcriptional mechanism.[156] The isozymes induced by TCDD have been identified and are active in the conjugation of a variety of phenolic substrates, including the thyroid hormone thyroxine (T4).[157] It has been proposed that the reduced serum T4 levels (potentially via an induction in conjugation by UGT and subsequent elimination) may lead to a decrease in the negative feedback inhibition on the pituitary gland. This would then lead to a rise in secreted thyroid-stimulating hormone, resulting in chronic hyperstimulation of the thyroid follicular cells, followed by thyroid follicular cell hyperplasia and hypertrophy of the thyroid gland and neoplasia.[158] This basic mechanism of rodent follicular cell carcinogenesis, increased clearance of T4, leading to chronic stimulation of the thyroid gland, leading to hyperplasia, hypertrophy, and eventually neoplasia, has been demonstrated for a variety of inducing agents.[159,160] However, the human is not believed to be as sensitive as the rat to the effects of chronic follicular cell stimulation.[87]

Mechanistic Models In support of this mechanism, Kohn et al. modeled the effect of TCDD on UGTs, and thyroid hormones in female rats within the framework of a physiologically based pharmacokinetic (PBPK) model.[161] This mathematical model described release and uptake of thyroid hormones, metabolism, AhR-dependent induction of UGT1 by TCDD, regulation of TSH release from the pituitary by T4, and feedback on TRH and somatostatin, which inhibits TSH release. The model reproduced successfully the observed effects of TCDD on serum T3, T4, and TSH, and UGT1 mRNA and enzyme activity, suggesting that this is a plausible mechanism for an indirect role of TCDD on the thyroid. However, it is important to note that a direct effect of TCDD on the thyroid gland has not been investigated and an effect on thyroid cell proliferation cannot be ruled out as a possible mechanism for thyroid carcinogenesis.

11.5 IMPACT OF MECHANISTIC WORK ON THE RELEVANCE OF THE CARCINOGENICITY OF TCDD TO HUMANS

Animal studies are the major source of information regarding the carcinogenic potency of TCDD. Bioassays conducted in rodents have demonstrated the carcinogenicity of TCDD in multiple species, both genders, and in multiple organ systems.[78] From a precautionary perspective, the strong carcinogenic response observed in the rodent bioassay supported the hypothesis that TCDD could be carcinogenic in other mammals, such as the human. In rodents, the most consistent sites where a carcinogenic response is observed are the liver, lung, and thyroid. By comparison, studies of dioxin-exposed human populations have reported increased risk for all cancers combined, respiratory cancers, soft tissue sarcomas, and non-Hodgkin's lymphoma.

The relevance of the rodent bioassay data to humans can be evaluated in light of both human epidemiological data (see Chapter 18) and rodent mechanistic data. There are two limitations to be overcome in the current state of knowledge. The first is the elucidation of a complete mechanism of action for TCDD, such that the components of the mechanism and functionality in the human can be determined. The second is high confidence estimates of the potency of TCDD at environmentally relevant doses.

Of the 10 organs exhibiting a positive carcinogenic response to TCDD in the rodent bioassays (Table 11.3), mechanistic work has been conducted in three of them: the liver, lung, and thyroid gland. The rodent lung contains functional AhR capable of ligand-activated induction of CYP1A1 and CYP1B1 in response to AhR agonists, including TCDD.[145,146] Induction of these enzymes is observed in Clara and type II alveolar epithelial cells, the two lung stem cells[162,163] present in the region where tumors are observed,[144] at TCDD doses (125 ng/kg per day) similar to those (125 ng/kg per day) that result in the development of preneoplastic changes (hyperplasia, metaplasia)[144] and tumors (100 ng/kg per day).[25] These data imply that an AhR-CYP induction-cellular response linkage in the lung is consistent with the generally accepted mechanistic elements of TCDD-mediated carcinogenesis in the liver.

Disruption of thyroid hormone homeostasis is an established mechanism of thyroid carcinogenesis for phase II enzyme–inducing compounds such as PCBs and phenobarbital.[164,165] Induction of UGTs increases clearance of T4, resulting in chronic hyperstimulation of the T4-producing thyroid gland, hyperplasia, and so on.[164] In the rat, TCDD has been shown to induce the expression of UGTs and cause alterations in TSH levels in a dose range (1 to 3.5 ng/kg per day)[165] which is equivalent to that which induces thyroid tumors in rats ($\geq$ 1.4 ng/kg per day).[26] Induction is believed to be AhR-dependent.[166] Alternative mechanisms of thyroid carcinogenesis have not been ruled out, but the effects observed are entirely consistent with a well-established mechanism of action for thyroid follicular cell carcinogens—disruption of thyroid hormone homeostasis and chronic overstimulation of the thyroid gland.

The bulk of mechanistic work has targeted the rodent liver, where

TCDD binds to the AhR, resulting in biochemical and organ-level effects, including induction of phase I and II enzymes, hypertrophy, toxicity, cell proliferation, and disruption of several cell signaling pathways and hepatic tumor promotion. Hepatic tumor promotion in the rodent is dominated by a nongenotoxic mechanism and is likely to be AhR-dependent.[91] The large number of effects resulting from administration of TCDD, a strong tumor promoter with effects on several hormonal systems (estrogen/ER, glucocorticoids, thyroid-stimulating hormone), confound attempts to attribute the tumor-promoting effects of TCDD to single biochemical responses (i.e., up/down regulation of a single gene). Our current understanding of these processes, although substantial, is not sufficient to construct a complete mechanism of action for hepatic tumor promotion.

The most informative mechanistic data for TCDD-mediated carcinogenesis for an organ system positive in the rodent bioassay is that of the thyroid gland. Here a relatively high confidence mechanism has been proposed which involves AhR-dependent induction of UGTs, subsequent increases in systemic clearance of T4, and TSH-mediated hyperstimulation of the thyroid gland. The relevance of this general mechanism of thyroid follicular cell carcinogenesis to humans has been reviewed. The risk assessment forum of the U.S. Environmental Protection Agency (USEPA) concluded that the human was likely to be less sensitive than the rat.[87] This conclusion would extend to TCDD-mediated follicular cell carcinogenesis. Mechanistic arguments provide a basis for concluding that humans are likely to be less sensitive than rats to the induction of thyroid tumors by TCDD.

The biology of TCDD-mediated carcinogenesis in the rodent lung has correlates in the human. Humans express functional AhR[151] and have a similar stem cell population in the bronchiolar-alveolar region of the lung where TCDD associated tumors arise in the rat.[167,168] Both Clara cells and type II pulmonary epithelial cells are present in this region. Human Clara cells also express inducible forms of P450s.[168] Mechanistic linkages between the effects observed in the rodent lung and TCDD-mediated lung tumorigenesis have not been made and so cannot be extended directly to the human through similarities in the underlying biology. Similarities do exist, highlighting the value of future mechanistic work for determining the relevance of the rodent bioassay data to human carcinogenesis.

The only clear element of the mechanism of hepatic tumor promotion in the rodent is binding to the AhR. A range of binding affinities has been reported for the human, including those that are similar to and less than those for the rat[169] (see more details elsewhere in this volume). To the extent that AhR binding alone determines potency of promotion, humans may be more or less sensitive to the promotional effects of TCDD in the liver. The potency of dioxinlike compounds in rodent tumor promotion studies is correlated with binding affinity. However, it is possible that downstream molecular interactions with less species similarity (homology) may also modulate the promotional response. One such response, reduction in hepatic EGFR, has been observed in

humans.[170] However, without a complete mechanism of action for TCDD-mediated hepatic tumor promotion, the species concordance of induction of hepatic tumors, as observed in the rodent bioassay, is limited to speculation. The difference in sensitivity expected for thyroid follicular cell carcinogenesis inferred from mechanistic data is a case study for the value of mechanistic data in the evaluation of interspecies sensitivity and the interpretation of rodent bioassay results in the absence of supporting human epidemiological data.

11.6 SUMMARY

Rodent bioassays and suitable animal mechanistic models have been used extensively to provide qualitative and quantitative assessments of the hazard posed by human exposure to chemicals such as TCDD. Such efforts directed toward evaluation of the carcinogenic potential of TCDD have led to its characterization as a multispecies, multisite, multigender carcinogen. Experimental studies indicate that TCDD is not directly genotoxic but is a highly potent tumor promoter, and as such, can give the appearance of being a complete carcinogen. The mode of action for TCDD-mediated carcinogenesis in the thyroid and liver appears to be dominated by nongenotoxicity, and it may reasonably be expected that the mode of action in the lung will also be predominantly nongenotoxic in nature. Of the organ systems positive for TCDD-mediated carcinogenesis in the rodent bioassay, a complete mechanism of action has only been proposed for thyroid follicular cell carcinogenesis. The elucidation of a proposed mechanism for thyroid follicular cell carcinogenesis in the rodent facilitates the evaluation of the relevancy of this tumor type to the risk posed by human exposure to TCDD and would suggest that humans are expected to be less sensitive to the induction of thyroid follicular cell tumors by TCDD. Less is known about the mechanism in the lung and liver, although all three organs appear to share dependency on the binding of TCDD to the cytosolic AhR. Inadequate mechanistic characterizations preclude assessment of the relative sensitivities at other sites based on a specific mechanism of action. The finding that TCDD reduces the incidence of some spontaneous tumors in rodents in some sites is also less well understood or the specific mechanisms by which specifically TCDD causes these reductions. Development of a complete mode of actions for the liver and lung and an understanding of the comparative biology of the mechanistic components in the human will be a significant future step toward reducing some of the uncertainties in the use of experimental carcinogenesis studies for the determination of human cancer risk.

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CHAPTER 12

Ah Receptor: Involvement in Toxic Responses

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12.1 INTRODUCTION

Thirty years ago several investigators first observed that the ability of certain polycyclic aromatic hydrocarbons (PAHs), such as 3-methylcholanthrene (3-MC), to induce aryl hydrocarbon hydroxylase (AHH) activity segregated with a single genetic locus. This was called the *Ah locus*.[1,2] However, while 3-MC failed to induce AHH in "nonresponsive" strains of mice, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) induced this activity within these same strains, although with approximately 10-fold lower potency than in "responsive" mice.[3] These data led to the hypothesis that nonresponsive mice contained a defective "receptor" that had lower affinity for these chemicals and that somehow this receptor mediated the induction of AHH activity. In 1976 Poland and his group reported the identification of a protein, the product of the *Ah* locus, contained in mouse hepatic cytosol that was able to bind TCDD and had the characteristics of a true receptor protein.[4] Some of these characteristics of the mouse Ah receptor (AhR) include (1) high affinity binding ($K_d \sim 0.7$ nM; although at infinite dilution this may be in the pM range[5]) that approximated the ED_{50} value (ca. 1 nmol/kg) for induction of AHH in responsive mice, (2) lower affinity binding in nonresponsive, mice,[4,6] and (3) low capacity binding (ca. 10^5 sites/mouse liver cell[4]). Additionally, an excellent stereospecific structure–activity relationship is observed between the ability of a variety of PCDDs, PCDFs, and PCBs to bind to the AhR and to induce AHH activity.[7,8] Finally, the identification of AhR antagonists has also substantiated the activity of the AhR as a mediator of AHH induction.[9,10]

Since these initial investigations there has been much work from a number of laboratories on the molecular and functional characteristics of the AhR.

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To date, these data have been consistent with the hypothesis that the binding of TCDD and certain other PAHs to the AhR leads to the induction not only of the *cytochrome P4501A1* gene (*CYP1A1*) and its associated AHH activity but also of several other genes. Furthermore, these data are consistent with the hypothesis that the signal transduction pathways activated by the AhR mediate the toxicity of TCDD and its dioxinlike congeners. While much experimental work has focused on the ability of the ligand-activated AhR to bind to specific DNA sequences and modulate the expression of particular genes, more recent information suggests the possibility that modulated cellular functions leading to TCDD-elicited toxicity could occur by several other, and perhaps more complex, molecular pathways which are also AhR dependent. In addition, there is accumulating evidence that the AhR has some normal cellular function.

In this chapter we highlight our current understanding of the structural and functional aspects of the AhR and the potential pathways by which inappropriate stimulation of the receptor by exogenous ligands may affect normal cellular functions. In addition, we offer some perspective of how the AhR may have some essential role in regulating these processes. In this regard, a further understanding of this function and factors that control the expression and activity of the AhR will undoubtedly add to our knowledge of the processes that lead to TCDD-elicited toxicity and may help to explain the diversity of toxic effects and differential sensitivity observed in numerous animal species including humans.

12.2 AhR AS A GENE REGULATORY PROTEIN

12.2.1 Mechanism of *CYP1A1* Induction

Analyses of the mechanism of induction for *CYP1A1* have continued to enrich our understanding of how AhR activation by small molecular weight chemicals leads to modulation of gene expression. The mechanistic model (Figure 12.1) indicates that binding of TCDD and related halogenated aromatic hydrocarbons to the AhR, dimerization of the AhR with a nuclear protein (AhR nuclear transport protein; ARNT), and the interaction of this complex with specific DNA sequences [aryl hydrocarbon-responsive elements (AhREs) or dioxin-responsive elements (DREs)] present in the 5′ upstream regions of responsive genes lead to transcription initiation. Many of the details of these events have been uncovered within the past several years.

In the absence of ligand, the AhR appears to be maintained in an inactive state as a cytosolic complex with two molecules of 90-kDa heat shock protein (hsp90), a single molecule of a protein of approximately 38 kDa, and probably several other proteins. Hsp90 is needed to maintain the unliganded receptor in a conformation that facilitates ligand binding,[11] although some evidence suggests this might not be the case in some species.[12] Association with hsp90 is also thought to limit nuclear uptake of the AhR by blocking a nuclear local-

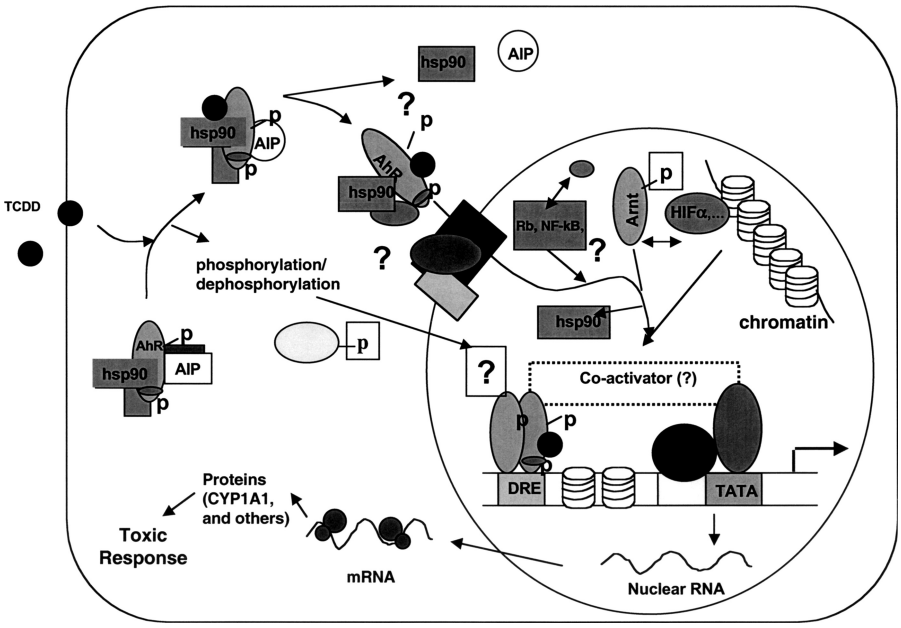

Figure 12.1 Postulated mechanisms of AhR action. AIP, AhR interacting protein; Rb, retinoblastoma protein; HIFα, hypoxia-inducible factor α.

ization sequence (NLS) found in the N-terminal region (see Figure 12.2 and below).[11,13–15] However, since complete hsp90 dissociation does not appear to be essential for AhR nuclear localization,[16] it is likely that significant conformational changes occur while the molecules remain associated. The dissociation of the two hsp90 molecules may also occur during separate events in the cytosol and the nucleus initiated by ligand binding and aryl hydrocarbon nuclear translocator (ARNT) association, respectively. The 38-kDa protein, ARA9/AIP/XAP2, has been shown to enhance transcriptional activity of the AhR-ARNT complex in several different cellular systems.[17–19] Although this protein does not appear to be required for the interaction between AhR and hsp90, it appears to stabilize this interaction and have some function in regulating the rate of AhR turnover in the cytosol or intracellular localization.[20–24] Ligand binding to the AhR initiates a transformation process that is not yet fully characterized. However, this process appears to involve ligand-induced conformational changes which result in the release of one molecule of hsp90, the unmasking of the NLS in the AhR, recognition of the NLS by the protein karyopherin α, docking of the AhR-karyopherin α complex to the nuclear transporter, and translocation of the liganded AhR-hsp90 complex to the nucleus at the expense of GTP hydrolysis.[15,16] An additional protein factor, p23, is thought to stabilize an intermediary complex that contains the ligand-occupied hsp90-associated AhR.[25,26] It is clear from accumulated evidence

that the AhR alone does not exhibit DNA-binding activity and must interact with ARNT protein to form a heteromeric, DNA-binding complex that can activate *CYP1A1* gene transcription.[27–31] Immunohistochemical studies indicate that ARNT is a nuclear protein and nuclear accumulation of the ligand-activated AhR can occur in ARNT-defective cells.[32] Thus, ARNT does not appear to have a primary role in nuclear translocation of the AhR.

The AhR-ARNT complex specifically recognizes DREs (5′-TNGCGTG-3′) located in the upstream regions of the *CYP1A1* gene.[33,34] The receptor heteromer lies within the major DNA groove and contacts the four guanines of the recognition sequence, with the AhR recognizing the 5′-half site (5′-TNGC-3′) and ARNT recognizing the 3′-half site (5′-GTG′3′).[35–38] Notably, few proteins bind in the major groove of DRE-containing enhancer regions in the absence of TCDD, due to the inaccessibility conferred by the configuration of nucleosomal proteins.[39,40] Activation of the AhR leads to occupancy of DRE sites on the enhancer, accompanied by loss of specifically positioned nucleosomes and increase in accessibility of the promoter region. This results in the binding of other transcription factors and initiation of *CYP1A1* gene transcription.[41–44]

12.2.2 Structural and Functional Features

The cloning and sequencing of the AhR cDNA[45–47] further solidified our appreciation of this molecule as a gene regulatory protein. However, these data also added complexity to our understanding of the possible roles that this protein may have in mediating the toxicity of TCDD. The AhR belongs to the bHLH-PAS (basic helix-loop-helix, Per-ARNT-Sim) transcription factor family. The members of this family include Per (a protein that appears to be involved the regulation of circadian rhythms), ARNT [also known as hypoxia-inducible factor (HIF)-1β], Sim (a regulator of CNS development), HIF1α (which regulates hypoxia-inducible genes), EPAS (endothelial-specific PAS protein), and NPAS (neuronal-specific PAS protein).[48–54] Both the AhR and ARNT have several distinctive functional domains that interact with different molecules. In the AhR, the PAS domain consists of approximately 300 amino acid residues containing two copies of a degenerate repeat of about 50 amino acids, referred to as the PAS-A and PAS-B repeats (Figure 12.2). In the absence of TCDD exposure, the PAS-B region of the AhR associates with one hsp90 molecule, permitting binding of a second hsp90 to the HLH region.[13,14,55–57] TCDD has been shown to interact with a ligand-binding pocket near the PAS-B region, the conformation of which is maintained by hsp90.[14,45,55,58] Dimerization between AhR and ARNT is mediated through their HLH regions, but is further stabilized by PAS–PAS interactions.[57,59] The C-terminal segment of the AhR contains multiple domains that synergistically potentiate its transcriptional activity. These domains are rich in glutamines (Q-rich) and acidic amino acids (i.e., aspartate and glutamate) or in prolines, serine, and/or threonines. ARNT contains a single transcription acti-

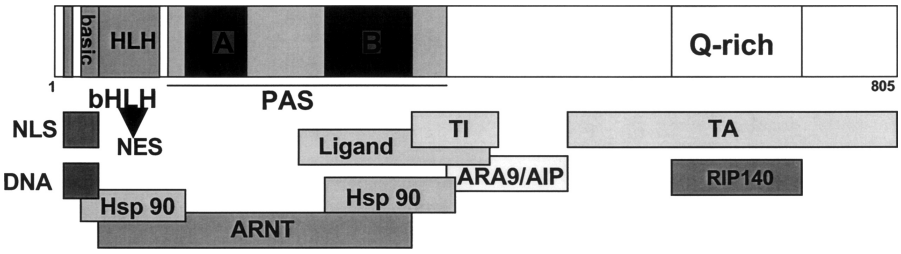

Figure 12.2 Functional organization of the murine AhR. The regions involved in AhR function or interaction with other molecules are shown with the domain structure of the AhR. Q-rich, glutamine-rich; NLS, nuclear localization sequence; NES, nuclear export sequence; TI, transcription inhibitory; TA, transcription activation domain.

vation domain (TAD) encompassing amino acids 582 to 774.[44,59,60] As indicated above, the AhR also contains a NLS in the N-terminal basic region that is masked by hsp90 when the AhR is not occupied by an agonist such as TCDD.[15]

Association of the AhR-ARNT complex with the DRE sequence requires multiple intermolecular interactions between the N-terminal bHLH-PAS domains of AhR and ARNT, which coordinate each DNA binding motif into an elaborate quaternary structure.[57,59,61,62] The actual contact with DNA appears to occur through the basic regions of AhR and ARNT. Deletion of this region in either protein results in complete loss of DNA binding without any effect on dimerization.[57,59] The AhR contains a "nominal" basic region at amino acids 27 to 39 which plays a crucial role in contacting DNA,[57,63] and a cluster of positively charged residues located about 20 amino acids upstream of the nominal basic region which is essential for DNA binding.[64] Both basic regions appear to be engaged in direct DNA contact, as mutation of these positively charged residues in either cluster abolished the formation of a complex with the DRE.[63–65] Interestingly, there are several proline residues between these two basic segments, accounting for a non-α-helical structure surrounding this relatively long DNA-binding domain of the AhR. These data suggest that the AhR may adopt a different and relatively unique DNA binding conformation which confers specificity to its own half site, 5′-TNGC-3′, in the DRE, in contrast to other bHLH "E-box" binding proteins that recognize the 5′-GTG-3′ half site.[28]

As indicated above, binding of the AhR-ARNT dimer to DREs in the enhancer region influences chromatin structure of the promoter region located hundreds of base pairs downstream, resulting in transcripton initiation of the *CYP1A1* gene. Under conditions in vitro AhR and ARNT have been found to interact directly with several proteins which make up the basal transcriptional machinery, such as TBP (TATA-box binding protein), TFIIF, and TFIIB.[66,67] However, in whole cells, communication between the enhancer and promoter elements does not appear to involve direct transmission of chromatin structural

changes since the DNA separating these regions retains nucleosomal organization during TCDD-stimulated transcription, and increased DNA accessibility at the enhancer elicited by AhR-ARNT binding of the DRE is not sufficient to induce *CYP1A1* gene expression. It has been hypothesized that this may be mediated by the ability of the AhR-ARNT complex to recruit other proteins that may assist in the alteration of chromatin structure.[41,43] Studies using a histone deacetylase inhibitor indicate that histone acetylation plays an important role in AhR-mediated activation of the *CYP1A1* gene.[68] The AhR-ARNT complex also interacts with several coactivator repressor proteins (RIP140, CBP/p300, Sp1, ERAP140, SMRT) that have been found to modulate TCDD-induced transcriptional activity in several cell lines.[69–72]

As noted above, many of these data emphasize the distinct functional nature of the domains in the AhR. However, it is important to point out that the exact functional and regulatory relationships between these domains have yet to be fully clarified. As such, sequences outside the ascribed functional domains may modulate the actions of these domains in both a qualitative and a quantitative manner.[73]

12.2.3 Some Structural Features Unique to the AhR

Some of the characteristics above are common to other members of the bHLH–PAS family of proteins as well as other transcripton factors. However, there are several unique structural features of the AhR that probably serve an important role in regulating its normal function as well as determining cell/tissue responses to TCDD. Several of the bHLH–PAS proteins appear to be involved in sensing changes in the cellular environment (e.g., HIF1α activity is affected by changes in oxygen tension), and the activation of these may occur by biochemical processes such as phosphorylation and change in oxidation state. However, the AhR is one of two members of this family whose activity is known to be dependent on binding with a ligand (the other being the juvenile hormone-binding protein Met in *Drosophila* (see Chapter 14 for additional discussion).[74] The AhR belongs to the α group of bHLH–PAS proteins. According to in vitro protein–protein interaction studies, members of the α group cannot homodimerize nor heterodimerize with members of the same group.[75] However, they can heterodimerize with members of the β group, of which ARNT is a member. In fact, ARNT appears to be a common dimerization partner for many bHLH-PAS family members.[76,77] The makeup of these interactions has several important functional consequences for the AhR. Each heterodimer complex, including AhR-ARNT, has its own consensus DNA-binding sequences and battery of genes that are directly regulated. Furthermore, while ARNT can form multiple heterodimer combinations, and thus be involved in the regulation of several gene batteries, the AhR is known to form a functional partner only with ARNT. If, in fact, this is the case, the recognizable enhancer sequences, and thus genes that are controlled directly by the AhR, are more limited (at least as compared to ARNT). On the other hand, multiple

forms of ARNT have been detected in several species.[78–80] Mouse and rat ARNT1 and ARNT2 are 83% identical in amino acid sequence and have the ability to dimerize with the AhR.[79] Furthermore, the expression patterns of these isoforms appear to be different.[78,79,81] Their respective roles in determining the gene battery affected are not yet clear. In addition, the AhR possesses a transcription inhibitory domain (TI) that overlaps with the PAS-B region and which appears to be active in a cell-specific manner.[82,83] The actual factors that interact with this domain are presently unknown. Together, the relative presence of these factors and ARNT isoforms represents a mechanism to determine diversity of tissue-, cell-, and gene-specific regulation.

12.3 AhR AS A MEDIATOR OF THE TOXICITY OF DIOXIN

12.3.1 Weight of Evidence to Support a Role of the AhR in TCDD Toxicity

Two lines of evidence suggest that most, if not all, of the toxic responses elicited by TCDD are mediated through the AhR. Structure–activity relationship (SAR) studies indicate that within groups of structurally related compounds, the general rank-order potency of dioxin congeners to produce a broad spectrum of biochemical, morphologic, immunologic, neoplastic, developmental, and reproductive effects correlates with their rank-order binding affinity for the AhR.[7,84] Second, the toxicity of TCDD to mice in which the AhR is genetically defective or altered clearly segregates with the presence of a high-affinity receptor. Just as mice carrying a "defective" low-affinity receptor (the *Ahrd* allele or variant) are less susceptible to TCDD-elicited *CYP1A1* induction than those carrying the high-affinity *Ahrb* allele, these mice are less susceptible to the toxic effects of this and related chemicals.[85–87] Furthermore, *Ahr−/−* mice, in which a functional AhR has been "knocked out," exhibit resistance to TCDD-induced toxicity.[88–93]

Is there any evidence to support a contention that the toxicity of TCDD and related chemicals is not mediated through the AhR? A very critical evaluation of the weight of evidence would have to indicate a possibility that some toxic effects might be mediated by another effector molecule. Even though data from SAR and genetic studies are consistent with the notion that the AhR mediates most toxic effects, clearly not all effects have been evaluated by these parameters. Similarly, *Ahr−/−* mice have not been thoroughly examined for susceptibility to all known toxic effects of TCDD. There are some in vitro and in vivo studies that could be interpreted to indicate that some toxic effects are AhR-independent.[87,94–98] However, some of these data obtained from the use of inbred mouse strains are probably due to well-documented TCDD-induced changes in the tissue distribtion of this chemical (see Chapter 6). In addition, some of the in vitro data conflict with those obtained in vivo and have been shown to be dependent on culture conditions.[96–98] Thus, there are no toxic

responses to TCDD that have been demonstrated conclusively to be independent of the AhR, and certainly many more investigations are consistent with its role in TCDD-induced toxicity. On the other hand, and despite the wealth of indirect evidence, a clear causal link between any AhR-induced biochemical event and a particular toxic effect has not been established (see Chapter 13). Furthermore, although the presence of the AhR appears to be necessary for toxicity to occur, several types of data, including those documenting the cell-, tissue-, and species-specific nature of these responses, indicate that AhR presence alone is not sufficient for these events to occur. What are the molecular pathways by which the AhR transduces signals leading to biochemical changes, and how do these subsequently lead to altered cellular function and toxicity? What other factors/processes regulate AhR activity? Do differences in these pathways/factors explain the observed tissue- and species-specific susceptibility to TCDD? These are questions that have yet to be clearly addressed.

12.3.2 AhR May Regulate Cellular Signals by Different Pathways

Based on the model presented above for the induction of *CYP1A1*, it is logical to hypothesize that the binding of TCDD to the AhR, AhR dimerization with ARNT, and the recognition of DREs by this complex leads to the inappropriate modulation of responsive genes as the initial steps eventually leading to a series of biochemical, cellular, and tissue changes that result in toxicity. Although the promoter regions of many genes, including *CYP1A1*, have been found to contain DREs,[99] only a few of these are known to be directly regulated by the AhR-ARNT complex (see also Chapter 13).[30] As yet, however, the modulated expression of these genes alone does not appear to completely explain any one or the diversity of toxic effects elicited by TCDD in a variety of animal species. It seems likely that there are other, as yet unidentified genes that are regulated by this pathway, and it is the inappropriate regulation of these that are directly related to particular toxic responses. Recent work indicated that the expression of between 100 and 300 genes may be modulated in human hepatoma cells following treatment with TCDD.[100,101] Although additional work in this area is clearly needed, the precise dissection of these events represents a considerable challenge. One can easily envision, for example, that a toxic response may depend on the timely modulation of several genes rather than just one particular gene, and possibly the modulation of these genes in several rather than just one cell type. Furthermore, since individual genes are most often coregulated by several transcription factors, the initiation of a toxic pathway by TCDD may be codependent on the presence (or absence) of these other factors as well as the AhR.

Although much attention has focused on the ability of TCDD to *induce* specific genes by the ability of the AhR to interact with DREs, AhR agonists, including TCDD, are also known to demonstrate inhibitory effects on gene expression. Evidence indicates that this may occur by targeting inhibitory DREs (iDREs) in the promoter regions of some genes. In this case, the DRE

may be in close proximity to a DNA binding site for another transcription factor, and AhR-ARNT binding to this site blocks the accessibility of the other factor, resulting in the inhibition of inducibility. This appears to be one mechanism for the ability of TCDD to exhibit antiestrogenic properties.[102–106]

Is it possible that TCDD could modulate gene expression by pathways that do not involve interaction of this protein with either ARNT or DREs? Although recent data indicates that conditional disruption of the ARNT gene results in the inability of TCDD to induce several responsive genes including *CYP1A1*,[107] there are no firm data yet proving that ARNT is required for any toxic effects elicited by TCDD. Although, no other functional heterodimer partner for the AhR has been identified, the AhR could interact with another protein and recognize DNA elements that are distinct from the consensus DRE identified for the AhR-ARNT complex. This is certainly very plausible considering the multiple dimerization partners identified for other bHLH-PAS proteins.

It is also conceivable that the ligand-elicited activation of the AhR and/or the formation of a complex with ARNT could inappropriately divert ARNT and possibly other proteins from other signaling pathways. Although recent data suggest a functional interference between hypoxia-induced and AhR-mediated signaling pathways,[108,109] other results indicate this is not due to competition between AhR and HIF1α for ARNT.[110] Thus, although it is an attractive hypothesis, there is no clear evidence, at least at present, to indicate that TCDD-induced toxicity is due to the "highjacking" of ARNT from its other heterodimeric partners. On the other hand, there are several studies indicating that the ability of AhR or the AhR-ARNT complex to interact with other proteins may disrupt normal cell functions. For example, direct interaction between the AhR and retinoblastoma (Rb) protein has been shown.[111,112] The proliferation of cells, and in particular the progression through G1 phase of the cell cycle, is regulated, in part, by Rb. Exposure of cells in vitro to TCDD has been shown to block proliferation by causing an arrest at the G1/S phase.[113–115] These observations suggest that the TCDD-activated AhR might disrupt normal proliferation processes by binding to the Rb protein. The murine AhR also has been shown to associate physically with the transcription factor NF-κB, and this interaction appears to produce a mutual functional repression of their actions, at least within cultured cells.[116] The ability of the activated AhR to divert NF-κB from its other functions may provide a mechanistic explanation for some of the toxic resonses elicited by TCDD. Other data suggest that AhR agonists may actually activate specific NF-kB subunits.[117,118] A recent report indicates that the AhR associates with the RelA subunit of NF-κB to activate the *c-myc* promoter in breast cancer cells.[117] One could also hypothesize that the ability of the AhR-ARNT complex to interact with several coactivator or suppressor proteins[69–72] (see above) may modulate their ability to regulate other genes. Nevertheless, at this time the relative importance of these interactions in the gene- and tissue-specific responses elicited by TCDD in vivo is unknown (see Chapter 13 for further discussion.)

Several publications have implicated that ligand binding to the AhR initiates a phosphorylation/dephosphorylation cascade resulting in modulated activity of other transcription factors.[119–126] Several of these reports suggest that activation of the cytosolic AhR, without nuclear translocation, may result in increased c-src kinase activity.[119,120,125,126] However, the precise components and consequences of these modulated pathways within intact cells have yet to be delineated. On the other hand, it would not be unreasonable to hypothesize that a ligand-induced change in the AhR conformation and subsequent dissociation of hsp90, and possibly other proteins, could initiate a cascade of events leading to changes in other signal transduction pathways that would not depend on the interaction of the AhR with DNA.

12.4 REGULATION OF AhR ACTIVITY: POSSIBLE FACTORS ASSOCIATED WITH DIFFERENTIAL TISSUE AND SPECIES SENSITIVITY

As we learn more about the AhR, the relationship between its structure and function, and the variety of proteins with which it may interact, we realize that there are multiple pathways that may be modulated by TCDD-initiated activation of the AhR. Similarly, there are number of mechanisms that may regulate the expression of this protein, its ability to be activated by ligand, and the type of intracellular signal produced. Together these mechanisms probably play a major role in determining the variety of responses observed following TCDD exposure as well as the tissue- and species-specific nature of these responses.

12.4.1 Regulation of Ahr Gene Expression and Tissue AhR Levels

The mouse *Ahr* gene is located in the centromeric region of chromosome 12 and consists of 11 exons spanning more than 30 kb of DNA.[127,128] The promoter region contains several Sp1 protein-binding sites, a potential cyclic AMP response element, AP-1 and E box sites, but no TATA or CCAAT boxes.[128,129] The AhR is expressed in most, if not all, mammalian tissues, but the level of expression is relatively tissue-specific. In general, the degree of enzyme induction does not appear to be strictly dependent on the level of AhR within a particular cell type or tissue. As indicated above, the relative presence of other factors may regulate the response. Furthermore, it appears that for many tissues there are "spare" AhR molecules; only a small percentage of the total AhR molecules present may need to be bound by ligand and activated to produce a maximal response.[130–132] In addition, a number of factors, including developmental and differentiation stage, diurnal cycle, cell activation, and presence and activation of other transcription factors and growth factors, have a significant influence on the relative expression of the *Ahr* gene and AhR protein.[133–149] The molecular pathways responsible for the regulation of the *Ahr* gene and the functional significance of this regulation remain to be determined.

Several investigations have demonstrated that prior exposure to AhR agonists elicits a sustained depletion of AhR protein, without an effect on AhR mRNA, in a variety of tissues.[147,150–153] This induced loss of the AhR requires TCDD-elicited nuclear uptake of the ligand-bound receptor, subsequent nuclear export by the recognition of a nuclear export sequence (NES) in the AhR, ubiquination, and proteosomal-mediated degradation.[154–159] Notably, AhR protein level has been reported to decrease during ongoing adipose differentiation in 3T3-L1 cells, and cellular responsiveness to TCDD was decreased concomitantly.[136] In contrast to some of the studies above, which were performed using cells in culture, a recent investigation observed an upregulation of AhR protein and mRNA in three rat strains following TCDD treatment at doses of 5 and 50 μg/kg.[160] However, subchronic exposure to relatively low levels of TCDD (10 or 30 ng/kg per day) did not substantially alter AhR levels in rats.[161] Together, these data suggest that the type of response observed in AhR expression following exposure to the dioxinlike chemicals may be dependent on dose, the type of tissue examined, and whether the treatment is performed in vivo or with intact cells in culture. Nevertheless, these data indicate that normal cellular processes regulate AhR protein and responsiveness to its signal transduction pathway(s). It would be of interest to determine whether for a given toxic response, an initial exposure to TCDD would decrease (or increase) the sensitivity of that response to a subsequent exposure, and whether this might be dependent on the type of response.

12.4.2 Differences in AhR Structure

In the past several years the *Ahr* gene from several species and strains of species have been cloned and sequenced. Ah receptor homologs have also been identified in several invertebrates. (The comparative sequences between a variety vertebrate and invertebrate species are discussed in Chapter 14.) As indicated above, detailed analysis of mouse *Ahr* variants has provided evidence for a role of this protein in the toxicity elicited by TCDD. Other strain- and species-specific variants have been observed, and a further analysis of these is likely to provide additional clues to the gene-, tissue- and species-specific activity of this protein.

One of the most fundamental processes regulating AhR activity within vertebrates is ligand binding. As indicated earlier, the responsiveness of certain mouse strains to TCDD is determined, to a large degree, by the presence of different *Ahr* alleles. The difference in affinities between these variant AhR forms can be partially attributed to a specific amino acid substitution, alanine 375 to valine, which lies in the ligand-binding domain.[160,161] It is of interest that an equivalent amino acid in the human AhR is valine 381, which, like valine 375 in the mouse AhR, also appears to explain partially the lower binding affinity of the human AhR for TCDD.[161,162] When alanine is substituted for valine 381, the affinity for TCDD is increased approximately twofold.[161] Although these data might suggest that the human AhR may be comparatively

less sensitive to the dioxins than several other species in terms of transducing a response, clearly a variety of other factors regulate the activity of this protein. Some data suggest the presence of more than one functional receptor form in humans,[163] although it is not clear whether this may be due to the presence of a protein with a different sequence or posttranslational modification. In earlier investigations, an analysis for human AhR polymorphisms identified only one amino acid exchanging polymorphism (arginine/lysine at position 554), and this is thought to have little or no functional significance.[164] Additional investigations found a polymorphism leading to a methionine 786-to-valine substitution and another polymorphism in the 5-untranslated region.[165] Neither were found to play a role in *CYP1A1* inducibility. However, a recent study identified an additional polymorphism leading to a valine 570 to isoleucine substitution, which when combined with the 554 variant fails to support TCDD induction of *CYP1A1* expression.[166] The combination of these variants is rare and appears to be confined to persons of African descent. The AhR from the beagle dog and cynomolgus monkey also has a lower affinity for TCDD.[167] However, other species with divergent sensitivities to TCDD-induced toxicity have AhRs with high affinity.[168] In particular, hamsters and Han/Wistar (H/W) rats are highly resistant to the acute toxicity of TCDD but possess an AhR in which the ligand-binding domain is highly conserved with that of the mouse and the corresponding affinity for TCDD is high.[168–170] Thus, although within a given species, receptor variants which alter ligand affinity appear to determine, to a large degree, sensitivity to TCDD, the comparative relationships between ligand affinity and responsiveness between species is less clear.

The Han/Wistar (H/W) rat strain has been shown to be at least 1000-fold less sensitive to the acute lethal effects of TCDD than most other rat strains, although it appears to be just as sensitive to some other biological effects, including *CYP1A1* induction.[171] Recently it was found that the apparent lower mass of the AhR from this rat strain is due to a loss of about 40 amino acids within the transactivation domain.[170] The glutamine (Q)-rich region of the transactivation domain has been found to be lacking in one form of the trout, killifish, and zebrafish AhR.[172–174] However, the Q-rich transactivation domain of the hamster AhR form is expanded substantially.[169] The hamster is one of the least sensitive species to the lethal effects of TCDD. In contrast, the C-terminal Q-rich region of the AhR from the guinea pig, one of the most sensitive species to the lethal effects of TCDD, is about half the size of that in the hamster but more similar to that of the human AhR.[175] Although the functional consequences of these differences remain to be characterized, the data suggest that this region of the AhR may designate differential sensitivity in the responses elicited by TCDD by regulating the ability of the AhR to interact with other proteins functioning as gene-specific activators or repressors. Thus, it might be hypothesized, for example, that while the expanded Q-rich region of the hamster AhR may not affect *CYP1A1* induction, this region may interact with some tissue-specific factor to prevent activation of a particular gene or

genes that might be important in mediating certain acute toxic effects of TCDD as observed in other species. Leucine-638 in this domain has been shown to be critical for efficient transcriptional activity of the human AhR by disrupting recruitment of coregulators.[176] Recently, a protein was identified that may act as a selective suppressor of *CYP1B1* gene expression.[177] The relative presence of this factor may account for the selective expression of *CYP1A1* or *CYP1B1* in many cell types with and without TCDD exposure. Further complexity for this type of regulation would occur if the expression of this putative factor were time-dependent.

It should be pointed out that the discussion above refers exclusively to AhR1, the most predominant form of this protein in vertebrates. With the exception of the AhR1 found in zebrafish,[178] this protein from different species binds TCDD and other AhR ligands with high affinity. Another form of the AhR, AhR2, that also binds TCDD has been found in bony fishes. (See Chapter 14 for a more detailed discussion of these AhR forms and their homologs.)

12.4.3 Regulation of AhR Activity by Other Molecules

It is possible that nonfunctional AhR-like proteins may be expressed, and these may compete with a functional AhR. For example, a factor was identified in human fibroblasts that acts by binding with ARNT to form an inactive complex.[179,180] Recent studies also characterized an AhR repressor (AhRR) in mice that competes with the AhR for ARNT dimerization.[181] Notably, the gene for this repressor is activated by the AhR-ARNT complex, and the sequence of this gene exhibited a great degree of similarity to the AhR. However, the PAS-A domain was variable and the PAS-B domain, which functions in ligand binding and interaction with hsp90, is missing. Additional work identified a proline 185 to alanine polymorphism of the AhRR gene in humans.[182] Furthermore, these investigators noted a weak but statistically significant association between this polymorphism and the presence of micropenis, and these data were interpreted to indicate that the ^{185}proline allele increases the suscecptibility to the undermasculinizing effects of dioxin exposure in utero (see Chapter 9), presumably through decreased inhibition of AhR-mediated signaling. It will be of further interest to determine whether differential expression of the AhRR might account for, at least in part, for tissue-, temporal-, and/or species-species differences in the sensitivity to TCDD and other dioxin-like chemicals.

In a similar manner, a variety of other signal transduction pathways might modulate AhR function, and this might even be gene-specific. One could envision several possibilities in which other molecules could bind to either the AhR, ARNT, coactivator proteins, or the upstream regulatory regions of certain AhR-responsive genes to modulate transactivation function initiated by dioxinlike chemicals. One or several of these mechanisms might explain a recent finding indicating that the AhR in the human hepatoma cell line, SK-Hep-1, is

unable to induce genes normally responsive despite its ability to be activated by agonists, transported to the nucleus, and bind to DREs.[183] Recent data also suggest that 12-*O*-tetradecanoylphorbol-13-acetate (TPA) suppresses TCDD-mediated induction of *CYP1A1* and *CYP1B1* through a mechanism that involves transforming growth factor-β (TGFβ) and mitogen-activated protein kinases.[184] Although it is well established that hormonal status and treatments modify certain TCDD-induced toxic endpoints,[185–190] the specific mechanisms have not been defined clearly. Indeed, they may be very complex, involving several factors working simultaneously,[191,192] making their definition a considerable challenge. Nevertheless, the dissection of how these factors regulate specific AhR responses differentially remain important goals if we are to determine if, and how, the dioxinlike chemicals affect human tissues.

12.4.4 Ligand Efficacy

The AhR may be regulated in both a positive and a negative manner by the type of ligand bound. As indicated above, it has long been recognized that in groups of structurally related compounds, the rank-order binding affinity to the AhR generally corresponds to the rank order of potency to transform the AhR to a transcriptionally active form and elicit a variety of biological responses. This rank order is largely dependent on several structural constraints. Relatively planar aromatic compounds with approximate van der Waals dimensions of $14 \times 12 \times 5$ Å with few bulky substituent groups and electron acceptor capability have, in general, the highest binding affinity.[7,8,193,194] A diverse number of xenobiotics, drugs, agricultural, and dietary chemicals and naturally occurring compounds have been found to bind to the AhR and act as agonists/antagonists in a number of biological systems.[195] It is of particular interest that some AhR ligands have been found to have varying degrees of antagonist activity (see Table 12.1), suggesting that not all ligands have the same degree of efficacy (or ability to form an active ligand-receptor complex).[198] Having some basis for predicting the relative efficacy of these agents would be extremely useful for several practical and theoretical reasons. However, the structural requirements and mechanisms of AhR antagonists are poorly understood. Whereas some chemicals that have antagonist properties may block transcriptional activation function of the AhR,[10] others appear to inhibit the dissociation of hsp90 from the AhR complex in the cytosol, thus inhibiting nuclear uptake and DNA binding.[9,10,204,216] In the latter case, the data suggest that particular substituent groups, such as those possessing high electron density that can form hydrogen bonds and/or electrostatic interactions with receptor amino acids, promote antagonist activity.[204,205] However, the ability of any of these ligands to act as an agonist or antagonist may also depend highly on the as yet undefined structural constraints of the AhR protein itself, especially in different species,[206] the cell type and relative presence of other coactivator or repressor proteins, the particular gene regulated, and the assay being used.[220,221]

TABLE 12.1 Chemicals Shown to Have AhR Antagonist Activity

Chemical	Refs.
Chlorinated biphenyls (2,2′,4,4′5,5′-hexachlorobiphenyl, 2,2′5,5′-tetrachlorobiphenyl, 2,2′,3,3′,4,4′-hexachlorobiphenyl, 2,3,3′,4,4′-pentachlorobiphenyl)	196–198
6-Methyl-1,3,8-trichlorodibenzofuran	10, 199
7,8-Benzoflavone (α-naphthoflavone)	9, 200
Natural flavones (e.g., flavone, apigenin)[a]	201
Synthetic flavones (e.g., 3′-methoxy-4′-nitroflavone, 4′-amino-3′-methoxyflavone, 4′-azido-3′-nitroflavone, 3′,4′-dimethoxyflavone)[a]	9, 201–206
Flavonols (e.g., flavonol, galangin, quercetin, kaemfperol)[a]	201, 207, 208
Flavanones (e.g., flavanone, naringenin)[a]	201
Catechins [e.g., (−)-epigallocatechin gallate, (−)-epicatechin gallate][a]	201, 209
4,7-Phenanthroline	210
9-Hydroxyellipticine[a]	205, 211
Indole-3-carbinol	212
Diiindolylmethane	212
3,5,4′-Trihydroxystilbene (resveratrol)	213–217
[2-(2′-Amino-3′-methoxyphenyl)-oxanaphthalen-4-one] (PD98059)	218
2-(4-Morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one (LY294002)	219

[a]Additional chemicals in this group also have been shown to possess some AhR antagonist activity. These can be found in the references given. Only the most potent are shown.

12.4.5 Phosphorylation and Oxidation–Reduction Processes

There have been several reports indicating that phosphorylation processes influence the ability of the AhR-ARNT complex to induce dioxin-responsive genes. In most mammalian cells, a mutual crosstalk appears to exist between the AhR and protein kinase C (PKC)–stimulated signaling pathways. Exposure to TCDD increases PKC activity,[222–224] and phosphorylation by PKC enhances TCDD-elicited induction of AhR target genes.[225–229] Although earlier studies suggested that PKC enhances transcriptional activity of the AhR-ARNT complex by increasing DNA-binding activity,[228,229] more recent studies indicate that increased PKC activity did not influence either nuclear uptake or DNA-binding activity.[226,227] Rather, PKC appears to enhance DRE reporter gene induction through the PAS region of the AhR probably by facilitating recruitment of an as yet unidentified PAS-specific coactivator.[230] Consistent with this, AhR was shown to be activiated to a DNA-binding form in cytosolic extracts lacking any detectable PKC activity.[231] Although both AhR and ARNT have been shown to be phosphoproteins,[232,233] the actual sites of PKC phosphorylation that are responsible for this enhanced stimulation of responsive genes have not been identified.

Although PKC may not affect the DNA binding activity of the AhR-ARNT complex, other data have underscored the importance of phosphor-

ylation for this process. Dephosphorylation of nuclear extracts from TCDD-treated cells abolishes the capacity of the AhR-ARNT complex in these extracts to bind to the DRE.[232,234,235] Additional data have indicated that at least one type of phosphorylation occurs on the AhR and positively regulates DNA binding. Phosphorylation on ARNT appears to contribute to dimerization between AhR and ARNT.[236,237] Although tyrosine phosphorylation in particular appears to be important for regulation DNA binding of the AhR-ARNT complex,[235] the exact amino acid residue, the mechanism of regulation, and if this phosphorylation may be partially responsible for tissue- and developmental stage-specific regulation of AhR activity have yet to be determined.

The DNA-binding activity of the AhR has also been shown to be sensitive to changes in oxidation–reduction conditions in vitro and in intact cells.[238–240] Together these data indicate that cysteine sulfhydryl residues within the AhR may play a role in the regulation of this activity. This also might suggest that AhR activity may be regulated under conditions of oxidative stress as may occur during several types of immune system responses as well as in aging.

12.5 EVIDENCE IMPLICATING A NORMAL FUNCTION OF THE AhR

It seems clear there are a number of cellular pathways present to control *Ahr* gene expression, AhR protein levels, and AhR signal transduction activity. It is reasonable to speculate that these mechanisms are in place to control a normal function of this protein. This would also suggest that exogenous ligands, like the dioxins, are toxic by virtue of their ability to activate the AhR at an inappropriate time or for an inappropriate length of time. Given the ability of AhR ligands to induce the synthesis of several enzymes involved in metabolism of a variety of chemicals, drugs, and several endogenous substrates, one could conjecture that the AhR is present simply to control the levels of these agents and protect the organism against their toxicty. However, the findings that other genes, but not necessarily these enzymes, are inducible in a variety of tissues where the AhR is present argues against this being the sole function of this protein. On the other hand, an attractive hypothesis is that the AhR may regulate a battery of genes and signal transduction pathways necessary for the normal growth, proliferation, and/or differentiation of cells, and that in addition, it regulates another battery of genes that are responsible for finely regulating the cellular concentrations of the normal endogenous ligand.

Several reports have shown stimulation of the AhR signal transduction pathway under certain conditions without the known addition of ligand.[113,115,135,241–244] For example, CYP1A1-deficient mouse hepatoma c37 cells possess transcriptionally active AhR-ARNT complexes in the absence of exogenous ligands. Similarly, the AhR was found to be activated following the treatment of Hepa-1 cells with an inhibitor of CYP1A1 activity.[242] These data suggest that a CYP1A1 substrate, which accumulates in cells lacking this enzyme activity, may be an endogenous ligand for the AhR. Finally, in mouse

hepatoma cells and fibroblasts the AhR appears to shuttle between nucleus and cytosol in the absence of exogenous ligands.[244,245] However, it is not clear whether this occurs via some endogenous ligand or another biochemical ligand-independent process that exogenous ligands may mimic. It is known that at least under cell-free conditions, the dissociation of hsp90 from the unliganded AhR will activate the receptor to a DNA-binding form.[11] Notably, an AhR mutant that lacks a minimal PAS-B domain, which contains the ligand-binding region, is constitutively active.[246] Furthermore, the targeted knockout of the *CYP1A1* gene in mice does not appear to alter the expression of other AhR-regulated genes.[247] Thus, although in most cases the transcriptional activity of the AhR appears to be dependent on ligand binding, it is not yet clear whether this binding merely amplifies a pathway already stimulated at some low level by some other process.

Identified candidates for endogenous agonist ligands include certain indole-related chemicals such as indirubin, indigo and tryptophan derivatives, carotinoids, arachidonic acid metabolites and certain prostaglandins, and tetrapyrroles or their derivatives.[247–254] These chemicals have been found to bind to the AhR and/or stimulate its transcriptional activity. Some very recent data suggest that 7-ketocholesterol may be an endogenous ligand that acts an AhR antagonist, and that some differences in species susceptibility to TCDD may be explained, at least in part, by their relative levels of this steroidal compound.[255] However, it has not yet been firmly established whether or how physiological levels of these may regulate AhR activity in an intact animal. As suggested above, a reasonable hypothesis might be that the AhR may regulate biochemical pathways involved either in the synthesis or degradation of an endogenous ligand so that effective, but not toxic, levels of such a ligand could be maintained during critical processes. In this regard, it is of particular interest that nearly all of the AhR ligands identified are also substrates for several of the CYP isozymes. Interestingly, TCDD exposure also appears to alter the expression of enzymes in prostaglandin synthesis.[256–259] Nevertheless, an endogenous AhR ligand has yet to be identified.

The best evidence that the AhR has a normal function comes from experiments in which the expression of a functional receptor has been knocked out. Inactivation of the AhR in mice results in developmental defects in the liver and the immune system.[260,261] Some evidence suggests that altered liver pathology in these animals is related to an abnormal accumulation of retinoic acid, resulting in increased activation of TGFβ and stimulation of apoptosis.[262–264] A role of the AhR in B-cell maturation processes has also been suggested.[265] These *Ahr*$-/-$, or *Ahr*-null allele, animals also demonstrate a decreased life span with abnormal and/or abnormally accelerated aging processes.[89] The females were reported to have difficulty maintaining the conceptus, surviving pregnancy and lactation, and rearing pups to weaning. However, the gender ratios of the offspring were comparable to those of wild-type animals.[266] A further analysis of serial ovarian sections revealed a twofold-higher number of primordial follicles in *Ahr*-null allele animals at day 4 post-

partum. Additional investigations suggested that this may result from a defect in the death rate of the developing germ line since AhR deficiency attenuated oocyte cell death in fetal ovaries maintained in culture.[267] These data are consistent with a recent investigation suggesting that the AhR may function in regulating the number of ovarian follicles.[268] Other studies indicate that normal mammary gland development in the mouse may be dependent on the presence of the AhR and that exposure to exogenous ligands during this period may alter normal AhR function to suppress development.[269–271] Recent investigations suggest that AhR signaling pathways may be involved in vascular remodeling.[272] It would be of particular interest to determine whether other developmental abnormalities found in *Ahr*−/− animals may be secondary to altered vascular development.

Since cells, tissues, and organisms lacking a functional AhR are viable, a conclusion can be drawn that the AhR is not required for life, but it strongly influences the manner in which development progresses and is maintained. Data from *Ahr*-null allele animals and toxicity studies with TCDD (see Chapter 4) together suggest that the AhR has an important role in the regulation of cellular growth and differentiation. These data also suggest that this role is highly dependent on the tissue and the developmental stage of that tissue. The cell cycle is one of the most fundamental processes integrating regulatory signals for cells to proliferate, differentiate, or die. There have been several studies implicating a subtle role of the AhR in those pathways controlling cell cycle. The exposure to AhR ligands has been shown to arrest cultured cells in G1/S phase.[113,114,273,274] AhR-defective hepatoma cells have a prolonged G1 phase of the cell cycle,[115] and embryonic *Ahr*-null allele mouse fibroblasts showed a slower growth rate than wild-type cells.[275] The exact molecular mechanism by which the AhR may influence these processes remains unclear. However, the AhR has been shown either to interact directly with or alter the expression of several proteins, such as retinoblastima protein, p300, and p27Kip1, which are known to be intimately involved in cell cycle control.[111,112,114,276,277] (A more detailed discussion is given in Chapter 13.) If an endogenous ligand exists, it might be generated as an intracellular signal to finely control these processes. This ligand also may be produced by a nearby cell type to communicate signals during tissue development or repair. On the other hand, as tissue development, remodeling, and processes such as angiogenesis take place, it is possible that the endogenous ligand may be a metabolic product generated by changes in the tissue environment that necessarily occur. The latter is particularly intriguing since several other bHLH-PAS protein family members, such as ARNT and HIFα, are involved in the sensing of these environmental changes (e.g., hypoxia).[278] Under some conditions AhR agonists may inhibit the activation of the hypoxia responsive enhancer and induction of erythropoietin by hypoxia.[109] In addition, the promoter for the *erythropoietin* gene contains DREs and appears to be a AhR-regulated gene.[109] Furthermore, hypoxia has been shown to inhibit TCDD-induced induction of the *CYP1A1* gene.[109,279]

Recently, the AhR sequence and signal transduction pathways have been

compared in mammalian and nonmammalian species.[224] Together, the information suggests that the AhR is an ancient protein and that its evolution stemmed from functions not related to the regulation of *CYP1A* genes and divergent from the ability of the mammalian AhR to bind TCDD and related xenobiotics (see Chapter 14). Thus, it seems likely that the AhR has some function in addition to that endowed by the binding of dioxinlike ligand, endogenous or exogenous.

12.6 AhR IN HUMANS: IMPLICATIONS FOR POTENTIAL SUSCEPTIBILITY TO CHEMICAL CONTAMINANTS AND HUMAN DISEASE PROCESSES

Human cells contain the AhR, and its properties, sequence, and identified molecular actions resemble those of the AhR identified in other species.[47,148,161,280–287] However, the human AhR appears, at least under cell-free conditions, to have a several-fold lower affinity for TCDD.[161,288] Additional data from cultured embryonic palatal cells suggest that the human AhR may be many times less sensitive in terms of eliciting a response.[289] However, based on these data, it is inappropriate to conclude that human tissues are less sensitive to the toxic effects of TCDD. The human AhR may be more labile during tissue preparation and cell fractionation procedures.[288] Furthermore, other available data suggest some heterogeneity of AhR concentrations and characteristics in the human population.[163,283,290] In addition, since, as indicated above, there are a number of factors and pathways regulating AhR activity in a gene- and tissue-specific manner, it is reasonable to consider the possibility that although the AhR in humans might have lower affinity for TCDD than that of other species, differences in other regulatory factors might actually increase the relative responsiveness under certain conditions. At present, there are no clear data on the molecular properties of the AhR to indicate whether humans would be more or less susceptible than other animal species to TCDD.

In some sense, what we have learned about the AhR indicates that it will be a considerable challange to draw firm conclusions regarding human susceptibility based on animal data or even studies of human cells in culture. The findings that many AhR-modulated genes and responses are regulated in a species-, cell- and developmental stage-specific pattern suggest that molecular and cellular pathways leading to any particular toxic event are extremely complex. Clearly, it is difficult to conclude that a dose–response relationship for the induction of any particular gene or even a toxic endpoint may be meaningful with respect to a different gene and endpoint, or even the same gene in a different species. Complex responses probably involve multiple events, genes, and signal transduction pathways. Indeed, the dose–response curve for any particular biological response (e.g., cancer) might be considered as an integration of a series of dose–response curves each dependent on the concentrations of

molecules involved in each particular step. It also seems likely that for some adverse effects of TCDD, the population at risk might be limited to those with a particular genetic disposition. There is some evidence for this in both animals and humans.[291–293] Thus, while there might be few polymorphisms in the human *Ahr* gene that affect AhR function, polymorphisms at other genes (e.g., *ARNT* and *AhRR*, which regulate the responsiveness of the AhR signal transduction pathway) could have profound effects on human susceptibility. Many studies also indicate that developing tissues are especially sensitive to TCDD, and that the hormonal status may often determine relative responsiveness to certain endpoints (see Chapter 9). In addition, while some animal studies suggest that TCDD exposure and the induction of detoxifying enzymes by the AhR may have beneficial effects for protecting against the toxicity of certain chemicals, other data indicate that these induced enzymes bioactivate different chemicals to mediate or potentiate their toxicity. For example, several recent studies indicate that the AhR has an important role in the genetic damage and cancer caused by tobacco smoke constituents.[92,294,295] Whether this information is meaningful for human susceptibility has yet to be determined.

Despite the complexity of the pathways that may regulate and be regulated by the AhR, it is reasonable to draw the conclusion, based on what is known about the human AhR, that some biological endpoints will be affected adversely at some concentration of TCDD. A clearer understanding of these endpoints will rely, to a large degree, upon our further understanding of the molecular pathways regulated by the AhR and the factors that may modulate these pathways in human tissues.

12.7 FUTURE DIRECTIONS

The identification of endogenous AhR ligand(s) and the normal function(s) of the AhR will have a great impact on our understanding of the mechanisms, dose-dependent relationships, and potential tissue targets of TCDD toxicity in humans and wildlife. However, it is not clear whether we yet have sufficient insight as to when and where to look for the presence of the endogenous ligand, if indeed one exists. The examination of cell lines or tissues in which the AhR is active in the absence of exogenous ligand treatment may be a useful strategy to approach these issues. Likewise, continued analysis of *Ahr*-null allele animals and animals in which AhR function is conditionally inactivated is also likely to provide useful clues for the identity of an endogenous ligand and a normal function of the AhR. In the latter case, it is clear that considerable work has yet to be performed to identify the various signaling pathways the AhR may stimulate. Are all the responses of the AhR mediated through DREs, or can the AhR activate other pathways through the interaction with other proteins and/or stimulation of phosphorylation/dephosphorylation? If there are multiple pathways by which the AhR can transduce signals, it will be challenging to determine which may mediate specific endpoints of TCDD toxicity. Discrimi-

nation of these pathways is likely to be particularly important for our understanding of the tissue- and species-specific responses to TCDD.

It is also likely that regulatory mechanisms controlling the activity of the AhR determine tissue- and species-specificity responses to AhR ligands. Some of the processes include *Ahr* gene expression, AhR protein stability and turnover, AhR and ARNT phosphorylation, and cellular concentrations and activities of coactivators and repressor proteins. The identification and characterization of gene polymorphisms in the AhR, AhRR, ARNT, or other proteins taking part in or modulating AhR signal transduction pathways will also provide new insight into how these factors may affect responsiveness to TCDD. Analyses for these polymorphisms in humans have the potential to identify which genotypes exhibit or lower sensitivity to particular dioxin-related effects.

We now appreciate that the AhR is a member of a family of proteins that is conserved through evolution and involved in growth and differentiation processes. The expression and activity of the AhR appear to be regulated in a differentiation- and cell cycle stage–dependent manner. We also know that genes regulated by the AhR are involved not only in the metabolism of xenobiotics and endogenous substrates, but also in growth and differentiation. Furthermore, it is likely that the AhR has some subtle but significant role in cell cycle regulation. Dissection of the pathways involved will probably help us to understand why developing tissues and neoplastic processes may be so sensitive to the dioxins. In addition, these data might provide clues regarding whether and how the AhR might play some role in other disease states.

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CHAPTER 13

Biochemical Responses to Dioxins: Which Genes? Which Endpoints?

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13.1 INTRODUCTION

The prototypical dioxin, tetrachlorodibenzo-*p*-dioxin (TCDD), is a ubiquitous environmental pollutant that originates as a by-product of incineration, industrial waste, incomplete combustion of fossil fuels, and forest fires. Exposures to dioxin occur primarily through the food chain and result in a low level of accumulation in the organism.[1,2] Despite its environmental persistence, this compound is purposefully synthesized only for use in laboratory studies and is present in the environment only as a by-product of other processes. The primary mechanism by which dioxin and dioxinlike compounds are believed to exert their toxic effects is through the specific alteration of gene expression patterns, leading to critical changes in the normal physiology of the exposed organism. Many of the biological effects of dioxin exposure have been well studied, but to date, the exact mechanisms leading to these effects are still an enigma.

The effects of dioxin exposure in humans are the subject of much debate because of the lack of sufficient information to arrive at a precise mechanism of action. Most epidemiological data on the human health effects of dioxin are derived from accidental industrial exposures, including the two well-known accidents in Seveso, Italy in 1976[3] (see Chapter 20) and the BASF spill in Hamburg, Germany in 1953,[4] which are discussed extensively elsewhere in this book. Despite the large numbers of persons exposed at these two sites, only a limited number of significant acute health effects or pathologic laboratory findings have been reported other than some 200 cases of chloracne.[5,6] Prolonged exposure of BASF workers to TCDD resulted in increased incidence of

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thyroid disease, appendicitis, increased infectious diseases (intestinal, upper respiratory tract), peripheral nervous system disorders, and chronic liver disease.[4] Workers exposed between 1968 and 1972 to trichlorophenol and 2,4,5-trichlorophenoxyacetic ester in two unrelated sites in Verona, Missouri and Newark, New Jersey, respectively, had elevated γ-glutamyl transferase enzyme levels and altered high-density lipoprotein levels as well as elevated levels of testosterone in males, and elevated lutenizing hormone and follicle stimulating hormone in females.[4,7]

Body burdens of dioxin that are frankly toxic in experimental rodents do not seem to cause much toxicity in humans. Current human body burdens of dioxin are in the range of 13 to 7000 ng/kg, approaching or exceeding concentrations in the range 10 to 12,500 ng/kg, associated with developmental toxicity in laboratory animals exposed in utero.[8] By comparison, TCDD levels for the Seveso exposures were approximately 10,400 ng/kg in adults (56,000 ng/kg in blood fat).[6] Even in this highly exposed population, body burdens that caused chloracne in some people did not cause chloracne in others, underscoring the high variability of dioxin health effects in exposed humans. The highest blood fat level of TCDD ever found in a human is 144,000 ng/kg, found in a 30-year-old woman in March 1998 after developing acute acne in late 1997.[6] This level of TCDD corresponds to 25 μg/kg of body weight; a level considered extremely high even for in vivo challenged animals.[6,9] This woman had moderately elevated levels of blood lipids.

Like humans, laboratory animals exposed to dioxin present a variety of biological responses. Adverse outcomes observed in these animals include wasting syndrome, thymic involution, immune system dysfunction, reproductive and developmental effects, teratogenesis, epithelial hyperplasia and metastasis, and cardiovascular disease.[10–12] For unknown reasons, TCDD is a potent hepatocarcinogen in female but not in male rats. The carcinogenic potential of TCDD is believed to be related to its tumor promoter properties,[13,14] possibly the result of altered gene expression and interference with cell cycle–related events.[15–18] Paradoxically, TCDD has also been found to be chemoprotective against breast cancer in both rats and mice,[19,20] and based on epidemiological data from Seveso, it appears to cause a reduction in breast cancer incidence in humans.[21] The fact that dioxin exposure in rodents results in tumors has largely overshadowed its ability to inhibit tumor growth. Despite the clear difference between dioxin effects in animals and humans, it is evident that pathological alterations can result from prolonged and/or chronic exposure. Although the cause of these disorders in humans is not completely understood, it should be pointed out that responses to dioxin exposure are different among different people in the human population, even in the case of chloracne, as indicated previously. Our laboratory is particularly interested in studying the role of dioxin-mediated deregulation of gene expression patterns in the development of cardiovascular disease, which is prevalent in fish, avian, and mammalian embryos as suggested by edema, hemorrhage, and mortality. TCDD is associated with dilated cardiomyopathy and with symptoms asso-

ciated with congestive heart failure.[22] We believe that dioxin exposure, and exposure to other dioxinlike environmental agents, also plays an important role in exacerbating the incidence or the severity of ischemic heart disease.

It is generally accepted that the majority of the effects of dioxin arise from dioxin binding to the aryl hydrocarbon receptor (AhR), a member of a family of transcription factors containing basic helix-loop-helix and PAS homology domains (bHLH–PAS). The biology of the AhR and its transformation to a transcription factor by ligand-dependent activation is reviewed extensively in Chapter 12. Binding of the heterodimeric AhR-ARNT complex to AhR response elements [AhREs; also known as dioxin response elements (DREs) and xenobiotic response elements (XREs)] in the promoter region of dioxin-responsive genes results in an increased, and in some cases, in a decreased rate of transcription. Many genes respond to dioxin-mediated activation of the AhR in this manner, including several phase I and phase II detoxification genes, such as CYP1A1,[23] CYP1A2,[24] CYP1B1,[25] GST-Ya subunit,[25a] NAD(P)H menadione oxidoreductase, UDP-glucoronosyltransferase,[23] and aldehyde-3-dehydrogenase.[26] In the presence of dioxin, the AhR shuts down the proliferation and metastasis of tumor cells,[20,27,28] most likely by altering apoptotic and cell cycle regulatory events.[15–18,29] One of the major mysteries of its biology is the paradox that TCDD can act to promote cell growth and proliferation in some cases and to arrest cells and block cell cycle progression in others. Differences in cell lineage, modular signal transduction pathways, and activation status of critical regulatory genes may be crucial to deciding in which direction a particular cell will go. It is likely that the carcinogenic effects of TCDD result from complex interactions between the genes that are deregulated as a result of exposure. Understanding the biochemical and molecular basis of these interactions could lead to the development of less toxic TCDD congeners or analogs with a specificity for proliferating tumor cells.[30]

Since gene regulation by dioxin occurs as a result of binding of AhR-ARNT complexes to cis-acting promoter sequences, researchers have focused on the temporal and spatial expression of the AhR to gain a perspective of its functions. Constitutive expression of the gene encoding AhR occurs in a tissue- and developmentally specific manner.[31] The promoter of the AhR is G-C rich and contains no TATA or CAAT boxes; however, sequence analysis has shown several binding sites for the transcription factor Sp1, a cAMP response element, AP-1, E-box sites, and two elements demonstrated in other genes to confer placenta-specific expression.[32,33] In addition to these elements, which are present in many housekeeping genes, the AhR can also regulate its own expression. The ligand-bound AhR activates the transcription of a novel repressor protein, the AhR repressor (AhRR), which interacts with ARNT and represses the transcription of the AhR. The AhR and AhRR form a regulatory circuit in the xenobiotic signal transduction pathway and provide a novel mechanism of regulation of AhR function that may determine tissue-specific sensitivity to environmental pollutants.[34]

Some of the highest levels of AhR expression are found in the lung,

while moderate levels are found in liver, thymus, placenta, brain, heart, and spleen, and low levels in skeletal muscle.[35] AhR expression can be altered by TCDD,[36] phenobarbital,[37] serum and mitogens,[38] retinoic acid,[39] and TGFβ.[40] AhR expression is also dependent on chromatin structure, as shown by treatment with chemicals that affect histone deacetylase (HDAC) activity in cells in culture. HDAC inhibitors such as sodium butyrate and trichostatin A increase the constitutive activation of the AhR gene promoter. Blocking histone acetylation with E1A oncoprotein, a negative regulator of the CBP/p300 histone acetylator complex, resulted in a decrease in AhR promoter activity.[41]

This short introduction has touched on the surface of the complex biology of the Ah receptor and its role in specific aspects of the health outcome of dioxin exposure. More detailed explanations of AhR biology and dioxin-mediated disease processes are discussed elsewhere in this book. In the remainder of this chapter we focus on dioxin-mediated gene expression and the biological outcomes resulting therefrom. The wide range of biological consequences of dioxin exposure attests to the vast number of mechanisms by which dioxin and dioxinlike compounds can exert toxicity. Clearly, activation of gene transcription through the Ah receptor is an important effect of dioxin exposure, but it is only the tip of the iceberg, and we now know that exposure to TCDD results in altered profiles of gene expression through direct and indirect mechanisms requiring multiple protein components. In this chapter we discuss recent findings on dioxin-mediated alterations in metabolic, cell cycle, and apoptotic gene expression and current results of global gene expression analyses of cells and tissues exposed to dioxin.

13.2 DIOXIN-REGULATED GENES AND METABOLISM

In toxicology, induction of the cytochrome P450 CYP1A1 by TCDD is one of the best-studied gene–toxicant interactions. Much of what is known about Ah receptor biology is the result of studies designed to analyze the mechanism of *CYP1A1* transactivation by the ligand-activated AhR. The molecular biology of the AhR and its role in P450 induction has been studied extensively and well characterized and has been described in many excellent reviews (see, e.g., Refs. 42 and 43). The development of transgenic and gene knockout mice has fostered many studies designed to analyze the effects of dioxin exposure in relation to specific genotypes. Of particular interest has been the development of *Ahr*-null mice, lacking AhR,[44–46] and of *Cyp1a1*-, *Cyp1a2*-, and *Cyp1b1*-null mice, lacking expression of the cytochromes P450 CYP1A1,[47] CYP1A2,[48] and CYP1B1,[49] respectively. Several laboratories are attempting to generate the quadruple knockout mice, lacking all the genes above, an effort hindered by the close linkage between the *Cyp1a1* and *Cyp1a2* genes[50] (D. W. Nebert, personal communication). $Ahr^{-/-}$ mice are resistant to TCDD-mediated toxic and pathological effects at doses 10 times higher than those that result in severe toxicity in $Ahr^{+/+}$ mice.[51] Similarly, Fuji-Kuriyama's laboratory has found

that B[*a*]P, a potent initiator and promoter of carcinogenesis, does not cause liver or skin lesions in $Ahr^{-/-}$ mice.[52] The development of mice that lack AhR, as well as other genes in the CYP1 family, will further define the role of these genes in both PAH- and dioxin-mediate disease processes.

One reason why it is often difficult to define target genes for TCDD is that different cell types and cells at different stages of the cell cycle show a certain degree of selectivity for induction of TCDD-responsive genes. For example, in murine hepatoma Hepa1c1c7 cells, steady-state CYP1A1 mRNA content is reduced by 45 to 90% in TCDD-treated cultures arrested in G2/M, relative to TCDD-treated asynchronous cultures.[53] The accumulation of mRNAs corresponding to NAD(P)H quinone oxidoreductase-1 (NQO-1), another TCDD-inducible gene of the Ah battery, is also reduced in TCDD-treated G2/M cultures.[53] Interestingly, this decline in CYP1A1 transcription is independent of AhR protein levels and AhR translocation to the nucleus, but when these cells are released from G2/M, TCDD responsiveness is restored. In addition, TCDD-mediated CYP1A1 expression levels during G1/S is much greater than in early G1 or in G2/M.[53] Induction of CYP1B1 or CYP1A1 mRNA in vascular smooth muscle cells (vSMCs) is less responsive to TCDD than to benzo[*a*]pyrene (BaP), the prototypical PAH AhR ligand.[54] In the vasculature, endothelial cells (vECs) preferentially express inducible CYP1A1, whereas vSMCs express CYP1B1.[54,55] TCDD exposure does not result in increased aryl hydrocarbon hydroxylase (AHH) activity in vSMCs, while AHH activity is increased in $Ahr^{-/-}$, as well as $Ahr^{+/+}$ vSMCs by BaP in a cell cycle–dependent manner.[54] TCDD upregulates the expression of CYP1A1 and CYP1B1 in the human mammary carcinoma MCF-7 line and in the human uterine cancer line RL95-2, but not in lymph node cancer of the prostate (LNCaP) cells.[56] The difference in expression patterns of these two TCDD-dependent genes in various cell types appears to be related to methylation patterns located within the AHRE in their 5′ promoter regions.[56] When pretreated with the methylase inhibitor AZA-C, CYP1A1 expression was restored only in LNCaP cells.[56] In addition, in LNCaP cells, TCDD treatment has been reported to inhibit testosterone-mediated transcriptional activity, whereas testosterone inhibits TCDD-mediated upregulation of CYP1A1 and its related ethoxyresorufin-*O*-deethylase (EROD) activity.[56,57]

Our laboratory has been interested in the study of dioxin-dependent gene expression for the past several years. In addition to examining the role of TCDD in CYP1A1 mRNA induction, we have examined key enzymes in prostaglandin (PGs) biosynthesis, in particular cyclooxygenase-2 (COX-2), the key rate-limiting enzyme for the production of PGs from arachidonic acid. This enzyme is bifunctional, converting arachidonic acid into PGG2, and concomitantly PGG2 into PGH2. We examined mouse hepatoma Hepa-1, and its AhR-, ARNT-, and CYP1A1-defficient derivatives for alterations in prostaglandin levels, to evaluate the potential role for dioxin, and the AhR, in eicosanoid metabolism. Analysis of prostaglandins released from cells treated with TCDD revealed major changes in the levels of several of the immediate cyclo-

oxygenase products, particularly 12-HHT.[58] The metabolite changes observed in TCDD-treated cells could best be explained if they resulted from an increase in PGH2 levels. Accumulation of COX-2 mRNA, but not of COX-1, after TCDD treatment was clearly significant. Accumulation of COX-2 mRNA was similarly induced in Hepa-1 cells deficient in CYP1A1, but not in AhR-deficient cells, suggesting that the AhR, but not CYP1A1 AHH activity, was required for the induction.[58] Upregulation of COX-2, an enzyme important in the metabolism of immunoregulatory eicosanoids, does nothing to impair the immunosuppressive activity of dioxin. One study that addressed this concern found that blocking the effects of arachidonic acid metabolites did not block the onset of TCDD immunotoxicity.[59] This work has not yet been confirmed in other laboratories, but it might suggest that more complex biochemical mechanisms, such as thymic atrophy or apoptosis, were at work.[60,61] This example illustrates how in many cases, gene expression changes resulting from a dioxin challenge, may show no obvious relationship to the ultimate outcome of TCDD exposure.

Dioxin's ability to alter lipid metabolism was first suggested almost 20 years ago. Administration of 20 μg TCDD/kg to New Zealand rabbits maintained on a normal or in a 0.5% cholesterol diets resulted in a significant increase in plasma triglyceride levels, particularly in the LDL fraction, suggesting that TCDD may inhibit the breakdown of triglycerides and lead to hypertriglyceridemia.[62] In this context we have reported that TCDD increases serum triglyceride levels, particularly the LDL fraction, in $Apoe^{+/+}$ and $Apoe^{-/-}$ mice. We also observed an increase in vascular lesions in $Apoe^{-/-}$ mice challenged with 150 ng TCDD/kg three times a week for 7 to 26 weeks. In vitro treatment of vascular smooth muscle cells with 1 n*M* TCDD was associated with altered gene expression of caspase-1, $p21^{waf/cip1}$, cyclin D1, mdm2, p15, and p57 by twofold or greater. Therefore, dioxin could increase the incidence of cardiovascular disease through altered gene regulation as well as through increases in serum lipid levels. The mechanism for this increase is not yet known, but it may take place through inhibition of lipid metabolism.[12]

In the environment, it is unlikely that individuals will ever come into contact with just one toxicant. It is becoming evident that exposure to chemical mixtures may have gene regulatory effects unpredictable from the patterns observed for each of the components of the mixture. Recent data from our laboratory suggest that coexposure to pro-oxidant metals or metalloids and dioxin, which is common in the environment, can disrupt the coordinate regulation of phase I and II detoxification genes, leading to imbalances in gene expression that may have important consequences for the toxicity of complex mixtures. Of the metals and metalloids tested, neither cadmium nor arsenic alone interfered with CYP1A1 mRNA expression; however, chromium-inhibited TCDD-induced CYP1A1 gene expression and arsenic, cadmium, and chromium showed differential effects on the induction by TCDD of *Nqo*-1; arsenic greatly enhanced Nqo-1 expression, cadmium mildly upregulated it, and chromium completely blocked it.[63] Many sources of environmental expo-

sure to AhR ligands involve metal coexposure. For example, carcinogenic metals and AhR ligands are common contaminants of hazardous waste sites and are coreleased from sources such as fossil fuel combustion, municipal waste incineration, and as components of cigarette smoke. Cadmium, chromium, arsenic, BaP, and polychlorinated biphenyls, including the AhR-activating mixture Arochlor 1254, continue to be high priorities for evaluation due to potential risks to human health.[64] The effect of metals on the balance between phase I and II gene expression may have significant practical implications for assessing the risk of environmental exposures.

13.3 ACTIVATION OF SIGNAL TRANSDUCTION PATHWAYS BY DIOXIN

Many of the effects of dioxin result from the activation of intracellular signaling pathways. In some cases, activation of signal transduction pathways contributes to the overall TCDD toxicity, but this may not be always the case, and often, the ultimate endpoint of the signals triggered by TCDD is unknown. Toxicity stemming from TCDD-induced AhR activation may be a direct consequence of AhR-regulated gene expression in response to the toxic agent, or it may result from perturbations of normal AhR-mediated gene expression impinging upon multiple signaling pathways. Most experimental evidence in this area is still purely descriptive. The pathways that connect TCDD with its ultimate effect on signal transduction are diverse and largely uncharacterized, and the signaling mechanisms are very poorly understood, if at all. Much more work is needed to bring into focus this extensive body of evidence that, if properly interpreted, might shed light into the molecular mechanisms of the toxic response and help design potential targets of intervention. Nonetheless, the limited amount of information available suggests that the combinatorial interaction between activated AhR and signal transduction pathways potentiates at some unknown level TCDD toxicity.

TCDD induces immediate increases in protein kinase C and tyrosine kinase activities in hepatocyte membranes.[65–67] In particular, TCDD activates epidermal growth factor receptor (EGFR)–associated tyrosine kinases and induces the molecular interactions of EGFR-associated adaptor proteins, including SHC, GRB2, and SOS.[67,68] This adaptor protein complex further transmits receptor and nonreceptor phosphotyrosine kinase signals to the activation of the small GTPase p21Ras.[68] Both TCDD and B[*a*]P are capable of the induction of RAS activity, although the relationship between RAS and AhR activity is somewhat confusing, showing both tissue-specific[69] and opposing effects.[70–73] Independent work using microarray global expression analyses has provided strong confirmation of the AhR-dependent activation of the RAS mitogen-activated protein (MAP) kinase pathway in human hepatoma cells.[74] In addition, in mouse lung tissues, K-RAS activity is enhanced by TCDD treatment in a manner not fully dependent on the AhR,[75] suggesting

that there are multiple mechanisms for AhR ligands to affect signal transduction pathways. TCDD also stimulates ERK activity in experimental animals.[76,77] ERKs, along with the Jun-N-terminal kinases (JNKs) and p38, are serine/threonine kinases that belong to the family of MAP kinases regulated through conserved protein kinase cascades consisting of the MAP kinase kinase kinases and the MAP kinase kinases.[78]

In rat primary hepatocytes, TCDD induces the association of the various effector proteins, including SHC, CBL, GRB2, and SOS, which form the signaling complex that transmits EGFR-originated signals,[68] although TCDD itself is not an EGFR ligand.[79] Crosstalk between AhR and EGFR signaling pathways has been shown to be critical for the hepatocarcinogenicity[80] and the developmental toxicity[81] of TCDD. Mechanistically, it is possible that TCDD activates EGFR tyrosine kinase in the cytoplasmic membrane, leading to ERK activation by the RAS-RAF-MEK signaling pathway. Alternatively, signaling molecules downstream of the EGFR, such as PK-C, PLC-γ, and PI-3K, known to be involved in induction of ERK activity by various stimuli, may be responsive to TCDD. All three enzymes are involved in AhR signaling and play an essential role in AhR activation and expression of CYP1A1.

Several studies have demonstrated that the PK-C pathway is required for AhR activity.[82–84] TCDD exposure leads to an increase in extracellular calcium flux and to the elevation of intracellular calcium by a process that is AhR-dependent and may require *CYP1* gene expression.[58,85–87] Although it is not yet understood how TCDD modulates calcium mobilization, it is possible that it activates PLC-γ, a phospholipase that plays a major role in the regulation of intracellular calcium by controlling inositol 1,4,5-triphosphate-gated intracellular calcium stores.[88]

The tyrosine kinase activity of c-SRC is also activated by xenobiotics in experimental animals, following one or both of two alternative pathways. Along one pathway, SRC may be activated by signals initiated from cell surface receptors, such as EGFR, integrins, G protein-coupled receptors, or intracellular receptors, such as the hormone receptors,[89–91] whereas along an alternative activation pathway, SRC, which may be functionally associated with the AhR/HSP90 complex,[92,93] could be activated by ligand-induced disruption of the complex. AhR-dependent c-SRC activation has been confirmed in vitro, using AhR blockers to prevent TCDD-induced SRC kinase activity.[94] Activation of c-SRC contributes to the maximal toxicity of TCDD and mice deficient in c-SRC expression exhibit reduced toxic responses.[92]

Work from several laboratories has shown that Ah receptor agonists can induce the expression of immediate-early proto-oncogenes in the *FOS* and *JUN* gene families and the concomitant increase of transcription factor AP-1 DNA-binding activity. Induction has, however, been observed in guinea pig and macaque liver cells and in mouse hepatoma cells[77,95,96] but not in human HepG2 hepatoma cells,[97] suggesting that immediate-early proto-oncogene induction by TCDD may be cell type–dependent. Transcriptional induction of mouse c-*Jun* and *Jun*-D has been described to result from Ah receptor complex

binding sites in the proximal promoter of these genes, while induction of c-*Fos* is Ah receptor–independent and is mediated by transcriptional responses at the serum response element (SRE) motif in the c-*Fos* promoter.[98] The SRE motif binds the ternary complex formed by ELK-1 and TCF,[99–101] implicating TCDD exposure in one pathway of activation of the ERK MAP kinases responsible for ELK-1 phosphorylation and transcriptional activity. Direct evidence for ERK activation by TCDD has recently been obtained.[101a]

13.4 DIOXIN EFFECTS IN CELL CYCLE REGULATION

Based on the observation that TCDD is a powerful tumor promoter,[102,103] it was reasonable to hypothesize that TCDD and other AhR ligands might act as modulators of second messengers and activate the expression of genes involved in proliferation and differentiation, much like the tumor promoters of the phorbol ester family. The starting point for this hypothesis was to test the role of Ah receptor ligands on the regulation of immediate-early response genes, responsible for bringing cells out of G_0 into the cell cycle.

Recent work to address this question has determined that expression of the immediate-early c-*MYC* gene in human breast cells is induced by an AhR/RelA DNA-binding complex through a NF-κB element in the c-*MYC* promoter, suggesting that the AhR may contribute to entry into the cell cycle indirectly.[104] The Ah receptor impact on NF-κB signaling is more complex; however, since formation of AhR/RelA complexes may account for the mutual functional repression of AhR/ARNT and RelA/p50 (NF-κB)-mediated transcriptional activities.[105,106] Sequestration of RelA by the AhR may also be responsible for the observed increase in p50 homodimer binding to NF-κB binding sites resulting from TCDD treatment of hepatoma cells.[107]

The activated Ah receptor has been implicated in other cellular processes, through its interactions with other gene products involved in cell cycle regulation, signal transduction, and apoptosis. Our laboratory, as well as others, has found that the AhR interacts with the retinoblastoma protein (RB) and that this interaction is critical for the transcriptional regulation of cell cycle–specific genes.[15–17,29] Due to this interaction, the AhR appears to be involved not only in signaling mechanisms of xenobiotic detoxification, but also in transcriptional responses resulting from complex protein interactions. The cyclin D-like LXCXE motif in the AhR protein is needed for RB binding to AhR, which is necessary for maximal TCDD-induced G1 arrest in rat 5L hepatoma cells. The data are consistent with the concept that RB regulates G1 arrest by a mechanism distinct from the direct repression of E2F-mediated transcription. At least two separate domains of the AhR are responsible for binding to hypophosphorylated RB.[15,16] In human mammary carcinoma MCF-7 cells, interaction with RB does not require ARNT or transcriptional activation of AhR-dependent genes. Transient transfection assays with AhR and RB

expression vectors in Saos-2 cells, which lack both AhR and RB endogenous expression, has shown that AhR synergizes with RB to mediate repression of E2F-dependent gene expression and leads to cell cycle arrest. Furthermore, the AhR partially blocks T-antigen-mediated E2F release from RB. Taken together, these data suggest a role for the activated AhR as a cell cycle regulator, activation being the result of either an endogenous ligand or of environmental signals.[16] Earlier work by Wang and co-workers[108] demonstrated that TCDD alone had little effect on expression of cell cycle proteins in MCF-7 cells, although it interfered with estradiol-mediated gene expression and cell cycle progression. TCDD significantly inhibited estradiol-induced hyperphosphorylation of RB, cyclin D1 protein, and cdk2-, cdk4-, and cdk7-dependent kinase activities.[108] Collectively, these sets of data demonstrate that the AhR has a role not only in directing gene regulation, but also in mediated cell cycle alterations. Given the potent repressive capacities of the complexes formed by hypophosphorylated RB and E2F, particularly during G0 and G1, the synergy of the AhR with these complexes can only mean that there will be a significant number of genes in the cell for which activation of the AhR by ligands will have repressive effects. This is a rather paradoxical function for a protein commonly described as a "ligand-activated transcription factor" but that in fact may be a repressor of some genes at some stages of the cycle and an inducer of the same or of other genes at other stages.

There are other genes involved in cell cycle regulation affected by dioxin exposure. In endocervical cells isolated after necropsy from TCDD-challenged macaques, changes in growth factor receptor signaling, cytosolic signaling proteins, tumor suppressors, and cell cycle proteins were observed, including increases in H-ras expression and in the activities of c-SRC kinase, receptor-associated protein tyrosine kinase, ERK2, cdk4, and AP-1 DNA binding. Conversely, there were decreases in p53, $p21^{cip1/waf1}$, and Cdc2 p34.[77] TCDD-activated AHR has been shown to induce $p27^{kip}$ directly, and $p27^{Kip}$-deficient mice were partially resistant to TCDD-mediated thymic proliferation inhibition and reduction of thymocyte recovery.[18]

Activation of the AHR by dioxin can also downregulate gene expression, as is the case of fibrinogen γ chain and plastin mRNA synthesis,[109] by pathways that cannot be explained by direct transactivation mechanisms. One of the most extensively studied cases of altered gene expression by dioxin is the upregulation of Il-1β and the downregulation of TGFβ production. Sutter and co-workers[110] first identified that Il-1β was upregulated in response to dioxin in a human keratinocyte cell line. The induction of this gene was later described in the endometrial adenocarcinoma cell line, RL95-2,[111] thymus,[112] liver,[33,112,113] and lung.[112] The TCDD-mediated response in keratinocytes appears to involve additional growth factors, such as those found in bovine pituitary extract,[114] indicating that secondary signals, such as phosphorylation events, may contribute to the regulation of this gene by TCDD. In contrast to the effects of dioxin, Il-1 has been shown to inhibit *CYP1A1* and *CYP1B1* expression in rat hepatocytes by a transcriptional mechanism,[115] suggesting

a complex mechanism, possibly involving negative feedback through genes altered by dioxin. TGFβ2 is transcriptionally repressed by dioxin,[116] by mechanisms controlled in part by a tyrosine kinase–dependent pathway.[117] In mouse embryo fibroblasts lacking the Ah receptor, there is an elevated expression of TGFβ2 that results in an increase in cdc2 and plk protein levels. Concomitantly, these cells have a prolonged G2/M transition.[118] Conversely, expression of the AhR is downregulated in response to TGFβ1.[119] TCDD-mediated induction of CYP1A1, CYP1B1, and NQO-1 are inhibited by TGFβ1, possibly as a result of the altered cell cycle pattern, since TGFβ also downregulated c-myc and cyclin A expression.[40] It is likely that the altered TGFβ signaling is responsible for the low proliferation and increased apoptosis rates of $Ahr^{-/-}$ cells.[118] The case of this cytokine is a prime example of how a molecule affecting the cell cycle can have pleiotropic effects that indirectly alter the regulation and expression of numerous other genes.

The effects of dioxin on cell cycle gene expression and regulation could very well be the mechanism by which this chemical leads to pathological alterations. Using an in vitro model, the Ramos laboratory reported a 40% reduction in peak DNA synthesis in vascular smooth muscle cells only when TCDD was added during the G0/G1 transition of the cell cycle. Increased tyrosine kinase phosphorylation was seen as early as 15 min following exposure but was not observed during the G1/S or S phase.[120] More recently, dioxin and other AhR agonists have been shown to alter cell cycle gene expression[12] as well as the critical GTP-associated signaling molecule, RAS,[72,73] in vascular smooth muscle cells in a cell cycle–dependent manner. These data indicate that dioxin's primary effects could be at the level of cell cycle alteration, thus making cells more susceptible to proliferative or apoptotic events, as well as priming the cells for genomic instability.

13.5 DIOXIN AND APOPTOSIS

The findings in the field of dioxin-induced apoptosis are extremely contradictory. On the one hand, dioxin enhances apoptosis in specific cell types and organisms. For example, in zebrafish (*Danio rerio*), TCDD, as well as β-naphthoflavone (β-NF), another AhR agonist, induce apoptotic cell death in the dorsal midbrain, as determined by terminal dUTP transferase-mediated nick-end-labeling (TUNEL) assay.[121] These effects were inhibited by pretreatment with the AhR antagonist, α-naphthoflavone (α-NF).[121] Apoptosis in response to TCDD was also observed in the vasculature of medaka embryos,[122] liver,[123] and erythrocytes,[124] as well as in thymocytes.[125–127] In T-cells, TCDD enhances activation-induced cell death (AICD) in a Fas-Fas-ligand-dependent manner, suggesting a possible mechanism of TCDD-mediated immunotoxicity.[60,128] Recently, Ah receptor–dependent induction by PAHs of the pro-apoptotic *BAX* gene has been found to be responsible for induction of a cell death pathway that culminates in ovarian failure. Interestingly, dioxin is

not cytotoxic to oocytes, and the difference in the action of PAHs versus dioxin is due to a single base pair flanking each AhR response element in the *BAX* promoter.[129]

TCDD has also been shown to increase apoptosis in structures where cell death normally occurs, including the outflow tract, endocardial cushion of the atrioventricular canal, and dorsal mesocardium. The reduction in cardiac myocyte proliferation by TCDD preceded the reduction in coronary artery number and size, suggesting that changes in coronary development may be a consequence of reduced myocyte proliferation and a thinner ventricle wall. The peak of TCDD-induced increase in apoptosis occurred even earlier in embryo development and thus may contribute to changes in myocyte proliferation, coronary development, and cardiac structural malformations.[130]

On the other hand, TCDD has been shown to protect from apoptotic events. TCDD inhibits apoptosis in MCF-10A cells in an autocrine manner,[131] possibly through a mechanism involving stimulation of the epidermal growth factor receptor signaling pathway and upregulation of TGFα mRNA and protein. In human keratinocytes in culture, TCDD treatment does not induce apoptosis, as determined by nucleosomal fragmentation, nuclear morphology, and caspase-3 activity, but results in the disruption of normal cell homeostasis.[132] The inability of TCDD challenge to result in apoptosis may be related to the spatial and temporal expression of the AhR. Mouse embryos challenged with TCDD at the morula stage of development, at which time AhR expression is low, do not show any signs of apoptosis or of inhibition of their ability to form blastocysts.[133]

A role for the Ah receptor in apoptosis in the absence of TCDD has been proposed by Reiners and Clift.[134] The relationship between the AhR and apoptosis was examined using Hepa1c1c7, which have normal levels of AhR, and Tao cells, which have only 10% of the normal level of AhR expression. *N*-Acetylsphingosine (c2-ceramide) caused apoptosis in direct relationship to AhR levels, independent of TCDD or α-NF, an AhR antagonist, exposure. Furthermore, ARNT, the AhR heterodimeric partner, did not appear to be involved in the effect. The evidence suggests that in addition to having a role in cell cycle regulation and metabolic gene expression, the AhR plays an important role in apoptotic events in a tissue- or developmental-specific manner.

13.6 USE OF GLOBAL GENE EXPRESSION ARRAYS TO STUDY DIOXIN EFFECTS

Global gene expression is quickly becoming a powerful tool in toxicology for studying the adverse effects of environmental chemicals, such as dioxin. Altered gene expression patterns observed by microarray analysis in TCDD-treated cells or tissues may be thought to be the direct result of the toxic response, but this may be a misconception, because the outcome of the exposure need not always be deleterious. Altered gene expression can result from toxic or from

adaptive responses, and generally it is difficult to determine whether a certain effect caused by dioxin belongs in one or the other category. Functional assays, such as determination of cell proliferation, and toxicity and apoptotic assays complement the microarray data to define the *cause* of the *effect* observed. In most cases, many of the altered genes have apparently unrelated functions. C*lustering* sets of genes found to be altered by microarray is a vital step in identifying regulatory pathways that could lead to the development of mechanistic data. Developing gene hierarchies based on known mechanisms of transcription, function, location, systemic effects, and disease processes may facilitate gene grouping. At this point, the data guide the researcher to interpret the results correctly rather than to discard unknown genes based on lack of annotation.

Ligand-dependent activation of the AhR upsets the regulation of many complex biological processes. Based on the concept that a complete signature of transcriptional regulatory mechanisms affected by AhR activation might shed light on the mechanisms responsible for its many biochemical, physiological, and biological effects, global changes in mRNA accumulation were analyzed in human hepatoma HepG2 cells and whole mouse livers treated with TCDD using commercially available high-density human and mouse DNA microarrays. Three laboratories have reported these studies.[74,135,136] It is worth noting that many effects taking place in the whole organism or in a different cell lineage or tissue may not be observed in HepG2 cells and that, vice versa, effects in these cells may not happen in other tissues or cell lineages. Results from this work revealed many possible clusters of interacting gene functions[74] and pointed at the possibility that interactions between these clusters could provide an explanation for the multiple effects of TCDD in particular and of AhR activation in general. We exposed HepG2 cells to 10 n*M* TCDD for 4 or 8 h with and without a previous cyclohexamide exposure to block protein synthesis. Expression of 310 genes was affected by TCDD treatment. Of these, 114 were upregulated by a factor of 2.1 or higher, and 196 were downregulated by the same magnitude. Several hierarchical groups of genes that were coordinately up- or downregulated by TCDD could be identified in these experiments, as well as seven gene groups or *clusters* that corresponded to well-defined cellular or biological processes. Of these clusters, four were of particular interest to cardiovascular biology, because they included genes involved in (1) Ras/MAP kinase signaling pathways, (2) calcium regulation, (3) cardiovascular and pulmonary functions, and (4) cell cycle regulation and apoptosis. In addition, we identified genes involved in (5) development, cell adhesion, cancer, and metastasis, (6) drug metabolism and DNA stability, (7) protein traffic and membrane integrity, (8) receptor-associated kinases and phosphatases, and (9) transcription factors.[74]

Altered transcriptional patterns in response to toxicants are initiated through a variety of signaling mechanisms. This domino effect originates at the surface of the cell and transmits its response to second messengers through kinases, phosphatases, or redox reactions that activate cytoplasmic receptors

and kinase cascades. The ultimate outcome is a transcriptional response, detectable by gene analysis as a "signature" of gene expression. Induction of many of the genes by TCDD was inhibited by cyclohexamide, suggesting that the effect of dioxin on these genes is secondary to a primary transcriptional response. However, the effects of dioxin on other known targets, such as the genes for PAI-2 and IL-1β,[110] *FOS* and *JUN* immediate-early gene families,[96,98] *COX-1* and *COX-2*,[58,137–142] and TNFα,[113,143–145] could be the result of a primary response or of a secondary or even higher-order response, resulting from initial signaling events.

Similar results have been obtained using B[*a*]P, in an effort to determine whether the genes affected by dioxin treatment would also respond to other AhR ligands. The results are very similar for the two ligands. Like TCDD, BaP affected the expression of many critical genes for vascular functions, such as endothelial NO synthase, PAI-1, VEGF, FGL-2, troponin, and calmodulin-binding proteins, and for cell cycle regulation, signal transduction, and apoptosis, such as various caspases and cyclins, extracellular matrix proteins, and G2/M-regulatory kinases.[74]

The study performed by Frueh and co-workers[136] was very similar to the study in this laboratory described above. These authors examined gene expression in HepG2 cells challenged with 10 n*M* TCDD for 18 h. As with most microarray experiments, twofold or greater induction or repression of a gene was accepted as statistically significant. Of the 12,412 genes analyzed, 85 were upregulated and 27 genes were downregulated. Several of the genes that responded to TCDD were confirmed by Northern blot analysis and RT-PCR, including the genes coding for CYP1A1, cot kinase, XMP, HM74, human enhancer of filamentation-1 (HEF-1), metallothionein, PAI-1, and HM74.[136] HEF1, XMP, and metallothionein displayed kinetic differences in maximal induction. The addition of cycloheximide completely blocked TCDD-mediated induction of XMP and metallothionein mRNA, indicating that for these genes, TCDD acted through a secondary mechanism as the one described above. There is a high degree of similarity between these two studies, including the cell type and the concentration of TCDD examined. As a consequence, many of the same genes were identified in both studies.

A third gene array study on the effects of dioxin was carried out by Thomas and co-workers.[135] The goal of this study was to identify common predictive genes that were altered as a result of 12 different toxicant exposures in mice. TCDD, the prototypical AhR agonists, was injected with a single dose of 10 μg/kg into C57BL/6J mice, which carry the Ahr^{b-1} allele, coding for the high-affinity form of the AhR. Other toxicants from various classes of chemicals, including noncoplanar PCBs (PCB-153, Arochlor 1260, phenobarbital), peroxisome proliferators (Cipro, Wy-16,463), inflammatory molecules (TNFα, lipopolysacharide, IL-6), and hypoxia-inducible agents (cobalt, phenylhydrazine) were also examined. At 6, 12, 24, 48, 96, 192, 288, 384, and 480 h postinoculation, the livers from the TCDD-challenged mice were harvested. RNA isolated from the liver was compared to vehicle control RNA,

and these data were cross-compared with all the toxicants examined. A set of 12 predictive genes for the various classes of chemicals were identified: CYP1A2, FMO5, CYP4A14, IL-18, CYP2B10, CYP4A10, mouse BMMT, CYP2C29, CYP1A1, SAA112, and two unknown transcripts. As would be expected, the two genes with the highest expression levels after TCDD challenge were *Cyp1a1* and *Cyp1a2*, which were expressed at equally high levels regardless of time of treatment. One important dimension of this study was that it used livers from normal mice treated in vivo rather than a transformed human liver cell line like HepG2 cells, which may have responses dependent on mutations accumulated during the process of in vitro propagation. Undoubtedly, other forthcoming experiments in mice with defined genetic backgrounds will address organ-specific gene profiles induced by different doses of TCDD or of other AhR ligands and will provide, in combination with arrayed libraries harboring the whole genome, a complete picture of the molecular signature of TCDD exposure, identifying tissue- and chemical-specific gene responses that might be used as toxicological markers.

13.7 CONCLUSIONS

Understanding the biochemical and biological processes that result from dioxin exposure is a complex task that requires extensive knowledge of mechanisms of de novo gene transcription and translation, the outcome of protein–protein interactions, the regulation of signal transduction and cell cycle progression pathways, and the physiology of multigene interactions: all of these in a scenario of yet-to-be-discovered mechanisms of species-, cell lineage-, and tissue-specific physiologic regulation.

Human populations are more resilient than most laboratory animals to the effects of dioxin. Could this be due to a more complex gene regulatory system and the lack of a controlled, highly inbred genetic background? Or could it be that the effects observed outside the laboratory are the result of dioxin's interaction with the hundreds, if not thousands, of other chemicals to which we are exposed on a daily basis? If the answer to either of these questions were "yes," we would have to develop much more sophisticated methods of studying chemical–gene and gene–gene interactions if we wished to decipher dioxin's "message" from laboratory animals to humans.

While the technology for studying biochemical responses to toxicants is advancing at a rapid, almost unbelievable pace, mechanistic studies in toxicological research, albeit many and important, move at a slower pace. With regard to dioxin toxicity, much still needs to be learned from descriptive analyses of exposure effects, and a great deal of information may be derived from fishing expeditions designed to study every possible aspect of developmental timing of exposure, genotype, dose responses, and target tissues in a combinatorial matrix of gene profiling experiments. Eventually, this analytical approach, which will depend heavily on DNA array technology, will open the

way for a synthetic approach in which every cluster of genes found to be deregulated by dioxin will give raise to a set of studies to fit their functions into the mechanistic and physiologic responses elicited by exposure. It is everyone's hope that as the arrays become more complete and the bioinformatics tools more sophisticated, it will be possible to create complete clustering maps of gene regulatory events and transcriptional processes that will be associated with certain biochemical or physiological events happening as a consequence of dioxin exposure. Fortunately, the task of sorting through the data, understanding the results, and relating those results to the biology of the organisms will still be in the hands of investigators for many years to come.

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CHAPTER 14

Evolutionary and Physiological Perspectives on Ah Receptor Function and Dioxin Toxicity

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14.1 INTRODUCTION

For more than 40 years, scientists have been intrigued by the extreme biological potency of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and related planar halogenated aromatic hydrocarbons (PHAHs), by the diversity of toxic responses that result from TCDD exposure, and by the dramatic species differences in sensitivity and effects. It was realized early that TCDD must be interfering with fundamental cellular processes and thus that this environmental toxicant could be used as a molecular probe to study normal cell physiology.[1] At the same time, it was recognized that understanding the mechanism and consequences of TCDD toxicity would require elucidation of the physiological pathways through which it acts.

Identification of mouse strain differences in sensitivity to polynuclear aromatic hydrocarbons (PAHs) and TCDD[2,3] and the elucidation of PHAH structure–activity relationships[4–6] led to the prediction[7] and then discovery[8] of the aryl hydrocarbon receptor (AhR) as a key protein involved in PHAH toxicity. The subsequent cloning of the AhR cDNA[9,10] and gene[11] led to the generation of AhR-null mice[12,13] and the demonstration that the AhR is necessary for most, if not all, of the effects of TCDD and (presumably) other PHAHs,[14–20] as well as certain effects of PAHs.[21–23] Detailed descriptions of the structure, function, and regulation of the mammalian AhR can be found in several recent reviews[24–29] and elsewhere in this volume (see Chapter 12).

Despite the important advances in our understanding of the AhR and its role in the mechanism of TCDD/PHAH toxicity, the normal physiological

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function of this protein has remained elusive, or at best incompletely understood. Ultimately, a comprehensive view of AhR function and TCDD toxicity will require a multidisciplinary approach, combining information from such fields as pharmacology, toxicology, pathology, molecular biology, genetics, endocrinology, developmental biology, and evolutionary biology. Characterization of the AhR and its similarities and differences in diverse model systems will be an integral part of this effort. Advances in comparative and genomic biology over the past 10 years have spurred the realization that innumerable molecular pathways involved in gene regulation and development are broadly conserved over vast phylogenetic distances.[31–33] Even features thought not to be homologous (e.g., eyes of mammals and insects) now have been shown—as a result of comparative studies—to share underlying genes and genetic pathways.[34–36] Thus, studies of the AhR in diverse taxa will help illuminate the unity and diversity of its physiological functions and the mechanisms by which it mediates toxicity. In this chapter we summarize several approaches to understanding the normal functions of the AhR and then describe recent insights obtained through research using a comparative/evolutionary approach with nontraditional species.

14.2 ADAPTIVE VERSUS PHYSIOLOGICAL FUNCTIONS OF THE AhR

Much of the early research on TCDD action and AhR function focused on the induction of xenobiotic-metabolizing enzymes such as cytochrome P450 1A1 (CYP1A1). These efforts led to a detailed understanding of the AhR-dependent transcriptional activation of this gene[37] and to the identification of the *Ah gene battery*, a set of xenobiotic-metabolizing enzymes that are coordinately regulated through a regulatory loop involving the AhR and CYP1A1/2.[38–40] Induction of these enzymes by TCDD can have protective or deleterious consequences,[41] but even 20 years ago it was evident that the toxicity of TCDD was due, at least in part, to changes in gene expression beyond those involved in drug metabolism.[42]

In 1982, Poland and Knutson proposed that PHAHs evoke two distinct pleiotropic responses: a *limited pleiotropic response* (induction of genes in the Ah gene battery) and the *restricted pleiotropic response* (induction of genes involved in the regulation of cell division and differentiation).[42,43] This remains the dominant paradigm concerning functions of the AhR.* Schmidt and Bradfield, for example, define the AhR-mediated induction of xenobiotic-metabolizing enzymes as the *adaptive response pathway*, equivalent to Poland's *limited pleiotropic response*.[24] They further divide Poland's *restricted pleiotropic response* into a *toxic response pathway* and an *endogenous response pathway*. Although these distinctions are no doubt an oversimplification of what are

* However, see Matsumura and colleagues[312–314] for a different perspective.

probably extremely complex regulatory networks, such models provide a useful conceptual framework within which to investigate dioxins and the AhR. Here, in discussing the functions of the AhR, I will retain Poland's dichotomy, combined with some of Bradfield's terminology, in classifying AhR functions as either adaptive or physiological, as described below.

14.2.1 Adaptive Functions

Most animals are exposed daily to a multitude of chemicals in the air, water, or food. Some of these are signaling molecules that carry valuable information about the animal's environment (e.g., the presence of food, conspecifics, or predators); other chemicals are toxic, either by design or by accident. Animals have evolved a variety of mechanisms to detect these chemicals and respond appropriately. Like the adaptive immune system, which is capable of recognizing and responding to a wide variety of antigens, chemical surveillance systems have evolved as mechanisms for recognizing a broad range of chemical structures and initiating appropriate responses. For example, vertebrate and invertebrate animals possess large families of olfactory/chemosensory receptors for detecting and interpreting chemicals in their environment[44]; chemicals recognized through this system often elicit behavioral responses in the animal exposed to them.

In addition to the immune and chemosensory systems, animals have evolved inducible enzymatic defenses to facilitate the biotransformation and elimination of toxic environmental compounds or endogenous metabolites.[45–47] Monooxygenases in the cytochrome P450 superfamily are well-known components of this inducible biotransformation system. The sensory component of this system consists of soluble receptors that regulate certain P450s and other enzymes in response to environmental chemicals. These receptors include several members of the steroid/thyroid/retinoid receptor (nuclear receptor) superfamily[48–52] as well as the AhR, a member of the bHLH-PAS gene family. Although originally thought to have a relatively narrow structural specificity,[42] the AhR is now known to recognize an impressive range of chemical structures, including nonaromatic and nonhalogenated compounds.[53] Such promiscuity is understandable in the context of this adaptive function.

14.2.2 Physiological Functions

Although it seems clear that the AhR has a sensory/adaptive function involving xenobiotic chemicals, there is evidence that this protein may have additional physiological roles, possibly involving the regulation of cell growth and differentiation. This may occur indirectly, through its regulation of CYP expression,[54,55] as well as through more direct mechanisms. Not all of these mechanisms need involve changes in gene expression as a primary effect of the AhR; some may involve protein–protein interactions that modulate other signaling pathways.[56–59] The evidence for the proposed physiological functions of the

AhR is as yet incomplete, but it includes information from several independent lines of inquiry, as described below.

14.3 APPROACHES AND INSIGHTS CONCERNING POSSIBLE PHYSIOLOGICAL FUNCTIONS OF THE AhR

Several lines of evidence provide information that can be used to make inferences and generate hypotheses concerning the physiological roles of the AhR and the mechanisms by which activation of the AhR by exogenous ligands* leads to toxicity. This evidence comes from the nature of the toxic syndromes caused by TCDD, the identity of the genes that are regulated by the AhR, the phenotype of cells and animals lacking AhR expression, the nature of AhR ligands, characteristics of homologous proteins, and evolutionary/phylogenetic comparisons.

14.3.1 Toxic Endpoints Involving Altered Cell Proliferation and Differentiation

Among the varied lesions produced by TCDD in different mammalian tissues, one can find hyperplasia (skin, gastric epithelium), metaplasia (liver, skin), and hypoplasia (thymus, bone marrow).[42] Thus, most of toxic responses produced by TCDD appear to represent altered growth and differentiation. In addition, TCDD and other AhR agonists† can also cause cells to undergo programmed cell death or apoptosis.[39] This occurs in the immune system,[60,61] reproductive tissues,[62] neural tissues[63] and vascular endothelium.[64,65] TCDD may also inhibit apoptosis in some systems.[66,67] Taken together, the effects produced by TCDD and other PHAHs strongly suggest that these compounds interfere with the control of cell growth. This conclusion, coupled with the altered cell growth and cell cycle observed in AhR-deficient cells,[68–71] implicates the AhR in the normal control of cell proliferation and/or differentiation. Further support for this notion comes from recent studies demonstrating interactions of the AhR with the product of the retinoblastoma tumor suppressor gene,[56,57,72] which is involved in regulation of cell cycle progression and AhR-dependent regulation of the *bax* gene,[22] which stimulates programmed cell death (apoptosis).

14.3.2 Identification of Genes Regulated by the AhR

Although the details of AhR functions are not yet well understood, it is clear that at least some of those functions involve the well-known action of

* Ligands are small molecules that bind with specificity (high affinity, low capacity) to receptor proteins.

† Agonists are ligands that bind to the receptor and convert it to an active form.

the AhR as a transcription factor. Like the other members of the bHLH-PAS gene family,[27,73,74] the AhR is able to directly regulate the expression of target genes. There are many approaches that can be used to discover differentially expressed genes[75] and several of these have been used to identify genes altered by TCDD: subtractive hybridization,[76–78] differential display PCR (dd-PCR),[79–86] genomic analysis of gene expression using cDNA microarrays,[87–89] and other approaches.[59,90–92] In addition to the members of the Ah gene battery,[39,93] the AhR regulates numerous genes not involved in xenobiotic metabolism.[25] These include genes involved in many other signaling pathways. The large number and great variety of TCDD-inducible genes identified by these studies, especially the recent microarray studies, support the idea that the response to dioxins is complex and suggests that it will not be possible to identify a single gene or even a small number of genes that are responsible for dioxin toxicity.[87,88] A more detailed discussion of AhR-regulated genes can be found elsewhere.[25,87,88,94,95]

14.3.3 AhR-Deficient Mice and Cells

The long-awaited generation of AhR-null mice has provided a window on the toxicological and possible physiological roles of the mammalian AhR. Three AhR knockout mouse lines have been produced independently.[12,13,16] All three strains are insensitive or nearly so to a variety of biochemical and toxic effects of TCDD (and presumably other AhR agonists).[12–17,19] In AhR-null mice that have not been exposed to exogenous dioxin, several abnormalities have been noted. There are some differences in phenotype among the three lines that may be due to the gene targeting strategy or to genetic background.[96,97] However, each of the changes seen is potentially important in terms of what it tells us about the possible physiological roles of the AhR. Among the features noted in AhR-null mice are changes in liver size and morphology,[12,13] lymphocyte depletion,[12,98] cardiomyopathy,[98] altered retinoid metabolism,[99] decreased weight of testes and epididymis,[100] increased numbers of ovarian primordial follicles,[101,102] altered mammary gland development,[103] and poor reproductive success.[104,105] In addition, a recent study of liver development in AhR-null mice provided evidence that the AhR may play a role in the remodeling (maturation) of the fetal vasculature in the liver and other organs.[105] This finding is extremely interesting in light of the demonstrated role of the bHLH-PAS proteins HIF1α, HIF2α, ARNT1, and ARNT2 in vasculogenesis and angiogenesis[106–112] and suggests that the AhR and HIFs play opposing or perhaps sequential roles in the maturation of the vasculature. These and other studies provide strong evidence for a developmental role for the AhR.

Although less powerful than whole animal studies, research using cultured cells that are deficient in AhR signaling have also provided clues to the AhRs role in cellular physiology. The results of these studies suggest a role of the AhR in regulation of the cell cycle and cell proliferation.[68–71]

14.3.4 Natural and Endogenous Ligands for the AhR

The search for an *endogenous ligand* for the AhR has been the Holy Grail of dioxin toxicology for many years. The AhR was initially discovered by virtue of its ability to bind planar aromatic molecules such as halogenated dioxins, halogenated biphenyls, and PAHs,[8,42] and recognition of these compounds is consistent with the AhR's role in an adaptive response to xenobiotics. However, the existence of an endogenous regulator of the AhR was suggested soon after the identification of this protein[42,54,113] and such an endogenous ligand has been sought ever since. As the list of structures that can bind the AhR has grown,[53] a variety of natural compounds that are AhR ligands, including some that are known to exist in human cells, have been identified (Figure 14.1).

Some of the first endogenous ligands identified were ultraviolet photoproducts of the amino acid tryptophan,[114,115] subsequently identified as 6,12-diformylindolo[3,2-*b*]carbazole, and 6-formylindolo[3,2-*b*]carbazole.[116] These are similar in structure to acid condensation products derived from the dietary chemical indole-3-carbinol, which have also been identified as AhR ligands.[117–120] Similarly, the endogenous tryptophan metabolites tryptamine and indole acetic acid also act as AhR agonists.[121,122] More recently, the indole-derived P450 metabolites indigo and indirubin[123] were shown to be AhR ligands.[124]

The linear tetrapyrroles bilirubin and biliverdin can act as agonists for the AhR.[125–127] It is tempting to include these in the adaptive responses mediated by the AhR, because the CYP1As induced via this mechanism are able to oxidize these heme degradation products, which would otherwise accumulate to toxic levels.[128,129] Another set of potentially important physiological ligands are fatty acid metabolites, especially metabolites of arachidonic acid. One such metabolite, lipoxin A4, is a potent AhR agonist,[130] and several prostaglandins also have been shown to activate the AhR signaling pathway.[131] Given the postulated role of the arachidonic acid pathways in TCDD toxicity,[55,91,132–136] continued investigation of metabolites of arachidonic acid or other fatty acids as AhR ligands may be especially illuminating, as it has been for the peroxisome-proliferator-activated receptors.[137–140] Although the existence of these and other endogenous ligands for the AhR is certainly intriguing, whether any of these are *physiological ligands* is not yet clear. Other natural chemicals that are ligands for the AhR but would not be considered as possible endogenous ligands include brevetoxin,[141] flavonoids (some of which are antagonists),[142–147] and methylenedioxybenzenes.[53]

The chemicals described above have all been shown to bind to the AhR. There are other compounds that exhibit effects resembling those of AhR ligands but for which AhR competitive binding studies have been negative. For example, the carotenoids canthaxanthin, astaxanthin, and β-apo-8′-carotenal all induce CYP1A1 and several other enzymes in the Ah gene battery, in an AhR-dependent manner,[148–150] but they fail to displace [^{3}H]TCDD specific binding in mouse hepatic cytosol. Negative results in competitive bind-

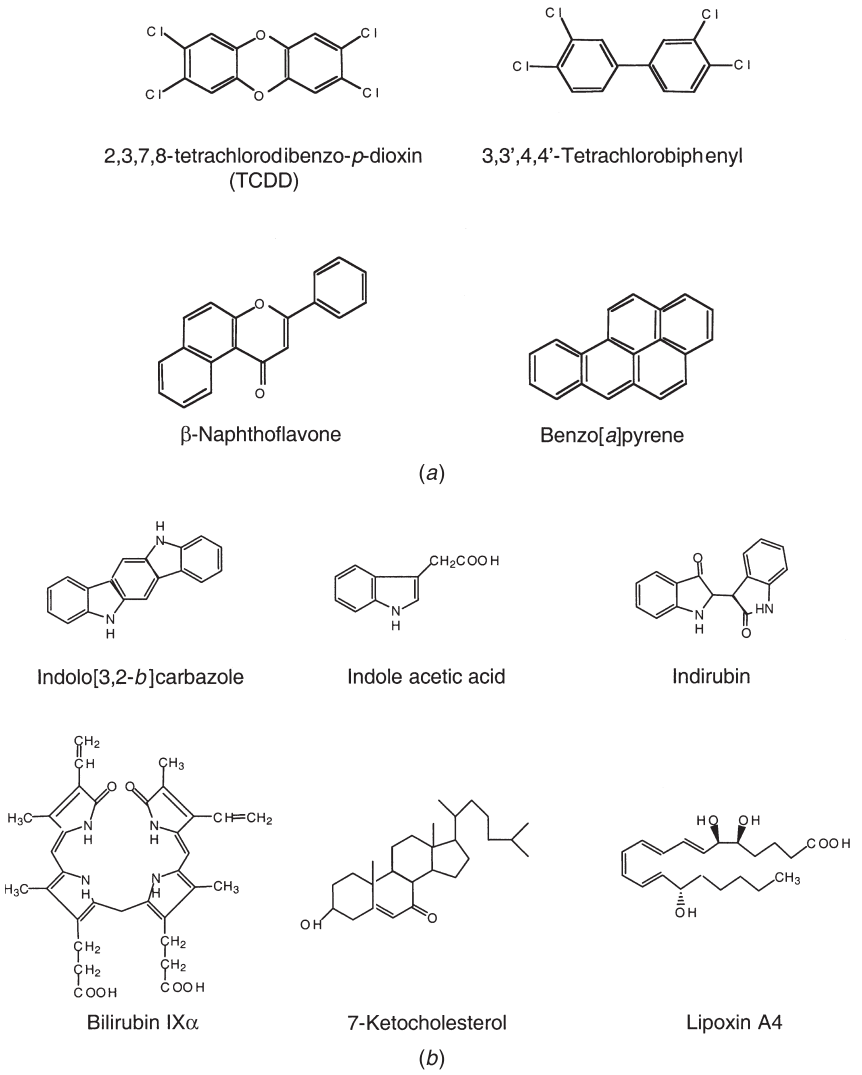


Figure 14.1 (*a*) Classical and (*b*) natural ligands for the AhR. For references, see the text and Denison et al.[53]

ing assays should be interpreted cautiously, however, because for weak AhR ligands further analysis may reveal the ability of these compounds to bind the AhR.[151,152] Alternatively, these compounds could interact with the AhR at a different binding site or activate the AhR indirectly, for example through metabolites or through altered phosphorylation.[153,154]

Although the search for endogenous AhR ligands has focused on agonists, it is also prudent to consider the possibility that endogenous AhR ligands might

act as antagonists or inverse agonists.* This is the case for a recently discovered nuclear receptor, the constitutive androstane receptor (CAR), for which both agonists and inverse agonists now have been described.[155] Recently, the oxysterol 7-ketocholesterol was found to be an endogenous modulator, and possibly an endogenous antagonist or inverse agonist, of the mammalian AhR.[156] The AhR-binding affinity of 7-ketocholesterol ($K_I \sim 2\ \mu M$) and other possible endogenous ligands might be considered too low for these compounds to be of physiological significance. Traditionally, toxicologists and pharmacologists have sought receptor ligands with affinities in the nanomolar range, typical of the affinities with which steroids and other hormones bind to their respective receptors, and similar to the affinity with which TCDD binds to the AhR. However, recent findings with some nuclear receptors (PPAR, FXR, LXR) have highlighted the potential for lower-affinity ligands to be physiologically relevant, due to high local concentrations that can be achieved at some sites.[155] Similarly, low-affinity ligands should not be ignored as possible endogenous modulators of AhR function.[53,131,156]

One approach to identifying endogenous ligands and functions for the AhR is to look for sites or systems where AhR activation occurs in the absence of exogenous chemicals. Evidence for this may include increased expression of CYP1A or altered AhR localization. CYP1A induction in the absence of exogenous chemicals has been shown to occur during the retinoid acid–induced differentiation of F9 mouse embryonal carcinoma cells,[157] Ca^{2+}-induced differentiation of mouse keratinocytes,[158] mitogen activation of human or mouse leukocytes,[159,160] suspension of human keratinocytes,[161,162] exposure of hepatoma cells to hydrodynamic shear,[163,164] in fertilized mouse ova,[165] in teleost chondroid cells,[166] and after inhibition of proteosome function.[167] In many of these experiments, the increased CYP1A expression was AhR-dependent or associated with other evidence for AhR activation. Related to this, Hankinson, Nebert, Puga, and colleagues,[40,168,169] in characterizing AhR-dependent signaling in Hepa-1 and CV-1 cells, described an autoregulatory loop involving a putative, endogenous AhR ligand that is also a substrate for CYP1A; the identity of this ligand is not yet known. Apparent AhR activation, as suggested by nuclear localization of the receptor, has also been observed in HeLa cells[170] and developing mouse embryos.[171] A system that shows great promise for identifying additional sites of endogenous AhR activation in vivo is the lacZ transgenic mouse line, which expresses β-galactosidase under control of AhR response element (AhRE) sequences.[172] It is important to keep in mind that endogenous AhR activation could reflect either the presence of an endogenous AhR ligand or activation of the AhR by another process, such as phosphorylation, proteolysis, or protein–protein interactions. Understanding possible ligand-independent functions of the AhR is one of the important challenges for future work.

*Inverse agonists are ligands that act to inactivate spontaneously active receptors.[298,315] Antagonists are ligands that are not active as either agonists or inverse agonists, but bind to a receptor and prevent the action of agonists.

14.3.5 Insights from Homology: The PAS Gene Family

Additional insight into the physiological functions of the AhR can be obtained through the identification and characterization of evolutionarily related genes. Members of multigene families often share functional characteristics. Thus, the common functional themes revealed by study of several members of a gene family can provide important clues to the activity of a newly identified or poorly understood protein. Such has been the case with the nuclear receptor superfamily, in which discovery of the physiological functions and ligands for ''orphan'' receptors has been facilitated by knowledge of the ligands and roles of many other superfamily members.[51,173–175]

The AhR was originally predicted to be a member of the nuclear (steroid/thyroid/retinoid) receptor superfamily based on biochemical characteristics. However, the cDNA cloning of the murine AhR in 1992[9,10] revealed it to be the fourth member of a new gene family, named PAS for the first three members, period (Per), AhR nuclear translocator (ARNT), and single-minded (Sim). The PAS gene family is now known to include more than 20 genes in vertebrates (primarily mammals), 11 in *Drosophila*, and 5 in *Caenorhabditis elegans* (Table 14.1). This family has been reviewed.[27,28,73,74,176]

PAS proteins possess several functional features in common. They are all transcription factors, and most act as heterodimers. Many of them have important roles in development, as demonstrated by the phenotype of null mutants.[106–112,177,178] Others appear to have important homeostatic roles in adults.[179–185] Many PAS proteins are directly regulated by, or are part of pathways that are dependent on, environmental cues. This is true of the meta-

TABLE 14.1 bHLH–PAS Gene Family in Animals[a]

Caenorhabditis elegans (5)	*Drosophila melanogaster* (11)	Vertebrates (23)
ahr-1	Spineless	AhR1, AhR2, AhRR
aha-1	Tango	ARNT1, ARNT2
hif-1	Similar	HIF-1α, HIF-2α, HIF-3α
T01D3[b]	Single-minded (Sim)	SIM1, SIM2
	Period (Per)	Per1, Per2, Per3
	Clock	CLOCK, CLOCK2
	Cycle	BMAL1, BMAL2
T01D3[b]	Trachealess	NPAS1, NPAS3
	Taiman	NRC-1, NRC-2, NRC-3
C15C8[b]	Met, Met-like	MOP22[c]

[a] Known members of the bHLH–PAS gene family in *C. elegans*, *D. melanogaster*, and *Homo sapiens*, as extracted from the literature and from searching completed genomes. For a complete list of abbreviations, citations, and synonymous gene names, see Refs. 28 and 203 and *http://www.whoi.edu/science/B/people/mhahn/hahnm.html*.

[b] For these *C. elegans* genes, orthology to specific *Drosophila* and vertebrate genes is uncertain.

[c] New bHLH–PAS sequence identified by genome searching (M. E. Hahn, unpublished).

zoan clock proteins and hypoxia-inducible factors, as well as the PAS-related proteins in plants, fungi, and bacteria.[176,186–190]

The participation of many PAS proteins in environmental sensing seems particularly relevant for understanding the adaptive function of the AhR, especially in light of its recognition of diverse chemical structures[53] and its well-known role in regulating xenobiotic-metabolizing enzymes. Additional insight regarding the ligand-binding characteristics of AhRs may be obtained by considering the variety of "ligands" or bound cofactors that exist for nonanimal PAS proteins. The plant and bacterial phytochromes (red photoreceptors), for example, have as their chromophores the linear tetrapyrroles phytochromobilin, phycocyanobilin, or biliverdin,[191,192] which are identical or structurally related to bilirubin and biliverdin, identified recently as AhR ligands.[126,127] Plant blue-light photoreceptors (phototropins) use FMN,[193] whereas PAS-associated cofactors of the bacterial proteins FixL and PYP are heme and 4-hydroxycinnamic acid, respectively.[194–197] A recent report suggests that NAD is a ligand for the mammalian bHLH–PAS proteins CLOCK and NPAS2.[198] Structural studies[194,196,199,200] indicate that PAS domains fold into a conserved three-dimensional structure (despite low overall sequence identity[200]), which might indicate strict limitations on the structures of ligands capable of fitting within this site. However, the cofactors noted above bind in different orientations with the PAS fold,[200] suggesting a more diverse structure-binding relationship, such as that emerging for the AhR.[53] Alternatively, the same ligand might bind in multiple orientations, as shown for PXR.[201] An understanding of AhR structure-binding relationships will be greatly facilitated once the three-dimensional structure of the AhR ligand-binding pocket is solved.

Overall, the emerging understanding concerning the physiological functions of other PAS proteins is consistent with possible roles of the AhR both during development (as suggested by AhR-null mice) and in mediating adaptive responses to environmental chemicals. Which of these reflects the original function of the AhR? Are there organisms in which AhR function is limited to one of these, or in which these functions can be distinguished?

14.3.6 Insights from Phylogeny: Comparative and Evolutionary Studies

The identification of AhR homologs in early vertebrates[202,203] and subsequently in invertebrates[203–205] has provided an opportunity to consider AhR function in the broader context of vertebrate and metazoan evolution. Characterization of these AhR homologs has revealed both shared and distinct features; these can be used to infer the ancestral and possibly present-day functions of the mammalian AhR and suggest ways in which chemicals may interfere with those functions to cause toxicity. The comparative and evolutionary biology of the AhR is considered in the following section.

14.4 COMPARATIVE AND EVOLUTIONARY BIOLOGY OF AhR SIGNALING

Identification of the original physiological roles of the AhR has been hindered by our inability to study the first metazoans (animals) directly. However, ancestral AhR functions and their evolutionary history can be inferred by characterizing the shared and unique features of AhR homologs in phylogenetically diverse, living taxa. In general, the study of PAS-related proteins in organisms as diverse as animals, plants, fungi, bacteria, and archea has provided valuable insights into the physiological and ecological roles of this group of proteins.[73,74,187,189,195,206–208] AhR homologs are known only from metazoans, but they are present in several phyla and in model species for which powerful genetic and developmental approaches have been established. Thus, there is much to be learned from a comparison of AhR structure, function, and regulation among these species. The comparative biology of the AhR is described below; specific features are summarized in Table 14.2.

14.4.1 Invertebrate Homologs of AhR and ARNT

The *C. elegans* (nematode) genome project[32,209] provided the first strong evidence for an AhR homolog in invertebrates.[203,204] Soon thereafter, an AhR homolog was identified in *Drosophila melanogaster*, an arthropod.[205] More recently, AhR homologs have been found in several mollusks.[210,211]

The *C. elegans* AhR (AhR-1; referred to here as CeAhR) is a 602-amino acid protein that shares 38% amino acid identity with the human AhR (HsAhR) over the first 395 amino acids.[204] (The N-terminal half of AhRs, containing the bHLH and PAS domains, is the region of greatest sequence identity.[212,213] Beyond this region, alignments cannot be generated with confidence except among closely related species.) The CeAhR protein contains a bHLH domain, within which specific residues required for AhRE-binding of mammalian AhRs are conserved. CeAhR also contains a PAS domain with PAS-A and PAS-B repeats. In vitro-expressed CeAhR is able to form a sequence-specific and ARNT-dependent complex with a mammalian AhRE (5′-T/GNGCGTG-3′).[204] Interestingly, the AhRE-binding complex forms in the absence of exogenous ligand. However, the significance of this finding is not clear, because similar results have been seen with mammalian,[212,214] fish,[213] and other invertebrate AhRs,[210] and could be due to tetrapyrroles or other ligands present in the rabbit reticulocyte lysate used for the in vitro transcription and translation.[213] Like mammalian AhRs, CeAhR is found associated with hsp90.[204] However, it does not form a complex with the mammalian AhR-associated protein Ara9 (also known as XAP-2 and AIP).[215] A glutamine (Q)-rich domain like that found in the C-terminal half of mammalian AhRs is not obvious in CeAhR. However, the C-terminal half of the CeAhR can function as a transcriptional activator, although it is constitutively repressed by the

TABLE 14.2 AhR Properties in Diverse Animal Groups[a]

	Nematode AhR (*C. elegans*)	Mollusk AhR (*M. arenaria*)	Arthropod AhR (*D. melanogaster*)	Vertebrate		
				AhR1	AhR2	AhRR
bHLH domain	+	+	+	+	+	+
PAS domains	PAS-A, B	PAS-A, B	PAS-A, B	PAS-A, B	PAS-A, B	PAS-A only
Q-rich domain	—	+	+	+	—	—
Specific binding						
[^{125}I]N_3DBDD	—	nd	nd	+	+	nd
[^{3}H]TCDD	—	—	—	+	+	—
[^{3}H]BNF	—	—	—	+	+	—
Bind hsp90	+	nd	nd	+	nd	—
Bind mouse Ara9[b]	—	nd	nd	+	+	nd
Dimerize with ARNT	+	+	+	+	+	+
Bind AhRE	+	+	+	+	+	+
Transcriptional activation	+	nd	+	+	+	—
References	203, 204, 210	210	205, 210, 219	9, 10	203, 213, 245	230, 250

[a] +, Present; —, not present; nd, not determined.

[b] Ara9 data from Ref. 215.

PAS domain and may require some form of posttranslational modification for its function.[204]

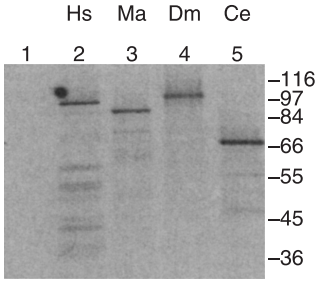
The ability of CeAhR to bind ligands has been assessed in several ways. In experiments using the photoaffinity ligand [^{125}I]N_3Br_2DD, specific labeling of CeAhR was not observed,[204] in contrast to the ability of this ligand to label mammalian[216] and fish[217] AhRs. Similarly, β-naphthoflavone (BNF) failed to activate the CeAhR in a yeast expression system.[204] Consistent with this, studies using the reversible radioligands [^{3}H]TCDD and [^{3}H]BNF in velocity sedimentation analyses also found that the CeAhR was unable to bind these prototypical AhR ligands (Figure 14.2).[210]

Recently, the in vivo expression and function of CeAhR have been assessed. CeAhR : lacZ and CeAhR : GFP reporter genes are expressed in several cells, including some chemosensory neurons. CeAhR mutant animals are viable, but they have subtle defects in neuronal development (J. A. Powell-Coffman, personal communication).

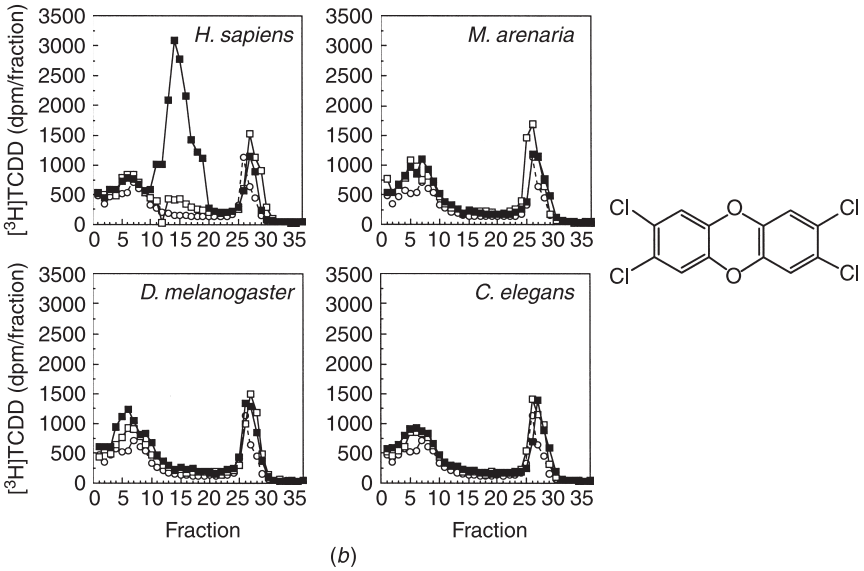
A *C. elegans* ARNT homolog (AHA-1; CeARNT) has also been identified.[203,204] This protein appears to function much like mammalian ARNTs, as a heterodimerization partner for AhR, HIF, and possibly other PAS proteins. CeARNT is expressed in most, if not all, cell types[218] and appears to have essential functions during both embryonic and larval development that are independent of its role in AhR or hypoxia signaling (J. A. Powell-Coffman, personal communication).

The *D. melanogaster* (fruitfly) AhR homolog spineless (ss; here referred to as DmAhR) is an 884-amino acid protein that, like the *C. elegans* AhR homolog, contains a bHLH domain that is substantially conserved as compared to mammalian AhRs (71% amino acid identity) and a PAS domain that also exhibits significant sequence identity (45%) with mammalian AhRs.[205] Like the mammalian AhRs, but unlike CeAhR, DmAhR contains a Q-rich region in its C-terminal half. DmAhR dimerizes with the tango protein (DmARNT) in a yeast two-hybrid assay and DmAhR-DmARNT dimers are able to activate an AhRE-dependent reporter gene in insect cells[219]; this response does not require exogenous ligand. The expression of DmAhR protein has not yet been localized, but DmARNT protein, which is normally cytoplasmic, is localized in nuclei of several cell types expressing DmAhR transcripts. These results have been interpreted as indicating that DmAhR is active in the absence of tissue-specific ligands.[219] In the only direct ligand-binding studies reported for DmAhR, this protein failed to show specific binding of [^{3}H]TCDD or [^{3}H]BNF (Figure 14.2).[210]

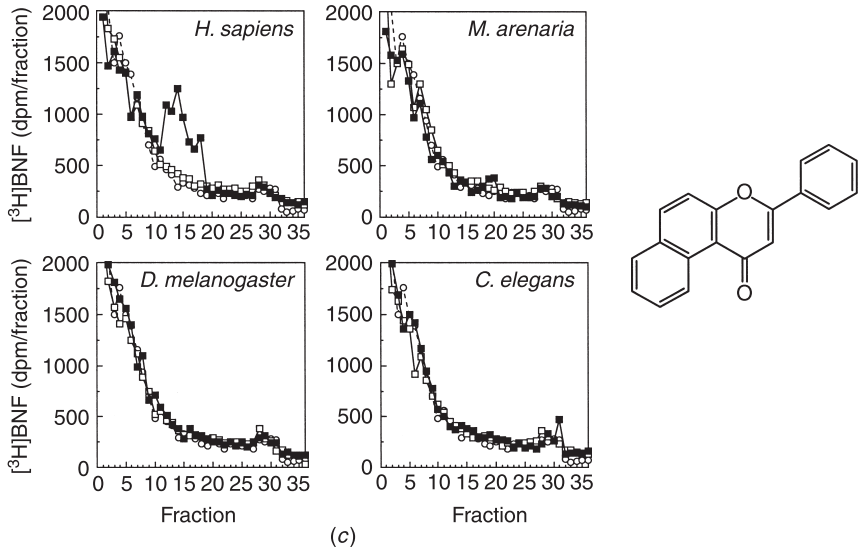
Characterization of DmAhR mutants has revealed that this protein plays an important role in defining the distal regions of the antenna and leg in *D. melanogaster*.[205] In flies lacking DmAhR, the distal part of the antenna (arista) is transformed to a distal leg segment. These mutants also show loss of distal leg segments and a reduction in the size of bristles. Consistent with these phenotypes, DmAhR transcripts are expressed in the distal part of the antennal disc, in the distal leg discs, and in bristle precursor cells.[205] Ectopic expression


Hs
Ma
Dm
Ce
1
2
3
4
5
116
97
84
66
55
45
36

(*a*)


[3H]TCDD (dpm/fraction)
H. sapiens
M. arenaria
D. melanogaster
C. elegans
Fraction
Cl
O
Cl
Cl
O
Cl

(*b*)


[3H]BNF (dpm/fraction)
H. sapiens
M. arenaria
D. melanogaster
C. elegans
Fraction
O
O

(*c*)

of DmAhR causes transformation of distal leg segments to antennae and the formation of ectopic antennae. Additional studies[205] showed that DmAhR is regulated by *distal-less* (*dll*), a master regulator of appendage formation in flies, and that DmAhR in turn controls the expression of *bric-a brac* (*bab*), a transcription factor that regulates development of appendages and ovaries and is involved in the control of sexually dimorphic characters in *D. melanogaster*.[220,221] It is not yet known whether *DLX* genes, the vertebrate orthologs of *dll*, are involved in regulating AhRs; a vertebrate ortholog of *bab* has not yet been identified.

In light of the results described above, Duncan and colleagues[205,219] speculated that the first function of DmAhR was in antennal specification and elongation, related to the chemosensory function of this structure. In its broad outline, this idea provides a thread with which to tie together the expression of CeAhR in chemosensory neurons (see above) and to the more clearly defined role of vertebrate AhRs in chemical sensing and adaptation. The details of how an ancestral AhR could have evolved both developmental and chemosensory functions remain to be elucidated. However, one might imagine an ancestral mechanism for the chemical-inducible development of chemosensory structures, which later evolved into a constitutive developmental pathway in some lineages (arthropods), and in other lineages became associated with the regulation of inducible biotransformation systems (vertebrates).

Recently, AhR homologs have been identified in mollusks, including the softshell clam *Mya arenaria*,[210] the zebra mussel *Dreissena polymorpha*,[211] and the blue mussel *Mytilus edulis*.[211] Like AhRs from *D. melanogaster* and *C. elegans*, the mollusk AhR homologs have bHLH and PAS domains, interact with mammalian AhRE sequences, and fail to bind [^{3}H]TCDD or [^{3}H]BNF (Figure 14.2).[210]

The presence of AhR homologs in these three invertebrate phyla (nematodes, arthropods, mollusks) suggests that an AhR was present in early metazoans (Figure 14.3). Nematodes and arthropods are both members of the ecdysozoan clade, while mollusks are lophotrochozoans.[222] These results imply

Figure 14.2 Lack of [^{3}H]TCDD and [^{3}H]BNF specific binding by invertebrate AhR homologs. (*a*) Expression of AhRs from human (lane 2), *M. arenaria* (lane 3), *D. melanogaster* (lane 4), *C. elegans* (lane 5) by in vitro transcription/translation in the presence of [^{35}S]methionine; unprogrammed lysate (lane 1) under identical conditions. (*b*) The specific binding of [^{3}H]TCDD to AhRs from human, *M. arenaria*, *D. melanogaster*, and *C. elegans* was analyzed by velocity sedimentation on sucrose gradients using a vertical tube rotor. In vitro-expressed AhRs were incubated with [^{3}H]TCDD (10 nM) (filled squares) or [^{3}H]TCDD (10 nM) + TCDF (1 μM) (open squares). The binding of [^{3}H]TCDD to unprogrammed lysate (UPL) was also assessed, as an independent measure of nonspecific binding (open circles). (*c*) The binding of [^{3}H]BNF (10 nM) (filled squares) or [^{3}H]BNF (10 nM) + unlabeled BNF (1 μM) (open squares) was measured as in (*b*). (Adapted from Ref. 210, with permission of Elsevier Science.)

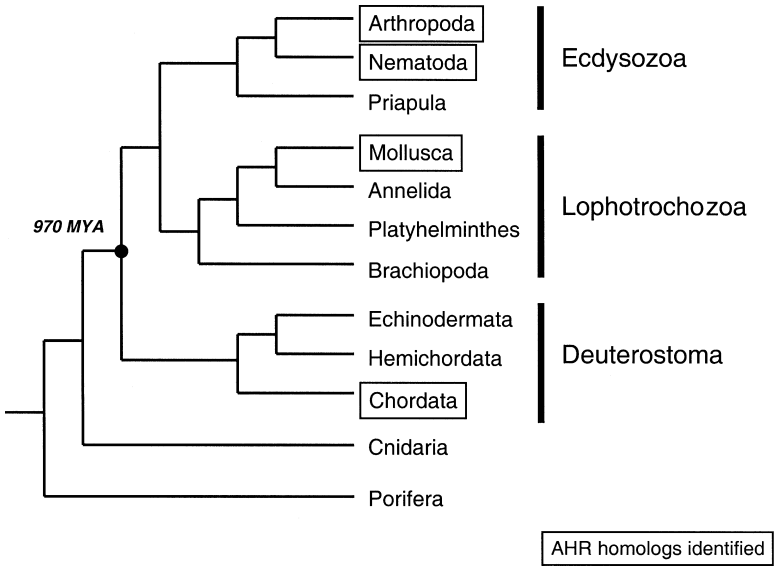


Figure 14.3 Tree showing relationships of animal phyla and the existence of AhR homologs. Phylogenetic relationships are derived from recent studies.[222,223]

that an AhR was present in the common ancestor of Ecdysozoa, Lophotrochozoa, and Deuterostomes (chordates and echinoderms), which lived near the end of the Proterozoic Era, 543 to 970 million years ago[222,223] (Figure 14.3).

14.4.2 Duplication, Diversification, and Evolution of Ligand Binding in Vertebrate AhRs

The complement of bHLH–PAS genes in the complete genomes of *D. melanogaster* and *C. elegans* as compared to that in humans and other vertebrates (Table 14.1) suggests that there were nine or ten original members of this family in the earliest chordates and that subsequently these genes underwent duplication and diversification within the chordate lineage, so that in extant vertebrates there are at least 22 bHLH–PAS genes, with two or three members of each "paralog group" descended from the original members.[28,203,224] Existing evidence suggests that this diversification is associated with the extensive gene duplication that occurred early in the evolution of the vertebrates.[225–229]

The AhR resembles other bHLH–PAS genes in undergoing diversification in vertebrates. The existence of a single AhR homolog in present-day invertebrate phyla suggests that a single AhR was present in the first chordates. Current evidence, summarized below, suggests that this ancestral *AhR* gene underwent duplication and diversification during chordate evolution, so that in verte-

brate animals today there are at least three genes that together form an AhR subfamily within the bHLH–PAS family. The first clue to this came with the discovery of a second vertebrate *AhR* gene, designated *AhR2*.[202,203,213] Subsequently, a third AhR-related gene was identified and named AhR repressor (*AhRR*).[230,231]

AhR1 The first AhR to be identified[8] and subsequently cloned[9,10] has been the subject of detailed biochemical and molecular characterization[24,25,30]; it is referred to here as AhR1. AhR1 forms are widely distributed among vertebrate taxa. AhR1 cDNAs have been cloned from several mammalian species, including mouse,[9,10,232,233] rat,[234,235] human,[212] hamster,[236] guinea pig,[237] beluga whale,[214] and harbor seal.[238] AhR1 orthologs are found in birds,[239,240] amphibians,[240] bony fishes,[203,213,241,242] and cartilaginous fishes.[203,243,244] With one exception[242] AhR1 proteins exhibit high-affinity ($K_D \leq 20$ nM) binding of TCDD and other classical AhR ligands.

AhR2 First identified in the Atlantic killifish, *Fundulus heteroclitus*,[202,203] AhR2 is now known to be a common, and perhaps the predominant, form of AhR in bony fishes.[213,245–248] A key finding was the identification of AhR2 orthologs in cartilaginous fishes,[203,243] which showed that the gene duplication from which AhR1 and AhR2 arose occurred early in the gnathostome (jawed vertebrate) lineage. AhR2 resembles AhR1 in its ability to support high-affinity binding of TCDD,[213,246] sequence-specific binding to mammalian AhRE sequences,[213,246,248] and transcriptional activation.[246,248–250] However, differences in tissue-specific expression and the lack of some sequence motifs that are present in AhR1 forms[213,245,246,248] suggest that AhR2 may possess some functions that are distinct from those of AhR1.[213]

The completion of the human genome projects[251,252] provided an opportunity to determine if there is a human AhR2 ortholog. A search of both the public and private databases using both AhR1 and AhR2 sequences from fish and AhR1 sequences from mammals revealed no AhR2 in the human genome (Hahn, unpublished), although the existence of an AhR2 pseudogene cannot be ruled out. Similarly, efforts to clone AhR homologs from a variety of mammalian species have not yet revealed any evidence for an AhR2 in mammals.[28,224] Whether an AhR2 exists in other tetrapod groups is still uncertain, but there are preliminary data suggesting a possible AhR2 in one avian species (J. Lapseritis and M. Hahn, unpublished).

AhRR Fujii-Kuriyama and co-workers identified a mouse protein closely related to the AhR; it was initially referred to as "AhR2"[253] but later designated AhR repressor (AhRR).[230] Although in some features it closely resembles the AhRs, it differs in several interesting ways. AhR and AhRR share a high degree of sequence identity in the bHLH and PAS-A domains, but the AhRR PAS-B domain is highly divergent.[230] Consistent with this lack of conservation in a region that comprises part of the ligand-binding domain of

AhRs,[9,254–256] AhRR does not bind [^{3}H]TCDD or [^{3}H]BNF.[250] Like AhR, AhRR is able to interact with ARNT and bind AhRE sequences,[230,231] but AhRR-ARNT dimers are not transcriptionally active.[230] The murine AhRR promoter contains three AhRE sequences, all of which mediate the induction of AhRR expression by 3-methylcholanthrene.[230,231]

Recently, AhRR orthologs have been cloned from killifish[250] and zebrafish (B. Evans and M. Hahn, unpublished). The killifish AhRR can inhibit the TCDD-dependent transactivation function of both AhR1 and AhR2.[250] As seen with the mammalian AhRRs, killifish AhRR does not bind [^{3}H]TCDD or [^{3}H]BNF.[250] AhRR mRNA is inducible by TCDD or polychlorinated biphenyls, consistent with the presence of three functional AhREs in its promoter.[250]

The presence of AhRR orthologs in mammals and fish indicates that AhR diversification into AhR1, AhR2, and AhRR occurred prior to the vertebrate radiation. Phylogenetic analysis of all AhRR and selected AhR sequences[250] suggests the following scenario (see Figure 14.4). A single AhR existed prior to the emergence of the vertebrate lineage. This ancestral AhR, represented by the AhRs described in present-day invertebrates, was a transcriptional activator. It lacked the ability to bind dioxinlike compounds and perhaps also PAHs, but it may have had high affinity for other types of structures, such as indoles or tetrapyrroles. Early in the evolutionary history of the vertebrates, a gene (or

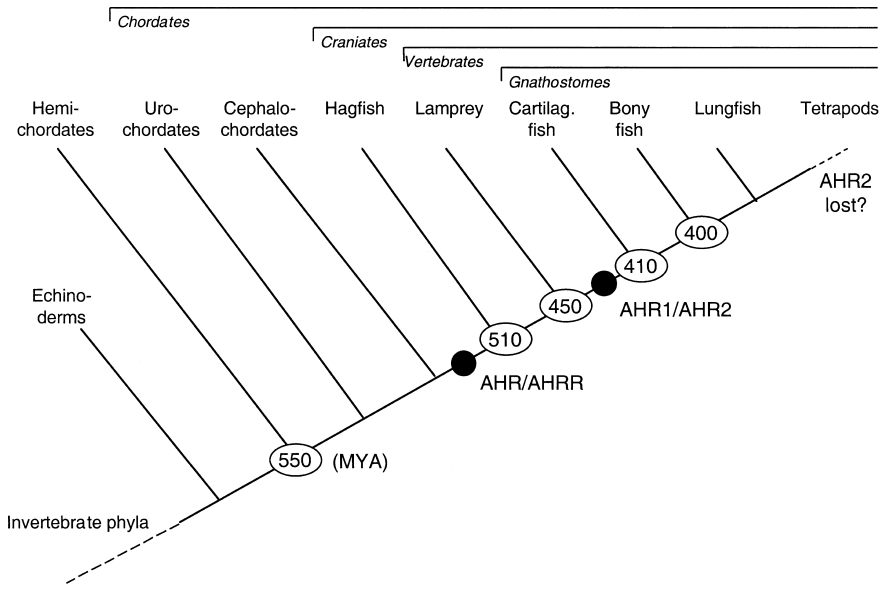


Figure 14.4 AhR gene duplications in chordate evolution. Numbers at nodes represent estimated divergence times, in millions of years ago (MYA). The solid circles indicate the hypothesized times of AhR gene duplications, based on phylogenetic analyses presented elsewhere.[203,213,250] (Adapted from Ref. 28, with permission of Elsevier Science.)

whole genome) duplication produced two AhR paralogs. One of these subsequently diverged, losing its transactivation function and acquiring activity as a repressor (AhRR). The other paralog evolved the ability to bind halogenated and nonhalogenated polycyclic aromatic hydrocarbons (AhR). As we have speculated,[28,257] the emergence of halogenated marine natural products may have contributed to the development of the ligand specificity of the AhR. Subsequently, a second gene or genome duplication produced two AhRs. These have been retained in most fish (AhR1 and AhR2), but in other vertebrate groups (e.g., mammals) the AhR2 form has been lost. Further studies in early diverging vertebrates (jawless and cartilaginous fishes) will more precisely indicate the timing of these duplications and whether there is additional AhR subfamily diversity.[203,243,244]

Phylogenetic Diversity of Other bHLH–PAS Proteins Involved in AhR Signaling Like the AhR, other bHLH–PAS proteins also exhibit differences in number and characteristics among species. For example, two ARNT proteins occur in mammals: ARNT1, which is widely expressed, and ARNT2, which is restricted to kidney and neural tissues.[258,259] In contrast, ARNT2 is the predominant, and perhaps only, ARNT form in some fishes,[260–262] whereas an ARNT1-like protein has been found in others.[263,264] A single ARNT homolog exists in invertebrate species.[203,204,265] Similar differences in diversity may occur with hypoxia-inducible factors (HIFs), proteins that share dimerization partners (ARNTs) with the AhR[266] and may influence AhR signaling.[59,267] Three HIF paralogs exist in mammals: HIF1α, which is widely expressed;[266] HIF2α, which is expressed primarily in endothelial cells;[268,269] and HIF3α, which is expressed in adult thymus, lung, brain, heart, and kidney.[270] A single HIF ortholog occurs in invertebrates.[218,271] HIF1α[272] and HIF2α[273] orthologs have been identified in fish. In the latter case, HIF2α transcripts are expressed in many tissues, but whether they are endothelium specific is not yet known.

Coevolution of AhR and CYP1A? The earliest diverging animals for which there exists definitive evidence for an AhR-regulated CYP1A form are the bony fish, which diverged from the human lineage more than 400 million years ago. CYP1As[274–276] and AhRs[203,213,245,246] have been cloned from several teleost species, and functional AhR-responsive regulatory elements have been identified upstream of the fish CYP1A genes.[277–279] An AhR-CYP1A pathway can also confidently be inferred to exist in cartilaginous fish, based on induction by model AhR agonists of P450s with immunochemical and catalytic characteristics of CYP1A.[280–283] In contrast, there is evidence that jawless fish (lamprey and hagfish) and invertebrates lack an AhR-regulated CYP1A.[28,257,283–285] Less is known about the regulation of other members of the Ah gene battery in these early diverging groups.

Thus, from current knowledge it appears that the role of the AhR in regulating xenobiotic-metabolizing enzymes first evolved in early vertebrates. The

emergence of this function appears to coincide with the evolution of the ability to bind halogenated and nonhalogenated polycyclic aromatic hydrocarbons, suggesting that the adaptive function of the AhR may have been a vertebrate innovation. Thus, the physiological functions of the AhR may be ancestral to the adaptive function, although new physiological functions appear to have evolved as well in the vertebrate lineage. One might conclude that the emergence of the adaptive function of the AhR is responsible for dioxin toxicity, because it coincided with the ability of this protein to bind PHAHs and PAHs, which could then interfere with its physiological functions.

14.5 SOME POSSIBLE MECHANISMS OF DIOXIN TOXICITY

Our understanding of the molecular and physiological mechanisms by which dioxins cause toxicity remains incomplete. However, the findings reviewed above suggest some possible scenarios and inspire testable hypotheses.

1. *Sustained induction of biotransformation enzymes.* Although the induction of CYP1A has been studied more than any other aspect of AhR function and dioxin action, its role in toxicity has often been questioned or dismissed.[42,286] However, recent studies implicating oxidative stress in the mechanism of dioxin toxicity,[39,65,287–292] coupled with evidence for reactive oxygen production via CYP1A[293–295] suggest that the sustained induction of CYP1A or CYP1B1 by PHAH exposure could contribute to some forms of dioxin toxicity.

2. *Sustained alterations in expression of genes involved in the regulation of cell growth or other aspects of cellular physiology.* The endogenous ligand, if one exists, is likely to be ephemeral, causing only transient activation of the AhR pathway.[54,168] PHAHs, in contrast, are persistent and therefore cause sustained activation of AhR signaling.[289,296,297] Such sustained stimulation of physiological processes could underlie some of dioxin's effects. These endogenous processes remain to be identified.

3. *Recruitment of AhR away from endogenous pathways.* If some physiological functions of the AhR are ligand-independent, binding of exogenous ligands and the accompanying changes in AhR conformation and protein–protein interactions could affect the ability of this protein to participate in its normal functions. In this respect, could TCDD act as an inverse agonist[298] for some physiological response, even as it acts as a traditional agonist for others (e.g., induction of CYP1A1)?

4. *Recruitment of ARNT or other proteins (e.g., NRC, Rb, NF-κB) away from endogenous pathways.* The sharing of dimerization partners (e.g., ARNT) and coactivators (e.g., NRC, p300) among bHLH–PAS proteins such as AhR and HIF and even between AhR and other receptor families (nuclear receptors) provides another avenue by which AhR ligands could cause toxicity. If AhR

activation reduces the available pool of interacting proteins, the ability of those proteins to participate in other signaling pathways could be compromised (e.g., "squelching"). There is evidence, for example, of interactions between dioxin and hypoxia signaling.[59,267,299,300] Whether these interactions involve competition for ARNT or coactivators or occur through other mechanisms is not yet clear. However, these interactions, and crosstalk between the AhR and other pathways,[301–303] provide additional, albeit indirect, mechanisms by which dioxins might cause toxicity.

It is likely that dioxin toxicity results from a complex series of events involving multiple pathways and endpoints. The relative importance of these endpoints varies among species, just as the spectrum of effects[42] and the relative sensitivity for each effect[304] vary among species. Understanding this complexity and its implications for human sensitivity to the various forms of dioxin toxicity will require studies in humans and in a variety of model systems, using multiple approaches.

14.6 SUMMARY AND CONCLUDING REMARKS

What have comparative studies taught us about AhR signaling and dioxin toxicity? Certainly, it is clear now that the AhR is an ancient protein that was present in early bilateral metazoans and exists today in at least three extant invertebrate phyla. In the vertebrate lineage, however, the *AhR* gene has undergone duplication and diversification resulting in a family of AhR-related genes (*AhR1*, *AhR2*, *AhRR*), all apparently involved in dioxin signaling.

The adaptive function of the AhR may have first evolved in early vertebrates. This function of the AhR is highly conserved in most modern vertebrates, and thus the AhR is likely to be an important component of cellular defenses against exogenous and perhaps also endogenous toxicants. In this regard, the AhR functions together with the PXR/SXR, CAR, and other xenobiotic-sensing receptors to mount an enzymatic response to the presence of toxic chemicals.

The AhR is more than just a xenobiotic sensor, however. It also has physiological roles, which may differ in evolutionarily divergent taxa. In early metazoans and some extant invertebrate species, the AhR may have played a role in the development of sensory structures or neurons. In mammals, it appears to be involved in the development of a variety of tissues and in vascular maturation.

The diversity of *AhR* and related genes differs among animal species. Many fish species possess at least two AhR paralogs, while mammals have a single AhR. Fish are among the most sensitive species to dioxin toxicity,[305] suggesting that differences in AhR diversity may underlie some species differences in the sensitivity to dioxin toxicity.[224] AhR properties, including ligand-binding affinity and specificity, also differ among species and strains and are known to

contribute to differences in responses.[214,233,235,306–310] In the future it may be possible to predict species-specific sensitivity by in vitro characterization of the AhR and other components of the AhR pathway.[214,233,235,238,240,306,311]

Ultimately, a complete understanding of the physiological roles of the AhR will provide the information necessary for assessing the risk of dioxins to humans and other animals exposed to these chemicals. Comparative and evolutionary studies that identify the unity and diversity in AhR function will make an important contribution to achieving that objective.

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NOTE ADDED IN PROOF

In addition to the natural and endogenous AhR ligands discussed in Section 14.3.4, the compound 2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE) has been identified recently as an AhR ligand isolated from mammalian lung (Song, J., Clagett-Dame, M., Peterson, R. E., Hahn, M. E., Westler, W. M., Sicinski, R. R., and DeLuca, H. F., A ligand for the aryl hydrocarbon receptor isolated from lung, *Proc. Natl. Acad. Sci. U.S.A.* **99**, 14694–14699 (2002).

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CHAPTER 15

Dioxin Toxicity and Aryl Hydrocarbon Receptor Signaling in Fish

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15.1 EXPOSURE AND BIOACCUMULATION OF DIOXIN AND RELATED COMPOUNDS BY FISH

Polychlorinated dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs), and biphenyls (PCBs) belong to a family of lipophilic halogenated aromatic hydrocarbons that have similar structures, resist chemical and biological degradation, and persist in the environment, posing a potential risk to aquatic organisms. The more potent PCDD, PCDF, and PCB congeners are planar or coplanar molecules with lateral chlorine substitutions and are approximate isostereomers of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Adult fish are exposed to TCDD and related chemicals via water, sediment, and food. Bioaccumulation is dependent on the physical and chemical characteristics of the individual PCDD, PCDF, and PCB congeners and on their biotransformation and elimination rates.[1–4] Fish preferentially bioaccumulate TCDD and the TCDD-like PCDD and PCDF congeners,[5–8] and PCB congeners with higher chlorine content (i.e., penta-, hexa-, and hepta-chlorinated biphenyls).[9,10] In fish embryos and sac fry larvae, the primary route of exposure to TCDD-like PCDD, PCDF, and PCB congeners is by transfer of these lipophilic chemicals from maternal tissues to oocytes during vitellogenesis.[4,11–16] The concentration of these TCDD-like congeners in the egg, expressed as TCDD equivalents, is

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the key determinant of developmental toxicity; the route of egg exposure is not a factor.[17–19]

15.2 DIOXIN TOXICITY IN JUVENILE AND ADULT FISH

The toxicity of TCDD and related chemicals in fish have been assessed following laboratory exposure to a single PCDD, PCDF, or PCB congener or commercial PCB mixtures, or by correlating PCDD, PCDF, and/or PCB concentrations in feral fish populations with adverse effects. While both field and laboratory research are important in understanding the toxicity of TCDD-like PCDDs, PCDFs, and PCBs; in this chapter we review laboratory studies only, focusing on acute toxicity in juvenile and adult fish species and developmental toxicity in embryos and larvae.

15.2.1 Acute Toxicity

Fish are among the most sensitive vertebrates to lethality following TCDD exposure.[20–22] The guinea pig[21] and hamster[23] are the most and least sensitive mammals, with LD_{50} values of 1 and 5000 μg TCDD/kg. Juvenile fish approach the sensitivity of the guinea pig, with LD_{50} values of 3 and 16 μg TCDD/kg in yellow perch (*Perca flavescens*)[22] and bluegill (*Lepomis macrochirus*),[22] respectively. Acute toxicity of TCDD to juvenile fish is dose-dependent and characterized by decreased food consumption, decreased body weight gain (wasting), and delayed mortality. However, decreased food intake and wasting are probably not the cause of lethality in fish, because at a sufficiently high dose of TCDD (LD_{90}), juvenile fish die before reducing their feed intake and losing weight.[22] Mortality is delayed in onset, with the length of the delay being related to TCDD dose. That is, a LD_{20} causes a longer latency period prior to death than a LD_{90}. Chronic exposure to TCDD also produces delayed mortality,[4,24–27] and it is not necessarily averted if the exposure ends prior to the onset of lethality.[4,24,25,27] Another endpoint of acute TCDD toxicity in juvenile fish is fin necrosis. Fin margins become necrotic and fin rays fragment,[22,28,29] often leading to increased susceptibility to fungal infection.[26,30] Other endpoints of acute TCDD toxicity in fish are species-specific, consisting of cutaneous hyperpigmentation, hemorrhage, and/or ascites.

15.2.2 Histopathology

TCDD-exposed juvenile fish typically exhibit lesions in epithelial and lymphomyeloid tissues. The liver often exhibits hepatocellular glycogen depletion and cytoplasmic vacuolation, and hepatocellular hypertrophy and lipidosis have also been described.[29] Other TCDD-induced epithelial lesions tend to be species-specific and include hyperplasia of the gills in yellow perch,[29] and hyperplasia and necrosis of the gastric mucosa and pancreatic lesions in rainbow

trout (*Oncorhynchus mykiss*).[28,31] Decreased hematopoiesis in the head kidney of rainbow trout is among the most sensitive TCDD-induced lesions reported in juvenile fish.[28]

15.3 DIOXIN TOXICITY IN EMBRYONIC AND LARVAL FISH

Early life stages of fish are at higher risk of TCDD toxicity because they are up to 50 times more sensitive than adults.[17,31–37] The lowest observable adverse effect level (LOAEL) for larval toxicity in lake trout (*Salvelinus namaychus*, 40 pg TCDD/g egg[33]) is $\frac{1}{25}$ of that for overt toxicity in juvenile rainbow trout (1000 pg TCDD/g fish[28]). The range of sensitivity between fish species for TCDD-induced mortality at the larval stage of development also varies significantly (Figure 15.1). Lake trout larvae are generally considered the most sensitive and zebrafish (*Danio rerio*) larvae the least sensitive. However, bull trout (*Salvelinus confluentus*), which are closely related to lake trout, appear to be even more sensitive than lake trout to TCDD-induced early life-stage mortality.[38] Significant differences in sensitivity have also been observed between different strains of rainbow trout[32] and different feral populations of Atlantic killifish (*Fundulus heteroclitus*).[39] The molecular explanations for such strain and species differences have not been elucidated.

The hallmark signs of TCDD early life-stage toxicity in fish are edema, hemorrhage, craniofacial malformation, growth retardation, and posthatch

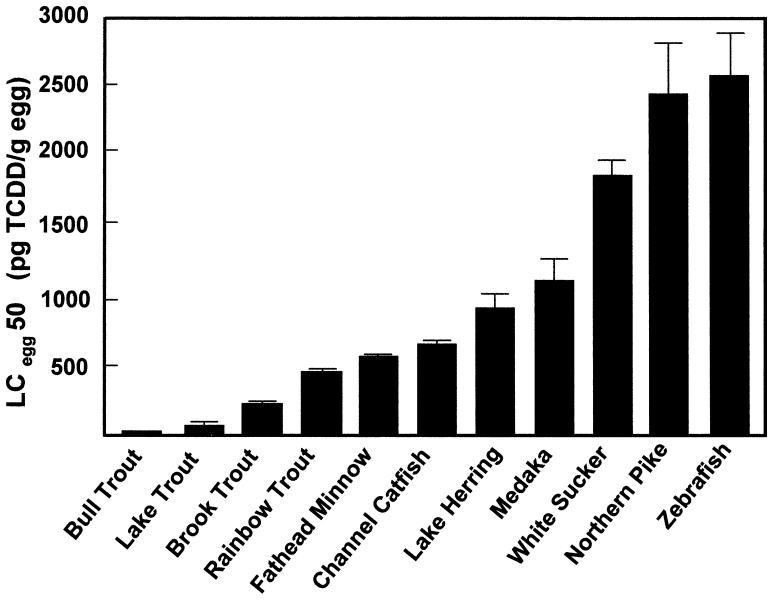

Figure 15.1 Species differences in sensitivity to TCDD-induced mortality at the larval stage of development. (Data from Refs. 38 and 42.)

mortality. These responses have been observed in lake trout,[17,34,40] bull trout,[38] brook trout (S*alvelinus fontinalis*),[40,41] rainbow trout,[17,31,32] northern pike (*Esox lucius*),[35,42] Japanese medaka (*Oryzias latipes*),[15,36,42,43] killifish,[44,45] zebrafish,[16,46] fathead minnow (*Pimephales promelas*),[42] lake herring (*Coregonus artedii*),[42] white sucker (*Catastomus commersoni*),[42] and channel catfish (*Ictalurus punctatus*).[42] The similar shape of the TCDD dose–response curve for larval mortality across several fish species suggests that a common mechanism is involved.[17–19,40,42] The developmental stage at which TCDD toxicity is typically manifested in lake trout, rainbow trout, northern pike, and zebrafish is after hatching. However, at elevated egg concentrations of TCDD (three to four times above LC_{99}), embryos can die prior to or during hatching. At lower egg concentrations (< 3 times below LC_{99}), TCDD toxicity is generally manifested after hatching during the sac fry stage of development.[19,33]

15.3.1 Lake Trout

Lake trout sac fry typically die with severe yolk sac edema, exophthalmia, petechial hemorrhages, disruption of the vitelline vasculature, cessation of blood circulation to the tail, head, and gills, and arrested development of skeletal and soft tissues.[19,33] Histologically, moribund lake trout sac fry also exhibit intraocular hemorrhage and severe congestion and hemorrhages in the capillary bed behind the eye.[33] TCDD-induced lesions during rainbow trout and lake trout early development resemble blue-sac disease,[17,19,31,33] an edematous syndrome observed in some hatchery-raised salmonids.[47,48] The etiology of blue-sac disease is poorly understood, but many exogenous factors also induce the edematous syndrome, including reduced water flow, increased water ammonia concentrations, and low dissolved oxygen levels.[47,48] In lake trout, TCDD appears to affect the cardiovascular system first, causing congestion of vascular beds in a variety of sites, leading to severe fluid accumulation and cessation of blood circulation in the yolk sac and body.[33,49] The yolk sac edema is dose-dependent and becomes so severe that it increases sac fry wet weight.[34,49] One explanation for the increased weight is that loss of fluid from the blood in producing edema causes the affected sac fry to take up excess water from the external environment in a futile attempt to restore blood volume to normal. Polyacrylamide gel electrophoresis of edema fluid proteins suggests that it is an ultrafiltrate of blood[49] caused by increased permeability of the vascular endothelium—not endothelial necrosis. This is evident by the endothelium displaying increased vacuolation, separation of intercellular junctions, and cytoplasmic blebbing, without detectable necrosis, in a dose- and time-dependent manner similar to the signs of cardiovascular toxicity.[49] Exposure of fertilized lake trout eggs to TCDD also induced cytochrome P4501A protein in vascular endothelial cells of the larvae prior to the development of signs of cardiovascular toxicity, suggesting a role for the aryl hydrocarbon receptor (AhR) pathway in the vascular toxicity.[50] Thus, TCDD may cause hemodynamic and/or vascular permeability changes during lake trout early

development, subsequent to AhR activation in the vascular endothelium, ultimately leading to edema and mortality.[50]

15.3.2 Rainbow Trout and Northern Pike

Rainbow trout[17,31] and northern pike[35] exposed to TCDD as eggs exhibit the same pattern of toxic responses after hatching as lake trout,[34] including reduced growth rate, yolk sac and pericardial edema, exophthalmia, and sac fry mortality.[31] Northern pike exhibit some hatching mortality, but primarily sac fry mortality associated with generalized edema and hemorrhages.[35] Histologically, rainbow trout and northern pike exposed to TCDD show disruption of blood capillaries,[31,35] and as with lake trout,[34] mortality occurs prior to swim-up. Perfusion of the vasculature is reduced progressively in posthatch rainbow trout exposed as embryos to TCDD[51] and is followed by pericardial and yolk sac edema, craniofacial malformations, and death.[51] The reduced blood flow is not associated with endothelial necrosis or apoptosis but is associated with reduced heart size.[51]

15.3.3 Zebrafish

Zebrafish exposed to waterborne TCDD as embryos or through maternal transfer exhibit signs of larval toxicity similar to salmonids (Figure 15.2).[16,46] Egg mortality and time to hatch is not affected by TCDD exposure.[42,46] As

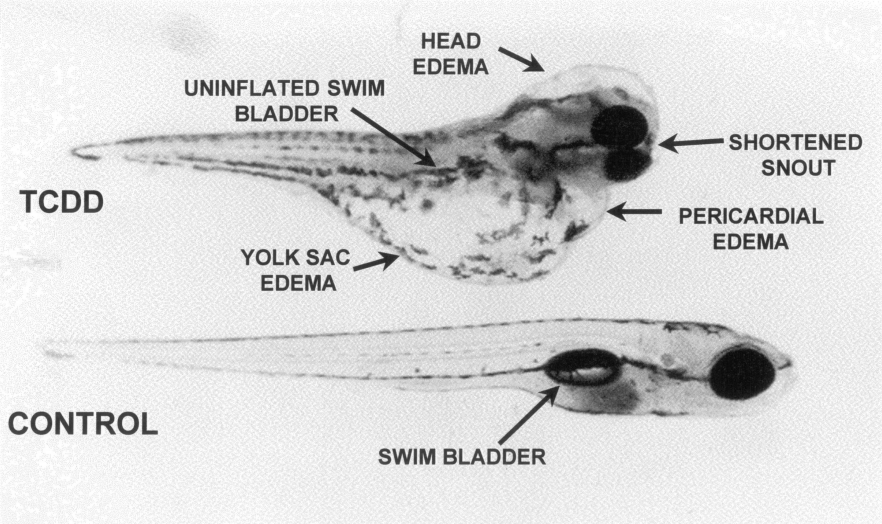

Figure 15.2 Representative zebrafish larva (5 days post fertilization) exposed as a fertilized egg for 1 hour to either DMSO vehicle (control, bottom) or TCDD (top). TCDD concentration in the egg was approximately 2500 pg TCDD/g egg.

with salmonids, TCDD exposure reduces blood flow in various vascular beds coincident with or followed by edema in the head, trunk, pericardium, and yolk sac.[46,52] Other signs of toxicity include impaired lower jaw development, anemia, inhibited swimbladder inflation, and decreased growth. While the decrease in peripheral blood flow is probably associated with decreased cardiac output, heart rate is not decreased until 144 h postfertilization when death, associated with massive edema, is imminent.[46,52] When embryos are exposed to TCDD concentrations that cause 100% mortality, the first detectable sign appears at 50 h, consisting of decreased blood flow to the dorsal midbrain.[53] Upon gross visual inspection of the embryo, a few overt signs of toxicity can be detected as early as 60 h[16,46]; however, most are not obvious until 72 to 96 h.[16,46]

Since toxicity is delayed until after hatching, a number of studies have characterized how late in embryo development TCDD exposure can occur and still produce the same toxicity syndrome. Zebrafish embryos exposed to a lethal dose of TCDD beginning at 4, 24, 48, or 72 h postfertilization develop the same toxicity syndrome, whereas those exposed beginning at 96 or 120 h do not. This suggests that TCDD produces toxicity by disrupting certain developmental processes or events that are completed before approximately 72 h. Although certain endpoints of TCDD toxicity appear to be secondary to the reduction in blood flow, this is not true for all endpoints. The initial impairment in lower jaw growth is not caused by a decrease in blood flow to the lower jaw, nor is it associated with increased apoptosis.[52] Alternatively, at 144 to 168 h postfertilization, moribund larvae exposed to a lethal dose of TCDD develop necrosis in several organs secondary to ischemia.[16,46] TCDD also causes anemia in zebrafish larvae between 72 and 96 h postfertilization in association with other endpoints of toxicity.[54] Hematopoiesis occurs in two phases in zebrafish. The embryonic phase, from 18 to 48 h, occurs in the intermediate cell mass, and the adult phase is initiated in the dorsal aorta at 36 h.[55] TCDD causes anemia by blocking the adult phase of erythropoiesis.[54] However, a direct link between the reduction in circulating mature erythrocytes and the evolution of overt signs of toxicity could not be made. This is because larvae exposed to TCDD beginning at 72 h, well after the switch to the adult phase of erythropoiesis had occurred, still developed the toxicity syndrome characteristic of TCDD.[54] With respect to vascular development, it has been found that development of blood vessels in the posterior trunk is not altered by TCDD from 48 to 96 h postfertilization.[54] However, it has yet to be determined if the vitelline vasculature and blood vessels in the head develop normally and are maintained properly following TCDD exposure.

15.3.4 Medaka

In Japanese medaka, the time course of TCDD-induced toxicity during early development is different than that for rainbow trout, lake trout, northern pike, and zebrafish. TCDD-induced lesions in medaka develop after organogenesis

but prior to hatching.[36] Following exposure of eggs to TCDD on the day of fertilization, embryos develop normally until day 4 or 5 of development, when blood flow in the caudal vein is decreased. Subcutaneous hemorrhages and pericardial edema develop subsequently, followed by collapse of the yolk sac, failure of heart chamber formation, circulatory shutdown, and mortality.[36] The sensitivity of medaka to TCDD is reduced when embryos are exposed after formation of the liver rudiment (day 4 to 5 postfertilization).[36] Medaka exposed to TCDD between fertilization and day 5 of development exhibit severe edema, hemorrhages, and mortality. When exposed on day 6 of development, mortality and the percentage of embryos with severe lesions (edema and hemorrhage) are reduced. This suggests that sensitivity of the medaka embryo to TCDD toxicity depends on events or processes occurring between days 5 and 9 postfertilization.[36]

15.3.5 Comparative Toxicity

Fish are among the most sensitive vertebrates to the lethal effect of TCDD, and early life stages of fish represent the most sensitive developmental stage, one to two orders of magnitude more sensitive than juveniles, with egg concentrations of TCDD in the low parts per trillion range producing mortality in the most sensitive fish species. Signs of toxicity and histopathologic lesions produced by TCDD in juvenile fish are similar to those seen in higher vertebrates and include decreased food intake, wasting, delayed mortality, and lesions in epithelial and lymphomyeloid tissues. Signs of toxicity and histopathologic lesions produced by TCDD during fish early development are characterized primarily by cardiovascular dysfunction, edema, hemorrhages, and mortality. It is noteworthy that the same pattern of lesions has been observed in birds and mammals.[21,56–58] Chickens[21,56] exposed to TCDD exhibit chick edema disease, which is characterized by ascites and pericardial, subcutaneous, and pulmonary edema. Chicken embryos,[59] injected with TCDD as newly fertilized eggs, develop abnormalities in the vitelline vasculature, including a decrease in the area of yolk vascularized and short, abnormally bent vitelline blood vessels, as well as a reduction in the branching and lumen size of the coronary arteries. Mice[57] treated with TCDD exhibited subcutaneous edema, ascites, fluid accumulation in the thoracic cavity, and submucosal edema in the stomach and small and large intestine. Mice[57] also show intraorbital hemorrhage and subcutaneous hemorrhages in the eyelids following TCDD exposure. Monkeys[58] typically develop periorbital and facial edema, ascites, subcutaneous edema in the lower abdomen, as well as pericardial hemorrhages, focal hemorrhages in the lungs, and petechial hemorrhages over the entire body surface following TCDD exposure. Although adult rats do not exhibit edema in direct response to TCDD, exposure to TCDD makes them supersensitive to edemagenic agents.[60,61] Taken together, these findings suggest that TCDD affects the vascular endothelium and/or mediators of vascular permeability in all vertebrate classes.

15.4 AhR SIGNAL TRANSDUCTION PATHWAY IN FISH

The AhR is a ligand-activated transcription factor that resides in the cytoplasm associated with two molecules of HSP90 and the AhR interacting protein (AIP), also known as XAP2 and ARA9.[62–64] AhR translocates to the nucleus following agonist binding, then dimerizes with the aryl hydrocarbon receptor nuclear translocator (ARNT) (reviewed in Refs. 65 and 66). The ligand-receptor complex binds to specific DNA sequences termed AhR-responsive enhancers (AhREs) that are present in genes such as cytochrome P4501A1, whose transcription is regulated by AhR ligands. Recently, the AhR repressor (AhRR) has been identified and may be an important modifier of the AhR pathway in a tissue-dependent manner.[67] In the past few years there has been significant progress in the molecular and biochemical characterization of components of the AhR signaling pathway in fish. It is apparent that the overall AhR pathway is conserved between mammals and fish; however, there are some notable differences.

15.4.1 Fish Aryl Hydrocarbon Receptors

AhR genes have been identified in both bony and cartilaginous fishes, including killifish,[68] zebrafish,[69,70] rainbow trout,[71] Atlantic tomcod (*Microgadus tomcod*),[72] medaka,[73] European flounder (*Platichythys fleus*),[74] smooth dogfish (*Mustelus canis*),[75] spiny dogfish (*Squalus acanthias*),[75] Greenland shark (*Somniosus microceophalus*),[76] and skate (*Raja erinacea*).[75] The studies of fish AhRs have revealed that a gene duplication event has occurred in the chordate lineage since there are two AhRs in fish (AhR1 and AhR2), while mammals apparently have only one (for reviews, see Refs. 75 and 76 and Chapter 14). Phylogenetic analysis demonstrated that the *Fundulus* and zebrafish AhR1s are orthologs of the mammalian AhRs, while the AhR2 forms are paralogs to the type 1 receptors.[75] The functional significance of having two AhRs in fish is unknown, but determination of the role that each receptor plays in TCDD toxicity is under way. To date, only a few of the fish AhRs have been functionally characterized. Full-length AhR1 and AhR2 cDNAs have been cloned in *F. heteroclitus* (FhAhR1, FhAhR2). Both FhAhR1 and FhAhR2 exhibit high-affinity binding of dioxin, interact with ARNT, and recognize AhREs. The two *Fundulus AhR* genes, however, display different tissue-specific patterns of expression; FhAhR2 transcripts are abundant in many tissues and FhAhR1 transcripts are expressed primarily in brain, heart, ovary, and testis.[68] Both receptors also possess transcriptional activity[77] in transient transfection experiments.[77]

The zebrafish AhR1 and AhR2 have recently been functionally characterized (zfAhR1 and zfAhR2).[69,70] Both zfAhR2 and zfAhR1 dimerize with zfARNT2b and bind with specificity to AhREs, although zfAhR1 binding is weak.[70] Only zfAhR2 exhibits high-affinity binding to [^{3}H]TCDD and [^{3}H]β-naphthoflavone. In transient transfection experiments only zfAhR2

stimulates transcription in response to ligands.[70] zfAhR1 and zfAhR2 mRNAs are expressed in early development, but they are expressed differentially in adult tissues, where zfAhR1 is expressed primarily in the adult liver, and zfAhR2 is more widely expressed.[70] These studies demonstrate that in zebrafish, it is zfAhR2 that mediates TCDD toxicity, and the functional role of zfAhR1 remains unclear.[70]

A type 1 AhR has not been identified in rainbow trout; however, two type 2 AhRs have been characterized and they are 98% identical to each other at the amino acid level, rtAhR2α and rtAhR2β.[71] Both proteins bind TCDD, dimerize with ARNT, and bind AhREs. Both rtAhR2 mRNAs are expressed in multiple tissues, and rtAhR2β is highly expressed in the heart.[71] Like the zebrafish AhR2 mRNA,[69] expression of rtAhR2α and rtAhR2β mRNAs are positively regulated by TCDD. Functionally, rtAhR2α and rtAhR2β produce TCDD-dependent activation of a reporter gene driven by AhREs. However, the two receptors have distinct preferences for reporter genes, suggesting that they may regulate a specific repertoire of genes.[71] A single amino acid, position 111 in rtAhR2β, underlies the differential activities of rtAhR2α and rtAhR2β. The importance of this residue for tranactivation activity was confirmed in both the human and zebrafish AhRs.[78]

15.4.2 Fish Aryl Hydrocarbon Receptor Nuclear Translocator

The nuclear dimerizartion partner for AhR is ARNT. In mammals there are two different forms of ARNT, encoded by different genes, ARNT1 and ARNT2.[79,80] The type and activities of ARNT(s) expressed in various vertebrates may contribute to the cross-species differences in sensitivity to AhR ligand toxicity. In mammals the widely expressed ARNT1 is the endogenous partner for AhR. ARNT2, which is largely restricted to the brain, does not play an important role in AhR signal transduction[80] and is an in vivo partner of SIM1. SIM1/ARNT2 are involved in hypothalamic development in the mouse.[81,82] ARNT cDNAs have been isolated and characterized from fish, including rainbow trout,[83] *Fundulus*,[84] and zebrafish.[85] An ARNT1 has yet to be identified in fish.

Rainbow trout possess an ARNT (rtARNT) that shares sequence homology with both mammalian ARNT1 and ARNT2.[83] Two splice variants of rtARNT have been described, rtARNTa and rtARNTb, that are identical for the first 533 amino acids but differ in their COOH-terminal domains due to the presence of an alternatively spliced exon in rtARNTb. Both rtARNT splice variants are expressed in RTG-2 trout gonad cells and in trout liver and gonad tissue and the proteins are expressed in RTG-2 cells. Both rtARNTa and rtARNT2b dimerize with AhR; however, only cells that express rtARNT2b can exhibit TCDD-mediated induction of endogenous P4501A protein.[83] The decreased function of rtARNTa results from inefficient DNA binding of the rtARNTa/AhR complex.[83] Importantly, the rtARNTa splice variant acts as a dominant negative regulator of rtARNTb in vivo and in vitro. Recent studies

suggest that the repressive properties of rtARNTa result from masking or misfolding of the DNA-binding domain by the rtARNTa C-terminal hydrophobic amino acids.[86,87] A full-length ARNT cDNA homolog has been identified in *Fundulus*, and phylogentic analysis indicates that the cDNA encodes an ARNT2 (FhARNT2).[68] FhARNT2 mRNA is ubiquitously expressed in tissues,[88,89] and the protein dimerizes with AhR, binds to AhRE, and induces reporter gene transcription in cells expressing AhR in response to TCDD exposure.[77]

Several ARNT2 mRNA splice variant cDNAs have been identified and characterized from zebrafish, zfARNT2a, zfARNT2b, zfARNT2c, and zfARNT2X.[85,90,91] zfARNT2a and zfARNT2b proteins are identical over the first 403 amino acids but differ in their C-terminal domain. zfARNT2a is truncated to 424 amino acids compared to the 737 amino acid zfARNT2b.[85,90] zfARNT2b and zfARNT2c are identical, with the exception of an in-frame 15-amino acid deletion near the basic region of zfARNT2c.[85] zfARNT2X is another truncated ARNT2 cDNA, which differs from zfARNT2a just N-terminal to the basic region.[91] Functionally, these splice variants are distinct. Transient transfection experiments in COS-7 cells expressing zfARNT2b and zfAHR2 show that TCDD causes significant dioxin-responsive reporter gene induction. In similar experiments, COS-7 cells expressing zfARNT2a or zfARNT2c fail to induce reporter gene expression in response to TCDD. Importantly, all three zfARNT2 proteins function with endothelial-specific PAS protein 1 (EPAS-1, also known as HIF2α and MOP2)[92,93] to induce reporter gene activity under control of hypoxia-responsive elements.[85] In transiently transfected zebrafish liver epithelial (ZLE) cells, expressing zARNT2a or zfARNT2X, TCDD-dependent CYP1A transcription was repressed. This suggests that the truncated zfARNT2s may function as dominant negative factors of AhR signaling.[90,91] Misexpression of zfARNT2X during early zebrafish development also results in severe developmental malformations, suggesting that ARNT2 activity is essential for normal zebrafish development.[91] zfARNT2b and zfARNT2c are expressed in the adult brain, eye, and skeletal muscle.[85] zfARNT2a mRNA is most highly expressed in the adult eye, skeletal muscle, gills, and brain and to a lesser extent in the liver, gonads, skin, and fins.[85,90] zfARNT2X mRNA is expressed in the retina and neural tube until hatching, and in the brain, eyes, hypothalamus, pharyngeal skeleton, heart, liver, pronephric duct, pectoral fin, and epithelial cells of the swimbladder of zebrafish larvae.[91]

15.4.3 Fish AhR Interacting Protein

The importance of the AhR interacting protein (AIP) in AhR signaling in fish is unclear. AIP has been identified in a number of mammalian species, including the mouse,[63] human,[62] and monkey.[64] AIP binds to the C-terminal end of HSP90,[94,95] and the C-terminus of AIP is required for binding of AhR.[95] It

has been suggested that AIP stabilizes AhR into a ligand-binding conformation, thereby enhancing AhR-mediated transcription.[63] Recently, an AIP-like sequence has been identified and characterized from zebrafish (zfAIP).[96] Overall, the zfAIP amino acid sequence is 66% identical and 81% similar to the human AIP and is expressed in adult gills, fins, heart, liver, brain, intestine, kidney, and eye. Functionally, the zfAIP protein does not interact with zfAhR1 or zfAhR2 and does not affect AhR function in transient transfection assays.[96] These results suggest that the role of AIP in AhR signaling may not be conserved in all vertebrates. The other possibility is that there exists another AIP gene in zebrafish that still functions in AhR signal transduction. To determine the importance of AIP proteins in fish AhR signaling, further gene identification in other fish species is required.

15.4.4 Fish AhR Repressor

Recently, the AhRR was identified in mice[67] and humans[97] and has been classified phylogenetically as a member of the AhR family,[76] suggesting that AhR1, AhR2, and AhRR have descended from a single invertebrate AhR (see Ref. 76 and Chapter 14). The *Fundulus* AhRR (FhAhRR) has been identified and is approximately 50% identical to the mammalian proteins, over the N-terminal half of the protein. Like the mammalian proteins, FhAhRR does not bind [^{3}H]TCDD or [^{3}H]β-naphthoflavone, and in transient transfection experiments, FhAhRR inhibits both FhAhR1 and FhAhR2 TCDD-dependent transactivation.[77] In the adult killifish, AhRR mRNA is widely expressed and is inducible by TCDD and a PCB mixture. The zebrafish AhRR has also been identified (M. Hahn, personal communication). The importance of AhRR as a modulator of AhR activity during early life stages and in adult organs of fish remains to be determined.

15.4.5 Tissue-Specific Expression of AhR, ARNT, and Dioxin-Induced CYP1A in the Zebrafish Embryo and Larva

Before we can understand the role of AhR signaling in mediating dioxin developmental toxicity in fish, it is necessary to demonstrate that the key components of the pathway are expressed during early development. In the zebrafish embryo and larva, AhR2 and ARNT2 transcripts are expressed from 12 to 120 h postfertilization when TCDD developmental toxicity is manifested.[98] Spatial expression of AhR2, ARNT2, and CYP1A mRNA has also been determined in zebrafish embryos and larvae exposed to vehicle (control) or TCDD.[98] AhR2 and ARNT2 mRNAs are colocalized in specific tissues.[98] In some of these tissues, but not all, CYP1A mRNA is induced by TCDD.[98] Whole mount in situ hybridization images illustrating colocalization of AhR2, ARNT2, and TCDD-induced CYP1A mRNA expression in certain tissues of the zebrafish embryo is shown in Figure 15.3.

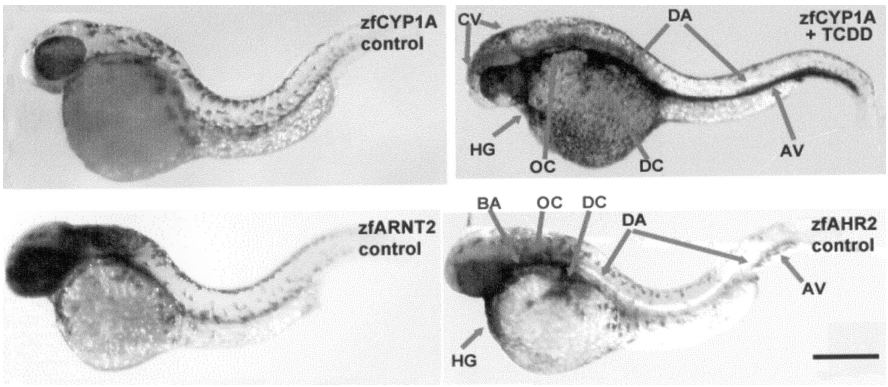

Figure 15.3 Whole mount in situ hybridization using the indicated gene-specific digoxigenin-labeled antisense RNA riboprobes in embryos treated at 3 to 4 h postfertilization with vehicle (control) or TCDD.[14] Lateral view of representative embryos hybridized at 36 h postfertilization. CV, cerebral veins; HG, hatching gland; OC, otic capsule; DC, duct of Cuvier; DA, dorsal aorta; AV, axial vein. Scale bar = 200 μm. (Reproduced with permission from Ref. 98.)

High-quality antibodies are not yet available for specific immunolocalization of fish AHRs and ARNTs. However, an excellent antibody is available for detecting CYP1A, and it has been used extensively to demonstrate induction of CYP1A in the cardiovascular system and other tissues of jawed fish exposed to AhR agonists.[99,100] This same type of response is observed in the zebrafish embryo and larva following TCDD exposure.[53,101] Specific immunolocalization of CYP1A in the head of a TCDD-exposed zebrafish larva at 120 h postfertilization is shown in Figure 15.4.[101] This is a ventral view and demonstrates from left to right increased immunostaining of CYP1A in arteries and veins, branchial arches, heart atrium and ventricle, and liver.[101]

15.5 MECHANISMS OF DIOXIN TOXICITY IN FISH

It is likely that TCDD toxicity results from the activities of numerous cellular factors with the underlying mechanisms of toxicity being complex and specific for given endpoints. It is largely accepted that most, if not all, dioxin toxicity is mediated, or initiated, by AhR. Therefore, characterization of the AhR signal transduction pathway in several species and across vertebrate classes will provide a basis for rational comparative studies. Furthermore, the advantage of certain fish models is that they may provide unique insight into these complex mechanisms. With our current understanding of AhR function, two major mechanisms can be proposed. Both are dependent on AhR either directly or indirectly.

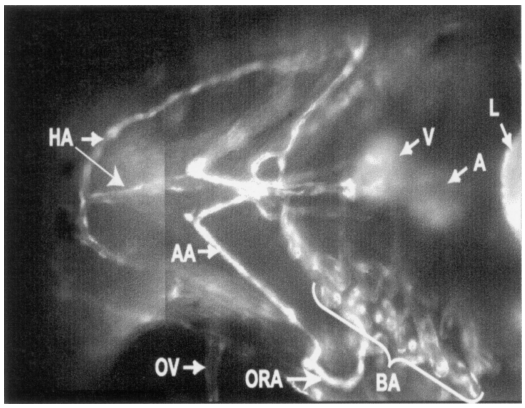

Figure 15.4 Tissue-specific immunolocalization of CYP1A at 120 h postfertilization in a zebrafish larva exposed to TCDD at 3 to 4 h postfertilization. Composite view of two ventral images of the head. HA, hypobranchial artery; OV, optic vein; AA, aortic arch; ORA, opercular artery; BA branchial arches; V, ventricle; A, atrium; and L, liver. (Reproduced with permission from Ref. 98.)

15.5.1 Direct AhR Mechanisms

It is well established that the AhR is a cytoplasmic protein that upon ligand binding translocates into the nucleus and dimerizes with ARNT. In the nucleus expression of genes containing AhREs are affected. If there is a physiological function for the AhR, the activation of AhR by xenobiotics could inappropriately induce or repress the expression of these AHR-dependent genes during critical periods of development or in specific tissues of adult animals. The consequence of this sustained and inappropriate regulation of genes would be expected to be developmental stage-specific as well as species- and tissue-specific, and this is precisely what is observed in dioxin toxicity studies. For example, sustained activation of CYP1A1 by TCDD and related compounds can generate oxidative stress in mammals.[102–108] Reactive oxygen species can be generated as a result of elevated CYP1A activity,[109,110] and in fish early life stages, oxidative stress may be involved in causing certain endpoints of TCDD toxicity. It has been demonstrated in medaka that TCDD-induced DNA damage and subsequent apoptotic cell death occurs in the vascular endothelium prior to the first signs of developmental toxicity.[101,111] The medial yolk vein was the initial site of TCDD action, as evidenced by increased expression of CYP4501A protein.[101] The involvement of CYP4501A activity is suggested by the protective effect of antioxidants or a CYP450 inhibitor that reduced both the overt signs of developmental toxicity and DNA damage in the vasculature.[111,112] TCDD exposure in *Fundulus* also leads to increased CYP1A expression and apoptotic cell death in several tissues. Importantly, CYP1A expression and apoptosis were colocalized in some, but not all, cell types.[45]

Recent studies also demonstrate that TCDD induces apoptotic cell death in the dorsal midbrain of the zebrafish embryo.[113] The vascular endothelium in this brain region expresses zfAhR2 and zfARNT2 and responds to TCDD with induction of CYP1A.[98] Well before blood flow is reduced to most vascular beds, an association between a decrease in blood flow to this brain region and subsequent apoptosis was observed.[52,53] The TCDD dose response for decreasing blood flow to the dorsal midbrain of the zebrafish embryo at 50 h postfertilization was similar to that for increasing apoptosis in the same brain region at 60 h, suggesting that the initial decrease in flow plays a role in increasing apoptosis.[53] Both responses were inhibited by coexposure to an AhR antagonist, antioxidant, or CYP450 inhibitor.[53] This implies that the two responses are AhR-mediated and that they may possibly involve increased production of reactive oxygen species by CYP4501A in the endothelium of the midbrain blood vessel where blood flow was decreased[53] and expression of CYP4501A increased: namely, the mesencephalic vein.[53,98] However, in rainbow trout, there is no correlation between early life stage vascular apoptosis and vascular toxicity. Furthermore, there is no evidence for vascular or cardiac myocyte apoptosis in chick embryos following exposure to TCDD. However, there is an increase in the number of apoptotic cells in structures where apoptosis is already occurring.[59]

15.5.2 Indirect AhR Mechanisms

The AhR interacts with a growing list of cellular proteins, including HSP90,[114] ARNTs,[79] AIP,[62,63,115] and RB,[116] coactivators including P300[117] and SRC-1,[118] and NF-κB.[119] The importance of these interactions in AhR function is only partially understood. Each of these factors interacts with or is required for the activities of other proteins. The inappropriate activation of AhR by TCDD or other persistent AhR agonists could result in altered protein–protein interactions, thus affecting non-AhR transduction pathways. This *squelching model* requires that at least one cellular factor be common to AhR and another pathway. Interaction between HIF1α and AhR, by competition for their common dimeric partner ARNT, has been demonstrated in vitro,[120–123] but the in vivo relevance of this interaction remains unclear. Recent gene expression studies also have revealed that the abundance of a significant number of mRNAs is altered in response to TCDD,[124–126] indicating that TCDD exposure and AhR activation result in a significant transcriptional response. These responses would be expected to result from both direct and indirect AhR transcriptional activities.

15.5.3 Adaptive and Toxic AhR Responses

It has been proposed that the response of vertebrates to xenobiotics can be categorized as either adaptive or toxic (reviewed in Ref. 66). The adaptive response hypothesizes that xenobiotics transiently induce the expression of

genes whose enzyme products metabolize the invading chemicals to less toxic metabolites, thereby protecting the organism. In this adaptive response the alteration in gene expression is postulated to be short-lived. On the other hand, the toxic response results from the sustained activation of the adaptive response by AhR ligands that persist in the body and are only very slowly metabolized and eliminated, such as dioxin and certain PCBs.

What evidence is there for the AhR-dependent adaptive response in fish? In zebrafish, tissue-specific early developmental and adult expression patterns of zfAhR2, zfARNT2, and TCDD-induced zfCYP1A have been completed.[98] zfAhR2, has been detected early in development,[69,98,127] as has the functional ARNT2b.[85,98,127] If an adaptive function is involved, it would be expected that the AhR signaling pathway would be present at tissue surfaces of the embryo facing the external environment where exposure to xenobiotics would first be encountered. In general, this type of expression pattern is seen in the zebrafish embryo and larva.[98]

15.6 FUTURE DIRECTIONS

What contribution may comparative dioxin toxicity studies in fish contribute to our understanding of the physiological function of the AhR and the underlying mechanisms of dioxin toxicity? First and foremost, gene functions can be unraveled through comparative approaches and fish models are amenable to functional studies. Fish models such as zebrafish, medaka, and *Fundulus* are poised to permit hypotheses on mechanisms of dioxin developmental toxicity to be tested. Ongoing full-genome sequencing projects are under way in zebrafish,[128] medaka,[129] and the pufferfish (*Fugu rubripes*)[130] that will allow for rapid, cross-species, functional studies. In both zebrafish and medaka, inbred strains and genetic maps are available allowing for the mapping and cloning of mutations. Genome-wide phenotypic mutant screens can be conducted that will allow unbiased determination of gene function.[131–133] Utilizing this reverse genetics approach, it will be possible to identify unknown genes by an alteration in a reliable phenotype. For example, a genetic screen could be devised to screen for resistance to a specific developmental endpoint of TCDD toxicity.

Targeted knockout approaches, well established in the mouse, appear to be achievable in medaka because of the availability of embryonic stem cells.[134,135] Transgenic approaches including the expression of reporter genes in specific tissues,[136–142] and the overexpression of gene products have proven useful for the determination of gene function in vivo.[143–146] Finally, transient repression of gene expression made possible by antisense or morpholino techniques offer tremendous potential for the elucidation of gene function in zebrafish.[147,148] Since the syntenic relationship between zebrafish and higher vertebrates is significant,[149,150] the rapid elucidation of gene function in these fish models provides a targeted opportunity to accelerate our understanding of vertebrate AhR biology.

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CHAPTER 16

Exposure Assessment: Measurement of Dioxins and Related Chemicals in Human Tissues

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16.1 INTRODUCTION

Toxic chemical exposure assessment in humans has been approached in different ways. For example, useful indirect estimates of exposure can be calculated from monitoring the amount of time spent in a workplace known to be contaminated with polychlorinated biphenyls (PCBs), volatile organic chemicals (VOCs), or asbestos. Packs of cigarettes smoked per day for a given number of years can be used to estimate the dose of toxins from cigarette smoke. This approach assumes some knowledge of the metabolism of the compound of concern as well as a good exposure history. In recent years, however, it has become possible to determine directly even very low chemical levels in blood or other tissues. In a sensitive and specific manner, we can now measure the highly toxic and persistent, lipid-soluble, synthetic polychlorinated dioxin (PCDD), the closely related dibenzofuran (PCDF), and PCB congeners in human tissues by the use of high-resolution gas chromatography/high-resolution mass spectroscopy (GC-MS). This has been aided by the synthesis of specific congeners of interest and improved techniques for separation and extraction of these compounds (see Chapter 2).

In this chapter we review primarily our own work in developing and using

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improved measurement techniques to document exposure to the halogenated dioxins and structurally related compounds, including the chlorinated dibenzofurans and PCBs, which we refer to as *dioxins*. This review does not include biological[1–7] or immunological[8–10] methods, which are used less frequently. It further does not include clinical indicators, such as chloracne (a nonsensitive and nonspecific high-dose skin effect sometimes seen in sensitive individuals), elevated liver enzyme and lipid levels in blood, or light and electron microscopic changes in liver parenchymal cell structure.[11–14]

Chlorinated dioxins and related compounds, such as chlorinated dibenzofurans and PCBs, are currently ubiquitous and persistent environmental contaminants, particularly in industrialized countries. The primary route of exposure to the general public is from food, especially animal fats found in meat, poultry, fish, and dairy products. These account for well over 95% of dioxins found in humans[15–23] (see Chapter 3). Municipal and toxic waste incinerators, PCB fires, phenoxyherbicides, fungicides such as chlorinated phenols, wood preservatives such as pentachlorophenol, and chlorinated bleaching of paper or pulp are some well-known sources of these primarily synthetic chemicals[24–27] (see Chapter 2). Environmental and human levels of dioxins before the production and use of chlorine-containing chemicals in industrialized countries were very low, as documented by studies of ancient (100- to 400-year-old) frozen U.S. Eskimo tissues from Point Barrow, Alaska, and of preindustrial lake sediments.[28–31]

Analytic techniques developed during the past few decades make it possible to measure polychlorinated dibenzo-*p*-dioxins (PCDDs) and dioxinlike PCBs down to the parts per trillion (ppt) level in human tissues. Worldwide, the World Health Organization (WHO) has certified approximately 40 dioxin laboratories for analysis of PCDD, PCDF, and dioxinlike PCBs in human blood or milk. The increased sensitivity and specificity of the newer analytic techniques, which utilize improved cleanup and extraction methods, followed by GC-MS, make it possible to measure the 7 dioxins, 10 dibenzofurans, and approximately 12 dioxinlike PCB congeners commonly found in most general population adults in industrialized countries. Higher levels of dioxin contamination tend to be found in industrialized countries as compared to less developed countries. These levels and patterns are sometimes characteristic of a given geographical region or special chemical exposure (see Section 16.4).

Some dioxins have shown remarkable persistence in human tissues. Elevated 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) body burden (amount of the chemical in a person's body reflected by tissue levels) from relatively high occupational or environmental exposure has been documented in human tissues up to 35 years after initial exposure.[32–35] In cases of lower exposures or those involving some of the less persistent dioxins, dibenzofurans or PCBs, human tissue levels often return to general population levels in a shorter time period, ranging from days to months to several years.[36–40] Patterns and levels are sometimes characteristic of a certain type of contamination, such as elevated TCDD levels following Agent Orange or 2,4,5-T phenoxyherbicide

exposure in Vietnam,[41,42] phenoxyherbicide exposure in Ufa, Russia,[35,43] PCB contaminated rice oil poisonings in Japan and Taiwan,[44–50] elevated higher chlorinated dibenzodioxins and dibenzofurans (PCDD/F) following chlorophenol agricultural exposure in China,[51,52] various occupational exposures in Germany,[53–55] and elevated levels of certain lower and also higher chlorinated PCDD/F congeners following municipal or toxic waste incinerator exposure.[56–58]

16.2 BACKGROUND

The first measurement of a dioxin, TCDD, in biological tissues was performed by Robert Baughman in the early 1970s.[59,60] Human milk and fish samples were collected in Vietnam by John D. Constable, a surgeon at Harvard Medical School, and colleagues, and the dioxin analyses were performed between 1970 and 1973 in the laboratory of Mathew Meselson, Baughman's Ph.D. advisor at Harvard University. Baughman measured levels of up to approximately 1850 ppt TCDD in the lipid fraction of human milk from nursing mothers in the south of Vietnam who had been exposed to Agent Orange, a mixture of phenoxyherbicides contaminated with TCDD. Fish from Vietnam were found with substantial elevations of TCDD, up to 850 ppt on a wet weight basis, a clear elevation above current U.S. levels of less than 1 ppt. Validation of the results was later done by Schecter and Ryan[61] when analyses of remaining archived deep frozen milk samples from the south of Vietnam, performed with more modern techniques, found TCDD levels for these samples very similar to those originally reported by Baughman. Levels of TCDD in American women's milk analyzed by Baughman at that time were below his limits of detection.

Later in the 1970s and 1980s, Rappe, Masuda, Schecter, Tiernan, and others documented elevated dioxin and dibenzofuran congeners in persons exposed occupationally and environmentally.[62–65] In the early 1980s, Gross and colleagues were the first to measure elevated TCDD in adipose tissue from U.S. Vietnam veterans who had been classified as "exposed" to Agent Orange by U.S. government investigators.[66]

In a project organized at the Binghamton Clinical Campus of the State University of New York Upstate Medical Center, a collaborating group of scientists first began measuring adipose tissue and blood levels in U.S. workers exposed to dioxins, dibenzofurans, and PCBs after a PCB transformer fire in Binghamton, New York, in 1981.[65,67–76] This project was the first to report the existence of dioxins and dibenzofurans in blood and adipose tissue in the general population. At that time, the general population with no special or known dioxin exposure was expected not to have any dioxin body burden. The extent of environmental and food dioxin contamination was yet to be discovered. Elevation of PCDD/F congeners found in soot from the fire was also found in some of the potentially exposed workers 2 years after the exposure.

A conceptual basis for estimating total toxicity of mixtures of PCDDs and PCDFs was developed by New York State Health Department scientists in order to establish reentry guidelines to the contaminated Binghamton State Office Building. Based on in vivo and in vitro studies as well as on theoretical considerations, toxic PCDD and PCDF congeners were assigned a relative toxicity factor or weighting compared to TCDD.[77] This weighting is known as a congener's dioxin *toxic equivalency factor* (TEF). The level of each congener measured was then multiplied by its specific TEF value, and the resulting values for each congener were then added. This final sum represents the total dioxin *toxic equivalents* (TEQs) for a given mixture of PCDDs and PCDFs.

TEFs represent an order-of-magnitude consensus value for each dioxin and dibenzofuran congener set by various government agencies, such as the states of New York and California, the U.S. Environmental Protection Agency (USEPA), the German government, or the World Health Organization (WHO).[78–82] These change over time as new data become available and meetings are held to set updated consensus TEF values. The TEFs currently range from 1.0 for TCDD (by definition) and 2,3,7,8-penta-CDD to 0.0001 for OCDD and also OCDF. Non-2,3,7,8-chlorine-substituted dioxins and dibenzofurans, rarely detected in human tissues, are given a weighting of zero. TEF values are generally used for human toxicity estimates. Different TEFs are used for fish and wildlife.[82] The measured congener pattern can also suggest the source of contamination if it resembles a known chemical pattern.

16.3 METHODOLOGY

Tissue such as fat, milk, or blood is first collected in chemically cleaned containers (free of dioxins, dibenzofurans, and PCBs) and then frozen. Specimens are kept frozen at -20 to $-80^{\circ}C$ until analyzed. Analytical methodology involving cleanup, capillary column separation, and subsequent GC-MS is described in Chapter 2. The majority of laboratories involved in the studies reported in this chapter have participated successfully in the World Health Organization (WHO) or similar interlaboratory validation studies for dioxins and related organochlorine compounds.[83–86] On occasion, alcohol or dichromate is used as a preservative rather than freezing the specimen; at the time of chapter preparation, these methods had not been validated.

16.4 SELECTED CASE STUDIES

16.4.1 Occupational Exposures

Binghamton State Office Building Fire, Binghamton, New York In 1981, a PCB and chlorinated benzene-containing transformer fire occurred at the State of New York Office Building in Binghamton, New York. Fire-

TABLE 16.1 PCDD and PCDF Levels and Dioxin Toxic Equivalents (TEQs) from Workers Involved in the PCB Transformer Incident, Binghamton, New York (Adipose Tissue, Wet Weight ppt)

	I-TEF	Control (Mean of 8)	Exposed Worker[a] 1	2	3	4
2,3,7,8-TCDD	1	7.2	13.3	28.3	16.2	11.6
1,2,3,7,8-penta-CDD	0.5	11.1	2.2	11.1	5.7	15
1,2,3,6,7,8-hexa-CDD	0.1	95.9	66.5	181	193	73
1,2,3,4,6,7,8-hepta-CDD	0.01	164	72	531	325	209
1,2,3,4,6,7,8,9-OCDD	0.001	707	166	946	948	690
2,3,4,7,8-penta-CDF	0.5	14.3	nd (3)	24.3	45.6	74.7
1,2,3,4/6,7,8-hexa-CDF	0.1	31.3	nd (3)	13.5	97	261
1,2,3,4,6,7,8-hepta-CDF	0.01	16.5	8.6	14.5	17.7	39.3
Total PCDD		985	320	1697	1488	999
Total PCDF		62	9	52	160	375
Total PCDD/F		1047	329	1749	1648	1374
Total PCDD TEQ		24.7	21.9	58.2	42.5	29.2
Total PCDF TEQ		10.4	1.7	13.6	32.7	63.8
Total PCDD/F TEQ		35.1	23.7	71.9	75.2	93

Source: Data from Ref. 72.

[a]nd, Not detected.

fighters and cleanup workers were exposed to a mixture of PCBs, PCDFs, and PCDDs. The first congener-specific dioxin and dibenzofuran measurements of exposed U.S. workers and the general population emerged from this incident (Table 16.1).[65,67–69,71] The mean measured level of the comparison samples, discarded fat tissue from eight adult surgical patients at local hospitals, was surprisingly high, approximately 1047 picograms per gram or parts per trillion (ppt), on a wet or whole weight basis. These initial findings were confirmed by further analyses of adipose tissue and blood from the Binghamton workers in later studies by Schecter and colleagues.[72]

Table 16.1 presents adipose tissue levels of dioxin and dibenzofurans and conversion to TEQ values from the early Binghamton studies. The TEQs were calculated using international toxicity equivalency factors (I-TEFs),[78,79] which were widely accepted and used by the USEPA and similar agencies in many European countries until recently replaced by WHO's new TEF values.[82]

Human tissue dioxin levels were first reported on a wet or whole weight basis in fat tissue. This seemed reasonable for the lipophilic dioxins. Later, when it became clear that even fat or adipose tissue varies in lipid content, the measurements were reported on a lipid basis to better reflect exposure and body burden. In our experience, blood usually ranges from 0.2 to 0.8% lipid, and human milk usually ranges from 2 to 4% lipid. Thus, reporting on a lipid basis normalizes dioxin levels and is a useful means of estimating body burden and exposure to dioxins.

Some, but not all, of the Binghamton workers showed elevated PCDD/F levels when sampled two years following exposure. Elevated 2,3,4,7,8-pentachlorodibenzofuran (penta-CDF) and hexachlorodibenzofuran (hexa-CDF) levels were particularly noticeable in certain exposed workers. Buser reported these dibenzofuran congeners as well as TCDD and others among those detected in some soot from the fire.[87] One of the former workers (exposed worker 1 of Table 16.1) had PCDD/F levels lower than those of the general population comparison group, including low levels for those congeners that were found in the soot. However, this worker showed somewhat elevated levels of TCDD. It is difficult to characterize this worker's intake from the incident without knowing his tissue levels prior to exposure. He may have had a relatively small intake, he may have eliminated the PCDD/Fs more rapidly than the other workers, and/or he may have had relatively low levels before exposure. This illustrates the importance of combining tissue measurement with exposure history, since this worker's potential exposure was similar to that of other workers in this study. Although GC-MS tissue measurement of dioxin congeners is now considered the gold standard for dioxin exposure assessment, limitations of using tissue measurements alone to determine exposure to dioxins and related chemicals were also discussed in a National Academy of Sciences report.[88]

Figure 16.1 shows serial hexa-CDF levels in one worker, a supervising engineer for the cleanup, from seven adipose tissue or whole blood samples, the first taken 2 years after exposure and the remainder taken during the following 6 years.[37,76] These congeners were among those found in the soot from the fire.[87] The decrease over time in this patient's tissue levels of hexa-CDF (shown in

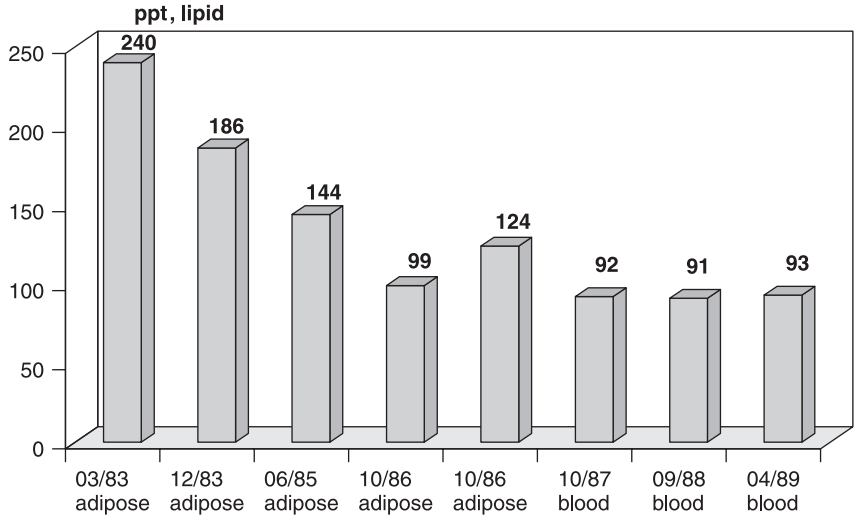

Figure 16.1 Serial levels of 1,2,3,4,7,8- and 1,2,3,6,7,8-hexa-CDF from a Binghamton State Office Building cleanup worker. (Data from Refs. 37 and 76.)

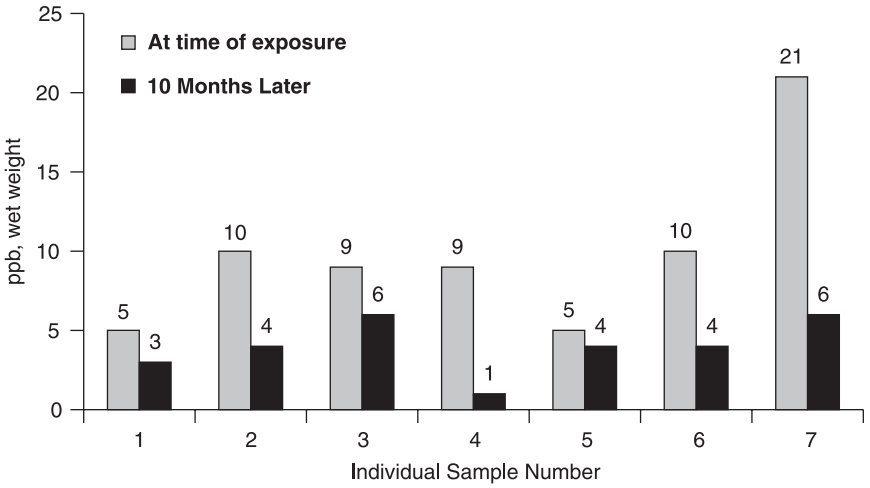

Figure 16.2 Repeated serum PCB levels in seven exposed firefighters, Binghamton, New York. PCBs reported as Arochlor 1254. (Data from Ref. 40.)

Figure 16.1) and other congeners illustrates a shorter half-life of elimination of these dibenzofurans, 2 months to 3 years, than for TCDD, 7 to 9 years.[89,90] Since dibenzofuran tissue levels return to baseline sooner than if the exposure were to TCDD, tissue samples should be collected as soon as possible after suspected PCDF exposure in order to document exposure. This is a reasonable approach for lower-level PCDD/F exposures as well as for exposures to some PCBs.

For the patients' and physicians' convenience and as analytic techniques improved, the use of fat tissue has generally been replaced by the use of blood. However, there is typically some difference in partitioning between blood and adipose tissue dioxin and dibenzofuran congeners, especially for the higher chlorinated congeners (some of the hexa, hepta, and octa congeners). Higher levels may be found in whole blood lipid than in fat or adipose lipid by a factor of up to twofold. For TCDD, the ratio between blood and adipose tissue is approximately 1.[91–93]

Figure 16.2 shows wet weight serum PCB levels in parts per billion (ppb) from seven firefighters who were involved in putting out the fire in the Binghamton State Office Building.[40] Blood samples were obtained in 1981 within days of the fire and were analyzed using a Webb–McCall packed column technique. Serum PCB levels were also obtained l0 months later. The use of serial values documented a decrease in PCB levels in the 10-month period for each of these seven firefighters, although some of their initial levels were within the usual range for serum then seen in New York State adults (5 to 10 ppb wet weight). This decrease was very rapid relative to the decrease in dibenzofurans for the worker shown in Figure 16.1. If serial measurements had not been uti-

lized or if blood had first been collected only at 10 months after the fire, it would have been difficult to determine intake of these chemicals from the incident from blood values alone. However, an occupational medical history would have provided convincing medical evidence of PCB exposure and almost certain intake of PCBs.

BASF Factory, Ludwigshafen, Germany A dioxin contamination incident at the BASF factory in Ludwigshafen, Germany, illustrates the persistence of TCDD in human tissue and the usefulness of tissue measurement to document high-level exposure long after initial exposure.[32] An uncontrolled reaction at the factory exposed workers to TCDD 32 years before their tissues were obtained for dioxin measurement. Exposed workers had elevated adipose tissue levels when tested more than three decades after exposure. Figure 16.3 shows elevated TCDD, ranging from 11 to 141 ppt in these six workers. German general population adult adipose tissue TCDD levels were approximately 3 to 8 ppt at that time, similar to U.S. levels. Many of these workers had chloracne, an easy-to-observe, although relatively insensitive, nonspecific, and rare biomarker of exposure to high levels of chlorinated organic chemicals. Some felt quite ill at the time of exposure; others become ill some time after exposure.

Approximate fat tissue TCDD levels at the time of exposure, calculated using a then-current 5-year half-life of elimination as our estimate,[94,95] ranged from approximately 917 to 11,750 ppt. Current estimates of average TCDD half-life of elimination are closer to 9 years.[96] These estimated levels are similar to measured TCDD blood lipid levels in exposed adults from the dioxin incident of 1976 in Seveso, Italy.[97,98] (See Chapter 20 for further discussion of the Seveso incident.) In Seveso, chloracne was frequently but not always seen

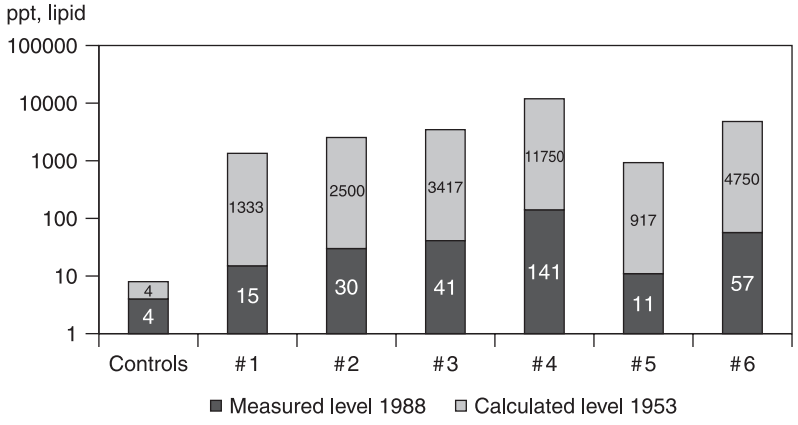

Figure 16.3 Adipose tissue levels of TCDD in six exposed workers and controls from Germany, measured 32 years after exposure. Estimated levels calculated for time of exposure with 5-year half-life assumed. (Data from Ref. 32.)

in exposed persons when blood levels were over 10,000 ppt and was usually absent below this level. The initial Seveso levels may not have adequately determined body burden because the blood was sampled soon after acute exposure rather than years later as in the Ludwigshafen incident, when more of an equilibrium among tissues would be expected.

German Pentachlorophenol-Exposed Workers Table 16.2 shows a characteristic congener pattern seen following pentachlorophenol (PCP) exposure. Samples were taken from 20 German workers at a PCP-producing plant, 5 years after last exposure. Higher chlorinated (five to eight chlorines) PCDDs and PCDFs were generally found at elevated levels. An exception is noted for octa-CDF, which was not elevated in the blood samples. PCP itself normally shows relatively high concentrations of octa-CDF. A relatively short biological half-life in humans may account for lower than expected levels of this congener. This pattern is different from that seen following other exposures with different dioxin-containing chemicals.[55] For example, TCDD alone is the major contaminant of the phenoxyherbicide 2,4,5-T, a major component of Agent Orange.[99]

Massachusetts and Michigan Vietnam Veteran Agent Orange Studies Over 19 million gallons of Agent Orange, a half-and-half mixture of the *n*-butyl esters of 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) herbicides, was sprayed by fixed winged aircraft in Vietnam in a defoliation project named Operation Ranch Hand between 1962 and 1971. Agent Orange was contaminated with TCDD during the production process. The average TCDD content of Agent Orange is believed to have been 2 to 3 ppm, although levels as high as 30 ppm were reported.[100] The name *Agent Orange* came from the orange identification band on the 55-gallon drums in which the herbicide was stored.

In the late 1980s, a Commonwealth of Massachusetts pilot study was conducted with the goal of determining whether U.S. troops, including ground troops, could be found with elevated levels of TCDD, the only dioxin contaminant of Agent Orange, several decades after their Vietnam service.[92,101–103] Elevated levels up to 55 ppt in blood lipids of TCDD were found in Massachusetts Vietnam conflict veterans. The general U.S. adult population adipose tissue and whole blood TCDD level at that time was between 5 and 10 ppt. The Massachusetts veteran study showed that decades after service in Vietnam, some U.S. Air Force Agent Orange sprayers and some Army ground troops still had elevated TCDD levels, which confirmed Agent Orange exposure, intake of TCDD, and the persistence of TCDD in humans.

In the subsequent Michigan Vietnam veteran Agent Orange study, blood levels of dioxins, dibenzofurans, and the dioxinlike PCBs, as well as semen levels of dioxins and dibenzofurans, were measured to better characterize total dioxin and dioxinlike chemical levels and toxicity.[104–107] Table 16.3 presents TCDD levels measured in whole blood lipid in 1991 and 1992, more than 20

TABLE 16.2 PCDD/PCDF Concentration in Blood of German Pentachlorophenol (PCP) Workers [$n = 20$, pg/g (ppt), Lipid Basis]

	PCP Workers					General Population[a]				
Congener	Min.	Max.	Mean	STD	Median	Min.	Max.	Mean	STD	Median
2,3,7,8-tetra-CDD	0.6	26	4.5	5.8	2.7	1	8.8	3.7	1.8	3.3
1,2,3,7,8-penta-CDD	5.5	119	28.3	27.8	19	2.8	20.8	8.3	3.7	7.7
1,2,3,4,7,8-hexa-CDD	7.3	391	47.9	87.4	14.5	3.6	19.4	10.2	4.6	9.0
1,2,3,6,7,8-hexa-CDD	23	1,087	241	295	111	7.5	99	35.5	17.6	30.7
1,2,3,7,8,9-hexa-CDD	7.7	754	110	182	32	1.8	15.8	5.9	2.5	5.6
1,2,3,4,6,8,9-hepta-CDD	78.8	22,407	2,514	5,091	666	16.7	159	56.7	30.9	48.6
Octa-CDD	2,807	284,224	33,192	62,621	11,999	123	1,267	462	225	418
2,3,7,8-tetra-CDF	0.6	6.3	2.6	1.7	2.2	1.2	3.8	2.1	0.7	2.1
1,2,3,7,8-penta-CDF	0.6	21	3.5	4.8	2.2	nd	2.5	0.4	0.7	nd
2,3,4,7,8-penta-CDF	7.7	151	48.6	36.8	39	6.8	48.2	18.8	10.5	16.3
1,2,3,4,7,8-hexa-CDF	9.6	399	69.1	101	23	4.4	24.5	10.9	4.9	9.7
1,2,3,6,7,8-hexa-CDF	7.7	383	63.7	98.8	21.5	3.1	20.7	7.8	4.1	7.0
1,2,3,7,8,9-hexa-CDF	0.6	1.6	1.2	0.3	1.1	nd	1.2	nd	0.2	nd
2,3,4,6,7,8-hexa-CDF	1.2	70	12.6	17.2	6.6	nd	9.9	2.9	2.3	2.4
1,2,3,4,6,7,8-hepta-CDF	19.0	1,369	238	345	96	nd	38.4	19.0	6.2	17.6
1,2,3,4,7,8,9-hepta-CDF	0.7	14	2.8	3.3	1.4	8.5	2.4	0.4	0.7	nd
Octa-CDF	1.5	24	8.7	6.9	6.4	nd	14.8	4.0	3.1	3.1
Total PCDD	3,057	309,008	36,137	68,180	12,948	182	1,627	582	260	547
Total PCDF	93.8	2,335	450	562	212	32.2	142	66.3	24.8	65.8
Total PCDD/PCDF	3,165	311,343	36,587	68,699	13,183	235	1,635	648	272	608
I-TEQ (NATO-CCMS)	31.2	947	94.7	210	83.1	12	61	26	11	24.1

Source: Data from Ref. 55.

[a]nd, Not detected.

TABLE 16.3 TCDD in Michigan Vietnam Veterans' Blood and Estimated Level at Time of Exposure

Veteran No.	Years Since Exposure	Measured Level, 1991–1992 (ppt)	TCDD at Time of Exposure (ppt) Assuming: 5-yr Half-Life	TCDD at Time of Exposure (ppt) Assuming: 10-yr Half-Life
1105	24	31	1133	219
1115	24	21.3	631	124
1107	24	131	5840	1112
0493	24	22.9	636	124
0500	23	54.5	1596	328
0111	24	20.4	645	127
Mean of 44 others	23–24	4.1		

Source: Data from Ref. 105.

years after exposure to Agent Orange in Vietnam, and estimated TCDD levels at time of exposure in the late 1960s for six individual and 44 other veterans from the Michigan study. Of the 50 veterans selected as having had possible Agent Orange exposure in Vietnam, six showed elevated TCDD, ranging from 20.4 to 131 ppt, while the remaining 44 had an average whole blood lipid level of approximately 4 ppt. Assuming a 5-year half-life of elimination, single-order kinetics, and a single-compartment model, estimated levels at time of exposure would have ranged from 631 to 5840 ppt, and assuming a 10-year half-life of elimination, from 124 ppt to 1112 ppt.[105] These estimated levels are similar to the 200 to 1850 ppt of TCDD measured in 1970 and 1973 samples of human milk lipid from Vietnamese women living in Agent Orange–sprayed areas at the time of exposure.[59,60,108] By way of comparison, 5 to 10 ppt of TCDD was then usually observed in fat, milk, or blood lipid from U.S. general population adults.[109–112] These Michigan data, shown in Table 16.4, omit the elevated TCDD levels shown in Table 16.3, and documented substantially higher total dioxin toxic equivalent (TEQ) levels from dioxins, dibenzofurans, and especially the dioxinlike (coplanar and mono-ortho) PCBs than was previously suspected in Americans.[106] This is shown in the Michigan veterans as well as in a comparison pooled blood sample from blood donors in Missouri. The large contribution of PCBs, PCDFs, and PCDDs other than TCDD to the TEQ levels in the general U.S. population became apparent from this and subsequent studies. The data also demonstrate that PCBs, especially mono-ortho PCBs, contribute more to the total TEQ than do dioxins and dibenzofurans. Because TCDD contaminates only Agent Orange, it is reasonable to conclude that all other congeners measured were probably similar to levels in the U.S. adult general population at that time.

TABLE 16.4 Mean Level of PCDDs, PCDFs, and PCBs in Individual Analyses from Michigan and Pooled Blood from Missouri (ppt, Lipid Basis)

Congener	I-TEF	Missouri[a]	I-TEQ	Michigan[b]	I-TEQ
1. 2,3,7,8-TCDD	1	3.4	3.4	3.8	3.8
2. 1,2,3,7,8-penta-CDD	0.5	7.1	3.4	9.3	4.6
3. 1,2,3,4,7,8-hexa-CDD	0.1	[c]	[c]	9.8	0.7
4. 1,2,3,6,7,8-hexa-CDD	0.1	67.5	6.8	72.1	7.2
5. 1,2,3,7,8,9-hexa-CDD	0.1	13.4	1.4	11.9	1.2
6. 1,2,3,4,5,6,7-hepta-CDD	0.001	155	1.6	119	1.2
7. Octa-CDD	0.001	1,208	1.2	794	0.8
8. 2,3,7,8-TCDF	0.1	3.2	0.3	2.3	0.2
9. 1,2,3,7,8-penta-CDF	0.05	nd (2.1)	0.06	1.2	0.06
10. 2,3,4,7,8-penta-CDF	0.5	7	3.5	8.8	4.4
11. 1,2,3,4,7,8-hexa-CDF	0.1	9.4	0.9	10.6	1.1
12. 1,2,3,6,7,8-hexa-CDF	0.1	6	0.6	6.9	0.6
13. 2,3,4,6,7,8-hexa-CDF	0.1	nd (5.7)	0.3	2.8	0.3
14. 1,2,3,7,8,9-hexa-CDF	0.1	nd (5.7)	0.3	2.8	0.3
15. 1,2,3,4,6,7,8-hepta-CDF	0.01	20.2	0.2	19.6	0.2
16. 1,2,3,4,7,8,9-hepta-CDF	0.01	nd (6.7)	0.3	3.1	0.03
17. Octa-CDF	0.001	nd (6.7)	0.01	9.3	0.01
18. 77 tri-PCB	0.01	34.2	0.3	78.6	0.8
19. 126 penta-PCB	0.1	49.8	5	104	10.4
20. 169 hexa-PCB	0.05	29.9	1.5	45.8	0.46
21. 28 tri-PCB	0.001	10,148	10.2	7,170	7
22. 74 2,4,4,5-tetra-PCB	0.001	7,602	7.6	14,330	14.3
23. 105 2,3,3,4,4-penta-PCB	0.001	3,200	3.2	6,928	6.9
24. 118 2,3,4,4,5-penta-PCB	0.001	11,346	11.4	16,213	16.2
25. 156 2,3,3,4,4,5-hexa-PCB	0.001	4,202	4.2	5,988	6
26. 99 2,2,4,4,5-penta-PCB	0.00002	5,328	0.1	11,361	0.2
27. 128 2,2,3,3,4,4-hexa-PCB	0.00002	1,200	0.02	2,104	0.04
28. 138 2,2,3,4,4,5-hexa-PCB	0.00002	14,784	0.3	26,297	0.5
29. 153 2,2,4,4,5,5-hexa-PCB	0.00002	23,666	0.5	4,005	0.8
30. 170 2,2,3,3,4,4,5-hepta-PCB	0.00002	4,260	0.09	6,620	0.1
31. 180 2,2,3,4,4,5,5-hepta-PCB	0.00002	12,728	0.4	19,034	0.4
32. 183 2,2,3,4,4,5,6-hepta-PCB	0.00002	1,402	0.03	2,534	0.05
33. 185 2,2,3,4,5,5,6-hepta-PCB	0.00002	852	0.02	1,284	0.03
34. 187 2,2,3,4,5,5,6-hepta-PCB	0.00002	3,588	0.07	7,378	0.2
Total PCDDs (1–7)		1,454	17.6	1,019	19.5
Total PCDFs (8–17)		64	6.5	67	7.2
Total coplanar PCBs (18–20)		114	6.8	229	13.5
Total mono-ortho PCBs (21–25)		36,498	36.5	50,629	50.4
Total di-ortho PCBs (26–34)		67,808	0	116,667	0
Grand total		105,938	67.4	168,611	90.6

Source: Data from Ref. 106.

[a] Pool $n = 6$; nd, not detected.

[b] TCDD is mean of 44 individual analyses, all other congeners are mean of 50.

[c] Level for 1,2,3,6,7,8-hexa-CDD is the sum of congeners 1,2,3,4,7,8-hexa-CDD and 1,2,3,6,7,8-hexa-CDD.

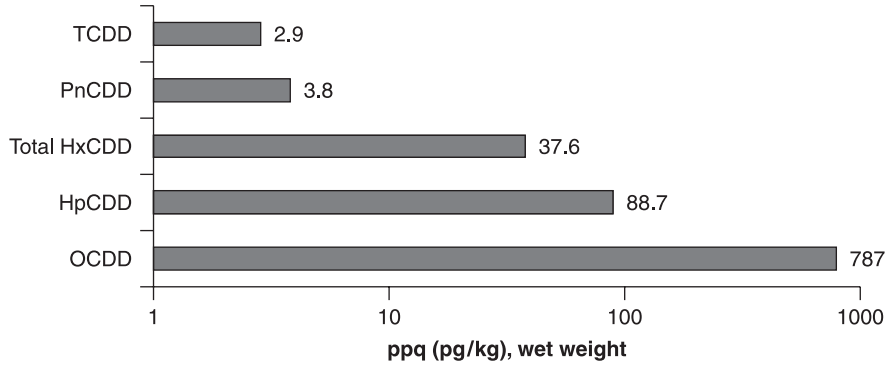

Figure 16.4 Mean dioxin levels in composite semen samples from 17 U.S. men. (Data from Ref. 106.)

PCDDs and PCDFs were identified in human semen samples from 17 veterans that were pooled in order to have sufficient sample amount for analysis.[105,106] Figure 16.4 (dioxins) and Figure 16.5 (dibenzofurans) present these semen PCDD and PCDF levels on a wet weight basis in parts per quadrillion (ppq). Lipid-adjusted data are not presented here because the low lipid content of the semen samples did not allow for accurate lipid measurements at that time. The pattern of the dioxins in semen resembles that seen in other tissues, with levels increasing as chlorination increases. However, the pattern of dibenzofurans does not appear to follow the usual pattern in human tissue (blood) because of the atypical large amount of OCDF present in this sample.

We have also reported dioxin semen wet weight levels in Vietnamese men.[113] The semen samples from southern Vietnam were collected in 1994 at Tan My village, Song Be Province, from 97 men of mean age 44.7 years. This village is in an area heavily sprayed by Agent Orange during the war. The lipid

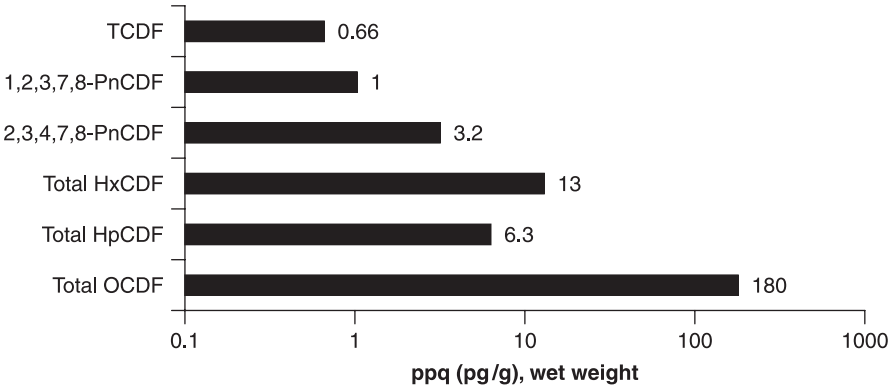

Figure 16.5 Mean dibenzofuran levels in composite semen samples from 17 U.S. men. (Data from Ref. 106.)

fraction was 0.12% for the Vietnamese, much lower than it is in blood, which usually ranges from 0.2 and 0.8%. As mentioned before, lipids were not determined for the Michigan Vietnam veterans. In this study we found that the men living in a southern Vietnamese village that had been sprayed with Agent Orange had semen levels of TCDD more than twice as high as the American veterans (7.6 vs. 2.8 ppq, wet basis). Levels of other PCDD congeners and of PCDF are higher for the Americans, consistent with exposure to PCDD/F incineration products and other environmental exposures. Total semen dioxin TEQ is nearly the same for Vietnamese and Americans in these samples, 13.6 versus 13.1 ppq wet weight. The finding of dioxins and dibenzofurans in semen is consistent with the hypothesis that male-mediated dioxin reproductive/developmental toxicity might involve dioxin transfer from semen to uterus, egg or zygote.[114]

It might be noted in passing that semen quality was altered after prenatal exposure to polychlorinated biphenyls and dibenzofurans in children of Yucheng women pregnant during and immediately following that poisoning in 1978–1979. (See Chapter 22 for further discussion of the Yucheng incident.) Sperm of individuals exposed as children had increased abnormal morphology, reduced motility, and reduced capacity to penetrate hamster oocytes. The authors concluded that further investigations are needed in respect to evaluating whether fecundity will be reduced in exposed men and how these effects can be extrapolated to the general population exposed to background levels of dioxinlike chemicals. Unfortunately, semen dioxins were not measured.[115]

Dioxin Exposure in Municipal Incinerator Workers Municipal incinerators, commonly used to dispose of household waste in industrial countries, have been found to generate dioxins and dibenzofurans during combustion.[116–118] For that reason, the question of hazards to workers and the general public has become an issue of concern. Because incinerator workers have much closer contact with incinerator ash, dioxins in the blood of incinerator workers from an older and presumably less environmentally safe New York City incineration facility were measured to determine whether bioavailability of dioxins from incinerator ash could be documented in humans.

Figure 16.6 and Table 16.5 summarize measurements of selected dioxin congeners in pooled whole blood from two municipal incinerator worker cohorts, one American and one German. The first was exposed to boiler ash containing PCDD/Fs at an older municipal incinerator located in New York City.[56] Dioxin levels in pooled blood were compared with control pooled blood of New York City residents, matched by gender and age. The elevated total dibenzofuran level is noticeable: 103 ppt in workers versus 47 ppt in controls. Total dioxins were also somewhat higher in workers than in controls: 904 versus 700 ppt, respectively. Congeners elevated in the worker's blood paralleled those found in the incinerator ash. These findings led to the implementation of more stringent worker protection measures at that incinerator.

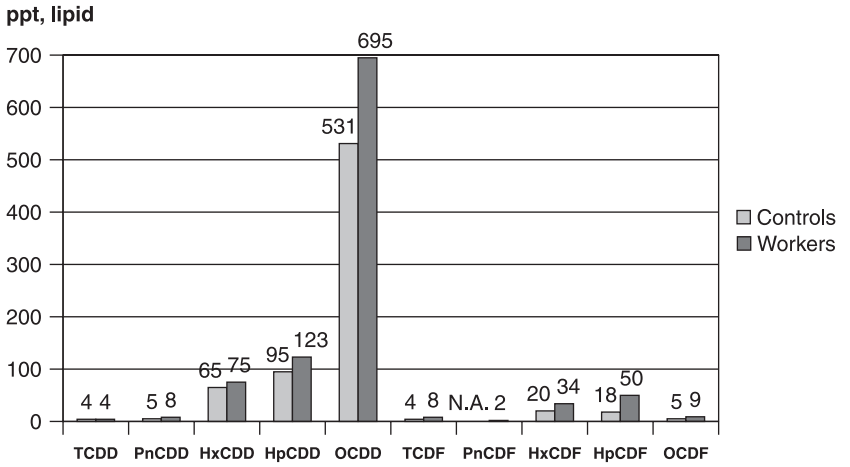

Figure 16.6 Measured dioxins and dibenzofurans in pooled blood of New York City municipal incinerator workers and matched controls (pg/g or ppt, lipid basis). N.A., due to external contamination PnCDF could not be measured. (Data from Ref. 56.)

TABLE 16.5 Selected Congeners and Total PCDD and PCDF Levels in Pooled Blood of U.S. and German Municipal Incinerator Workers and Matched Controls (ppt, Lipid Basis)

	U.S. Controls ($n = 14$)	U.S. Workers ($n = 56$)	German Controls ($n = 25$)	German Workers ($n = 10$)
2,3,7,8-TCDD	4	3.8	3.6	3.3
1,2,3,7,8-penta-CDD	5.2	7.7	15	11
Hexa-CDD[a]	65	75	94	86
1,2,3,4,6,7,8-hepta-CDD	95	123	95	111
Octa-CDD	531	695	637	1051
2,3,7,8-TCDF	3.5	8.1	2.6	2.7
Penta-CDF[b]	na	2	43	28
Hexa-CDF[c]	20	34	30	52
Hepta-CDF[d]	18	50	23	44
Octa-CDF	5	9	7	2.3
Total PCDDs	700	904	845	1262
Total PCDFs	47	103	106	129
Total PCDD/Fs[e]	747	1007	951	1391
Total I-TEQ	16.8	21.9	42.9	39.7

Source: Data from Refs. 56 and 57.

[a] Sum of 1,2,3,7,8-hexa-CDD, 1,2,3,6,7,8-hexa-CDD, and 1,2,3,7,8,9-hexa-CDD.

[b] Sum of 1,2,3,7,8-penta-CDF and 2,3,4,7,8-penta-CDF; na, not available.

[c] Sum of 1,2,3,7,8-hexa-CDF, 1,2,3,6,7,8-hexa-CDF, and 1,2,3,7,8,9-hexa-CDF.

[d] Sum of 1,2,3,4,6,7,8-hepta-CDF and 1,2,3,4,7,8,9-hepta-CDF.

[e] Sums of means for groups of congeners; Ref. 57 reports totals of individual congeners.

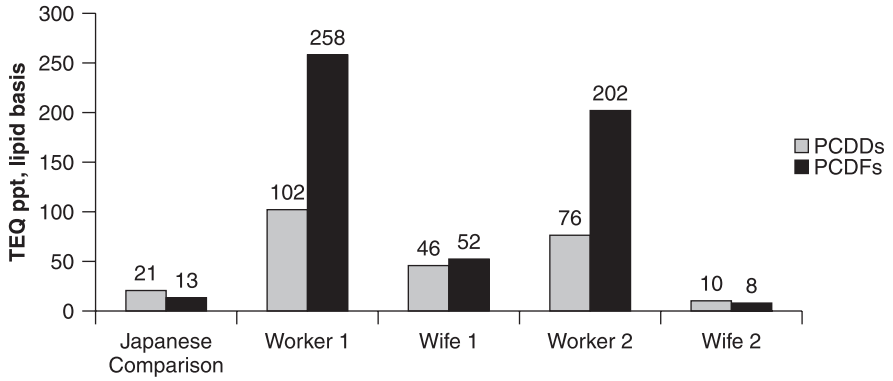

Figure 16.7 Dioxin and dibenzofuran WHO TEQ levels in blood of two Japanese incinerator workers and their wives, 1999, and in comparison Japanese pooled blood samples, 1991–1999 (TEQ ppt, lipid basis). (Data from Ref. 58.)

The fourth worker column of Table 16.5 represents mean dioxin levels from 10 individual blood samples from workers at an old, non-state-of-the-art German municipal waste incinerator. Their levels are compared with those of 25 matched controls. Statistically significant elevations ($p < 0.05$) are seen in the incinerator workers' blood for octa-CDD, hexa-CDF, hepta-CDF, total PCDD and total PCDF.[57,119] No statistically significant differences were found in dioxin blood levels in workers from a modern German municipal incinerator, where worker protection and incinerator technology had been improved (data not shown in Table 16.5).

A more recent Japanese incinerator study reveals somewhat different findings. The Nose incinerator in Japan was an older municipal solid waste incinerator that was shut down because of excessive dioxin and dibenzofuran emission. Dioxin (PCDD) and dibenzofuran (PCDF) TEQ values were compared for two workers at this facility, their wives, and the Japanese general population as shown in Figure 16.7.[58] WHO-TEQs of 21 and 13 ppt were found for PCDDs and PCDFs, respectively, in the general population. Worker 1 and his wife exhibited higher PCDD TEQ values than the general population as well as an even greater elevation of PCDF TEQ values compared to the general population levels. The wife is believed to have been exposed by cleaning her husband's contaminated clothing. Similar to worker 1, worker 2 also shows an elevation of his dioxin TEQ compared to the general population but exhibits an even greater elevation of dibenzofurans. His wife, wife 2, shows PCDD and PCDF TEQ values below the average background levels. No blood PCDD or PCDF TEQ values prior to potential exposure are available.

Secondary Exposure of Worker Spouses in Hamburg, Germany In Hamburg, Germany, certain chemical production workers exhibited elevated

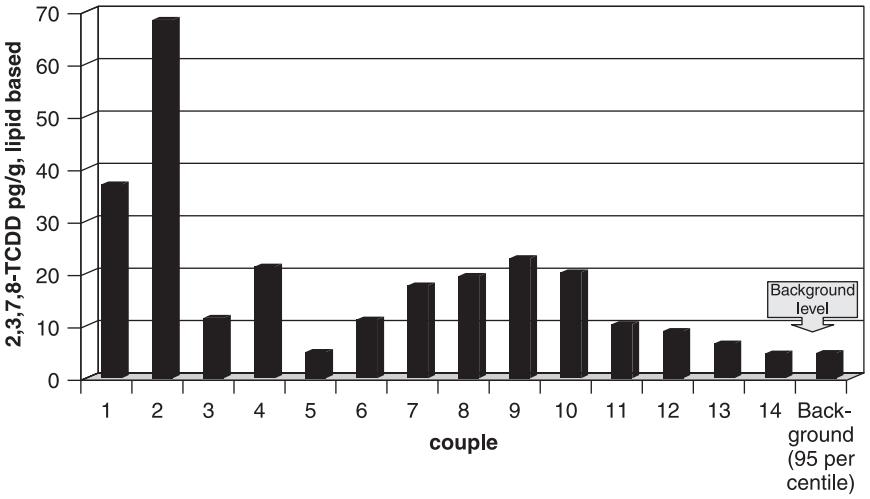

Figure 16.8 Elevated levels of TCDD in human blood in 14 spouses of German dioxin exposed workers. (Data from Ref. 120.)

dioxin levels. These people worked at a plant that produced herbicides until 1984. Interestingly, some spouses of randomly selected workers also exhibited elevated TCDD blood levels compared with levels in the general German population (Figure 16.8). The TCDD blood levels found in the female partners ranged from 4.7 to 68.4 pg/g, while the corresponding 95th percentile background value was determined to be 4.8 pg/g.[120]

Similar to the Japanese incinerator worker's spouse described previously, these data document transfer of PCDD/Fs from the occupationally exposed workers to their residential family contacts. This secondary exposure to dioxins could have occurred via clothing, skin, or other pathways. Similar findings of spousal contamination with PCBs have also been reported.[121]

Vienna TCDD Poisoning Incident Table 16.6 displays the whole blood levels of TCDD in two of five dioxin-poisoned Viennese workers, who were poisoned with dioxin in 1998. These two office workers exhibited the highest TCDD levels at this facility. Both women became sick, and worker 1 suffered from chloracne. Three other workers had elevated blood TCDD levels but were not followed as thoroughly as were the two women with the highest TCDD levels. Workers 3, 4, and 5 did not become clinically ill following exposure.

An astute dermatologist made the diagnosis of possible dioxin poisoning based on the chloracne and other skin and clinical reactions. This diagnosis was verified by blood dioxin levels. The first worker's measured TCDD blood level of 144,100 ppt on a lipid basis remains the highest dioxin measurement made in a human to date.[122,123] TCDD blood lipid levels in workers 3, 4, and 5 in August 1998 were 865, 149, and 93 ppt, respectively.

TABLE 16.6 TCDD in Human Tissue Samples, Vienna Incident

		Concentration (pg/g, lipid basis)	
Sampling Date	Day	Worker 1	Worker 2
4/27/1998	0	144,100	—
6/8/1998	42	—	26,000
6/29/1998	63	111,000	20,500
8/31/1998	126	80,900	16,100
9/21/1998	147	70,200	14,300
10/12/1998	168	89,900	18,500
11/16/1998	203	62,100	13,300
1/22/1999	270	68,300	19,300
3/23/1999	330	72,500	15,200
5/21/1999	389	73,900	15,700
9/9/1999	500	68,100	17,700
12/14/1999	596	47,100	14,100
3/27/1999	700	39,300	10,500
10/17/2000	904	30,300	10,100
3/16/2001	1,054	35,900	—
3/23/2001	1,061	—	9,500

Source: Data from Refs. 122 and 123.

Table 16.6 presents changes in TCDD blood lipid levels in workers 1 and 2 between April (June) 1998 and March 2001. Initially, TCDD blood levels decreased rapidly in workers 1 and 2, with a subsequent slower rate of reduction. Worker 1 was treated with Olestra®, first in potato chips and later in cookies, to increase fecal excretion following binding to this nonabsorbed fat substitute. Although fecal excretion of TCDD increased with this treatment, there was no convincing evidence of an increased rate of reduction of TCDD blood levels, which presumably reflect body burden. The source of the TCDD poisoning remains undiscovered, and this instance of chemical poisoning with dioxin received relatively little notice outside Vienna.

Chemical Workers from Ufa, Russia, and Their Children The Chimprom Manufacturing Complex in Ufa, Bashkortastan Republic, Russia, a large chemical company manufacturing many chlorinated compounds, produced 2,4,5-T and also 2,4-D from 1965 to 1967. As noted previously with respect to Agent Orange, 2,4,5-T is characteristically contaminated with TCDD. During that time, several hundred factory production workers were believed to have been exposed directly to the 2,4,5-T with its dioxin contamination. Many employees sought medical assistance at the time of exposure for evaluation of skin rashes and other medical problems; 137 employees out of approximately 231 involved in 2,4,5-T production were registered at the Occupational Medicine Institute in Ufa with chloracne. In 1991, blood from workers who had been employed at the plant during these years was collected and analyzed; their

TCDD blood lipid levels 25 years after exposure ranged from 36 to 291 ppt, whereas we had previously documented levels of 2 to 5 ppt of TCDD in blood in the Russian general population.[35]

Individual blood samples were collected from 65 residents of the city of Ufa, including the exposed workers and their children.[43,124] Table 16.7 presents mean selected dioxins and PCB levels of six 2,4-D workers, thirty-four 2,4,5-T workers, eight 2,4,5-T worker children, administrative factory workers, and the results of a pooled sample ($n = 100$) from residents of Ufa who had not worked in the factory and of other comparison groups. TCDD was elevated in chemical workers, workers' children, administrative workers, and some residents of the city of Ufa. TCDD was elevated more in 2,4,5-T workers than in 2,4-D workers but was elevated in both groups. Penta-CDD, hexa-CDD, 1,2,3,7,8-penta-CDF, and 2,3,4,6,7,8-hexa-CDF were elevated more in 2,4-D workers than in 2,4,5-T workers. 2,4,5-T characteristically contains elevated TCDD, and 2,4-D usually contains elevated higher chlorinated PCDD/Fs, hence the aforementioned pattern seen in blood. The workers all worked in one small building, so some were probably exposed to both 2,4-D and 2,4,5-T. Finally, the children of 2,4,5-T workers exhibited elevated tissue TCDD levels decades after their mother's exposure, yet these children had no other known special exposure to TCDD other than transplacental transfer in utero, intake from nursing, or possible exposure to contaminated clothing.

Pentachlorophenol Dioxin Exposure in China Certain rural areas in China have been sprayed with dioxin- and dibenzofuran-contaminated sodium pentachlorophenol (Na-PCP) to control the spread of snail-borne schistosomiasis. A sample of Na-PCP from China was shown to have a total dioxin and dibenzofuran content of just under 1000 parts per billion (ppb) and a TEQ value of 29.9 ppb.[51] The Na-PCP was contaminated with low levels of TCDD, 1,2,3,7,8-penta-CDD, 2,3,7,8-TCDF, penta-CDF, hexa-CDF, and hepta-CDF and higher levels of hexa-CDD, hepta-CDD, octa-CDD, and octa-CDF.

Age- and gender-matched pooled blood samples from sprayed and non-sprayed areas in a central mainland region were collected, as was a pooled sample from persons in direct contact with the pesticide. In each case, the comparison blood had a lower TEQ value than that of blood from exposed persons. The samples from those living in sprayed areas had a slightly higher TEQ value than the values from those who handled the pesticide directly, possibly from direct contact or from ingestion of contaminated food. The pattern of congeners in the exposed persons was consistent with intake of dioxins from PCP.[51]

PCDD/F levels in breast milk from exposed mothers were compared with those of control milk from the Chinese general population on a lipid basis in parts per trillion. In these samples, dioxin congeners, particularly TCDD, 1,2,3,7,8-penta-CDD, and octa-CDD, were noticeably higher in the exposed mothers, and the total PCDD/F TEQ value of the breast milk sample from sprayed areas was 5.4 versus 2.6 from the control group.[52] The low PCDD/F

TABLE 16.7 Selected Dioxins (PCDDs), Dibenzofurans (PCDFs), and Coplanar PCB Blood Levels and Total Dioxin Toxic Equivalents (TEQs) for Chemical Workers, Their Children, and the General Population from Ufa, Bashkortastan Republic, Russia (pg/g, Lipid Basis)[a]

		Ufa Chimprom Workers				General Population			
Congener	WHO-TEF	2,4-D Workers ($n = 6$)	2,4,5-T Workers ($n = 34$)	Children of 2,4,5-T Workers ($n = 8$)	Administrative Workers ($n = 5$)	Ufa 1992 ($n = 100$)	Russia 1990 ($n = 68$)	Ufa 1997 ($n = 44$)	Bashkorstan 1997 ($n = 264$)
PCDDs									
2,3,7,8-TCDD	1.0	68.9	166	39.5	31	12	4.4	18	4.8
1,2,3,7,8-penta-CDD	1.0	132	51.8	13	30.7	9.5	8.8	12.7	6.6
1,2,3(4/6)7,8(9)-hexa-CDD	0.1	181	44.4	11.4	27.3	7.5	10.6	18.8	13.1
PCDFs									
2,3,7,8-TCDF	0.1	—	—	—	—	nd (2.0)	2.3	—	—
1,2,3,7,8-penta-CDF	0.05	49.3	26.7	11	15.8	8	9.9	20.3	17.5
2,3,4,6,7,8-hexa-CDF	0.1	143	30.2	13.8	32.2	8.5	14.3	18.8	26.8
Coplanar PCBs									
3,3′,4,4′,5-PCB 126	0.1	105	160	77	158	65	—	—	—
3,3′,4,4′,5,5′-HCB 169	0.01	—	—	—	—	38	—	—	—
TEQ PCDDs		224	209	52.2	64.3	17.7	10.3	32.8	12.9
TEQ PCDFs		46	16.9	7	10.4	5	6.7	13.9	13.8
TEQ PCDD/Fs		257	230	57.7	74.7	22.7	17	46.7	26.7
TEQ coplanar PCBs		11.2	17.3	8.4	16.4	8.8	—	—	—
Total TEQ		274	253	66.6	86.6	31.5	—	—	—

Source: Data from Refs. 35, 43, and 124.

[a]nd, Not detected; —, Not analyzed.

and total TEQ values of all of these samples from mainland China are similar to levels previously reported in Chinese adipose tissue.[45] Since China is the world's most populous country, with over 1 billion inhabitants, the finding of relatively low dioxin and dibenzofuran tissue levels from China and other less developed countries studied, including Laos, Cambodia, and the north of Vietnam, suggests that at this time much of the earth's population has lower dioxin body tissue levels than those currently found in persons from industrial countries.

16.4.2 Environmental Exposures

Vietnamese Exposed to Agent Orange Studies of human exposure to Agent Orange contaminated with TCDD usually focus on U.S. or, more rarely, Australian or Korean veterans.[101–107,112,125–130] However, the persons with the highest and longest exposure to Agent Orange are Vietnamese living in the south of Vietnam. It was there that Agent Orange was sprayed over about 10% of South Vietnam between 1962 and 1971, with the heaviest spraying occurring between 1967 and 1970; this is reflected in elevated TCDD levels in Vietnamese living in these sprayed areas.[41,42,59,60,108,131–142] The U.S. military used Agent Orange and other herbicides during wartime primarily for defoliation of forests to prevent enemy troops from hiding as well as for crop destruction to interfere with enemy food supply.

TCDD tissue levels in Vietnamese have been shown to be low in the north and higher in certain central and southern regions, with some intraregional variations (Table 16.8).[137,142] In a more recent series of individual and pooled blood samples, TCDD varied from 1.2 ppt in whole blood from the nonsprayed city of Hanoi, the capital of Vietnam, which is located in the north, to 413 ppt from an individual blood sample from Bien Hoa City in the south, an Agent Orange–sprayed area (Figure 16.9).[41,42]

Our earlier Vietnamese samples were collected in 1970 and 1973, with later samples collected between 1984 and 1994. The majority of these later samples were collected in 1991–1992 and are presented in Table 16.8. Others are shown in Figure 16.10. Upon conversion to dioxin toxic equivalents,[78,79] we found total dioxin toxicity values in the pooled and individually analyzed samples ranging from 12 ppt in the nonsprayed north to 118 ppt in the sprayed central Vietnam area of Da Nang. These values resulted from all measured chlorinated dioxins and dibenzofurans, not from TCDD alone. TCDD contributed 19 ppt to the highest total TEQ value of 118 ppt from Da Nang blood, while TCDD contributed 28 ppt to a lower total TEQ value of 47 ppt from an early Bien Hoa sample. The higher levels of dioxins and dibenzofurans other than TCDD in central and southern Vietnamese reflect industrialization and possibly the use of chlorophenols in agriculture and wood preservatives.

TCDD was found to be slightly elevated in a pooled sample from veterans of the North Vietnam Army compared to the highest levels from the general population in the north, 6.1 ppt versus 2.9 ppt, respectively (Table 16.8). This is

TABLE 16.8 TCDD and Dioxin Toxic Equivalents in Pooled Blood from Vietnam, 1991–1992[a]

	Collection Date	Number	Mean Age	TCDD	TEQ	TCDD/ TEQ
Northern Vietnam (n = 168)						
Hanoi, Hospital 103	3/91	33	45	1.2	12	TCDD 1.2–2.9 (6.1)[b]
Tay Nguyen (veterans)[c]	11/91	35	48	6.1	40.3	TEQ 12–18 (40.3)[b]
Quang Binh, Dong Hoi	1/91	50	47	2.9	17.2	
Than Hoa	11/91	50	55	2.9	18	
Central Vietnam (n = 490)						
Thua Thien, Hue	1/91	30	57	11	57	TCDD 2.9–19.0
Quang Tri, Quang Tri	1/91	50	51	9.5	34	TEQ 23–118.2
Da Nang, Da Nang	2/91	49	59	18	77	
Thua Thien, A Luoi	1/91	35	52	15	23	
Khanh Hoa, Nha Trang	1/92	50	49	4.1	29.5	
Phu Yen, Phu Yen	1/92	43	51	6.2	26.4	
Ninh Thuan, Phan Rang	1/92	33	56	2.9	31.7	
Da Nang, Da Nang (18–40 y)	8/92	100	30	14	96.3	
Da Nang, Da Nang (> 40 y)	8/92	100	56	19	118	
Southern Vietnam (n = 2062)						
Dong Nai, Tri An (Ma Da Forest)	3/91	50	47	12	19	TCDD 1.0–33.0
Cuu Long, Vinh Long	8/91	51	59	4.3	16.9	TEQ 8.7–104.6
Dong Nai, Bien Hoa	3/91	50	51	28	47	
Ben Tre, Giong Trom	8/91	34	55	10.2	29	
Kien Giang, Go Quao	8/91	37	58	10.9	27.5	
Kien Giang, Rach Gia	8/91	48	58	4.9	17.3	
Minh Hai, Ca Mau	8/91	52	59	7.2	19.9	
Song Be, Song Be	3/91	47	47	9	48	
Song Be, Tan Uyen	3/91	48	54	32	55	
Tay Ninh, Tan Bien	2/91	50	60	5.3	25	
Tay Ninh, Tay Ninh	3/91	50	53	6.8	16	
Cuu Long, Tra Vinh	8/91	48	57	7.2	27.7	

TABLE 16.8 *(Continued)*

	Collection Date	Number	Mean Age	TCDD	TEQ	TCDD/ TEQ
Hau Giang, Can Tho	8/91	52	61	4.8	16.4	
An Giang, Long Xuyen	8/91	49	62	2.2	10.5	
An Giang, Chau Doc	8/91	46	56	3.5	16.8	
Ho Chi Minh, Cho Ray Hospital	2/91	48	54	10.8	30	
Minh Hai, Bac Lieu	8/91	50	60	10.3	34.8	
Gia Lai, Pleyku	1/91	50	57	4.2	34.2	
Tay Ninh, Chan Thanh	8/92	100	54	4.6	19.4	
Tra Noc, Can Tho	8/92	102	51	33	105	
Song Be, Tan Uyen (18–40 y)	8/92	100	32	9.4	25.4	
Song Be, Tan Uyen (> 40 y)	8/92	100	51	5.7	18.9	
Song Be, Ben Cat	8/92	100	32	12	49.8	
Dong Nai (18–40 y)	8/92	100	31	14	61	
Dong Nai (> 40 y)	8/92	100	53	19	53.7	
Tay Ninh, Hoa Thanh	8/92	100	50	1	38.8	
Song Be, Dong Xoai	8/92	100	50	3.1	8.7	
Tay Ninh, D. M. Chan	5/92	100	50	7	35.3	
Dong Nai, Bien Hoa (18–40 y)	5/92	100	27	7.3	22.8	
Dong Nai, Bien Hoa (> 40 y)	5/92	100	na	12	49	

Source: Data from Ref. 142.

[a] na, Not available; TCDD = 2,3,7,8-TCDD; TEQ = total dioxin toxic equivalent; lipid basis, parts per trillion.

[b] Figures for Tay Nguyen only, not included in range amounts.

[c] Although hospital is located in the north, these veterans were stationed in the south during the Agent Orange spraying.

consistent with some uptake of TCDD from Agent Orange by veterans serving for many years in the south, where Agent Orange was sprayed, and from living on or near a supply trail, the Ho Chi Minh Trail, which ran from the north to the south of Vietnam and which was heavily sprayed to discourage troop movement. However, not all of the elevated dioxins are from Agent Orange; total PCDD and PCDF levels in these former soldiers and in others were also elevated and contribute to higher total TEQ levels.

It should be noted that the highest Vietnamese tissue levels found between 1984 and 1994, 32 ppt of TCDD in blood (Table 16.8) and 103 ppt in adipose tissue,[108] were collected in 1991 and 1984, respectively, and presumably

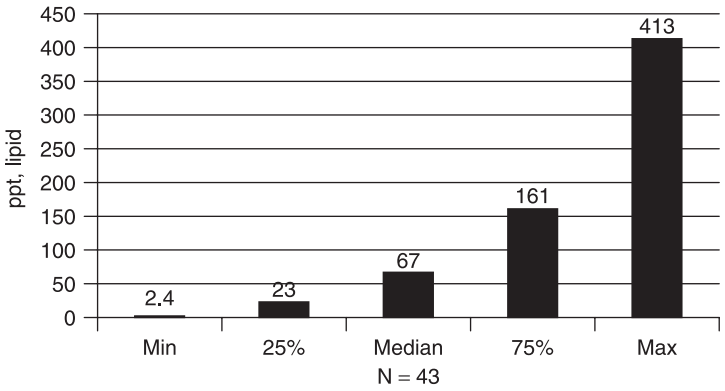

Figure 16.9 Summary of Bien Hoa City blood TCDD levels, ppt lipid basis, 1999–2001. (Data from Ref. 42.)

indicated prior Agent Orange exposure. Because of the scarcity of qualified dioxin laboratories, the large amount of blood (450 mL) or adipose tissue then needed for a dioxin analysis, and the high cost of analyses, pooled or combined blood or fat tissue samples from many persons were frequently used to provide average values for a given geographic region for many of the first 3000 Vietnamese we tested for dioxin.

The elevated tissue TCDD levels found over two and three decades after Agent Orange was initially sprayed demonstrate the persistence of TCDD in human tissue. Figure 16.10 illustrates breast milk levels of TCDD found during and after initial exposure. These samples were collected while Agent Orange was still being sprayed and are compared with both 1973 samples, collected 2 to 3 years after spraying ended, and with more recent samples.[59,60,108,138,142] The 1970 breast milk lipid-adjusted value of approximately 1832 ppt is the highest TCDD level measured in human tissue following Agent Orange exposure as well as the highest dioxin level reported in human breast milk to date. A decline in Vietnamese TCDD levels appeared to be occurring during the 1970s and the 1980s, presumably from a lack of continued exposure.

Recently, 95% of 43 individual blood samples collected in Bien Hoa City were found to have elevated TCDD (over 5 ppt) from Agent Orange exposure. Bien Hoa City is 35 km north of Ho Chi Minh City, formerly Saigon. Its airbase was used for Agent Orange storage and spraying, and a large leakage of Agent Orange into soil occurred there in 1970. Blood TCDD levels were elevated up to 206-fold (413 ppt) above background Hanoi levels of 2 ppt TCDD from samples collected in 105 persons between 1999 and 2001. Figure 16.9 depicts a summary distribution of 43 individual blood TCDD levels found in Bien Hoa City.

Elevated TCDD was found in parents and children in two families living in Bien Hoa City between 1999 and 2001 (Figure 16.11). A family of sustenance

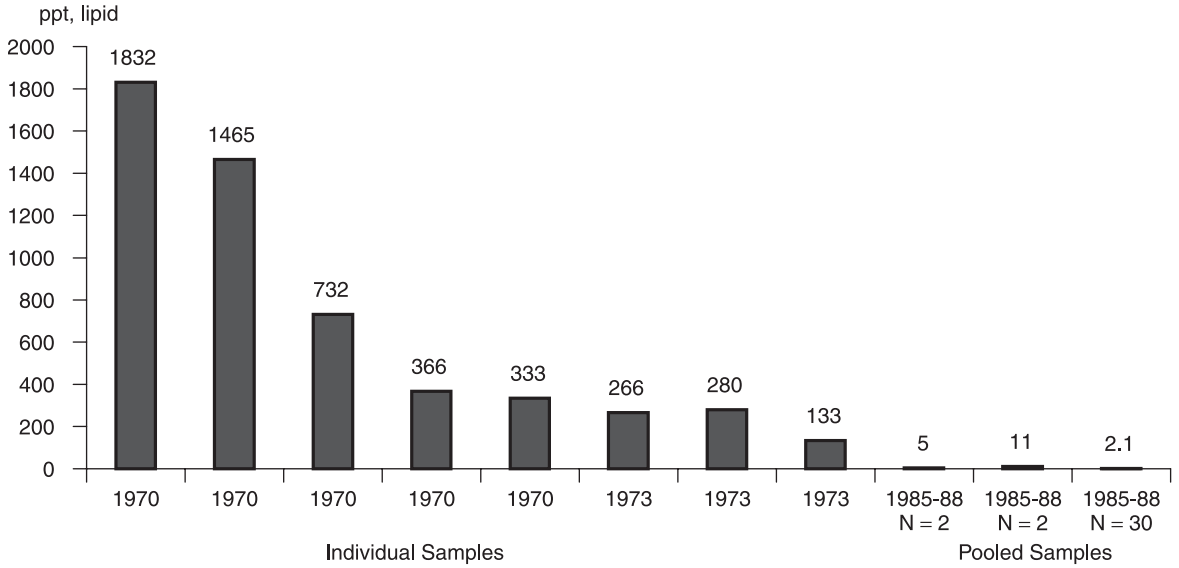

Figure 16.10 TCDD in human milk from the south of Vietnam, 1970–1988 (ppt, lipid basis). The 1970 and 1973 analyses were from individual samples and were calculated assuming 3% milk lipid. Collection sites (1970 and 1973): the villages of Tan Uyen, Can Gio, Quang Xuen, Dau Tieng, and Phu Cuong. (Data from Ref. 142.)

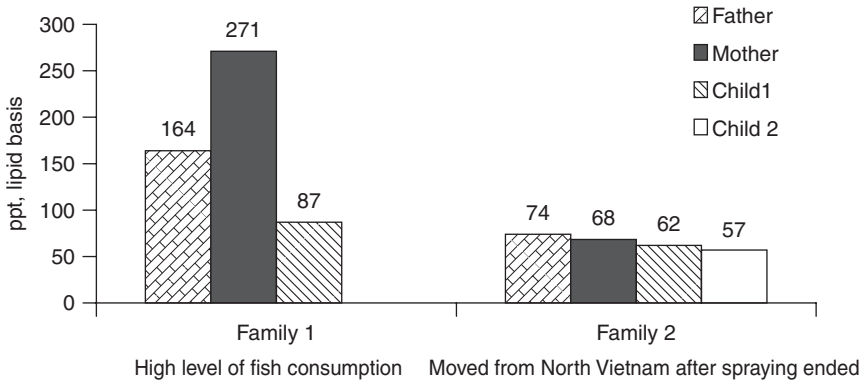

Figure 16.11 TCDD blood levels in Ben Hoa City, Vietnam, 1999–2001 (ppt, lipid basis). (Data from Ref. 41.)

fish eaters had TCDD blood levels of 87 to 271 ppt, lipid basis, and a family of new immigrants from the north had levels between 57 and 74 ppt.[41] Agent Orange was last used in Vietnam in 1971, yet both long-term and new residents of Bien Hoa City exhibited elevated blood TCDD levels. These findings suggests current as well as possible older exposure, presumably from fish and other animal foods, the usual route of intake of dioxins into humans. Elevated sediment TCDD levels in nearby streams and several very elevated soil TCDD levels at one location on the airbase (up to 1,100,000 ppt) support this belief.[41] Elevated dioxins, including elevated TCDD, had previously been documented in sediment samples from other regions in the south of Vietnam.[25,143]

Yusho and Yucheng Rice Oil Poisonings in Japan and Taiwan Rice oil contaminated with PCBs, PCDFs, and a small amount of PCDDs was used in cooking and ingested by over 1850 people in Japan in 1968 and over 2000 people in Taiwan in 1979 in remarkably similar incidents (Chapters 21 and 22). These exposures caused illnesses similar to those documented in animal studies of dioxins and PCBs.[144–147] The toxic effects were believed to be primarily from the dibenzofurans, especially the 2,3,7,8-substituted penta-, hexa-, and hepta-chlorinated congeners. PCBs are believed to have contributed a lesser amount of toxicity, and a small contribution is thought to have come from the dioxins. Elevated PCB and PCDF congeners were observed in human tissues for many years following exposure.

In a 1996 study of dioxin levels in human placentas from Yucheng patients in Taiwan, markedly elevated levels of dibenzofurans were found as compared with levels in U.S. placentas (Table 16.9). These high levels of PCDFs emphasize the possible role of intrauterine dioxin exposure on the developing fetus.[49]

TABLE 16.9 PCDDs and PCDFs in Taiwanese Yucheng Incident Placenta Samples Compared with 14 American General Population Placentas (ppt, Lipid Basis)[a]

	I-TEQ	American Samples	Taiwan Samples					
			1	2	3	4	5	6
2,3,7,8-TCDD	1	2.4	0.7	0.9	1.8	1.8	6.2	1.3
1,2,3,7,8-penta-CDD	0.5	4	4.8	8	23.9	12.8	36.4	14.9
1,2,3,4,7,8-hexa-CDD	0.1	2.4	1.3	—	—	—	—	—
1,2,3,6,7,8-hexa-CDD	0.1	15.9	19.3	40.2	411	194	395	200
1,2,3,7,8,9-hexa-CDD	0.1	3.2	2.7	2.7	13.9	22.8	73.6	19.2
1,2,3,4,6,7,8-hepta-CDD	0.01	36.2	17.4	14.8	46.2	47.8	63.4	75.3
Octa-CDD	0.001	282	194	121	596	945	815	923
2,3,7,8-TCDF	1	1.9	1.7	0.8	4.3	3.9	9.4	1.6
1,2,3,7,8-penta-CDF	0.5	3.6	1.7	1.2	10.5	5.7	0.4	5.7
2,3,4,7,8-penta-CDF	0.05	< 1.0	762	998	5,700	5,310	12,600	2,750
1,2,3,4,7,8-hexa-CDF	0.1	4	2,180	2,710	30,200	137,000	26,500	17,100
1,2,3,6,7,8-hexa-CDF	0.1	2	41.4	55.7	633	275	—	—
2,3,4,6,7,8-hexa-CDF	0.1	1.7	0.2	0.1	0.2	0.2	0.6	0.1
1,2,3,7,8,9-hexa-CDF	0.1	2	0.1	0.1	0.1	0.2	0.5	0.1
1,2,3,4,6,7,8-hepta-CDF	0.01	6.3	50	49.1	773	363	431	468
1,2,3,4,7,8,9-hepta-CDF	0.01	< 0.1	7.6	2.1	77.3	59.5	46.4	41.6
Octa-CDF	0.001	< 5.0	1.7	3.4	7.0	3.7	9.6	6.8
Total PCDD TEQ		7.2	5.8	9.5	57.2	31.3	72.7	32.3
Total PCDF TEQ		2.9	604	776	5,944	4,060	8,940	3,090
Total PCDD/Fs TEQ		10.1	610	786	6,011	4,091	9,013	3,122

Source: Data from Ref. 49.

[a]Totals are rounded. Half of "<" value used in totals. —, Peak was fused with other peak(s) on gas chromatography analysis. Taiwan samples obtained 5 to $5\frac{1}{2}$ years after exposure.

16.5 GENERAL POPULATION DIOXIN AND DIBENZOFURAN LEVELS

Dioxin human tissue levels and patterns vary in different geographical areas.[109,135–140,148–151] With some exceptions, in industrialized countries dioxin levels are considerably higher than dibenzofuran levels, and dioxin TEQ values are higher than dibenzofuran TEQs. General population background human tissue dioxin levels can be useful when evaluating a potentially exposed cohort. Based on current data, there is usually little geographic variation within a given country, with the exception of Vietnam, where dioxin levels are high in the south, reflecting Agent Orange and industrial contamination, and low in the north, reflecting their absence.

Somewhat different patterns and levels of dioxin, dibenzofuran, and PCB congeners are present in various tissues of the human body, even after adjusting on a lipid basis to normalize to each tissue's lipid content.[91,92,93,152] This can be more striking for the higher-chlorinated PCDD/Fs than for the lower-chlorinated congeners, as was noted previously for blood and adipose tissue. In estimation of actual exposure to and intake of PCDD/Fs, it is necessary to take into account exposure history, time after exposure, age, the tissue sampled, and wet weight versus lipid-adjusted values.

General population dioxin tissue levels have been shown to be considerably lower in certain less industrialized countries, including Thailand, Pakistan, Cambodia, China, India, Africa, the north of Vietnam, and parts of Russia.[136,138,139,141,148–151] More chemical use and contamination of the environment are the most likely cause of higher dioxins and dibenzofurans levels in human tissues from industrialized countries. European human tissue specimens at this time have a characteristic elevation of 2,3,4,7,8-penta-CDF, relative to U.S. and Canadian samples, possibly from the more common use of leaded gasoline in Europe. German blood generally has higher PCB content than does U.S. blood at the present time.[153,154,155]

16.5.1 PCBs in Human Tissue

The Michigan data mentioned previously, as well as data from Japan and Atlanta, Georgia, suggest that the relatively higher PCB human body burden and lower current levels of dioxins and dibenzofurans may have to be considered when characterizing total dioxin toxicity.[104–106,156–159] Because dioxins are now found in all humans, the term *nonexposed* or *unexposed* can no longer be considered an accurate designation of the general population. The important issue becomes the extent of the exposure and the toxicity of congeners.

Considering the large amount of PCBs currently found in human tissue relative to the smaller dioxin and dibenzofuran levels, the potential health effects of the dioxins could have been overestimated and that of PCBs underestimated relative to one another. However, because for many toxic endpoints the effects of PCDDs, PCDFs, and the dioxinlike PCBs are additive, the total dioxin and

dioxinlike toxicity may be more important to consider than that of dioxins and dibenzofurans alone. Recent work described in other chapters of this book suggests that the dioxinlike toxicity of some PCBs may be less than previously estimated, and also that some nondioxinlike PCBs may be more toxic than previously thought with respect to certain toxic endpoints.[160–162]

The most complete data we have obtained for human tissue levels of dioxinlike compounds, including dioxins, dibenzofurans, and the coplanar and mono-ortho dioxinlike PCBs, was from the 50 Michigan Vietnam veterans (see Section 16.3.1 and Table 16.4). These data also include a comparison pooled blood sample from a blood donor bank in Missouri ($n = 6$), with similar dioxin levels. The remaining six Vietnam veterans from the Michigan pool had elevated TCDD but no elevations of other congeners. The data, shown in Table 16.4, include the 2,3,7,8 chlorinated and, hence, toxic dioxins and dibenzofurans as well as the dioxinlike coplanar and mono-ortho PCBs. In addition, it lists the nondioxinlike di-ortho PCBs. At the time of analysis, it was believed that all were dioxinlike. The di-ortho PCBs are now believed to belong to the category of PCBs with toxicity unrelated to dioxinlike toxicity.

16.5.2 Trends over Time

Tables 16.10 and 16.11 report levels of PCDD/F congeners and dioxin toxic equivalents from U.S. and German blood and breast milk, respectively, over time. Table 16.12 provides a summary of PCDD/F TEQ levels in German blood over time from a series of published studies. Combined, they illustrate a decrease in total dioxins and dioxin toxicity (TEQ) over time in both countries. Substantial reductions are noted in U.S. values, and similar findings are seen in German studies.[153,155,163–175] This remarkable decrease in the persistent dioxins and dibenzofurans over a short period of time is usually attributed to the implementation and enforcement of strict government regulations that reduce emissions from incineration and other processes such as chlorine bleaching of paper and pulp. However, to some, this does not seem like a plausible explanation for the very rapid drop in human levels considering the long half-lives of elimination for many dioxins and dibenzofurans. The cause of this decline remains to be determined, but the decrease in human dioxin levels seen in many industrial countries despite differing sampling protocols seems real. There are no data for trends in amount of dioxin contamination of people in less industrial countries. The levels in developing countries may rise as they did during the twentieth century in industrial countries. Recent studies from Europe suggest that this rapid decrease in dioxin blood and milk levels may no longer be occurring.[171,172]

16.5.3 Special Populations: Vegans

Vegans are defined as persons consuming no foods of animal origin (i.e., no milk, cheese, eggs, butter, other dairy products, meat, poultry, or fish).

TABLE 16.10 Mean Dioxin, Dibenzofuran, and International Dioxin Toxic Equivalents (I-TEQs) Blood and Milk Levels in U.S. General Population Adults over Time (pg/g, Lipid Basis)[a]

		Blood								Breast Milk			
		1980s ($n = 28$)		1992 ($n = 44$)		1996 ($n = 100$)		2000 ($n = 200$)		1988 ($n = 43$)		2000 ($n = 50$)	
Congener	I-TEF	Measured	TEQ	Measured	TEQ	Measured	TEQ	Measured	TEQ	Measured	TEQ	Measured	TEQ
2,3,7,8-TCDD	1	3.5	3.5	3.8	3.8	4.3	4.3	2.6	2.6	3.3	3.3	1.1	1.2
1,2,3,7,8-penta-CDD	0.5	7.7	3.9	9.3	4.6	8.7	4.4	6.3	3.1	6.7	3.4	2.9	1.5
1,2,3,4,7,8-hexa-CDD	0.1	9.3	0.9	9.8	0.7	9.7	1	6.4	0.6	6.0	0.6	2.6	0.3
1,2,3,6,7,8-hexa-CDD	0.1	64	6.4	72.1	7.2	63.7	6.4	32.8	3.3	6.2	0.6	14.7	1.5
1,2,3,7,8,9-hexa-CDD	0.1	13	1.3	12	1.2	7.8	0.8	4.9	0.5	30.5	3.1	4.5	0.5
1,2,3,4,6,7,8-hepta-CDD	0.01	135	1.4	119	1.2	102	1	49.2	0.5	42	0.4	48.1	0.5
1,2,3,4,6,7,8,9-octa-CDD	0.001	1113	1.1	794	0.8	780	0.8	330	0.4	233	0.2	182	0.2
2,3,7,8-TCDF	0.1	nd	nd	2.3	0.2	nd (2.0)	0.1	nd (2.0)	0.1	2.9	0.3	0.8	0.08
1,2,3,7,8-penta-CDF	0.05	nd	nd	1.2	0.06	nd (1.4)	0.04	nd (1.0)	0.03	7.3	3.7	0.3	0.02
2,3,4,7,8-penta-CDF	0.5	9.2	4.6	8.8	4.4	11.1	5.6	4.5	2.2	0.5	0.03	2.6	1.3

1,2,3,4,7,8-hexa-CDF	0.1	13.3	1.3	10.6	1.1	14.1	1.4	5.9	0.6	5.6	0.6	1.9	0.2
1,2,3,6,7,8-hexa-CDF	0.1	7.4	0.7	6.9	0.6	7.9	0.8	3.5	0.4	3.2	0.3	1.3	0.1
2,3,4,6,7,8-hexa-CDF	0.1	2.0	0.2	2.8	0.3	nd (3.7)	0.2	nd (1.0)	0.05	1.9	0.2	0.7	0.07
1,2,3,7,8,9-hexa-CDF	0.1	nd	nd	2.8	0.3	3.5	0.4	1.5	0.2	nd	nd	0.1	0.01
1,2,3,4,6,7,8-hepta-CDF	0.01	27.0	0.3	19.6	0.2	12	0.1	6.7	0.07	4.1	0.04	3.1	0.03
1,2,3,4,7,8,9-hepta-CDF	0.01	nd	nd	3.1	0.03	nd (4)	0.02	nd (1.6)	0.01	4.1	0.04	0.2	nd
1,2,3,4,6,7,8,9-octa-CDF	0.001	nd	nd	9.3	0.01	nd (5)	0.003	nd (5)	0.003	4.1	0.002	0.4	0.002
Total PCDDs		1342	18.4	1016	15.7	977	18.6	537	11.1	328	11.6	255	5.5
Total PCDFs		58.9	7.1	67.4	7.1	52	8.6	25.4	3.5	33.7	5.1	11.2	1.8
Total PCDD/PCDFs		1401	25.5	1083	22.8	1029	27.2	562	14.6	362	16.7	266	7.3

Source: Data from Refs. 163, 164, and 165.

[a]nd, Not detected.

TABLE 16.11 Comparison of Mean Dioxin, Dibenzofuran, and International Dioxin Toxic Equivalents (I-TEQs) Blood and Milk Levels in General Population Adults from Germany over Time (pg/g, Lipid Basis)[a]

		Blood								Breast Milk					
		1989 ($n = 102$)		1994 ($n = 134$)		1996 ($n = 139$)		1998–1999 ($n = 85$)		1989 ($n = 185$)		1991 ($n = 111$)		1995 ($n = 38$)	
Congener	I-TEF	Measured	TEQ	Measured	TEQ	Measured	TEQ	Measured	TEQ	Measured	TEQ	Measured	TEQ	Measured	TEQ
2,3,7,8-TCDD	1	3.6	3.60	2.9	2.9	2.3	2.3	2.7	2.7	3	3	3.4	3.4	2.1	2.1
1,2,3,7,8-penta-CDD	0.5	13.8	6.90	6.3	3.2	5.9	3	4.9	2.5	9.3	4.7	8	4	5.7	2.9
1,2,3,4,7,8-hexa-CDD	0.1	10.9	1.09	6.9	0.7	5.7	0.6	4.9	0.5	7.8	0.8	7.8	0.8	4.8	0.5
1,2,3,6,7,8-hexa-CDD	0.1	54.6	5.46	26.7	2.7	22.6	2.3	19.5	2	32	3.2	29.3	2.9	21.8	2.18
1,2,3,7,8,9-hexa-CDD	0.1	10.6	1.06	4.9	0.5	3.8	0.4	3.6	0.4	6.3	0.6	4.6	0.5	2.9	0.3
1,2,3,4,6,7,8-hepta-CDD	0.01	92.4	0.92	45.3	0.5	33	0.3	38.7	0.4	46	0.5	35.8	0.4	21.9	0.2
1,2,3,4,6,7,8,9-octa-CDD	0.001	610.0	0.61	370	0.4	293	0.3	344	0.3	185	0.2	170	0.2	121.8	0.1
2,3,7,8-TCDF	0.1	2.3	0.23	1.9	0.2	1.2	0.1	0.9	0.09	2	0.2	0.8	0.08	0.6	0.06
1,2,3,7,8-penta-CDF	0.05	2	0.10	0.5	0.03	0.6	0.03	nd	nd	0.7	0.04	0.4	0.02	0.3	0.02

2,3,4,7,8-penta-CDF	0.5	37	18.50	12.8	6.4	10.9	5.5	10.5	5.3	24	12	19.2	9.6	13.5	6.8
1,2,3,4,7,8-hexa-CDF	0.1	15.4	1.54	7.9	0.8	6.3	0.6	6.2	0.6	7.3	0.7	7.5	0.8	4.9	0.5
1,2,3,6,7,8-hexa-CDF	0.1	13.3	1.33	5.8	0.6	4.7	0.5	4.3	0.4	5.9	0.6	5.4	0.5	3.7	0.4
2,3,4,6,7,8-hexa-CDF	0.1	4.3	0.43	2.6	0.26	nd	nd	nd	nd	3.2	0.3	2.6	0.3	1.7	0.2
1,2,3,7,8,9-hexa-CDF	0.1	1.7	0.17	nd	nd	2.4	0.2	2.3	0.2	nd (1.0)	0.05	nd	nd	nd	nd
1,2,3,4,6,7,8-hepta-CDF	0.01	23.4	0.23	11.4	0.1	8.1	0.08	7.8	0.08	5.2	0.05	4.4	0.04	2.8	0.03
1,2,3,4,7,8,9-hepta-CDF	0.01	1.5	0.02	0.6	0.01	0.9	0.01	nd	nd	nd (1.0)	0.05	nd	nd	nd	nd
1,2,3,4,6,7,8,9-octa-CDF	0.001	4.2	0.004	2.6	0.003	2.4	0.002	2.5	0.003	9.9	0.01	1.3	0.001	0.7	0.001
Total PCDDs		795.9	20.00	463	11	366	9.1	418	8.6	289	12.9	259	12	181	8
Total PCDFs		105.1	22.60	46.1	8.4	36.5	7	34.4	6.7	59.2	13.9	41.6	11.3	28.2	7.9
Total PCDD/PCDFs		901.0	42.60	509	19.4	403	16.1	452	15.3	349	26.9	301	23.3	209	16.9

Source: Data from Refs. 163, 164, and 165.

[a]nd, Not detected.

TABLE 16.12 Time Trend of PCDD/F Background Contamination in Human Blood, Germany

Year of Collection	*n*	I-TEQ Mean Values (pg/g, Lipid Basis)	Ref.
1988	10	45.8	166
1989–1990	102	40.8	55
1991	56	44.4	167
1991+	94[a]	42.7	168
1992	44	26.0	169
1993	70	21.7	153
1994	134	19.1	155
1996	180	16.5	170
1998	55	14.7	171, 172
1999	43	15.3	172

[a] Includes 56 persons from Kieselrot study.[167]

Figure 16.12 illustrates dioxin blood levels from two persons who had been vegans for over 35 years prior to sampling.[176] Compared to the U.S. general population, they were lower in dioxins, dibenzofurans, and coplanar PCBs. Limiting animal fat intake in this sort of dietary approach represents one way to decrease dioxin body burden voluntarily. However, environmental controls are considered the preferred way of decreasing these toxic chemicals in the environment, in humans and in wildlife and fish.

16.5.4 Partitioning of Dioxinlike Compounds

Partitioning studies comparing PCDD/F levels on a lipid or wet weight basis in different organs from the same person collected during autopsy show some

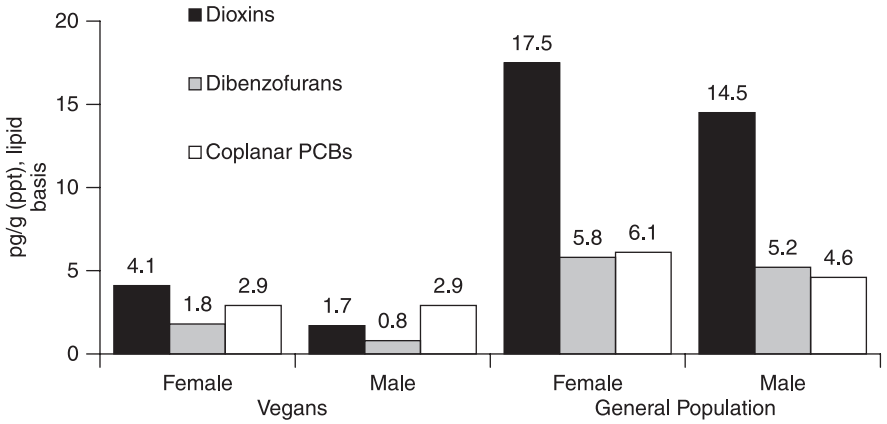

Figure 16.12 Blood I-TEQ levels of dioxins, dibenzofurans, and coplanar PCBs in strict vegans and the general U.S. population [pg/g (ppt) lipid basis]. (Data from Ref. 176.)

variations in congener levels. In general, adipose tissue and liver have higher levels than other tissues. Brain and kidney have much lower dioxin levels on a wet weight or lipid basis. This was shown in studies comparing PCDD/F and also PCB congeners in various organs in several autopsies.[152] In other partitioning studies comparing blood, milk, adipose tissue, and different cell fractions of blood,[92,93,177] the findings usually show almost 1 : 1 : 1 partitioning of TCDD in blood, milk, and adipose tissue lipid, but octa-CDD can vary by a twofold level in the tissues tested.

There are few published data concerning maternal and fetal tissue levels of polychlorinated dioxins, dibenzofurans, and coplanar PCBs prior to, during, and shortly after birth. Such information may be useful in understanding the metabolic changes that accompany pregnancy and the nursing state. In addition, better characterization of partitioning of these congeners may be useful in predicting levels in the fetus at birth from the levels of mother's blood prior to birth, in predicting levels in maternal milk from levels in blood before or after delivery, in extrapolating from milk to maternal blood levels, and in extrapolating from placental levels to maternal blood or cord blood levels. These predictions may be of some use to the clinician or patient if knowledge of any of these values is of concern.

To address some of these issues, we collected specimens from five New York women who ranged in age from 19 to 34 and who had undergone cesarean section deliveries between September 1995 and January 1996.[178] Maternal whole blood, placenta, maternal adipose tissue, and cord blood were collected during or shortly before delivery. Maternal milk and maternal blood were collected 13 to 63 days after delivery. As shown in Figure 16.13, the partitioning of dioxins, dibenzofurans, and coplanar PCBs is not passive in lipid. Thus, one cannot correctly extrapolate from blood or adipose tissue to body burden or to a specified target organ by making such an assumption. We found that for each woman, predelivery maternal blood total measure levels and TEQ values are higher than postpartum blood maternal blood levels (526 vs. 353 pg/g and 12.1 vs. 9.98 pg/g TEQ, respectively). The mean measured PCDD, PCDF, and coplanar PCB levels in placental tissue were lower than those of adipose tissue and whole blood. Also, measured cord blood levels and TEQ values were typically the lowest of the tissues studied. It should be noted that the difference in dioxin levels in tissues is greater on a measured basis than on a TEQ basis. It is possible that these values could be used in clinical medicine for predicting levels in milk from maternal blood or placental tissue.

16.6 CONCLUSIONS AND DISCUSSION

For the most part, dioxins, dibenzofurans, and PCBs are historically new synthetic chemicals.[25–31] Because of the lack of information concerning kinetics in humans and concerning the health effects of dioxins, dibenzofurans, and PCBs alone or in combination with similar and dissimilar chemicals, we are at an early learning stage in understanding how to use human dioxin measurements

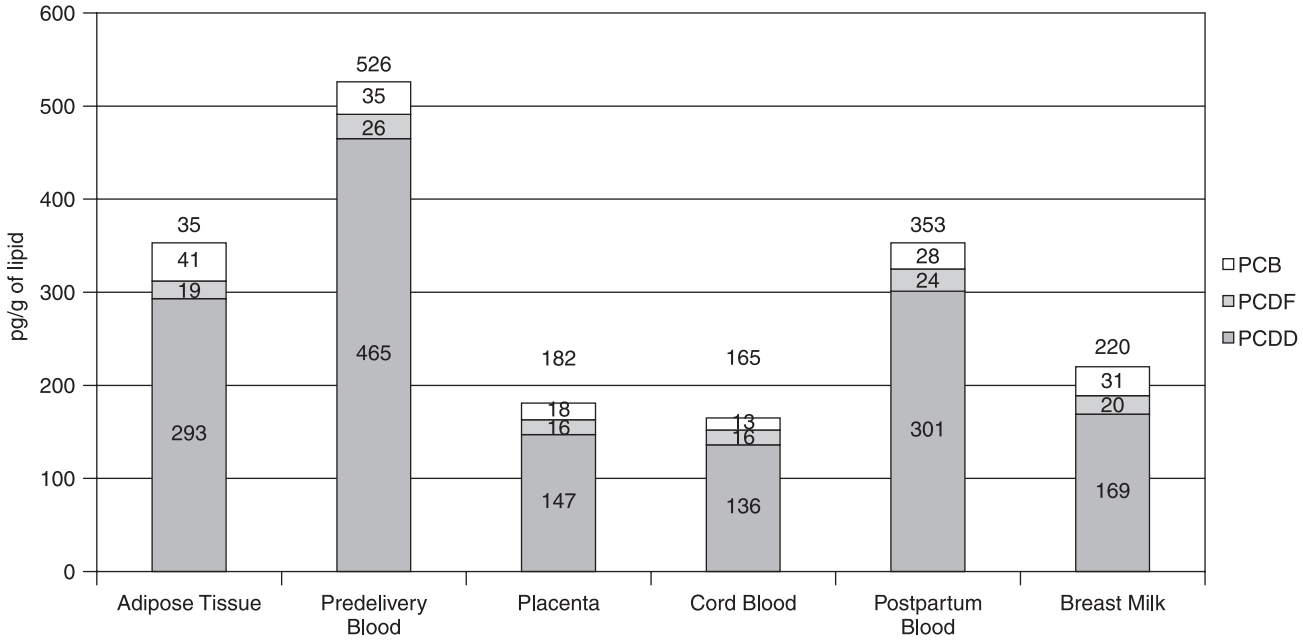

Figure 16.13 Mean measured PCDD, PCDF, and coplanar PCB levels in U.S. maternal and fetal tissues ($n = 5$; ppt, lipid basis). (Data from Ref. 178.)

in health evaluation. Metabolic, kinetic, clinical, and epidemiological studies, relying, as they must, on nondeliberate exposures of these toxic chemicals in humans, add to the body of knowledge essential for better use of these sensitive and specific biomarkers of exposure.

Compared with the extremely crude and nonspecific biomarkers available to estimate dioxin exposure several decades ago, such as chloracne, chemists, physicians, toxicologists, and epidemiologists have made considerable progress working together in this multidisciplinary field to relate dioxin exposure to human health. Hopefully, through continued multidisciplinary scientific collaborations on exposure assessment and health effects of dioxins, it will be possible to move more rapidly toward an understanding of associations between the levels of dioxins and related chemicals and adverse health consequences in humans. It is clear that these new tools for the exposure assessment of dioxins and related chemicals provide an advance over what is usual in the practice of occupational medicine with respect to most chemical exposures today.

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CHAPTER 17

Human Health Effects of Polychlorinated Biphenyls

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17.1 INTRODUCTION

In this chapter we review data on polychlorinated biphenyl (PCB) exposure in relation to health effects in humans. After the background material and summary of methodologic considerations affecting data interpretation, the order of the sections is as follows: reproductive outcomes, birth weight and growth, thyroid axis, neurodevelopment, immune system, breast cancer, other cancers, occupational exposures, and outcomes not covered elsewhere.

The health effects reported in occupational studies are considered apart from those from background-level PCB exposure, by which we mean exposure experienced by the general public, resulting primarily from normal diet,[1] and not from unusual circumstances of occupation, accident, or local contamination. We consider occupational effects separately because the level of exposure and nature of the exposure are markedly different from those in background-exposed populations.

Apart from the occupational studies, in this review we focus on results from studies where PCB levels have been measured. We do this because of several problems associated with use of fish intake as a surrogate measure of PCB exposure. A major problem is that fish intake may correlate poorly with measured PCB levels.[2,3] A second problem is that fish intake can be a better proxy

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measure of exposure to other toxic environmental contaminants than it is for PCBs.[4] Thus, use of fish intake as a surrogate measure of PCB exposure may be misleading. Limited data on health effects due to local contamination are available and are discussed in the section on outcomes not covered elsewhere. The Asian mass poisonings, discussed separately in this volume (see Chapters 21 and 22), reflect PCB exposure concomitant with exposure to PCDFs and related compounds.

The material covered in this review was intended to be comprehensive as of March 2000. We included material published in the peer-reviewed literature, with few exceptions. Throughout this review we often use the term *exposure* as being synonymous with the concentration of PCB measured in a human tissue, such as blood.

17.2 BACKGROUND

PCBs were first manufactured commercially in 1929 and have been used for a variety of applications, including use as dielectrics in transformers and capacitors and as cooling fluids in hydraulic systems. PCBs were also put in lubricating and cutting oils, pesticides, and flame retardants and used as plasticizers in paints, copying paper, adhesives, sealants, and plastics. Their resistance to chemical and biological breakdown contributed to their widespread commercial use.[5] The manufacture of PCBs was banned in the United States in 1977.

There are 209 different polychlorinated biphenyls; each specific PCB compound, or congener, is defined by the location of the chlorines on the phenyl moieties. The most bioaccumulating PCBs have five to seven chlorine atoms per molecule. These moderately chlorinated isomer groups (penta-, hexa-, and heptachlorobiphenyls) account for 112 of the 209 congeners. PCB congeners with five to seven chlorines were synthesized in high proportions in many commercial preparations and are the congeners found in the highest concentrations in background-exposed people.[6] The more highly chlorinated congeners are generally less available to organisms both because they bind more tightly with soils and sediments and because they are present in lower quantities in the environment. Congeners with fewer chlorines are more readily metabolized and eliminated and tend to bioaccumulate less.[7]

Evidence from laboratory studies demonstrates that PCBs have many biological effects.[8] Immunotoxicity,[9,10] tumor promotion,[11,12] interference with thyroid hormone metabolism,[13] and neurotoxicity[14] are among the more notable effects. PCB congeners that have no chlorines to interfere with both aromatic rings lying in the same plane (i.e., planar congeners) can bind with the aryl hydrocarbon (Ah) receptor (the biologic significance of Ah receptor binding is discussed in Chapter 12); PCBs with one chlorine in the ortho position also bind with the Ah receptor, but to a lesser extent than do most planar congeners. Not all PCB toxicity is due to Ah receptor binding, however.[15] Regardless of the precise mechanism, some PCB congeners induce cytochrome

P450 enzymes,[11,16] which in turn can be associated with estrogenic[17] or antiestrogenic effects.[18]

17.3 METHODOLOGIC CONSIDERATIONS

In observational epidemiologic studies, apart from the usual liabilities to bias, data on PCB effects raise their own special set of concerns because of the complexity of exposure, in background-exposed subjects because of the difficulties in measurement posed by the low levels present, and because exposure levels are usually characterized with a biomarker. Within subjects in a background-exposed population, levels of PCBs are typically correlated with those of other persistent halogenated organic pollutants. Thus, some PCB–health associations observed may be due, in part or wholly, to actions of other, correlated substances. An example is the correlation of levels of PCBs with those of polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and total toxic equivalents (TEQs), of which about half are attributable to PCBs. The correlation between total PCBs and TEQs (from PCBs, PCDDs, and PCDFs) was 0.77 in one study[19] and 0.87 in another.[20] The correlation between total PCBs and total hydroxlyated PCBs in another study was 0.84,[21] and hydroxlylated PCBs may be more toxic than PCBs in some respects.[22]

In nearly all human studies of PCB health effects, level of exposure to total PCBs was examined. As noted above, however, PCB congeners vary with respect to their toxic effects. Thus, expressing exposure as total PCBs rather than the level of a specific congener or group of congeners with similar effects,[7,23,24] may result in imprecision and decreased power to detect effects.

Several laboratories supporting epidemiologic studies have begun to quantitate large numbers of PCB congeners.[25] Deciding how to calculate total PCB level is a particular challenge in such studies because, for many congeners, a large proportion of subjects have a level below the limit of detection. The method of assigning concentration to these low values may affect the accuracy of classifying subjects. The degree of imprecision incurred by different approaches has not been evaluated systematically. Laboratories quantitating fewer congeners do not necessarily have less of a problem in this regard—they simply assume that levels of measured congeners are informative about levels of unmeasured congeners, which also leads to imprecision.

Although the exposure levels in background-exposed populations may be comparable in many instances, the specific PCB congeners present in a given subject or a given population can differ. We note, however, that the proportional contribution of the major PCB congeners to the total PCB level is generally similar within and across studies of background-exposed subjects.[6,26,27]

In evaluating the consistency of results across studies, an underlying assumption is that the range and nature of PCB exposure were comparable. Whether the exposures were, in fact, comparable across studies was often diffi-

cult or impossible to evaluate for many reasons; for example, different analytical methods yield different estimates of the amount of PCBs present, the number of congeners quantitated varies across studies, and the tissue in which PCBs were measured varies.

An evaluation of the comparability of exposure level in a large group of studies of PCB-associated health findings in background-exposed populations[28] revealed that the highest exposures were in the Faroe Islands,[29] Greenland,[30] and selected Swedish populations,[31] where consumption of contaminated marine foods was relatively high. In addition, subjects in Washington County, Maryland[32] had unusually high exposure levels. In addition, exposure levels among the background-exposed in more recent studies have tended to be lower than in earlier studies.[33] Furthermore, PCB levels have been shown consistently to increase with the age of subjects,[34] probably reflecting both the slightly higher exposure levels in the past and the greater length of time for persistent compounds to bioaccumulate. In a recent study among German children,[35] for example, exposure levels were quite low. Apart from the exceptions just noted and a few others for which there is less certainty about the levels reported, the range of exposure in background-exposed humans may, in fact, be fairly comparable across the studies reviewed, with a median total PCB level among adults equivalent to 2 to 5 μg/L serum on a wet weight basis, as measured by modern congener-specific methods.

In epidemiologic studies of PCB health effects, an assumption generally made is that the concentration of PCBs is in equilibrium across body compartments (tissues). Substantial evidence exists, for example, for high correlation of blood and adipose tissue levels.[36,37] Nonetheless, recent data call this assumption into question.[26] Measurement of levels, for example, in blood may lead to imprecision of true long-term exposure if adipose levels are, in fact, more relevant to risk. The tissue whose levels best reflect relevant exposure, however, is usually not known. Because PCB levels in blood are proportional to blood lipid levels,[38,39] taking blood lipids into account in classifying relative exposure is considered good practice.

As with any biomarker, occurrence and severity of outcome can affect the measure under consideration. In prospective studies this is usually not an issue, but in case–control and cross-sectional studies, especially of outcomes with metabolic consequences, an effect of disease on the level of PCBs may bias results.[40]

In human studies of PCB health effects, exposure is usually measured once, on the assumption that levels are consistent enough over time that additional measures add little. Data available among background-exposed populations support this belief.[41,42] These observations are in keeping with the half-life of the main PCB congeners found in humans, which ranges from months to years.[43]

To summarize the challenges posed in evaluating PCB exposure in relation to human disease, there are two main concerns. First, despite sophisticated laboratory measurements, exposure may be imprecisely characterized, thereby

decreasing the power to detect effects. Second, any effects observed may be due in part to correlated substances.

The latter point is an example of confounding. Experiments with PCBs cannot be done in humans; thus, as noted above, all available data are observational and therefore liable to confounding and other biases.

17.4 REPRODUCTIVE OUTCOMES

Data on reproductive outcomes in relation to background-level PCB exposure have been presented in three studies. In a small case–control study of preterm delivery in Israel, a threefold higher mean PCB level was found in cases ($n = 17$) than in controls ($n = 10$).[44] In a small case–control study in New York City ($n = 20$ cases, $n = 20$ controls), preterm birth was not associated with serum PCB levels.[45] In a larger case–control study of miscarriage in Italy,[46] samples from subjects were combined before analysis (10 subjects' material/group; 12 groups of cases, 12 groups of controls), thus raising questions about the appropriate methods needed to evaluate the statistical precision of the results and to adjust for potentially confounding factors. At any event, the mean PCB level among the case pools was 26% higher than among control pools. In a study of subjects from Triana (see Section 17.12), PCB levels were not associated with a history of miscarriage, stillbirth, or infant death, but no specifics were presented.[47] By virtue of modest size,[44,45] low exposure level,[45] or methods,[46] none of these studies was especially informative.

Great Lakes fish consumption has been examined in relation to spontaneous fetal death,[48] conception delay,[49,50] and menstrual cycle length[51]; in these studies an association was detected between greater fish consumption and shorter menstrual cycle length.

In a study of men with background exposure, PCB levels in seminal fluid were unrelated to sperm count or motility.[52] In a subgroup of infertile subjects in that study, however, sperm motility was inversely related to PCB level, but whether the reduction in motility was due to PCBs was unclear.

17.5 BIRTH WEIGHT AND GROWTH

Epidemiologic data on PCB levels in relation to birth weight are summarized in Table 17.1. The PCB levels were probably highest in the Greenland[30] and Swedish[58] studies. The exposure levels in the remaining studies were probably in the range 2.7 to 4.6 μg/L serum, roughly 40% lower. Yet neither the Greenland nor the Swedish study provided unequivocal evidence of an association. In the largest study with data on PCBs and birth weight,[59] the authors analyzed their data to determine whether the child's birth weight was a predictor of the concentration of PCBs in the mother's milk, conditional on other determinants of milk PCB levels. Birth weight was not a statistically significant predictor of

TABLE 17.1 Dietary Exposure Studies: Relation of Measured PCB Levels to Birth Weight

Study	Location	Number of Subjects	Association[a]	Comments
Fein et al. (1984)[53]	Michigan	241	↓	Higher PCB levels were associated with decreased gestational age and decreased head circumference.
Smith et al. (1984)[54]	Wisconsin	72	↑	
Rogan et al. (1986)[55]	North Carolina	912	None	
Vartiainen et al. (1998)[56]	Finland	167	None	Birth weight decreased for some PCDDs.
Patandin et al. (1998)[57]	The Netherlands	179[b]	↓	
Rylander et al. (1998)[58]	Sweden	192	↓±	
Schade et al. (1998)[59]	Germany	1553	None	
Bjerregaard et al. (2000)[30]	Greenland	180	None	

[a] A down arrow means that increased PCB levels were associated with decreased birth weight and that the association was statistically significant. A ± next to an arrow indicates that the association was not statistically significant.

[b] Number of subjects shown is from the cord blood analysis.

PCB levels, suggesting that had they examined predictors of birth weight, PCBs would not have been important. The next-largest study[55] also found no association. In comparison, the remaining studies were all much smaller. In two studies,[53,57] PCB levels were inversely associated with birth weight. In Wisconsin,[54] however, the opposite was found. Overall, most of the data are consistent with no association of birth weight with PCB exposure within the range considered.

In the Rotterdam group of the Dutch birth cohort,[57] the relation of PCB exposure to growth through 42 months was examined separately, according to whether the children were formula-fed or breast-fed. Among the formula-fed infants, higher PCB levels in maternal and cord plasma were associated with slower growth between birth and 3 months of age, but differences in growth from 3 to 42 months were not discernible. Among the breast-fed infants, prenatal exposure measures were unrelated to growth. Postnatal exposure to PCBs

and TEQ was associated with slower length growth between 3 and 7 months. In the Michigan cohort, prenatal PCB exposure was associated with lower weight at age 5 months and at 4 years.[60,61] In the North Carolina cohort,[62] growth by 1 year was unrelated to PCB exposure. In a later study of the North Carolina cohort[63] that included over 200 boys, early-life PCB exposure was not related to adolescent height, weight, or age at sexual maturation. Among the more than 300 girls in that study, transplacental PCB exposure was associated with greater height-adjusted weight, although this association was statistically significant only when the analysis was restricted to whites. Early-life PCB exposure was not related to the girls' height or age at sexual maturation.

17.6 THYROID AXIS

Epidemiologic data on associations between levels of polychlorinated biphenyls and measures of thyroxine and related hormones are summarized in Table 17.2. The level of PCB exposure in the Faroe Islands[29] is about four times greater than that in the first Dutch study[64] and in the North Carolina study[66]; in the second Dutch study,[65] levels were a bit lower, and the study among German children[35] had the lowest levels of the studies reviewed. Even though the German study was the largest, the most informative study was the one from the Faroe Islands, because of the higher exposure levels.

The most sensitive indicator of a thyroid disorder in humans is the serum level of thyroid-stimulating hormone (TSH). In animal models, however, PCBs cause low thyroxine (T4) levels in the absence of a disturbance in TSH. Investigators have tended not to show data on the size of the PCB–T4 association, and instead, the most frequent finding presented was that nothing of statistical significance was seen. For TSH, the only evidence that supports the hypothyroidism hypothesis was from the first Dutch study[64] of neonates. In the German study,[35] total PCBs showed no clear relation with TSH, although a congener-specific analysis showed TSH levels increased with level of PCB 118 (not shown in Table 17.2). As noted above, the levels of exposure in the German study were relatively low. The lack of association between PCBs and TSH or T4 in the Faroe Island study[29] weighs against the hypothyroidism hypothesis.

If we use statistical significance as a guide, three other results in Table 17.2 were notable. In the first Dutch study,[64] among mothers, higher PCB levels were associated with lower serum total triiodothyronine (TT3). Results for TSH were available but not mentioned in that report, so presumably there was no corresponding increase in TSH. Second, among school-aged children in Germany,[35] PCB levels were inversely associated with serum free triiodothyronine (FT3) but in the absence of a clear association of PCB with TSH. Third, in the Faroe Island neonates,[29] higher PCB levels were associated with

TABLE 17.2 Dietary Exposure Studies: Summary of Epidemiologic Data on Associations between Levels of Polychlorinated Biphenyl and Measures of Thyroxine and Related Hormones[a]

Study	Location	Mean Age[b] (yr)	n	Measure of Association					
				TT3	FT3	TT4	FT4	TSH	RT3U
Koopman et al. (1994)[64c]	The Netherlands	0	78	ns	—	ns	—	$r = 0.38^*$	—
Fiolet et al. (1997)[65]	The Netherlands	0	93	—	—	ns	—	—	—
Longnecker et al. (2000)[66]	North Carolina	0	160	—	—	$r = 0.07$	$r = -0.12$	$r = -0.08$	—
Steuerwald et al. (2000)[29]	Faroe Islands	0	182	—	ns	ns	ns	$r = -0.04$	$r = -0.21^*$
Osius et al. (1999)[35]	Germany	8	296	—	$\beta = -0.25^*$	—	~	~	—
Koopman et al. (1994)[64]	The Netherlands	29	78	$r = -0.36^*$	—	ns	—	ns	—
Steuerwald et al. (2000)[29]	Faroe Islands	28	182	—	ns	ns	ns	$r = -0.13$	ns

[a] n, number of subjects; TT3, total triiodothyronine; FT3, free triiodothyronine; TT4, total thyroxine; FT4, free thryroxine; TSH, thyroid stimulating hormone; RT3U, radioiodine triiodothyronine update; —, not measured; ns, association not statistically significant; *, association statistically significant ($p < 0.05$); r, value is a correlation coefficient; β, value following is a regression coefficient; ~, nonlinear association (no strong evidence of any association in either case).

[b] Mean age 0 indicates that the hormone levels were measured at birth or shortly thereafter.

[c] Results shown are for nonplanar PCB Teq, but results were described as being similar for nonplanar PCBs.

lower radioiodine T3 uptake, again in the absence of a corresponding PCB–TSH association.

Overall, data on the relation between PCB levels and thyroid economy were inconsistent, although the weight of evidence on the relation of PCB levels with TSH and T4 was null. Yet isolated positive findings are sprinkled throughout Table 17.2. We speculate that PCBs may alter thyroid economy, but in a way that has no obvious clinical interpretation. Nonetheless, adverse effects of PCBs on neurodevelopment may be a manifestation of a disturbance in thyroid economy. This topic is discussed in the following section.

17.7 NEURODEVELOPMENT

Recognition of PCBs and related compounds as potential human neurodevelopmental toxicants was largely a consequence of the two Asian mass poisonings, in Japan in 1968 and in Taiwan in 1979, the Yusho and Yucheng incidents, respectively (see Chapters 21 and 22, respectively). In both poisoning episodes PCB-contaminated rice oil was ingested, and many of those who consumed the oil and their children born during or after the episode became ill.[67–71] The developmental effects of substantial pre- and postnatal exposures resulting from these poisoning episodes are well documented in Taiwan.[72] In brief, Taiwanese children exposed prenatally during or after the poisoning episode were at increased risk for a syndrome of intrauterine growth retardation, hyperbilirubinemia, hyperpigmentation, acne, abnormalities of the nails, teeth, and gingiva, and in later childhood, lower weight and height, diminished IQ, and behavioral disorders.[67,71,73–76] The contaminated rice oil contained thermal degradation products of PCBs, including polychlorinated dibenzofurans (PCDFs), more potent toxins than PCBs. Studies in animals suggest that PCDFs are a likely source of some effects seen in the Yusho and Yucheng episodes.[77] As a consequence, the relative importance of PCBs versus PCDFs as the putative causal agent(s) in these poisoning episodes remains unclear.

As a result of the Asian mass poisonings, more than 10 studies of background-level PCB exposure in relation to neurodevelopmental outcomes were initiated (Table 17.3). Studies with published results include birth cohorts in Michigan, North Carolina, the Netherlands, Oswego (New York), the Faroe Islands (Denmark), and Duesseldorf (Germany). Because many reports have addressed the PCB-neurodevelopment hypothesis and because of its potential public health significance, these data have been reviewed in some detail (Tables 17.4 to 17.6). In addition, we note four birth cohorts for which data from peer-reviewed reports on neurodevelopmental outcomes are not yet available: New Bedford, Massachusetts[83]; a cohort of Inuit children from Canada and Greenland (NIEHS R01-ES07902, ''Environmental Contaminants and Infant Development''); the Collaborative Perinatal Project (U.S.)[84]; and the Child Health and Development Study (California).[85]

TABLE 17.3 Birth Cohort Studies of PCBs and Neurodevelopment in General Population Samples

Study	Location	*n*	Published Follow-up[a]	Birth Years
Rogan et al. (1986)[55]	North Carolina	931	Birth to adolescence	1978–1982
Jacobson et al. (1984)[78]	Michigan	313	Birth to age 11 years	1980–1981
Grandjean et al. (1997)[79]	Faroe Islands	1022	Birth to age 7 years	1986–1987
Sauer et al. (1994)[80]	The Netherlands	418	Birth to 42 months	1990–1992
Lonky et al. (1996)[81]	New York	316	Birth	1991–1994
Winneke et al. (1998)[82]	Germany	171	Birth to 7 months	1993–1995
Steuerwald et al. (2000)[29]	Faroe Islands	182	Birth	1994–1995
Korrick et al. (2000)[83]	Massachusetts	788	Pending	1993–1998
Jacobson et al.[b]	Canada/Greenland	300	Pending	1996–present
Longnecker et al. (2000)[84]	U.S./CPP[c]	1000	Pending	1959–1966
Hertz-Picciotto et al. (2000)[85]	California/CHDS[d]	400	Pending	1964–1967

[a] Does not include published abstracts.

[b] NIEHS R01-ES07902, *Environmental Contaminants and Infant Development*.

[c] Collaborative Perinatal Project.

[d] Childhood Health and Development Study.

TABLE 17.4 Neonatal Neurologic Examination in Relation to PCBs, by Age at Examination[a]

	Reflexes (days after birth)				Muscle Tone (days after birth)			
Birth Cohort	2	3	7	14	2	3	7	14
Michigan		↓[b]				—		
North Carolina			↓				↓	
The Netherlands				—				↓[c]
Faroes-2[d]				—				—
Oswego	—				—[e]			

[a] ↓, decreased; —, no association; see Table 17.3 and the text for references.

[b] Only when fish intake is used as measure of exposure.

[c] Among breast-fed infants only. Type of PCBs implicated depended on specific model used.

[d] The smaller of the Faroe Islands cohorts.

[e] Absence of association is implied.[119]

TABLE 17.5 Bayley Scales of Infant Development in Relation to PCB Exposure, by Age at Examination[a]

	Psychomotor Development Index (months of age)							Mental Development Index (months of age)						
Birth Cohort	3	5	6	7	12	18	24	3	5	6	7	12	18	24
Michigan		—							—					
North Carolina			↓		↓	—	↓			—		—	—	—
The Netherlands	↓			↓[b]		—		—			—		—	
Duesseldorf				—							↓[c]			

[a] ↓, decreased; —, no association; see Table 17.3 and the text for references.

[b] Among breast-fed only, with total TEQ as exposure measure.

[c] $p = 0.05$ using a one-tailed test.

TABLE 17.6 Overall Cognitive Functioning in Relation to PCB Exposure[a]

	Years of Age				
Birth Cohort	3	4	5	7	11
Michigan		↓			↓
North Carolina	—	—	—		
The Netherlands		↓			
Faroes-1[b]				—	

[a] ↓, decreased; —, no association; see Table 17.3 and the text for references.
[b] The larger of the Faroe Islands cohorts.

17.7.1 Michigan Birth Cohort

The mothers in this study were selected on the basis of consumption of Lake Michigan fish species that were contaminated with PCBs. 242 women who consumed moderate amounts of contaminated fish before their child's birth and 71 women who did not consume the fish were enrolled in 1980–1981.[86] The cohort was predominantly white and restricted to mothers age 18 and older who had completed at least a 10th-grade education.[78] Over half (61%) of the infants were breast-fed.[86]

An estimate of cumulative maternal consumption of contaminated fish was used as a proxy prenatal PCB exposure measure. In addition, total PCB levels were measured in cord serum, maternal serum after delivery, and maternal milk and quantitated with an adaptation of the Webb–McCall method.[86] Cord and maternal serum PCB measures were available for 198 and 196 infants, with mean PCB levels of 2.5 and 5.5 μg/L serum, respectively.[86] Of the breast-fed infants, 138 had perinatal milk samples for analysis with a mean PCB level of 0.8 mg/kg lipid.[86] Postnatal PCB exposure was estimated by the PCB levels in breast milk and the number of weeks of breast-feeding.[87] Cumulative maternal contaminated fish consumption was moderately correlated with maternal serum ($r = 0.29$) and milk ($r = 0.21$), but not cord serum, PCB levels.[86]

The children underwent extensive formal developmental assessments from birth to age 11 (Tables 17.4 to 17.6), beginning with the Brazelton Neonatal Behavioral Assessment Scale (NBAS), which measures 28 behavioral items and 18 reflex items in the neonate.[88] Although a single NBAS exam has limited predictive value for later development, it provides basic insight into neonatal behavior and neurologic function.

NBAS examinations were administered to 287 (92%) of the infants 48 to 72 h after birth. Maternal consumption of contaminated fish was associated with increased hyporeflexia, poorer autonomic maturity, and depressed lability of states, meaning that the infant's state of arousal tended to be more stable.[78] In addition, infants classified as worrisome on these NBAS measures had significantly higher maternal contaminated fish consumption. Cord serum PCB levels were unrelated to NBAS scores, although cord serum values were not

available for approximately 37% of the subjects.[78] The absence of confirmatory NBAS findings with cord serum PCB levels, however, suggests that these results should be interpreted with caution.

The Bayley Scales of Infant Development (BSID) were administered to the Michigan infants at 5 months of age. The BSID provides two standardized scores, the Mental Developmental Index (MDI), a measure of cognitive development, and the Psychomotor Developmental Index (PDI), a measure of motor development.[89,90] The MDI and PDI scores were unrelated to maternal contaminated fish consumption or cord serum PCB levels.[91,92]

At 7 months of age, 123 of the Michigan infants were assessed with the Fagan Test of Infant Intelligence (FTII)[93]; 81 and 67 of these infants, respectively, had cord serum and milk PCB levels available. The FTII is based on the principle that 3- to 8-month-old infants will preferentially look at a new image. The infant is shown a series of pictures, some of which have been shown previously and some of which have not. The infant's frequency of preference for the novel images is used to evaluate FTII performance. This exam has been shown to be predictive of IQ assessment in later childhood.[94] Higher cord serum PCB levels were associated with lower novelty preference scores (i.e., poorer performance) on the FTII.[87] There was no association of postnatal exposure via breast milk with FTII performance.

At age 4 years, 236 (75%) of the children were assessed with a number of exams, including the McCarthy Scales of Children's Abilities, the Beery Test of Visual-Motor Integration (VMI), the Peabody Picture Vocabulary Test–Revised (PPVT-R), and activity ratings were obtained.[61,95] In addition, 226 subjects underwent testing to assess cognitive processing efficiency and sustained attention.[96] Blood samples for PCBs were obtained. Cord serum and milk PCB levels were available for 146 and 120 of these children, respectively.[61]

The McCarthy Scales of Children's Abilities is a standardized test of cognitive and motor skill appropriate for children ages 2 to 8 years.[97] Scores are provided for six scales: Verbal, Perceptual-Performance, Quantitative, Memory, General Cognitive, and Motor. Prenatal PCB exposure as assessed by cord serum was inversely associated with performance on the Verbal and Memory McCarthy Scales.[61] This effect was mediated through significant associations of cord serum PCB levels with performance on Verbal Memory and Numerical Memory subtests of the McCarthy, both of which contribute substantially to the Verbal and Memory Scale indices and reflect short-term memory function. Similarly, higher maternal milk PCB levels were associated with poorer performance on the Memory Scale, primarily via poorer performance on the Verbal Memory and Numerical Memory subtests. Postnatal PCB exposure estimated with duration of nursing or total PCB intake was not associated with decrements in McCarthy performance. The VMI and PPVT-R tests were unrelated to PCB levels.

Study examiners used the Child Behavior Record (Activity Scale) adopted from the Bayley Infant Behavior Record to assess activity at two separate

home visits, and mothers completed an Activity Scale from the Emotionality Activity Sociability (EAS) Temperament Survey for Children. These three activity ratings were standardized and summed into a single composite rating.[95] In analyses combining the 236 children from this fish-eating birth cohort with a smaller ($n = 87$) sample of 4-year-old Michigan farm children, the composite activity rating was inversely related to serum PCB level at 4 years. Among the fish-eating birth cohort, composite activity was inversely related to milk PCB level, with the strongest association among children of mothers with the highest milk PCB levels and who were breast-fed the longest, consistent with a postnatal exposure effect. This PCB-associated lower activity level at 4 years is notable as the only PCB-related effect among the Michigan cohort that was associated with measures of postnatal, not prenatal, exposure.

Cognitive processing efficiency was assessed with a modified version of the Sternberg Memory Test and drawings from Kagan's Matching Familiar Figures Test.[96] In these two evaluations, the child was instructed to press a button in response to drawings previously seen in a memory set (short-term memory scanning task) and to identify drawings that were identical to, or discrepant from, a criterion picture, including correctly identifying the minor discrepant feature (visual discrimination task). Sustained attention was assessed with a vigilance paradigm in which each child pressed a button when a criterion stimulus (e.g., a cat) appeared as part of a computer screen image. Reaction time (i.e., response time), number correct, and number of errors were used in assessing performance of these tasks. Measures of higher prenatal PCB exposure were associated with more errors in short-term memory scanning and slower response time for visual discrimination tasks at 4 years. There was no significant relationship between PCB measures and sustained attention. Postnatal exposure measures, including total milk consumed and serum PCB level at 4 years, were unrelated to these outcomes.

The 4-year data were later reanalyzed using as the exposure measure an estimate of milk PCB level based on all available PCB measures (cord serum, maternal serum, and maternal milk).[98] Results of the reanalyses showed similar or stronger associations of PCB levels with McCarthy Verbal and Memory Scales, short-term memory scanning errors, and visual discrimination response time. In addition, the McCarthy General Cognitive Index and the McCarthy Quantitative Scale were both inversely and significantly associated with the composite exposure measure. Most of the effects demonstrated in the reanalysis, however, were evident only among the most exposed children (prenatal exposure ≥ 1.25 mg/kg lipid equivalents in milk),[98] whereas a more linear dose–response curve had been demonstrated using cord serum PCB levels as the measure of exposure.[61,96]

At 11 years, 212 (68%) of the children underwent IQ and achievement testing.[99] Fewer children ($n = 178$) had complete covariates for multivariate analyses. Blood samples were collected and analyzed for PCBs. As was done in the reanalysis of the 4-year assessments, a composite PCB measure using all avail-

able samples (cord serum, maternal serum, and maternal milk) was constructed to estimate prenatal PCB levels in mg/kg lipid equivalents in milk.[99] Participants in the 11-year follow-up had higher prenatal PCB exposure than those lost to follow-up. Each child was examined with the Wechsler Intelligence Scales for Children–Revised (WISC-R), the Wide Range Achievement Test–Revised (spelling and arithmetic subtests), and the Woodcock Reading Mastery Test–Revised (word- and passage-comprehension subtests). In factor analyses of the IQ subtests, three summary scales were also assessed: verbal comprehension, perceptual organization, and freedom from distractibility. Higher prenatal PCB levels were associated with lower full-scale and verbal IQ scores and poorer word comprehension on the Woodcock achievement test. These effects were largely among the most exposed children (prenatal PCB levels ≥ 1.25 mg/kg lipid for IQ and ≥ 1.00 mg/kg lipid for achievement). The most exposed children had approximately an IQ score 6 points lower and 7 months' delayed word comprehension compared with the less exposed. In analyses of the IQ summary scales from factor analysis, prenatal PCB levels were inversely associated with subtests reflecting general intellectual ability, memory, executive function, and focused attention. IQ and achievement test results were not associated with measures of postnatal PCB exposure.

17.7.2 North Carolina Birth Cohort

The mothers in this study were recruited from three clinical sites in central North Carolina: 880 women and their 931 infants born between 1978 and 1982 were enrolled[100]; 912 of the infants had neonatal information available for analysis, 856 children continued participation after the neonatal period, and over 700 children were available for assessments between ages 3 and 5 years.[55,100,101] The study mothers were predominantly white, well-educated (over half had a college education) professionals (41%); most of the infants (88%) were breast-fed.[100]

PCB levels were measured in cord blood, placenta, and postpartum maternal serum (at birth and 6 weeks) and milk samples (at birth, 6 weeks, 3 and 6 months, and periodically until lactation ended). Total PCBs were quantitated by a uniquely modified (two peaks) Webb–McCall method.[102] Relative to methods used for Michigan specimens, this analytic approach overestimates PCB levels by a factor of approximately 2.[103]

Prenatal PCB exposure was estimated as the average PCB level in all available maternal serum and milk samples and expressed as a PCB concentration in milk at birth.[100] Most cord blood and placental PCB levels were below the method detection limit. Median PCB concentrations in cord serum, maternal serum, and milk at birth were < 4 μg/L, 9 μg/L, and 1.8 mg/kg lipid, respectively, and the estimated median concentration for milk at birth (taking all serum and milk specimens into account) was 1.7 mg/kg lipid.[100] One hundred and four infant formula samples were tested, and all but one had PCB levels

below the detection limit.[100] For breast-fed children, postnatal PCB exposure was estimated as the product of milk PCB concentration, duration of lactation, quantity of milk consumed, and average milk fat content (2.5%).

During the first 3 weeks of life, performance on the NBAS was associated with prenatal PCB exposure but only among infants in the top 5th percentile of estimated maternal milk PCB levels at birth ($\geq$ 3.5 mg/kg lipid). These infants had an increased prevalence of hypotonia (reflected primarily in decreased overall tone and decreased activity) and hyporeflexia compared with the remaining infants.[55]

At ages 6, 12, 18, and 24 months, estimated maternal milk PCB levels at birth were associated with poorer performance on the BSID PDI, although this association was not significant among the 18-month-olds.[104,105] Furthermore, among older infants (18 and 24 months) the relationship of PCBs with PDI was nonlinear, with statistically significant declines in PDI apparent only among infants in the top 5th percentile of exposure. For example, 24-month-olds with milk PCB concentrations at birth $\geq$ 3.5 mg/kg lipid scored, on average, about 8 points lower on the Bayley PDI than did less exposed infants.[105] Maternal milk PCB levels were not associated with the Bayley MDI at any age tested. Finally, there was no association of either Bayley score with measures of postnatal PCB exposure.

At ages 3, 4, and 5 years, the children were assessed with the McCarthy Scales of Children's Abilities. Performance on the McCarthy Scales was unrelated to estimated maternal milk PCB levels at birth.[101] This null finding applied to all components of the exam, including the Verbal, Memory, and Motor Scales. There was no association of McCarthy Scale scores with postnatal PCB exposure.

Although no formal developmental assessments were done after age 5, school grades for English and mathematics were obtained for grades 3, 4, and 5 (ages 7.5 through 10.5 years). There was no association between maternal milk PCB levels at birth or postnatal PCB exposure and school grades in this cohort.[101] Information about whether the child was hyperactive was obtained at the same time as the grades. Assessment of hyperactivity was based on reports by parents, teachers, counselors, or physicians and whether the child was taking medication for hyperactivity, but no formal assessment tool was used. PCB exposure was unrelated to hyperactivity.[106]

17.7.3 Dutch Birth Cohort

Mothers were recruited from Rotterdam, an urban industrialized area, and Groningen, a more rural community. The study population was selected to include equal portions of breast-fed and formula-fed infants. Four hundred eighteen infants born in 1990–1992 were enrolled, about half from each center[107]; 94% of the infants participated in follow-up evaluations at 42 months. All subjects were white.

Levels of specific PCBs in maternal plasma in the last month of pregnancy, cord plasma, and maternal milk samples (2 weeks postpartum for the 209 breast-fed infants) were used to estimate pre- and postnatal exposures. In addition, dioxins (PCDDs and PCDFs) and coplanar PCBs were measured in maternal milk samples and used in pre- and postnatal exposure estimates. Four PCB congeners were measured (IUPAC PCB congeners 118, 138, 153, and 180)[7] in all biological samples. The concentrations of these individual congeners as well as their sum ($\sum$PCB) were considered in analyses. In milk, 17 PCDD and PCDF congeners, three planar PCB congeners (IUPAC PCB congeners 77, 126, and 169), and 19 additional nonplanar PCB congeners were measured.[80] TEQ concentrations for dioxinlike PCBs and dioxins (PCDDs and PCDFs) were calculated for breast milk.[108] The respective median $\sum$PCB concentrations in maternal and umbilical cord plasma specimens were 2.0 and 0.38 μg/L.[107] The mean TEQ levels of dioxins, planar PCBs, and nonplanar PCBs in milk were 30, 16, and 19.5 ng/kg lipid, respectively.[27] The study protocol included providing formula to mothers of formula-fed infants. PCB and dioxin levels were below the detection limits in this formula.[105] Milk TEQ levels correlated well with milk or maternal plasma levels of the PCB congeners 118, 138, 153, and 180 (*r* values were from 0.66 to 0.85).[27]

To estimate prenatal exposure to $\sum$PCB (118, 138, 153, and 180) levels in maternal and cord plasma and for dioxinlike PCBs and dioxins that could not be measured in plasma, levels in milk were used.[107] Among older infants and children, postnatal exposure was estimated by multiplying PCB and TEQ levels in milk by the number of weeks of breast feeding.[57,109]

At 10 to 21 days of age, the children were subjected to detailed neurologic examinations. The neonatal neurologic examination as described by Prechtl[110] was administered because it has better predictive value for later neurological function than does the NBAS.[107] Results of this examination were used to generate three separate scores reflecting performance on measures of postural tone, neonatal reflexes, and neurologic optimality. Neurologic optimality score (NOS) was defined as the sum of neurologic exam items for which the infant's performance met optimal criteria.[111] Among combined breast- and formula-fed infants, higher maternal or cord plasma $\sum$PCBs or individual PCB congener levels were not associated with significant differences in neonatal reflexes, tone, or NOS.[107]

Among the breast-fed infants at 10 to 21 days, high milk concentrations of $\sum$PCBs and of a number of individual PCBs (including PCBs 118, 138, and 153) and dioxin congeners, dioxin TEQ, PCB TEQ, and total TEQ (dioxin + PCB) were associated significantly with an increased risk of low NOS.[107] Similarly, higher planar PCB TEQ levels in milk were associated with significantly increased odds of hypotonia. In a model with milk $\sum$PCB levels and cord $\sum$PCB levels, the higher milk $\sum$PCB levels were associated with increased odds of hypotonia and reduced NOS.[107] Milk PCB, dioxin, or TEQ levels were not associated with neonatal reflexes.

At 3, 7, and 18 months of age, Bayley assessments were performed for the Rotterdam children.[109] At 3 months, PDI was inversely associated with prenatal PCB levels. For example, doubling maternal plasma $\sum$PCB levels (e.g., from 1.0 to 2.0 μg/L) was associated with a 3-point lower PDI score.[109] Also at 3 months, postnatal exposure measures were not associated with PDI. At 7 months there was no discernible effect of prenatal PCB levels on PDI; postnatal total TEQ was associated with decreased PDI, but other exposure measures were not. At 18 months, none of the exposure measures were associated with PDI. MDI was unrelated to PCB or TEQ levels in plasma, milk, or postnatal exposure measures at any age tested.

At 18 months, an age-specific neurologic examination was performed ($n = 418$).[112] Although overall findings were not presented, among children of nonsmoking fathers, higher maternal and cord plasma $\sum$PCB levels were associated with lower NOS values.[112] Milk PCB or dioxin levels were not associated with NOS. Motor fluency was not related to plasma or milk PCB or dioxin levels.

At 42 months of age, neurologic examinations were again performed.[113] Plasma PCBs and milk PCB or dioxin TEQ levels were related neither to NOS nor to motor fluency.[113] The Kaufman Assessment Battery for Children (K-ABC, Dutch version), a standardized cognitive assessment instrument appropriate for children between 2 and 4 years of age, was also given to 395 (94%) of the children.[114] Results of this exam are expressed as scores on two scales—sequential processing and simultaneous processing—designed to reflect two general types of cognitive processing. An overall cognitive score is generated by combining these two scales. Reynell Development Language Scale (RDLS) verbal comprehension examinations were given only to the Rotterdam children, 193 (93%) of whom participated. The RDLS (Dutch version) is a measure of language ability, including verbal comprehension, and is standardized for children between ages 2 and 6 years. The $\sum$PCBs in maternal plasma was associated with each of the three K-ABC scores.[114] On the K-ABC overall cognition scale, a doubling of maternal plasma PCB level (e.g., from 1.0 to 2.0 μg/L) was associated with a 3-point lower score ($p = 0.005$).[114] Among children formula fed in infancy, a doubling of maternal plasma PCB level was associated with a 6-point lower K-ABC overall cognitive scale score ($p = 0.0006$), compared with a 1.5-point decline ($p = 0.3$) in breast-fed children. Similarly, significant PCB-associated differences in verbal comprehension scores (RDLS) were demonstrated only among formula-fed children, in whom a doubling of maternal plasma PCB level was associated with a 4-point lower score.[114] Neither postnatal exposure nor current body burden (plasma $\sum$PCBs at 42 months) was associated with K-ABC or RDLS scores.

17.7.4 Faroe Islands Birth Cohorts

Two birth cohort studies of PCBs and neurodevelopment have been done in the Faroe Islands, islands in the North Atlantic whose population has a diet that

includes a substantial amount of fish, whale meat, and whale blubber. The whale meat is a source of mercury exposure, and the blubber is a source of PCBs.[115] The average PCB level in breast milk from Faroese women (e.g., 1.9 to 3.5 mg/kg lipid) is higher than in any of the other neurodevelopmental studies considered in this section.[79,115]

The first cohort included 1022 children born in 1986–1987.[79,116] Cord blood, maternal hair specimens, and questionnaire data were obtained for 997 of the children, and umbilical cord tissue was obtained for 436.[79,117] Cord tissue was analyzed for specific PCBs (congeners 138, 153, and 180) and their sum ($\sum$PCB), with total PCBs estimated as $\sum$PCB multiplied by 2.[79] Levels of cord tissue PCBs and cord blood mercury were correlated ($r = 0.41$).[79]

Neurodevelopment was assessed at 7 years for 917 (90%) of the children.[79,116] The 7-year evaluation included clinical assessment with a physical exam (including neurologic exam), visual acuity and contrast sensitivity testing, audiometry, maternal interview (including a child behavioral checklist), and an extensive battery of neurophysiological and neuropsychological tests. The neurophysiological tests assessed visual evoked potentials (VEPs; reflects the functional integrity of the visual afferent neural pathways), brain stem auditory evoked potentials (BAEPs; reflects the functional integrity of the auditory afferent neural pathways), postural sway (reflects the functional integrity of proprioceptive, vestibular, and other neural systems responsible for postural stability), and heart rate variability (as a measure of autonomic neurologic function). The neuropsychological battery included tests of: motor speed [Neurobehavioral Evaluation System (NES 2) Finger Tapping], manual motor coordination (NES 2 Hand–Eye Coordination), processing of tactile stimuli, sustained attention (NES 2 Continuous Performance Test), cognition (Wechsler Intelligence Scale for Children–Revised: subtests for Digit Spans, Similarities, and Block Design), visuospatial function (Bender Gestalt Test), memory (California Verbal Learning Test), language (Boston Naming Test), and mood (Nonverbal Analogue Profile of Mood States).

After adjustment for cord blood mercury level, an effect of PCBs was suggested for performance on the Boston Naming Test, but it was not statistically significant.[79] There was no evidence of significant interaction between prenatal PCB and mercury exposure measures.[118]

The second cohort included 182 children born in 1994–1995.[29] Third-trimester maternal serum, cord blood, and milk 4 to 5 days postpartum were collected and analyzed for specific PCB congeners. Total PCB level ($\sum$PCB) was calculated as twice the sum of congeners 138, 153, and 180. Geometric mean maternal serum and milk $\sum$PCB levels were 1.1 and 1.5 mg/kg lipid, respectively.

Prechtl neurologic examinations at approximately 2 weeks were used to measure NOS, including subscores for muscle tone and reflexes. Measures of prenatal PCB exposure were not associated with NOS, muscle tone or reflexes in these analyses.[29]

17.7.5 Oswego Birth Cohort

The mothers in this study were selected on the basis of consumption of Lake Ontario fish species that were contaminated with PCBs.[81] The population was enrolled in 1991–1994 and consisted of 316 mother–infant pairs representing two groups: a high-fish-consumption group ($n = 152$) and controls (no Lake Ontario fish consumption, $n = 164$). The mothers were predominantly white and of low-to-middle socioeconomic status.

Cord blood levels of PCBs were obtained for 141 (93%) of the high-fish-consumption group and 152 (93%) of the control group.[119] Eighty-three mothers provided milk samples for analysis within 6 months of their child's birth.[25] Cord whole blood samples were analyzed for specific PCB congeners.[25,119] PCB levels in over half (59%) of the samples were nondetectable.[119] The sum of all congeners measured ($\sum$PCB) and the sum of congener groupings based on the number of chlorine substitutions—lightly chlorinated (one to three chlorines), moderately chlorinated (four to six chlorines), and heavily chlorinated (seven to nine chlorines) PCB congeners—were considered in analyses.[119] The median cord blood total $\sum$PCB was 0.5 μg/L.

On the first and second day after birth, NBAS examinations were performed on the infants. In the first report about the NBAS results, the change in NBAS score between the two exams (day 1 and day 2) was used to assess performance. The high-fish-exposed infants had a greater number of abnormal reflexes, less autonomic maturity, and poorer habituation at the second than the first exam compared with the controls.[81] The second report used as the exposure measure cord blood PCB levels and examined the NBAS scores at each time separately. Higher cord blood PCB levels of the heavily chlorinated PCBs were associated with less autonomic maturity and poorer habituation at the second NBAS exam and with a higher proportion of poor NBAS scores.[119] Cord blood PCB levels were not significantly associated with abnormal reflexes. The level of highly chlorinated PCB congeners was more strongly correlated with Lake Ontario fish consumption and breast milk PCB concentrations than were levels of low and moderately chlorinated congeners.[25]

17.7.6 Duesseldorf (Germany) Birth Cohort

The mothers ($n = 171$) were recruited for study from three Duesseldorf hospitals in 1993–1995.[82] Most infants (83%) were breast-fed. Families were primarily middle or upper class. Cord plasma and maternal milk samples (at 2 and 4 weeks postpartum) were collected and analyzed for specific PCBs. Exposure was expressed as the sum of congeners 138, 153, and 180 ($\sum$PCB). The average PCB concentration of the two milk samples (at 2 and 4 weeks) was used in analyses. The mean levels of $\sum$PCB in cord plasma and milk were 0.55 μg/L and 0.427 mg/kg lipid, respectively.[82]

At age 7 months infants were evaluated with the BSID, version II[90] and the FTII. The mobile FTII used in this study had poor retest reliability ($r = -0.2$). PCB levels were inversely associated with performance on the BSID cognitive (MDI) scores ($p = 0.05$ in one-tailed probability), and this association was specific to $\sum$PCB levels in milk, but not cord plasma, specimens.[82] A relationship between cord plasma or maternal milk $\sum$PCB levels and performance on the FTII was not found.[82]

17.7.7 Discussion of Neurodevelopmental Studies

Among the neurodevelopmental measures that have been examined in relation to PCBs, none were consistently related to exposure (Tables 17.4 to 17.6). The BSID PDI and a measure of overall cognitive index were each unequivocally associated with prenatal PCB exposure in two studies. Most of the studies reviewed in this section had at least one finding that supports the hypothesis that prenatal PCB exposure has an adverse effect on neurodevelopment. In the poisoning with a PCB mixture in Taiwan (reviewed in Chapter 22), the precise level of exposure remains unclear. During the incident, however, the intake of PCBs exceeded the average U.S. intake by more than a 1000-fold.[120] The WISC IQ among the children poisoned in utero was 4 points lower than that in the control group.[68] In the Michigan birth cohort,[99] the difference in WISC IQ between those in the highest exposed group and those less exposed was 6 points. Possibly a threshold for PCB exposure exists above which no further decrement in IQ occurs. Such a threshold could explain the lack of unequivocal adverse effects among the relatively highly exposed Faroe Islands population. Nonethless, the absence of clear effects in the Faroes data weakens the data supporting the hypothesis that PCBs adversely affect neurodevelopment.

The reason for the inconsistency of results across studies published to date is unclear.[121,122] Proposed sources of variation among study results include differences in choice and timing of outcome assessment, breast-feeding, other exposures, and PCB exposure level, congener mix, and rate of intake during pregnancy. If a neurotoxin or other determinant of neurodevelopment is associated with PCB exposure, this could confound assessment of the relation between PCBs and neurodevelopment. PCB exposure, however, has been found to be associated consistently only with age and region.[34]

PCB doses that result in tissue levels comparable to those in background-exposed humans have detectable effects on neurodevelopment in animal models.[123] Furthermore, several specific domains of neurodevelopment are possibly the most vulnerable to PCBs in animals: attention, memory, and overall cognitive functioning, among others.[124] Although memory and cognitive function measures, for example, were not consistently associated with prenatal PCB exposure among the studies published to date, the overall data leave open the possibility that adverse neurodevelopmental effects can occur in background-exposed humans.

17.8 IMMUNE SYSTEM

17.8.1 Infections

The relation of PCB levels to frequency of infection has been examined in four studies. In a study of 59 infants in Wisconsin, PCB levels were associated with an increased frequency of infectious illness in the first 4 months of life.[54] In a study of 207 children in the Netherlands,[125] PCB levels were not associated with the frequency of infectious illness in the first 18 months of life. In studies of 171 Inuit infants from Quebec[126] and of 754 infants from North Carolina.[62] PCB levels were not associated with the frequency of infectious illness in the first 12 months of life. Exposure levels in the studies from the Netherlands,[125] North Carolina,[62] and Quebec[126] were roughly similar.

17.8.2 Antibody Titers

Antibody levels, presumably IgG, to mumps, measles, and rubella at 18 months of age, were unrelated to PCB levels in the same study from the Netherlands as that noted in Section 17.8.1.[125] In the study of Inuit children mentioned above,[126] the authors noted that levels of several classes of immunoglobulins (Ig), specifically IgG, IgA, and IgM at 3, 7, and 12 months of age, were unrelated to prenatal PCB exposure. Among 120 neonates in Slovakia,[127] placental PCB 118 level was correlated with cord serum IgE level (Spearman $r = 0.35$). DDE (the persistent metabolite of DDT) levels and urban location were also positively associated with IgE levels, and if the results had been adjusted for these potential confounders, the PCB 118-IgE association might easily have been unimpressive. Nonetheless, the association being found with PCB 118, but not with other PCB congeners (seven were measured), may indicate an Ah receptor–mediated effect, as PCB 118 binds to the Ah receptor more tightly than do the other congeners examined. Exposure levels were probably similar in the three studies considered here.

17.8.3 T Cells

In the study of Inuit children,[126] the authors found that levels of lymphocyte subsets were unrelated to prenatal PCB exposure. In the Dutch birth cohort noted above,[125] at 18 months of age, levels of T cells of the subtype positive for cell surface markers $CD3^+$ and $CD8^+$ were positively associated with prenatal PCB levels ($n = 43$). The stronger correlations seen for total TEQ suggest that the association with PCBs may have been due to Ah receptor binding. In a more highly exposed group of 12 Swedish adults, serum PCB 118 levels were inversely correlated with number and percent of natural killer lymphocytes, but the results were not adjusted for potential confounding by levels of DDE, study group, or fish intake.[31]

17.8.4 Other Immune System Findings

In the Dutch birth cohort,[125] another finding of note was that PCB levels were associated with fewer monocytes and granulocytes at 3 months of age. Whether the effects were more strongly associated with congeners that bind to the Ah receptor than those that do not was not specified.

17.9 BREAST CANCER

We divided the studies of background-level PCB exposure in relation to breast cancer risk into three groups—small, large, and nested—because their informativeness increased in that order. Overall results for the studies are discussed in the first three sections below. For the instances where an association between PCBs and breast cancer risk was present only in a subgroup, the effects are described in a separate section.

17.9.1 Small Case–Control Studies

In the seven small case–control studies (Table 17.7), PCB concentrations were measured in adipose tissue. In most, PCB levels were higher in breast cancer cases than in controls.[128,129,131–134] Some of these differences, however, were slight and not statistically significant,[129,131–134] or were limited to specific PCB congeners (e.g., coplanar PCBs).[134]

The informativeness of these seven studies was limited because of the small sample sizes (fewer than 50 cases and 50 controls), the possibility that disease affected PCBs levels among cases, possible biases due to the selection of controls, and the limited or absent consideration of potentially confounding variables.

17.9.2 Large Case–Control Studies

The majority of the seven large case–control studies used a blood specimen to determine PCB level (Table 17.8). Overall, these data provided no strong evidence to implicate PCBs in breast cancer etiology. Only one study demonstrated an overall increased risk of breast cancer in association with elevated PCB levels,[135] although the risk estimates were not statistically significant and there was no evidence for a linear dose–response relationship. In the Moysich et al. study,[136] the authors saw a modest risk elevation for women who had detectable levels of lower-chlorinated PCBs compared with women without detectable levels of these compounds. Lower-chlorinated PCBs have been associated with greater toxicological activity, including estrogenic activity, than that of some of the higher-chlorinated congeners.[142]

Overall, results from these seven case–control studies provide evidence of modest informativeness at best, because of the possibility that disease altered the PCB levels among cases or that the selection of the controls resulted in bias.

TABLE 17.7 PCBs and Breast Cancer Risk: Results from Small Case–Control Studies[a]

Study	Year	Location	Source of Controls	Number of Cases/ Controls	Cases: Mean PCB Levels (mg/kg)	Controls: Mean PCB Levels (mg/kg)
Wasserman (1976)[128]	1976	Brazil	Accident victims	9/5	9.1	3.0
Unger (1984)[129]	1982	Denmark	Autopsy series BBD patients	18/35	6.47	5.12
				14/21	3.89	3.93
Mussalo-Rauhamaa (1990)[130]	1985–1986	Finland	Accident victims	41/33	1.05	1.30
Falck (1992)[131]	1987	United States	BBD patients	20/20	1.67	1.11*
Dewailly (1994)[132]	1991–1992	Canada	BBD patients	20/17	ER+ 0.332	0.397
					ER− 0.405	
Güttes (1998)[133]	1993–1994	Germany	BBD patients	45/20	PCB153 0.624	PCB153 0.505
Liljegren (1998)[134]	1993–1995	Sweden	BBD patients	43/35	1.21	1.15

[a]Concentrations shown are mg/kg lipid. BBD, benign breast disease; ER+, estrogen receptor–positive tumors; ER−, estrogen receptor–negative tumors. PCB 153 refers to a specific (major) PCB congener.

*$p < 0.05$.

TABLE 17.8 PCBs and Breast Cancer Risk: Results from Large Case–Control Studies[a]

Study	Year	Location	Source of Controls	Number of Cases/ Controls	Mean PCB Levels		OR Highest vs. Lowest PCB Category (95% CI)
					Cases	Controls	
Blood studies							
Wolff (1993)[135]	1985–1991	United States	Mammography screening clinic attendees, New York City	58/171	8.0 μg/L	6.7 μg/L	4.4 (0.90–20.4)[b]
Moysich (1998)[136]	1986–1991	United States	Erie and Niagara Counties, New York	154/192	4.3 μg/L	4.1 μg/L	1.14 (0.6–2.2)[c]
Zheng (2000a)[137]	1995–1997	United States	BBD patients and Tolland County, Connecticut residents	475/502	0.733 mg/kg	0.748 mg/kg	0.95 (0.7–1.3)[c]
Demers (2000)[138]	1994–1997	Canada	Quebec City	315/	0.059 mg/kg		
			Hospital patients	219		0.053 mg/kg	1.07 (0.5–2.1)[b]
			City residents	307		0.057 mg/kg	1.28 (0.7–2.2)[b]
Millikan (2000)[139]	1993–1995	United States	24 North Carolina counties	748/659	2.79 μg/L	2.46 μg/L	1.03 (0.68–1.56)[c,d] 1.74 (1.00–3.01)
Adipose studies							
Aronson (2000)[140]	1995–1997	Canada	BBD patients	217/213	0.94 mg/kg	0.87 mg/kg	1.15 (0.6–2.3)[e]
Zheng (2000)[141]	1995–1997	United States	BBD patients	304/186	0.479 mg/kg	0.494 mg/kg	0.70 (0.3–1.2)[c]

[a]None of the results were statistically significant. Values in μg/L were expressed on a wet weight basis, values in mg/kg were expressed on a lipid weight basis. Results for Demers et al. (2000) were for PCB 153.

[b]First versus fifth quintile.

[c]First versus third tertile.

[d]First row is results for whites, second row is results for blacks.

[e]First versus fourth quartile.

17.9.3 Nested Case–Control Studies

The major distinctions between the case–control studies considered in earlier sections and nested case–control studies were that in nested case–control studies PCB levels were measured in biological specimens obtained long before the breast cancer diagnosis for cases, and the results are much less susceptible to biases that can be caused by method of selecting subjects or less than complete participation of subjects.

The major findings of the six nested case–control studies are presented in Table 17.9. Three studies reported inverse associations between blood PCB concentrations and breast cancer risk[144,146,147]; two studies reported no association of PCB exposure with risk[143,145]; and one study reported a nonsignificantly greater risk for women with the highest levels than those with the lowest levels.[148]

17.9.4 Associations Found in Subgroups

Moysich et al.[136] observed that among parous women (women who have given birth to one or more children) who never lactated, higher PCB levels were associated with a threefold increase in risk of breast cancer. But in two separate analyses of a case–control study in Connecticut, Zheng et al.[137,141] did not observe greater risk of breast cancer in association with elevated PCB levels among parous women who had never breast-fed.

Aronson and colleagues[140] observed that premenopausal women with the highest levels of PCB 105 and PCB 118 had a fourfold and threefold increase in breast cancer risk, respectively. These two congeners have dioxinlike activity and might therefore be more carcinogenic than other PCB congeners, but on the other hand, dioxinlike compounds have antiestrogenic activity.[149]

Two studies have evaluated the association of PCB levels with risk of breast cancer risk in African-American women. Krieger et al.[143] studied the association in 50 African-American breast cancer patients and 50 matched controls. The results indicated that compared with women in the lowest tertile of the PCB distribution, those in the middle and upper tertiles had a nonsignificant increase in risk. In Millikan and colleagues' study,[139] among the African-American women (292 cases and 270 controls) with the highest PCB levels, there was a borderline significant, modest increase in risk. The PCB-associated risk was more pronounced among African-American women with the highest body mass index. A similarly increased risk among heavier women was also observed for Caucasian women, although the estimate was not statistically significant.

Women with a variant of the cytochrome P4501A1 gene (*CYP1A1*) might be at increased risk of breast cancer if exposed to PCBs. In laboratory studies, PCBs are potent inducers of *CYP1A1*, a drug-metabolizing gene, involved in the activation of potentially toxic endogenous and exogenous substances.[150,151] There is wide interindividual variation in *CYP1A1* activity, and

TABLE 17.9 PCBs and Breast Cancer Risk: Results from Nested Case–Control Studies[a]

Study	Year (Place)	Location	Cohort	Number of Cases/ Controls	Mean PCB Levels		OR Highest vs. Lowest PCB Levels (95% CI)
					Cases	Controls	
Krieger (1994)[143]	1964–1991	United States	Kaiser Permanente	150/150	4.4 ppb	4.8 ppb	0.94 (0.5–1.8)[b]
Hunter (1997)[144]	1989–1990	United States	Nurses	230/230	5.08 ppb	5.16 ppb	0.66 (0.3–1.4)[c]
Høyer (1998)[145]	1976	Denmark	Copenhagen City Heart Study	240/477	1099.89 ng/g		1.11 (0.70–1.77)[c]
Dorgan (1999)[146]	1977–1987	United States	Breast Cancer Detection and Demonstration Project	105/208			0.70 (0.3–1.5)[d]
Helzlsouer (1999)[147]	1974/1989	United States	Washington County, Maryland				
			CLUE I	235/235	4.9 ng/mL	4.7 ng/mL	0.68 (0.4–1.3)[c]
			CLUE II	105/105	2.1 ng/mL	2.2 ng/mL	0.73 (0.4–1.5)[b]
Wolff (2000)[148]	1985–1991	United States	Breast cancer screening attendees	148/295	5.04 ng/mL	4.97 ng/mL	2.02 (0.8–5.4)[d]

[a]None of the results were statistically significant. Hoyer et al. (1998) presented levels only for cases and controls combined. Dorgan et al. (1999) did not present mean values for cases and controls.

[b]First versus third tertile.

[c]First versus fifth quintile.

[d]First versus fourth quartile.

several genetic polymorphisms exist. Approximately 10 to 15% of Caucasians carry a *CYP1A1* valine-for-isoleucine substitution allele.[152] A difference in enzymatic activity of this variant type compared with the wild type has not been demonstrated,[153] but *CYP1A1* activity is more inducible in lymphocytes with the *CYP1A1* variant genotype than in those with the wild genotype.[154] Greater activity may lead to enhanced carcinogen activation and steroid hormone metabolism and may therefore be related to risk of breast cancer. In Moysich et al.'s[155] study of postmenopausal women, an increased risk of breast cancer among those with the *CYP1A1* variant genotype compared with women without the variant was present among women with PCB levels above the median but not among those with levels below the median. The increase in risk among postmenopausal women with elevated PCB body burden and the *CYP1A1* variant genotype may result from a PCB-mediated enhanced induction of polymorphic *CYP1A1*, leading to increased activation of environmental carcinogens and resulting in the production of reactive intermediates and DNA damage. Thus, by inducing *CYP1A1*, PCBs could trigger the activation of xenobiotics, such as those found in tobacco, into mutagenic compounds.

Demers et al.[138] recently reported that in a case–case comparison, PCB body burden was associated with aggressiveness of disease. Breast cancer patients with the highest blood concentrations of PCB 153 were twice as likely to have lymph node involvement as were patients with the lower concentrations, which suggests that PCBs and other organochlorines play a role in disease progression. A biological mechanism for such an effect has not been established, although the authors suggested that these compounds may contribute to disease progression by mimicking or antagonizing the effects of endogenous sex hormones.

17.9.5 Summary of Results in Breast Cancer Studies

Overall, the epidemiologic data on background-level PCB exposure in relation to breast cancer risk indicate no relation. Although a relation has been reported among several subgroups, such as African-American women, the importance of the associations will depend on whether they are replicated.

17.10 OTHER CANCERS

Serum PCB levels among subjects with pancreatic cancer have been compared with levels among controls in two studies (Table 17.10).[156,157] In both studies, mean levels among cases were higher than among controls. Complications of pancreatic cancer could easily cause cases' tissue levels of PCBs to increase [e.g., due to contraction of the body lipid compartment (wasting) or interference with PCB excretion due to biliary obstruction]. In the Spanish study,[156] cases whose tumors had a K-ras mutation ($n = 34$) had higher PCB levels than

TABLE 17.10 Dietary Exposure Studies: Summary of Epidemiologic Data on Associations between Levels of PCBs and Risk of Selected Cancers

Study	Location	Case–Control Design	Number of Cases/Controls	Association[a]	Comments
Pancreatic cancer					
Porta et al. (1999)[156]	Spain	Hospital-based	51/26	↑±	Increased PCB 180[b]
Hoppin et al. (2000)[157]	California	Population-based	108/82	↑	
Non-Hodgkin's lymphoma					
Hardell et al. (1996)[158]	Sweden	Hospital-based	28/17	↑±	
Rothman et al. (1997)[32]	Maryland	Nested	74/147	↑	

[a]±, Increased relative risk associated with higher PCB levels not statistically significant.
[b]Among cases.

those of cases without this mutation ($n = 17$). Exposure levels among subjects in Spain[156] were higher than in those from the San Francisco area.[157]

One retrospective and one prospective study of PCB levels and non-Hodgkin's lymphoma have been reported.[32,158] For the retrospective study, as for pancreatic cancer, the possibility that disease affected PCB levels suggests caution in interpreting the association as causal. Results from the prospective study, however, are especially intriguing because a clear dose–response relation was seen and because the population had a relatively high level of background exposure. Furthermore, because PCBs cause immune dysregulation experimentally,[159] it is plausible that they might cause a malignancy in immune system cells. In a case–control study of endometrial cancer (90 cases and 90 controls), PCB serum levels were unrelated to occurrence of disease.[160]

17.11 OCCUPATIONAL EXPOSURES

17.11.1 Cancer

The mortality from specific cancers among capacitor and transformer workers has been reported in eight cohorts (Table 17.11). With the number of subjects with the same type of cancer summed across studies, the limited power of these data to detect associations was clear, especially when considering that not all cohort members were necessarily highly exposed to PCBs. The methods used to summarize these data have been presented elsewhere.[120] The standardized mortality ratio across studies for melanoma was increased (Table 17.11). Of the two studies with the most cases of melanoma, Sinks and colleagues[166] found no risk gradient with duration of exposure, cumulative exposure, or time since first employment; Kimbrough et al.[164] did not present results specifically for melanoma to address these issues.

The relation between PCB exposure and cancer mortality has also been evaluated in studies of other types of workers, who were probably less exposed to PCBs than were the capacitor and transformer workers.[164] Loomis et al.[169] found that among electrical utility workers, hours of exposure to PCBs showed a dose–response relation with the relative risk of melanoma, and the association was stronger when exposure levels 20 or more years in the past were considered. Emmett et al.[170] reported two incident cases of melanoma among a small group of PCB-exposed transformer repair workers and none in an unexposed comparison group. Among PCB-exposed workers in a petrochemical plant, Bahn et al.[171] reported an increased standardized mortality ratio (SMR) for melanoma. DeGuire et al.[172] reported an increased SMR for malignant melanoma among telecommunications workers employed at a plant where PCBs were used, but the confidence interval was wide. Magnani et al.[173] also found an association between PCB exposure and melanoma among workers from various industries, and the confidence interval was also wide. In other studies of mortality among electrical and petrochemical workers,

TABLE 17.11 Observed (O) and Expected (E) Number of Cancer Deaths among PCB-Exposed Capacitor and Transformer Workers in Eight Cohorts, for Selected Sites[a]

	Rectum ICD-9: 154		Liver, Biliary 155–156		Pancreas 157		Mela-noma 172		Breast 174		Prostate 185		Kidney 189		Brain 191–192		Lym-phoma[c] 200–203	
First Author[b]	O	E	O	E	O	E	O	E	O	E	O	E	O	E	O	E	O	E
Capacitor workers																		
Bertazzi[161]	0	0.41	2	0.4	2	0.43	0	0.14	2	1.99	1	0.47	0	0.20	2	0.28	5	1.36
Brown[162]	4	1.9	5	1.9	2	3.7	1	1.49	9	11.56	1	1.48	2	1.52	0	2.73	5	4.44
Gustavsson[163]	na	na	1	0.51	na	na	0	0.39	na	na	1	1.48	1	0.54	na	na	1	0.39
Kimbrough[164]	10	7.7	5	6.2	22	18.7	12	8.1	31	35.7	15	16.2	5	8.2	8	12.0	14	14.2
Nicholson[165]	2	1.20	0	0.55	1	2.02	1	0.56	5	3.76	2	2.3	1	0.78	0	0.97	2	1.94
Sinks[166]	1	1.2	1	0.8	2	2.8	8	2.0	2	3.97	1	2.3	2	1.5	5	2.8	5	4.35
Transformer workers																		
Liss[167]	na	na	0	0.5	1	1.37	0	0.38	1	0.23	5	1.2	3	0.6	4	0.8	3	1.3
Yassi[168]	0	0.82	0	0.70	8	1.21	2	0.66	0	0.01	0	1.24	1	0.73	9	2.92	7	4.4
Total	17	13.2	14	11.6	38	30.2	24	13.7	50	57.2	26	26.7	15	14.1	28	22.5	42	32.4
SMR	1.3		1.2		1.3		1.7		0.9		1.0		1.1		1.2		1.3	
(95% CI)	0.7–2.1		0.7–2.0		0.9–1.7		1.1–2.6		0.6–1.2		0.6–1.4		0.6–1.8		0.8–1.8		0.9–1.8	

[a]Data for males and females combined, except for cancer of the breast and prostate. na, not available.

[b]The data for Liss[167] are for those known to be exposed. The data for Yassi et al.[168] are for those employed for more than 3 months, with acceptance criteria 1, 2, and 3 (see Yassi et al.[168] for an explanation of the criteria). The cases classified as melanoma for Nicholson et al.[165] and Brown et al.[162] might have been nonmelanoma skin cancer, although this is unlikely. For all studies except Sinks et al.[166] and Kimbrough et al.,[164] ICD 173 (nonmelanoma skin cancer) was included when calculating the expected number of deaths (see Nicholson et al.[165]). Also, the subjects in Brown et al.[162] overlapped with those in the Kimbrough et al.[164] study. When the SMR across all eight studies was recalculated excluding the "melanoma" cases from the Brown and Nicholson studies, the results were still statistically significant (two-sided $p < 0.05$).

[c]For Kimbrough, data shown are for "other lymphatics and hemapoetic" malignancies.

increased rates of melanoma have been found, but exposure specifically to PCBs was not examined.[174,175]

Taken together, the data on occupational PCB exposure and risk of melanoma remain inconclusive, yet the relative consistency of the findings across studies is notable. Additional data that address dose–response and induction period and that examine the site of the lesions would be of particular interest. The SMR for total cancer mortality in the eight capacitor–transformer worker cohorts was 0.9 (not shown in Table 17.11).

17.11.2 Cardiovascular Disease

The SMR values for cardiovascular disease reported for the capacitor and transformer worker cohorts were: Bertazzi et al.,[161] 0.80; Brown,[162] 1.04; Gustavsson and Hogstedt,[163] 1.12; Kimbrough et al.,[164] 0.76; Sinks et al.,[166] 0.70; Liss,[167] 0.89; Yassi et al.,[168] 0.84. The International Classification of Disease codes included in the definitions of cardiovascular disease varied across studies, so an overall standardized mortality ratio was not calculated. The Kimbrough et al.[164] study was by far the largest. Cardiovascular disease SMR values are decreased by the healthy worker effect,[176] so there could be a modest effect of PCBs on the cardiovascular disease rates even with SMR values below 1. Nonetheless, these data provide little support for an effect of occupational PCB exposure on risk of cardiovascular disease.

17.11.3 Diabetes and Glucose Metabolism

Of the eight cohorts of capacitor and transformer workers (Table 17.11), only Kimbrough et al.[164] reported results for diabetes mellitus and from those results an overall standardized mortality ratio of 0.64 was calculated. Of the studies of occupational PCB exposure in relation to clinical blood test results,[177–184] Chase et al.[180] measured glucose but presented no results for it. Emmett,[183] however, found a PCB–glucose association that was rendered statistically insignificant after adjustment for serum lipids.

17.11.4 Liver Function Abnormalities

Subtle elevations of serum enzymes of hepatic origin, especially γ-glutamyl transferase (GGT), were a frequent finding among the PCB-exposed capacitor and transformer workers.[179–183] In those studies, however, the particular PCB mixture used, the level of exposure, the degree of contamination with PCDFs, and the concurrent exposure to other compounds varied. Whether this alteration merely reflects induction of liver enzymes and whether it has clinical significance is not clear. Because of the relatively small size of the capacitor and transistor worker cohorts exposed to PCBs, few investigators have reported findings for death from liver cirrhosis. SMR values for cirrhosis of 107,[162] 61,[164] and 9,[166] however, have been observed.

17.11.5 Induction of the P450 System

For many xenobiotics, the first step in metabolism is catalysis by the P450 enzyme system, primarily in the liver. Continued exposure to a given xenobiotic can cause an increase in the levels of the P450 enzyme(s) that metabolize the agent. The resulting increase in P450 enzymes can affect metabolism of related compounds (possibly endogenous), thus causing—at least in theory—effects other than those due to direct action of the xenobiotic that induced the enzyme(s).

The half-life of antipyrine, a drug metabolized by the P450 enzyme system like phenobarbital, was studied in PCB-exposed workers and controls and was found to be 50% shorter among the exposed.[185] A less dramatic but similar decrease was found in a second group of workers exposed to high-molecular-weight PCBs.[183]

17.11.6 Serum Lipid Levels

A frequent assumption in studies of the health effects of environmental exposure to PCBs is that levels are determined, in part, by the concentration of lipids in serum. This assumption is reasonable only if PCBs do not affect lipid levels. Therefore, we identified studies of workers occupationally exposed to PCBs in which serum lipid levels were compared with those for a group of unexposed subjects. In both studies identified,[180,183] the average levels of triglycerides and total cholesterol were not higher among the exposed workers. Although other studies have reported associations between serum lipid levels and serum PCB levels,[180–183] they are probably due to the partitioning of PCBs into serum lipids.

17.11.7 Thyroid Axis

In men occupationally exposed to PCBs,[183] serum thyroxine levels were 6% lower than in an unexposed group. In a multivariate model among the subjects in the same study, thyroxine levels were inversely related to PCB levels, but the relation was not statistically significant.

17.11.8 Immune System

Among workers with high-level exposure to PCBs studied by Emmett et al.,[186] the cutaneous delayed hypersensitivity response to mumps and trychophyton was found to be no different than in a control group. In another group of workers, PCB levels were related to higher lymphocyte counts when PCB exposure was ongoing; in addition, monocytosis was noted and was greater after exposure had ended.[182] Among another group, however, Maroni et al.[179] found that "... blood count [was] within the normal range in all workers."

17.11.9 Dermatologic Abnormalities

Studies of skin abnormalities in relation to occupational exposure to PCBs have been supportive of a relation but were often weakened by lack of a control group[177,179] or by small sample size.[170,178,180] Nonetheless, several skin abnormalities in addition to chloracne appear to be caused by occupational exposure to PCBs. For example, 10% of capacitor manufacturing workers had hyperpigmentation,[177] a prevalence presumably higher than in the general population. More important, Fischbein et al.[177] showed that workers with skin abnormalities had higher plasma PCB levels than did those without abnormalities. James et al.[187] have pointed out that at least one early report of a relation between PCB exposure and chloracne[188] could have been due to contamination with polychlorinated dibenzofurans, a more potent chloracnegen. High blood levels of PCBs are not always associated with chloracne. In any event, given the consistency of data that implicate aromatic chlorinated compounds as a cause of chloracne, a causal role for PCBs is highly plausible.

17.11.10 Reproductive Outcomes

Taylor et al.[189] examined birth weight and gestational age among offspring of female workers with and without direct exposure to PCBs. Maternal serum PCB levels were estimated on the basis of work history and an independent calibration study. Estimated serum PCB level was associated with lower birth weight and shorter gestation. The birth weight effect was mediated, at least in part, by the effect on length of gestation. The effects observed were small. Taylor et al.[189] noted that the dose–response relation implied by their data, if applicable to lower exposure levels, suggested that a serum PCB level increase from 10 to 20 μg/L PCBs would be associated with a birth weight decrease of 23 g. A typical birth weight is roughly 3500 g.

Among PCB-exposed transformer repair workers, sperm counts were nearly identical to those in an unexposed comparison group.[186]

17.11.11 Neurologic Abnormalities

Altenkirch et al.[190] reported three cases of a polyneuropathy among three men highly exposed to transformers filled with low-chlorinated PCBs; two of the subjects also did poorly on cognitive examination. A PCB level measured in one subject was only modestly elevated, but because exposure was primarily to di- and trichlorinated biphenyls, metabolism and excretion may have been relatively rapid.

17.11.12 Other Health Effects

An association between PCB exposure and decreased forced vital capacity was reported by Warshaw et al.[191] Emmett et al.[186] reported that there was no

association with forced vital capacity and that an association with forced expired volume (1 s) was no longer present after adjusting for smoking.

17.11.13 Summary of Findings from Occupational Studies

Occupational exposure to PCBs has consistently been associated with abnormal liver function tests and chloracne. The data relating occupational exposure to decreased weight of offspring at birth and shorter gestational age are suggestive. The data linking occupational PCB exposure to melanoma was fairly consistent but was based on a small number of cases.

17.12 OTHER OUTCOMES

The waterways adjacent to Triana, Alabama were found to be contaminated with PCBs during an investigation of exceptionally high DDT levels in the area and populace.[47] As part of the investigation of the potential health consequences of the local DDT contamination, PCB levels were measured in 458 subjects. PCB levels were associated with increased blood pressure.[47] The geometric mean serum PCB level among those subjects was 17.2 μg/L, measured by a Webb–McCall approach. Although Kreiss et al.[47] commented that the PCB levels in their subjects "... fall within the range seen in other communities in the United States," few data were available for comparison in 1981. The level of exposure may well have been relatively high.

In Triana, PCB levels were also associated with increased levels of serum GGT and serum cholesterol. GGT is a marker of cholestasis, a process that may decrease excretion of PCBs; thus, the direction of causality is not clear. Somewhat different concerns hold for the serum cholesterol association. Because PCBs are lipid-soluble, one would expect levels of serum lipids and PCBs to be correlated.

Among women who underwent laparoscopy (70 with endometriosis and 86 without), PCB levels were not associated with the presence of that disease.[192]

17.13 CONCLUSIONS

In populations with low-level PCB exposure, PCB levels were generally not related to the level of TSH, the most sensitive indicator of hypothyroidism in humans. In several studies, however, other measures of thyroid economy were associated with PCB levels, although the associated measure varied. With respect to data on background-level PCB exposure and neurodevelopment, despite the inconsistencies in specific findings, overall the data leave open the possibility that adverse effects can occur. The number of specific types of lymphocytes was associated with background-level PCB exposure in two small studies. Overall, the epidemiologic data on background-level PCB exposure in

relation to breast cancer risk indicate no relation. Background-level PCB exposure has been associated with cancer of the pancreas and non-Hodgkin's lymphoma, but these data are limited.

Occupational exposure to PCBs has been associated consistently with abnormal liver function tests and chloracne. The data relating occupational exposure to decreased weight of offspring at birth and shorter gestational age are suggestive. The data linking occupational PCB exposure to melanoma, although fairly consistent, were based on a small number of cases.

17.14 SUMMARY

The epidemiologic evidence supporting adverse effects of background-level PCB exposure is not strong. Nonetheless, the data are suggestive but inconclusive regarding alterations in thyroid economy, immune function, neurodevelopment, and non-Hodgkin's lymphoma. Evidence that workers occupationally exposed to PCBs have had life-threatening consequences is also not strong. Yet such exposure does appear to be related to altered hepatic function, adverse dermatologic effects, and possibly increased risk of selected cancers.

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CHAPTER 18

Epidemiological Studies on Cancer and Exposure to Dioxins and Related Compounds

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18.1 SCOPE OF EPIDEMIOLOGY

Any definite assessment of a causal relationship between exposure and disease usually requires epidemiological studies. Randomized trials on humans are rarely achievable but can sometimes be accomplished in a secondary follow-up of, for example, the long-term side effects of drugs. The association between smoking and lung cancer is a classical example of important knowledge discovered by epidemiological studies. Other important epidemiologic achievements include detecting the relationship between asbestos exposure and mesothelioma, arsenic exposure and lung cancer, and vinyl chloride and hemangiosarcoma of the liver. The epidemiologic approach has been criticized because of its nonexperimental nature, but there is no other ethically acceptable way to confirm whether toxicologic observations of an adverse health effect are relevant for humans. However, epidemiological studies must be conducted and evaluated carefully since methodologic limitations may influence the study results as well as the interpretation of the findings.

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Epidemiological investigations focusing on etiologic aspects are essentially of case–control or cohort design. Correlation (or *ecological*) studies regarding incidence or morbidity data in relation to some crude population exposure data are usually not persuasive. In case–control studies data on various exposures are considered among both persons with the disease and others representing the source population for the cases (i.e., the controls). This type of study tends to be the only alternative for rare diseases. The cohort approach, on the other hand, follows a defined population exposed to a certain agent and compares the disease outcome to that experienced by a reference population without exposure to the agent under consideration. It is necessary that the outcome diseases of interest are reasonably common; otherwise, very large cohorts are required to obtain numbers large enough to permit any conclusions.

18.2 EPIDEMIOLOGICAL STUDIES ON DIOXIN-EXPOSED POPULATIONS

Since the 1970s many epidemiological studies have been published on persons exposed to polychlorinated dibenzodioxins (PCDDs), mainly as occurring in various types of pesticides, especially some of the chlorinated phenoxy herbicides. These people being studied have been either producers or users of chemicals in which dioxins might have occurred as impurities. Several studies have been of the case–control design and have involved the quite rare group of malignant tumors known as soft-tissue sarcomas (STSs) but also the more common malignant lymphomas. Studies of these cancer types were initiated because of clinical observations about a possible association. Also, cohorts of pesticide applicators or persons involved in the production of dioxin-contaminated chemicals have been studied.

There have also been several cohort studies of other populations with potential exposure to dioxins, such as workers in paper and pulp production, Vietnam veterans, and members of the general population (e.g., after the Seveso and Yusho accidents; see Chapters 20 and 21, respectively). Presently, several studies have also been presented on cancer risks associated with exposure to related organochlorine compounds, some of which are referred to briefly in this overview.

18.3 CLINICAL OBSERVATIONS

The first report linking human malignant disease with dioxin exposure appeared in 1977 and concerned three male patients with STS treated at the Department of Oncology in Umeå in northern Sweden. All three had been occupationally exposed to phenoxyacetic acids in forestry. Subsequent review of the medical records of the department identified another four cases with exposure to phenoxyacetic acids.[1] At that time there was also some indication

that Swedish railroad workers who sprayed various herbicides, including phenoxy herbicides, suffered an increased risk of cancer.[2,3]

Furthermore, in 1979, 11 patients with non-Hodgkin's lymphoma (NHL) and exposure to phenoxyacetic acids and/or chlorophenols were described by the same Oncology Department as the case report on STS.[4] These various observations instigated two Swedish case–control studies on STS[5,6] and one on malignant lymphoma.[7,8] The results of these studies, described below, seem to have stimulated researchers in many countries to conduct similar investigations. Also a number of case–control studies on pesticide exposure and other cancer types have been reported. Subsequently, also, several cohort studies have been published on workers exposed to dioxins and/or phenoxy herbicides and chlorophenols. The results from these different studies are conflicting in certain respects. Not surprisingly, therefore, a debate on the somewhat discrepant results has followed, not only considering the scientific aspects but also with involvement of economical overtones, since phenoxy herbicides and chlorophenols have gained considerable profit for the chemical industry worldwide.[9]

18.4 CASE–CONTROL STUDIES

18.4.1 Soft-Tissue Sarcoma

Swedish Studies In addition to the above-mentioned investigations performed in the late 1970s, two more case–control studies of STS were conducted by the same research group[10,11] and additional studies have also been conducted in Sweden on STS.[12,13] In Swedish forestry the predominant herbicide exposure until 1977 has been a combination of 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) used to combat hardwoods. In agriculture, the predominant herbicide exposures up to the mid-1990s have been the phenoxyacetic acids 4-chloro-2-methylphenoxyacetic acid (MCPA) and 2,4-D, which have been used extensively in southern Sweden, where one of the Swedish studies was performed.[6]

Regarding chlorophenols, exposure to pentachlorophenols was reported by workers in sawmills, carpentry, and certain other occupations, as well as in leisure-time activities. On the other hand, no subject in the Swedish studies reported exposure to trichlorophenol. This compound is known to have been contaminated by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) but has been used very little in Sweden.

In general, similar methods were used in several of the Swedish studies.[5,6,10–12] The cases were drawn from cancer registers, and the controls were extracted from population registers. In two of the studies,[10,12] a second control group of malignant diseases other than STS was used. In one of these studies,[10] subjects with malignant lymphoma or nasopharyngeal cancer were excluded as controls since these types of tumors were at that time suspected to

be related to the exposure at issue.[7,8,14] Furthermore, for deceased cases, deceased controls were used to equalize exposure assessment. These control subjects were identified from the National Register on Causes of Death. The next-of-kin were traced by contacting the parishes where cases and controls were registered at the time of their deaths.

In the various studies referred to, an extensive self-administered questionnaire was used to obtain a complete working history for each case and control. Questions were asked as to specific job categories and exposures, smoking habits, leisure-time exposure to chemicals, and so on. These detailed questions elicited a broad exposure history and are therefore also likely to have obscured the hypotheses under investigation from the study subjects, so as not influencing the reporting of exposure. If the answers were unclear or incomplete, the respondent was phoned for further information by an interviewer.

In some of the Swedish studies,[5,6,10,11] a minimum exposure time of 1 day was required for subjects to be classified as having been exposed to phenoxyacetic acids or chlorophenols. Because of the generally suggested latency time applied in studies of chemical carcinogenesis, all exposure to these substances within 5 years before the diagnosis was excluded. Exposure to chlorophenols was classified into high grade—1 week or more continously or at least 1 month totally over the years—and low grade, with less exposure than that.

All four most similarly designed studies on STS[5,6,10,11] demonstrated an association between STS and exposure to phenoxyacetic acids or chlorophenols, as shown in Table 18.1, where the results of all Swedish soft-tissue sarcoma studies are presented. The odds ratios in the two later studies were somewhat lower than in the earlier studies. This may partly reflect a later time period of exposure for the subjects, with a possibility of other exposure conditions (e.g., less dioxin-contaminated phenoxy herbicides).

Another above-mentioned case–control study from southeastern Sweden enrolled 96 cases with STS, 450 randomly selected population controls, and 200 cancer controls.[12] Increased odds ratios (ORs) were obtained for gardeners

TABLE 18.1 Odds Ratios and 95% Confidence Intervals (in Parentheses) in Swedish Case–Control Studies on Soft-Tissue Sarcoma[a]

Study	Chlorophenols[b]	Phenoxyacetic Acids	Ref.
STS I	6.6 (2.4–18)	5.3 (2.4–12)	5
STS II	3.3 (1.3–8.1)	6.8 (2.6–17)	6
STS III	nc	3.3 (1.4–8.1)	10
STS IV	5.3 (1.7–16)	1.3 (0.7–2.6)	11
STS V		1.6 (0.8–3.3)[c]	12
STS VI	na	2.4 (0.8–7.0)	13

[a] nc, not calculated since few were exposed; na, exposure to chlorophenols not assessed.

[b] Only high-grade exposure is presented.

[c] 90% Confidence interval.

(OR = 4.1, 90% CI = 1.0 to 14), railroad workers (OR = 3.1, 90% CI = 0.6 to 14), construction workers with exposure to impregnating agents (OR = 2.3, 90% CI = 0.5 to 8.9), and unspecified workers with potential exposure to phenoxy herbicides and/or chlorophenols (OR = 1.7, 90% CI = 0.3 to 7.3). Notably, the classification of exposure in this study was relying mainly on occupational categories.[12]

Still another case–control study was performed as part of the Scandinavian Joint Care Program on STS.[13] It included 79 cases from Sweden and Finland and 226 controls, both males and females. Only cases with histopathologically high-grade STS were included. Exposure to phenoxyacetic acids yielded OR = 2.4 (95% CI = 0.8 to 7.0). No information was given regarding the various types of phenoxy herbicides, and chlorophenol exposure was not assessed.

Meta-analysis on STS and Potential Dioxin Exposure The most similarly designed Swedish case–control studies on STS[5,6,10,11] were aggregated for a meta-analysis and the results are presented in Table 18.2. The data were stratified by study, and only population controls were included in the analyses presented in the table (i.e., controls with other types of malignant diseases as used in one of the studies in addition to population controls were excluded).[10] It may also be remarked that the exposure to dioxins was assessed through exist-

TABLE 18.2 Mantel–Haenszel Odds Ratios (OR) and 90% Confidence Intervals (CI) Adjusted by Study for STS among Persons in Four Case–Control Studies

		Exposed	
	Unexposed	< 1 Year	≥ 1 Year
All dioxins			
Cases	352	58	24
Controls	865	74	9
OR	1.0	2.4	6.4
CI		1.7–3.4	3.5–12
TCDD			
Cases	352	40	6
Controls	865	39	2
OR	1.0	3.0	7.2
CI		2.0–4.5	2.6–20
Other dioxins			
Cases	352	18	18
Controls	865	35	7
OR	1.0	1.7	6.2
CI		0.98–2.9	2.9–13

Source: Data from Ref. 15.

[a]Exposed to all dioxins, TCDD, and dioxins other than TCDD; 434 cases and 948 controls. All subjects were exposed for at least 1 day, and a minimum latency period of 5 years was used.

ing knowledge about the contamination of phenoxyacetic acids and chlorophenols by different isomers. An increased risk for STS was associated with exposure to all dioxins (i.e., both for TCDD and dioxins other than TCDD). Furthermore, a dose–response effect for duration of exposure was obtained with a significant trend ($p < 0.001$). Although meta-analyses can be criticized on methodological grounds, it may be justified to use this approach here in order to obtain a summary view of these studies along with some information on dose–response relationships.

Comments on Design Issues Epidemiologic studies indicating new and unexpected associations between a disease and some specific exposure are usually subject to much criticism. The Swedish studies discussed here are no exception in this respect and as focusing on economically important compounds, criticism from representatives for the chemical industry is well understandable.[16–18] A good deal of this criticism has involved issues such as inappropriate choice of controls, recall and interviewer bias, and confounding. It may therefore be appropriate here to elucidate some of the methodological matters involved in these studies and the validity of the results presented.

Case–control studies using questionnaires or interviews for assessment of exposure usually attract criticism suggesting recall or observational bias. Hence, the underlying assumption is that cases might remember various exposures better than controls because of their cancer diagnosis, especially exposures subject to some concern regarding the health effects. It is unlikely, however, that there at a certain point in time should be any difference in this respect between studies considering various exposures as being associated with cancer. It is worth noticing, therefore, that two case–control studies on colon cancer,[19,20] performed in the same area in the same time period and with the same methodology as the studies debated, did not show increased risk for exposure to phenoxyacetic acids or chlorophenols.

Observational bias might, theoretically, have been introduced in the initial three Swedish studies[5–7] by the fact that no special precautions were taken to prevent the telephone interviewer from knowing the case–control status during the supplementary telephone calls. However, in an analysis based on questionaire information only, there was no substantially different result.[19] In two of the later studies on STS,[10,11] all telephone interviews and all coding of data collected were done blinded with respect to the case–control status of the subjects, although some persons may have revealed their status during the interviews. Furthermore, when the exposure was assessed mainly from occupation,[12] an effect was also observed, although weaker, as should be expected when exposure information is less specific.

Another question of concern is the matter of *confounding*: the introduction of a spurious association by some causative agent associated with the exposure in the study population, as not adequately controlled for in the design or analysis of the study. This other agent would then necessarily have to exert a stronger effect, or at least an equally strong effect as that appearing to be asso-

ciated with the exposure under study. No such strong confounding factor has ever been identified in connection with STS and exposure to phenoxy herbicides or chlorophenols, however, although the question of confounding has been echoing through the years.[16,17,21] The association observed with the exposure is therefore very unlikely to result from confounding.

The criticism of the Swedish studies has been met on several occasions,[9,22,23] and it may be noted that the U.S. National Academy of Sciences has concluded: "Although these studies have been criticized, the committee feels that there is insufficient justification to discount the consistent pattern of elevated risks, and the clearly described and sound methods employed."[24] Despite this, the same arguments casting doubt on the validity of the studies are still echoed in a recent publication.[25]

U.S. Studies

Kansas Study Researchers from the U.S. National Cancer Institute have conducted a case–control study in Kansas investigating the role of herbicides in relation to STS (and malignant lymphoma; see below). This study found no association between the phenoxyacetic acid herbicide 2,4-D and STS.[26]

Washington State Study This case–control study included 128 cases of STS, 576 of NHL (see below), and 694 controls.[27] The exposure assessment was based on categorizing job titles, activities, and chemical preparations by potential exposure reported during interviews. Therefore, there may be fairly objective but less specific exposure information compared with other case–control studies, where subjects themselves named specific exposures. No association with STS was found in the analyses based on the assessment of potential exposure to phenoxyacetic acids or chlorophenols. The report provides limited information on the exposure to TCDD and related compounds.

However, in light of the results in the Swedish studies, it is interesting that this study showed elevated risks for STS among exposed persons with Scandinavian surnames. Thus, high estimated potential for phenoxy acid exposure gave an OR of 2.8 (95% CI = 0.5 to 15.6), and for chlorophenol exposure an OR of 7.2 (95% CI = 2.1 to 24.7). Another interesting finding was elevated relative risks associated with self-reported histories of chloracne, which gave an OR of 3.3 (95% CI = 0.8 to 14.0) for STS.

Selected Cancers Cooperative Studies With the focus on the effects of exposure to Agent Orange and other possible health hazards among military personel in the Vietnam conflict, a series of case–control studies have been conducted. The study on STS included 342 STS and other sarcoma cases and 1776 control subjects obtained through random-digit dialing.[28] Restriction of analysis to the 254 STS cases yielded OR = 0.9 (95% CI = 0.5 to 1.6) for Vietnam veterans. However, only 26 STS cases had been stationed in Vietnam or off the coast of Vietnam.

Although rather detailed information was obtained about location in Vietnam, job duties, and self-perceived exposure to pesticides, there seems to be quite some uncertainty as to whether or not a person was really exposed. Moreover, the results were based on only 26 cases and 133 controls who had served in Vietnam.

To evaluate specifically the association of chlorophenol exposure independent of phenoxy herbicides within this study group, a subset of 295 male STS cases was used. The STS risk was associated significantly with ever having high-intensity chlorophenol exposure, OR = 1.79 (95% CI = 1.10 to 2.88). For subjects with 10 or more years of substantial exposure, the OR was 7.78 (95% CI = 2.46 to 24.65).[29]

Further analyses on this study regarding occupational risk factors for sarcoma subtypes showed an association between herbicides (not further specified) and malignant fibrohistiocytic sarcoma (OR = 2.9, 95% CI = 1.1 to 7.3).[30] For exposure to chlorophenols, the increased ORs were found independent of STS subgroup.

New Zealand Studies In New Zealand a research group has performed two studies on STS.[31,32] All controls were patients diagnosed with other cancers. This is essential to remember when interpreting the results, because more recent studies indicate a certain degree of general carcinogenic effects by dioxins.

Although these studies seem to have been initiated as a consequence of the findings obtained in Sweden, the cases were not fully comparable with those in some of the Swedish studies[5,6,10,11] because patients with STS in parenchymatous organs such as the stomach were not included. Notably, the Swedish cases with these locations of disease comprised about 40% of the total.

Exposure of the subjects was categorized as "potential," "probable," or "definite" (e.g., based on job titles, activities, or information on specific chemicals, which should be expected to decrease any existing risks by dilution of exposure).

The first New Zealand STS study[31] included 82 cases and 92 controls. It showed ORs between 1.3 and 1.6 for phenoxyacetic acids and chlorophenols, with the higher ratios if only probable or definite exposure of more than 1 day and at least 5 years of latency was included. The risk increased to 3.0 (95% CI = 1.1 to 8.3) if only exposure among farmers was considered. Moreover, work within tannery or meat workers pelt departments with potential exposure to chlorophenols gave an OR of 7.2.

The second New Zealand study on STS[32] encompassed 51 cases and 315 controls, which were derived from a parallel study on NHL[33,34] (see below). In contrast to the first study, this study yielded an OR of 0.8 (95% CI = 0.3 to 1.9) for exposure to phenoxyacetic acids. The report on this study was very brief, however, and no details were given on chlorophenol exposure, and no OR restricted to farmers can be calculated with the data available.

A somewhat strange pattern in the control groups in the New Zealand studies is the high percentage of railway workers (i.e., 7.6 to 12%). This may

raise the question of whether the controls represent the source population for the cases.[35] There was a remarkable and unexplained increased risk ratio for railway workers of 3.2 with regard to STS in the first study.[31]

The authors of the New Zealand studies discuss the fact that herbicide spraying is a full-time occupation in that country and that none of the STS or NHL (see below) cases had occurred among the approximately 1500 current and former commercial sprayers. However, only about 0.1 case would be expected among the commercial sprayers in each of the two STS studies, and about 0.3 case among the commercial sprayers in the NHL study as based on available incidence data.[36] Therefore, the absence of commercial sprayers among the cases is hardly evidence against the association. In fact, one case of STS occurred in a commercial sprayer in 1984 (i.e., 2 years after the end of recruitment to the second New Zealand STS study; letter dated January 15, 1984, Tasman Pulp and Paper Company Ltd, Medical Centre, Kawerau, New Zealand).

Italian Study An Italian case–control study on STS was published in 1986.[37] It included 37 male and 31 female cases, and 85 male and 73 female controls. The study was conducted in a region of northern Italy where the principal agricultural crop is rice. In this area rice weeding was a predominantly female occupation involving manual labor and therefore potential for exposure to phenoxy herbicides, mainly by dermal contact. Rice weeding among women was associated with a relative risk of 2.3 (95% CI = 0.7 to 7.7) in the study. No increased risk was seen in men.

Australian Study A rather small study on STS encompassing 30 males with STS (and cases with malignant lymphoma, see below) with one population control and one cancer control for each case has been published.[38] Definite or probable exposure to phenoxy herbicides or chlorophenols yielded an OR of 1.0 (95% CI = 0.3 to 3.1). For more than 30 days exposure the risk for STS increased to OR = 2.7 (95% CI = 0.7 to 9.6).

18.4.2 Malignant Lymphoma Studies

Swedish Studies A case–control study on malignant lymphoma was published in 1980. This study, which included both Hodgkin's disease (HD) and NHL,[7,8] was performed using the same methodology as in the STS studies by the same group.[5,6,10,11] The lymphoma study included 60 HD cases, 105 NHL cases, 4 unclassifiable lymphoma cases, and 335 controls. Statistically significant ORs were found for exposure to phenoxyacetic acids or chlorophenols (Table 18.3) as demonstrated also in further analyses for both HD[8] and NHL.[39] Regarding NHL, exposure to phenoxyacetic acids yielded an OR of 5.5 (95% CI = 2.7 to 11). High-grade exposure to chlorophenols resulted in OR = 9.4 (95% CI = 3.6 to 25) and low-grade exposure OR = 3.3 (95% CI = 1.6 to 6.8).

TABLE 18.3 Odds Ratios and 95% Confidence Intervals (in Parentheses) in Swedish Case–Control Studies on Malignant Lymphoproliferative Diseases

Study	Chlorophenols[a]	Phenoxyacetic Acids	Refs.
HD + NHL	8.4 (4.2–17)	4.8 (2.9–8.1)	7
−NHL	9.4 (3.6–25)	5.5 (2.7–11)	7, 39
−HD	6.5 (2.2–19)	5.0 (2.4–10)	8
NHL	nc	4.9 (1.3–18)[b]	40
HD	nc	3.8 (0.7–21)[b]	40
NHL	1.1 (0.7–1.8)[c]	1.5 (0.9–2.4)	42
Hairy cell leukemia	2.6 (1.1–6.2)[d]	2.7 (1.3–5.7)	43
Multiple myeloma	1.1 (0.6–1.9)[b,c]	2.2 (1.1–4.7)	51

[a] Only high-grade exposure is presented; nc, not calculated since few were exposed.

[b] 90% Confidence interval.

[c] All chlorophenol exposure.

[d] Pentachlorophenol.

Another case–control study on 54 cases of HD, 106 NHL cases, and 175 referents also indicated an excess risk from exposure to phenoxyacetic acids.[40] By logistic regression analysis, increased OR values were obtained for phenoxyacetic acids and HD (OR = 3.8, 90% CI = 0.7 to 21) as well as NHL (OR = 4.9, 90% CI = 1.3 to 18). Interestingly, farming as such appeared to protect against NHL, implying negative confounding. This protective effect may help explain why no excess risk for NHL was seen on a rather crude cohort basis in a study on Swedish farmers: that is, because of the lack of data on exposure to different agents in that study.[41]

More recently, a new case–control study from Sweden further analyzed the association between phenoxy herbicides or chlorophenols and NHL (Table 18.3).[42] The study included 442 male cases and twice as many population-based controls. Increased risk was found for exposure to herbicides and fungicides. Regarding phenoxy herbicides an OR of 1.5 (95% CI = 0.9 to 2.4) was obtained with increased risk both for MCPA (OR = 2.7, 95% CI = 1.0 to 7.0) and 2,4-D + 2,4,5-T (OR = 1.3, 95% CI = 0.7 to 2.3). The highest OR was calculated for exposure to phenoxyacetic acids during the 1970s (OR = 2.8, 95% CI = 1.3 to 5.6) and 1980s (OR = 4.0, 95% CI = 1.2 to 13), whereas no increased risk was found for exposure during the 1940s and 1950s. Chlorophenols were banned in Sweden in 1977, and no significantly increased risk was found for exposure to these substances, although an effect of the tumor induction period was shown also for these chemicals with highest risk for the most recent exposure. Thus, these results suggest a rather short malignant lymphoma induction period for exposure to these chemicals. Another conclusion from this study seems to be that restriction of the use may have had a preventive effect on NHL.

Hairy cell leukemia is a rare subtype of NHL with a marked male predominance. A recent Swedish study encompassing 121 cases with hairy cell leukemia and 484 controls found an increased OR of 2.7 (95% CI = 1.3 to 5.7) for exposure to phenoxy herbicides.[43] Exposure to pentachlorophenol gave an OR of 2.6 (95% CI = 1.1 to 6.2). Interestingly, these risks were increased further in cases with elevated levels of antibodies to Epstein–Barr virus (EBV) early antigen.[44] EBV is a human herpesvirus with a tropism for B lymphocytes, and the virus is ubiquitous worldwide. EBV has been associated with certain types of NHL, such as Burkitt's lymphoma and lymphomas occurring in immunologically compromised or HIV-infected persons.

U.S. Studies

Kansas Study The Kansas study described above included not only cases with STS but also studied NHL and HD[26] (Table 18.4). This population-based case–control study found a strong association between the phenoxyacetic acid herbicide 2,4-D and NHL but no association between herbicide use and HD. Among 2,4-D users, the risk of NHL increased with frequency of herbicide exposure to an OR of 7.6 (95% CI = 1.8 to 32) for farmers exposed more than 20 days per year (Table 18.4). Only three cases and 18 controls reported use of 2,4,5-T, and all but two of these controls has also used 2,4-D.

Nebraska Study In Nebraska, a population-based case–control study of NHL also found an association with 2,4-D.[45] Risks were lower than in Kansas, but the patterns were similar. Risk increased with frequency of exposure to an OR of 3.3 among farmers exposed more than 20 days per year (Table 18.4). Among 2,4-D users, the risk also increased the longer the farmers continued to

TABLE 18.4 Number of White Male NHL Cases and Controls and Odds Ratios of Exposure to 2,4-D in Kansas and Nebraska

State	Days/Year	Number of NHL Cases	Number of Controls	Odds Ratio
Kansas[a]	0	37	286	1.0
	1–2	6	17	2.7
	3–5	4	16	1.6
	6–10	4	16	1.9
	11–20	4	9	3.0
	21+	5	6	7.6
Nebraska[b]	0	54	184	1.0
	1–5	16	44	1.2
	6–20	12	25	1.6
	21+	3	4	3.3

[a] From Ref. 26; *p*-value for trend, 0.0001.

[b] From Ref. 45; *p*-value for trend, 0.051.

work in potentially contaminated clothing after pesticide application. For those farmers who changed clothing immediately, the OR was 1.1. Changing at the end of the day was associated with an OR of 1.5, while waiting to change until the following day or later was associated with an OR of 4.7 (95% CI = 1.1 to 22).

Iowa/Minnesota Study In this study modest nonsignificant increases in risk for NHL were observed for use of 2,4-D or 2,4,5-T.[46] If these agents had been handled at least 20 years prior to the interview, ORs increased to 1.3 (95% CI = 0.9 to 1.8) and 1.7 (95% CI = 0.8 to 3.6) for 2,4-D and 2,4,5-T, respectively. No information on frequency of use was presented.

Washington State Study In this study, as also referred to above regarding STS, elevated risks of NHL were found among men who had been farmers (OR = 1.3, 95% CI = 1.0 to 1.7) or forestry herbicide applicators (OR = 4.8, 95% CI = 1.2 to 19).[27] Furthermore, those potentially exposed to phenoxy herbicides in any occupation for 15 years or more during the period prior to 15 years before diagnosis of NHL also had an increased risk (OR = 1.7, 95% CI = 1.0 to 2.8). Considering self-reported histories of chloracne, the OR was 2.1 (95% CI = 0.6 to 7.0).

Selected Cancers Cooperative Study In a series of studies, as described above regarding STS, 1157 NHL[47] and 310 HD[48] cases were included. Among these, only 99 NHL and 28 HD cases had served in Vietnam, however. For NHL, the study showed an OR of 1.47 (95% CI = 1.09 to 1.97) and for HD an OR of 1.09 (95% CI = 0.62 to 1.91) if men who did not serve in Vietnam were used as the reference. However, no increased risk was found for any reported possible contact with Agent Orange.

Regarding NHL and the association between self-reported possible contact with Agent Orange, the reference group for these comparisons (''other Vietnam veterans'') included blue-water Navy servicemen who were found to be at a significantly elevated risk of NHL. Consequently, the inclusion of these sea-based men in the reference group would depress the relative risk estimates associated with self-reported exposure to Agent Orange.

Italian Study A population-based case–control study demonstrated an increased risk for NHL in female rice weeders with probable 2,4-D and 2,4,5-T exposure in a region of Italy with an OR of 1.9 (95% CI = 0.6 to 6.0).[49]

New Zealand Study This study on NHL[33] consisted of 183 cases and 338 controls. No significantly increased OR for exposure to phenoxyacetic acids or chlorophenols was obtained, but the risk estimates varied between 1.1 and 1.5 between different subgroups. The risk of NHL did not increase with duration of exposure to phenoxy herbicides but did increase to 2.2 (95% CI = 0.4 to 12.6) in the category with a frequency of use of 10 to 19 times per year. Then it dropped off to 1.1 (95% CI = 0.3 to 4.1) for those having used the herbicides

for more than 19 times per year.[34] Some methodological aspects of this study have been discussed above in the context of reviewing the New Zealand STS studies.[31,32,35]

Australian Study This study encompassed 52 males with malignant lymphoma.[38] For definite or probable exposure to phenoxy herbicides or chlorophenols an OR of 1.5 (95% CI = 0.6 to 3.7) was obtained. With more than 30 days' exposure, the OR increased to 2.7 (95% CI = 0.7 to 9.6).

18.4.3 Other Hematological Malignancies

Multiple Myeloma Multiple myeloma is a lymphoproliferative malignancy related to malignant lymphoma. Several reports have described an association with farming.[50] A Swedish case–control study on multiple myeloma[51] found an increased OR for exposure to phenoxyacetic acids but not to chlorophenols. This finding was not supported by a study from Iowa, however, in which a slightly elevated risk was seen for farmers but without any significant association with any class of pesticides.[52]

In an Italian study, an increased risk was found for several classes of pesticides.[53] Herbicide exposure among professionals only yielded an OR of 1.9 (95% CI = 0.5 to 7.6). Similar results were found for several other classes of pesticides. However, the only significantly increased risk was found for chlorinated insecticides.

Leukemia In a study from Iowa and Minnesota, leukemia was associated with only modest nonsignificant increases of risk for 2,4-D and 2,4,5-T exposure.[54] MCPA, however, was associated with an OR of 1.9 (95% CI = 0.8 to 4.3). When the analyses were restricted to persons first exposed at least 20 years before the interview, risks increased for MCPA (OR = 2.4, 95% CI = 0.7 to 2.8) and 2,4,5-T (OR = 1.8, 95% CI = 0.8 to 4.0).

18.4.4 Nasal and Nasopharyngeal Cancer

Nasal cancer has long been associated with exposure to hardwood dust, and different hypotheses regarding the responsible mechanism have been tested in epidemiological studies. However, in one Swedish study of nasal and nasopharyngeal cancer, both malignancies showed a rather strong association with exposure to chlorophenols (OR = 6.7, 95% CI = 2.8 to 16), and a weaker relationship with exposure to phenoxy herbicides (OR = 2.1, 95% CI = 0.9 to 4.7).[14] Chlorophenols have been used as wood preservatives, and exposure to these substances, including contaminating dioxins, has occurred in different occupational procedures where inhalation of sawdust from treated wood cannot be avoided.

In a study from the Selected Cancer Cooperative Study Group in the United States encompassing 43 nasal, 92 nasopharyngeal carcinoma cases, and 1909 controls, high-intensity chlorophenol exposure gave OR = 1.94 (95% CI = 1.03

to 3.50).[55] Among those with over 10 years in jobs assigned high intensity with high certainty of exposure OR increased to 9.07 (95% CI = 1.41 to 42.9). The initial purpose of this study was to investigate Agent Orange exposure in Vietnam.[48] No association was found, but only 2 of 48 included nasal cancer and 3 of 80 nasopharyngeal cancer cases had served in Vietnam.

18.4.5 Liver Cancer

An increase in the incidence of primary liver cancer has been reported from northern Vietnam, and an effect of contact with Agent Orange on the incidence has been postulated.[56] Soldiers who had stayed for more than 10 years in the south of Vietnam had a significantly increased risk for hepatocellular cancer after adjustment for matching variables, hepatitis B antibody status, and alcohol consumption (OR = 8.8, 95% CI = 1.9 to 41).

A case–control study in northern Sweden on primary liver cancer in men[57] yielded ORs of 1.7 (95% CI = 0.7 to 4.4) and 2.2 (95% CI = 0.7 to 7.3) for exposure to phenoxyacetic acids and chlorophenols, respectively. No association with Agent Orange exposure was demonstrated in a study from the Selected Cancers Cooperative Study Group in the United States including 130 liver cancer cases.[48] Only eight of the subjects with liver cancer had served in Vietnam.

18.4.6 Other Cancer Types

Gastric Cancer Supporting an early finding from a Swedish study on railway workers exposed to herbicides,[2,3] a more recent Swedish study found an association between exposure to phenoxy herbicides and gastric cancer.[58] Exposure to a combination of 2,4-D and 2,4,5-T yielded an OR of 1.73 (95% CI = 1.16 to 2.58) and for exposure to MCPA an OR of 1.84 (95% CI = 0.82 to 4.10) was obtained.

Miscellanous Cancers In a Swedish study on occupational risk factors for oral cancer, an increased risk for exposure to phenoxy herbicides was shown (OR = 1.7, 95% CI = 0.8 to 3.5).[59] Regarding colon cancer, no significantly increased risks have been found in those few studies from Sweden[19,20] or the United States[60] that considered exposure to phenoxy herbicides or chlorophenols.

18.5 COHORT STUDIES ON PRODUCERS AND USERS OF DIOXIN-CONTAMINATED CHEMICALS

18.5.1 Some Early Cohort Studies on Pesticide Users

The first studies of workers exposed to various pesticides appeared in the beginning of the 1970s. One of these studies concerned a cohort of 348 railroad

workers who had been spraying various herbicides along the Swedish railroads, especially amitrol, diurone, phenoxyacetic acids, both 2,4-D and 2,4,5-T, and potassium chlorate and monurone.[2] These workers were found to have some excess of malignant tumors, first thought to be related to amitrol exposure as of greater concern at the time for its potential carcinogenecity. In a further analysis, however, the greatest effect turned out to be among those with combined exposure to both amitrol and phenoxy herbicides, with a significantly increased risk of 3.4 for all tumors.[61] For those with exposure mainly to phenoxy herbicides, there were two gastric cancers with only 0.33 expected such cases.

Similar observations appeared from East Germany, where pesticide sprayers, again exposed to a mixture of both phenoxyacetic acids and other herbicides as well as various insecticides, were found to suffer from an excess of lung cancers.[62] However, a Finnish cohort of 1926 men who had been spraying phenoxy herbicides along the roadsides had no increase in total cancer mortality or morbidity.[63,64] Noteworthy in light of the above-referred results on multiple myeloma, the standard mortality ratio (SMR) for this malignancy was increased to 2.63 (95% CI = 0.54 to 7.68). No case of NHL or STS was found in the mortality study. The statistical power was, however, low to detect increased risks for rare tumor types in this cohort.

18.5.2 Later Cohort Studies on Pesticide Manufacturers and Users

Since the 1980s several cohort studies have been set up in Europe and the United States. Some of these cohort studies will be presented in more detail here, depending on their size or some noteworthy results. However, all these cohorts are by now included in an International Agency for Research on Cancer (IARC) pooled analysis,[65,66] discussed below.

Danish Cohort A follow-up study of cancer incidence among workers in the manufacturing of phenoxy herbicides was performed in Denmark.[67] In total, 3390 males and 1069 females were included in the study. In an updated report from this study,[68] 5 cases of STS were observed. Four of these STS cases were exposed to phenoxy herbicides yielding a standard incidence ratio (SIR) of 2.3 (95% CI = 0.6 to 5.8). Three of the cases occurred among men employed for at least one year in one factory. With a 10-year latency period a SIR of 6.4 (95% CI = 1.3 to 18.7) was found in this subgroup. For persons exposed to phenoxy herbicides a SIR of 1.3 (95% CI = 0.4 to 3.3) was reported for NHL.

U.S. Cohort A total of 5172 persons who had been involved in the production of TCDD-contaminated chemicals in any of 12 plants in the United States were included in a cohort established by the National Institute of Occupational Safety and Health (NIOSH).[69] Of these persons, 172 of the cohort members had been incorporated in previously published small cohort studies from two companies, whereas the remaining 5000 subjects in the cohort were identified as "assigned to a production or maintenance job in a process involving TCDD

contamination.'' The follow-up period ended in 1987, and comparisons were made with the U.S. population regarding the expected mortality.

A total of 265 cancer deaths were observed, which gave a SMR of 1.15 (95% CI = 1.02 to 1.30). In a subcohort of 1520 persons with a latency of 20 years or more, and exposure of at least 1 year, the SMR for all cancer was 1.46 (95% CI = 1.21 to 1.76). In the total cohort SMR for STS was 3.38 (95% CI = 0.92 to 8.65), and in the subcohort defined above the SMR was 9.22 (95% CI = 1.90 to 26.95).

Four STS deaths were found in the total cohort. Review of tissue specimens of these cases had been performed previously.[70] Two of the cases were thereby reclassified as not STS. However, erroneous information on death certificates is likely to have occurred in the death certificates of the reference group (i.e., the U.S. population). Thus, this type of directed reanalysis of death certificate diagnosis in only the cohort cases is problematic. It may be noted also that two more STS deaths were found in the NIOSH cohort, but according to classification principles, they were assigned to ICD codes (International Statistical Classification of Diseases and Related Health Problems; WHO) for other sites of malignant diseases and therefore not included in the SMR value for STS. Furthermore, the authors reported another STS death among a group of 139 workers with chloracne who did not meet the entry criteria for the cohort.

The SMR for NHL was 1.37 (95% CI = 0.66 to 2.54), but there was no increased SMR in the subcohort with exposure. The subcohort had an increased risk for respiratory system cancers with an SMR of 1.42 (95% CI = 1.03 to 1.92). To verify TCDD exposure in the cohort, dioxin levels in serum were measured in a sample of 253 persons. The levels of TCDD, adjusted for lipids, correlated well with years of exposure.

The cohort has now been updated with mortality data until 1993.[71] The results were similar to those in the previous report. For all cancer, SMR was 1.13 (95% CI = 1.02 to 1.25). SMR increased in the highest exposure group to 1.60 (95% CI = 1.15 to 1.82). Among other findings in the total cohort, increased mortality was found for multiple myeloma (SMR = 2.07, 95% CI = 0.99 to 3.80), larynx cancer (SMR = 2.22, 95% CI = 1.06 to 4.08), and bladder cancer (SMR = 1.99, 95% CI = 1.13 to 3.23). In the chloracne subcohort ($n = 608$), 3 STS cases were found, with a resulting SMR of 11.32 (95% CI = 2.33 to 33.10).

German BASF Cohort Persons employed at a German chemical manufacturing facility where 2,4,5-trichlorophenol was produced were enrolled in a cohort study after a 1953 reactor accident. [This cohort is not included in the IARC pooled analysis (see below).][72] A total of 247 workers potentially exposed to TCDD were followed from 1953 until 1987. In this mortality study, 78 persons had died at the end of the follow-up period, including 23 deaths from malignant diseases.

The SMR for all malignant diseases was 1.17 (90% CI = 0.80 to 1.66). When workers with chloracne were examined separately, the SMR for all

malignancies was 1.39 (90% CI = 0.87 to 2.11). Considering a latency period of at least 20 years after the first exposure to TCDD, the SMR was increased to 2.01 (90% CI = 1.22 to 3.15) for all malignancies and to 2.52 (90% CI = 0.99 to 5.30) for lung cancer based on five cases. Nonsignificantly increased SMRs were also found for cancer in the buccal cavity and pharynx, stomach, colon, and rectum, although based on very few cases.

No deaths from STS or NHL were reported, but only 0.1 STS case and 0.6 NHL case would be expected based on the NIOSH study data. Thus, the study had low power to detect an increased risk because of the low expected numbers. In a subsequent follow-up,[73] the estimated dose of TCDD was for 69 men $\geq$ 1 μg/kg body weight. Within this high-dose group total cancer mortality was increased $\geq$ 20 years after first exposure with SMR 1.97 (95% CI = 1.05 to 3.36), as was respiratory cancer, with SMR 3.06 (95% CI = 1.12 to 6.66). No case of NHL or STS was found.

German Boehringer Cohort A mortality follow-up of 1583 workers (1184 men and 399 women) employed in a chemical plant in Germany that produced herbicides, including processes contaminated with TCDD, has been reported.[74] The vital status of workers hired between 1952 and 1984 was determined as of 1989. As reference, both the national mortality statistics of West Germany and deaths in a cohort of male gas workers were used. Since the results did not differ by reference group, figures based on the general population mortality statistics are presented here.

The SMR for total cancer mortality was increased to 1.24 in men (95% CI = 1.00 to 1.52). Among men with 20 or more years of exposure the SMR was 1.87 (95% CI = 1.11 to 2.95). In a subgroup with high exposure to TCDD, the SMR for all malignant diseases was 1.42 (95% CI = 0.98 to 1.99) overall and 2.54 (95% CI = 1.10 to 5.00) for persons who had been employed for at least 20 years. The accuracy in the assigning of TCDD exposure was evaluated by analyses of TCDD in adipose tissue from 48 members of the cohort.

Increased SMRs for men were found for malignant diseases in the hypopharynx, esophagus, stomach, larynx, lung, prostate, kidney, and hematopoetic system. These results were based on comparatively small numbers, however, and are not significant. Among women, 20 deaths from malignant neoplasms were found, corresponding to an SMR of 0.94 (95% CI = 0.58 to 1.45). Only the risk for breast cancer was elevated, with an SMR of 2.15 (95% CI = 0.98 to 4.09) based on nine deaths.

An investigation of dose–response relationships for total cancer and estimated blood levels for TCDD and TEQ using data from this cohort with an additional 3 years of follow-up was published in 1995.[75] Using available blood levels for 190 workers together with the working histories in a regression analysis, the blood levels for all cohort members at the end of exposure were estimated, and these were used in a Cox regression model for total cancer. The analysis revealed a statistically significant trend for increasing cancer mortality

with increasing estimated blood levels. The relative risk in the highest decile of exposure ($\geq$ 344.7 ng/kg blood lipid) was 2.65.

To develop a risk estimate for the general population exposed at ubiquity background levels based on human data, this approach was refined further in a dose–response model incorporating additional blood data, modeling elimination kinetics, and using a more biologically based dose parameter: namely, the cumulative blood levels over time of follow up (area under the curve).[76,77] The interval estimate for the unit risk for a daily intake of 1 pg TCDD/kg body weight was $5 \times 10^{-4} - 5 \times 10^{-3}$.

The Boehringer cohort presented here has been aggregated with three other German cohorts.[78] One very small cohort with a similar production profile with regard to TCDD contamination as the Boehringer cohort was included along with two other cohorts with exposure mainly to 2,4-D and MCPA as supposed not to be highly contaminated with TCDD. Overall cancer mortality was elevated (SMR = 1.19, 95% CI = 1.00 to 1.41). Cancer mortality increased with latency. Statistically significant elevated SMRs were observed for cancer of the respiratory tract (SMR = 1.54), buccal pharynx (SMR = 2.95), and non-Hodgkin's lymphoma (SMR = 3.26).

The finding of elevated breast cancer mortality in the female Boehringer cohort was investigated further by a cancer incidence study.[79] The follow-up for this study ended in 1995. Using the same cumulative dose estimation techniques as described for the male cohort above, a trend of increasing breast cancer incidence with increasing TCDD and TEQ doses was observed. The standardized incidence ratio compared to a reference population was 2.56 (95% CI = 1.23 to 4.71) for women in the highest exposure tertile for toxic equivalencies. However, especially for the female cohort a high correlation of TEQs and exposure to hexacyclohexane was observed.

Netherlands Study Results from a cohort study encompassing 2310 workers from two Dutch companies involved in the production of phenoxy herbicides was first presented in 1993.[80] An increased risk was observed for NHL based on two deaths (SMR = 2.99, 95% CI = 0.36 to 10.78), but not for total cancer mortality (SMR = 1.37, 95% CI = 0.66 to 2.52). No case of STS was found. In an update from 1998, the mortality was increased for total cancer with an SMR of 4.10 (95% CI = 1.80 to 9.00), as well as for respiratory cancer with SMR = 7.50 (95% CI = 1.00 to 56.10), and for NHL with an SMR of 1.70 (95% CI = 0.20 to 16.50).[81] The results indicated exposure-related increases in risk with increasing TCDD level.

IARC Study In 1991 the International Agency for Research on Cancer (IARC) published a cohort mortality study which encompassed 18,910 production workers or sprayers from 10 countries, among them the cases from the studies from Denmark, the United States, Germany, and the Netherlands as described above, and who were potentially exposed to phenoxyacetic herbicides or chlorophenols.[65] Exposure data were collected through questionnaires, job

histories, and factory or spraying records. Workers were classified as exposed ($n = 13{,}482$), probably exposed ($n = 416$), unexposed ($n = 3{,}951$), and a group with unknown exposure ($n = 541$). Cause-specific national death rates were used as the reference.

In the exposed group, the SMR for all malignant neoplasms was 1.01 (95% CI = 0.92 to 1.10). An excess risk based on four observed deaths was noted for STS with an SMR of 1.96 (95% CI = 0.53 to 5.02). In a group with 10 to 19 years since first exposure, the SMR was 6.06 (95% CI = 1.65 to 15.52) for STS in the entire cohort, and among sprayers the corresponding SMR was 8.82 (95% CI = 1.82 to 25.79).

The risk also appeared to be increased for cancers of the testicle, thyroid, other endocrine glands, and nose and nasal cavity, but small numbers of deaths represented these cancer sites. Five additional cases of STS were recorded for cohort members who were alive at the end of follow-up or who had died with a certified cause of death other than the ICD code 171 (used for STS). Regarding NHL, 14 deaths were observed among men and 1 among women. These numbers did not represent significantly increased SMRs.

Cancer incidence and mortality among women was further analyzed.[82] Among workers exposed to phenoxy herbicides contaminated with TCDD, excess cancer incidence for all sites was observed with SIR = 2.22 (95% CI = 1.02 to 42.29). Updated results on cancer mortality, with some additional cohorts incorporated, have been presented.[66] Mortality from all malignant neoplasms was increased, SMR = 1.12 (95% CI = 1.04 to 1.21), also for NHL with SMR = 1.39 (95% CI = 0.89 to 2.06), and lung cancer with SMR = 1.12 (95% CI = 0.98 to 1.28). For STS an SMR of 2.03 (95% CI = 0.75 to 4.43) was obtained.

In a nested case–control study on STS and NHL within the IARC pooled cohort, exposure to phenoxy herbicides, chlorophenols, and dioxins were evaluated further.[83] STS was associated with exposure to any phenoxy herbicide (OR = 10.3, 95% CI = 1.2 to 91), any dioxin or dibenzofuran (OR = 5.6, 95% CI = 1.1 to 28), and to TCDD (OR = 5.2, 95% CI = 0.9 to 32). For any chlorophenol exposure the OR was 1.3 (95% CI = 0.2 to 6.9). Regarding NHL, exposure to phenoxy herbicide yielded an OR of 1.3 (95% CI = 0.5 to 2.9), for chlorophenol exposure the OR was 1.3 (95% CI = 0.5 to 3.1), for any dioxin or dibenzofuran exposure the OR amounted to 1.8 (95% CI = 0.8 to 4.3), and for TCDD exposure an OR of 1.9 (95% CI = 0.7 to 5.1) was obtained. For pentachlorophenol an increased risk was found, with an OR of 2.75 (95% CI = 0.45 to 17).

18.5.3 Studies on Vietnam Veterans

Both case–control and cohort studies have been performed to evaluate any health effects, particularly malignant diseases, among veterans who fought in the Vietnam conflict, where phenoxy herbicides were used in the warfare.[84–86] Common to all these studies are considerable difficulties in assessment of

exposure, and often indirect measures have been used (e.g., service in certain corps or areas with potential exposure to the sprayings).

TCDD levels in serum and adipose tissue clearly show that such indirect exposure criteria do not accurately identify or rank persons actually exposed to dioxin-contaminated herbicides.[87] Interpretation of all these studies must therefore be done with caution.

A study that may suffer from limitations due to the problem of assessing exposure is the mortality cohort of 1261 Air Force veterans participating in Operation Ranch Hand and responsible for the aerial herbicide spraying missions in Vietnam.[88] Serum TCDD measurements on a small subset of this cohort (i.e., those who were believed to be most exposed) show that very few had high TCDD levels; 200 ppt was exceeded in only five subjects. Thus, the absence of any increased cancer deaths in this cohort is not very informative.

An update of this cohort gave for all cancers an OR of 0.9 (95% CI = 0.6 to 1.3), for NHL an OR of 1.4 (95% CI = 0.0 to 7.7), and for STS the OR obtained was 2.4 (95% CI = 0.1 to 13.6), based on low numbers of cases, however.[89] However, a cohort study on Massachussets veterans[90] showed a significantly increased risk for STS among veterans with potential exposure. Information was obtained from death certificates and veterans' bonuses for 840 Vietnam veterans and 2515 Vietnam-era veterans who died in Massachusetts during 1972–1983.

18.5.4 Studies on Chlorophenol Manufacturers and Users

Mortality in a cohort of pentachlorophenol manufacturing workers during 1940–1989 was published from the United States.[91] With a 15-year latency period, the SMR for all malignant diseases was 1.05 (95% CI = 0.77 to 1.41), for gastric cancer 1.76 (95% CI = 0.36 to 5.16), for kidney cancer 3.00 (95% CI = 0.62 to 8.77), and for all lymphopoeitic malignancies 1.32 (95% CI = 0.42 to 3.07). No case of STS was found, but only 0.2 was expected.

In a cohort of more than 26,000 sawmill workers in British Columbia, Canada, a slightly increased incidence was found for all cancer, skin excluded, with SIR = 1.05 (95% CI = 1.01 to 1.10).[92] The incidence of NHL increased with increasing chlorophenol exposure. The highest risk was found in the category with cumulative hours of exposure $\geq$ 10,000 with SIR = 1.30 (95% CI = 0.91 to 1.80), and there was a significant trend over exposure categories (χ^2 test for trend with $p = 0.04$). In the group of workers with over 20 years of employment, SIR increased to 1.54 ($p = 0.04$). A significantly increased risk was found for all hematologic malignancies with SIR = 1.16 (95% CI = 1.01 to 1.34). The SIR was for STS = 1.17 (95% CI = 0.66 to 1.94), for nose and nasal cavities 2.03 (95% CI = 0.95 to 3.83), and for rectum 1.22 (95% CI = 1.03 to 1.44).

A series of studies on workers in the tanning industry with potential chlorophenol exposure have been performed. A mortality study from the United

Kingdom on 833 male tannery workers resulted in an SMR of 4.76 (95% CI = 0.12 to 26.53) for nasal cancer. One death from STS yielded an SMR of 14.58 (95% CI = 0.37 to 81.23).[93]

In an Italian study of 2926 male workers in tanneries, increases in deaths were found for cancer of the lung with SMR = 1.31 (95% CI = 0.88 to 1.82), bladder with SMR = 1.50 (95% CI = 0.48 to 3.49), kidney with SMR = 3.23 (95% CI = 0.86 to 8.27), pancreas with SMR = 1.46 (95% CI = 0.39 to 3.73), hematologic malignancies with SMR = 1.53 (95% CI = 0.73 to 2.88), and STS with SMR = 21.78 (95% CI = 2.50 to 80.23).[94]

In a Swedish cohort of tannery workers, significantly increased incidence was found for STS, as well as for multiple myeloma and sinonasal cancer.[95] A nested case–control study was performed within the same cohort, yielding for STS an OR of 3.79 (95% CI = 0.30 to 48), and for pancreatic cancer the OR amounted to 7.19 (95% CI = 1.44 to 36).[96]

18.5.5 Studies on Farmers and Related Occupations

A number of studies have used occupational titles such as *farmer* as a surrogate for exposure to pesticdes. In general, these studies lack individual exposure data and are less informative on a causal link between pesticides and cancer. Nevertheless, some studies are of interest and are briefly presented in the following.

A mortality study of forest and soil conservationists reported increased risk for all malignant neoplasms, with a proportional mortality ratio (PMR) of 1.10 (95% CI = 1.00 to 1.20), for NHL the PMR was 2.40 (95% CI = 1.50 to 3.60), for kidney cancer the PMR was 2.10 (95% CI = 1.20 to 3.30), and for colon cancer a PMR of 1.50 (95% CI = 1.10 to 2.00) was obtained.[97] An increased risk was reported for malignant neoplasms of the lymphatic tissue in a cohort of licensed pesticide users in Italy with a SIR of 1.40 (95% CI = 1.00 to 1.90).[98]

In a Canadian investigation on farmers with potential exposure to herbicide, an increased risk for NHL was indicated, but somewhat remarkably, only among those who had an ethnic origin in Germany and Ukraine.[99] In the 1960s, 90% and in the 1970s, 75% of the herbicides used in Canada was 2,4-D.

Cancer incidence was studied in a cohort of Danish gardeners and resulted in an SIR for STS of 5.26 (95% CI = 1.09 to 15.38), for chronic lymphatic leukemia the SIR was 2.75 (95% CI = 1.01 to 5.99), and for NHL the SIR was 2.00 (95% CI = 0.86 to 2.93).[100] Exposure to multiple types of pesticides had occurred among these gardeners.

Among male pesticide applicators an increased mortality in NHL was found in a U.S. study with an SMR of 1.63 (95% CI = 0.33 to 4.77). The risk increased with 3 or more years employment: SMR = 7.11 (95% CI = 1.78 to 28.42).[101] The pesticides used included phenoxy herbicides. Cancer incidence among women living on farms yielded for NHL an OR of 1.52 (95% CI = 0.96 to 2.39).[102] A meta-analysis of NHL and farming including 36 studies pub-

lished between 1982 and 1997 resulted in an OR of 1.19 (95% CI = 1.06 to 1.33).[103]

18.6 COHORT STUDIES ON THE GENERAL PUBLIC AFTER ACCIDENTS

18.6.1 Seveso Study

Through an accident in a chemical plant in Seveso, Italy in 1976, the general public in the area was exposed to TCDD (see Chapter 20). A 10-year mortality study was reported in 1989.[104] In the second 5-year period of follow-up there was some increase in mortality both from NHL and from STS, although statistically, rather imprecise. For all types of malignancies combined, no increase was seen.

A later 15-year mortality study showed elevated risk for STS in zone R (lowest exposure to dioxin) in males with an OR of 2.1 (95% CI = 0.6 to 5.4).[105] The risks for hematological malignancies were also increased. In zone B, with a medium-level dioxin exposure, increased risks were seen for male leukemia: OR = 3.1 (95% CI = 1.3 to 6.4), female multiple myeloma: OR = 6.6 (95% CI = 1.8 to 16.8), male HD: OR = 3.3 (95% CI = 0.4 to 11.9), and female HD: OR = 6.5 (95% CI = 0.7 to 23.5). Women had an increased risk for gastric cancer in zone B: OR = 2.4 (95% CI = 0.8 to 5.7), and men had increased mortality from rectal cancer with an OR of 6.2 (95% CI = 1.7 to 15.9).

Data on the occurrence of cancer have been been published in several reports.[106–108] Notably, an increase has been found in zone B regarding hepatobiliary cancer: OR = 2.8 (95% CI = 1.2 to 6.3), and extrahepatic bile ducts and gallbladder cancer in women: OR = 4.9 (95% CI = 1.8 to 13.6). For males an increased risk was found for NHL with OR = 2.3 (95% CI = 0.7 to 7.4). Females had an increased risk for multiple myeloma: OR = 5.3 (95% CI = 1.2 to 22.6), and myeloid leukemia: OR = 3.7 (95% CI = 0.9 to 15.7). In zone R, the incidence of STS was elevated, particularly among persons living in the area for > 5 years: OR = 3.5 (95% CI = 1.2 to 10.4), and of NHL: OR = 2.0 (95% CI = 1.2 to 3.6). For more details, see Chapter 20.

18.6.2 Yusho Study

In 1968, approximately 1900 persons accidentally consumed polychlorinated biphenyls (PCBs) and dibenzofurans (PCDFs) through poisoned rice oil (*Yusho* meaning oil disease) in Japan (see Chapter 21). In a follow-up of this cohort,[109] a significantly increased risk of liver cancer in males (SMR = 5.59, $p < 0.01$) and a nonsignificant increase of the same malignancy in females (SMR = 3.04) were noted. This finding is well in accordance with the known hepatocarcinogenicity of PCBs in animals. Furthermore, a significantly

increased risk of lung cancer was also seen in males (SMR = 3.26, $p < 0.01$). A similar accident (Yucheng) occurred in 1979 in Taiwan (see Chapter 22), but no follow-up of that cohort has been reported on.

18.7 COHORT STUDIES OF PULP AND PAPER MILL WORKERS

The bleaching of pulp and paper by chlorine may result in various chlorinated organic compounds, among them PCDDs. Several cohort studies of pulp and paper mill workers, who have a potential exposure to PCDDs in their work environment, have been published. Two cancer mortality studies from the United States[110,111] and a Finnish incidence study,[112] all from the 1980s, did not find any increase in cancer in general or in certain specified tumor types (e.g., NHL). STS was not studied specifically, however.

A study from Spain on workers in the pulp and paper industry showed excess mortality from all neoplasms in females: SMR = 1.68 (95% CI = 0.84 to 3.03), colon cancer in both sexes: SMR = 2.50 (95% CI = 1.15 to 5.25), and breast cancer in females: SMR = 2.86 (95% CI = 0.77 to 7.32).[113]

Among Danish paper mill workers an increased risk was found for male pharyngeal cancer: SIR = 1.99 (95% CI = 1.11 to 3.29), male Hodgkin's disease: SIR = 2.01 (95% CI = 1.19 to 3.18), and female STS: SIR = 2.33 (95% CI = 1.06 to 4.43), further increasing among female paper sorters to SIR = 3.98 (95% CI = 1.71 to 7.84).[114]

A Swedish case–control study encompassing 4070 men deceased during the period 1950–1970 revealed an increased mortality from secondary tumors of the liver and lung, and there was also a slightly increased risk from malignant lymphoma, leukemia, and cancer of the pancreas and stomach.[115] An excess of stomach cancer has also appeared in another small study from a Swedish paper mill.[116] Others have found an increased risk of lung cancer among paper mill workers, probably due to asbestos exposure, however.[117] In the interpretation of these findings, it is important to consider that the actual exposure to dioxins is not known in the various studies.

18.8 ENVIRONMENTAL EXPOSURE THROUGH POLLUTION

18.8.1 Human Exposure

Chlorophenols may contaminate areas surrounding sawmills where they are used. Thus, both the soil and water may be contaminated. The population may be exposed through the drinking water or by eating contaminated food (e.g., fish). In a Finnish study, an increased risk for both STS and NHL was reported in a municipality using drinking water and consuming fish from a lake contaminated with chlorophenols.[118] The OR for STS was 8.9 (95% CI = 1.8 to 44) and for NHL 2.8 (95% CI = 1.4 to 5.6). The risk for HD was 1.5 (95% CI = 0.4 to 5.0).

A higher incidence of NHL has been reported in males living in a rice-growing area where 2,4-D and 2,4,5-TP (the propionic acid of 2,4,5-T) have been identified in the soil and water.[49,119] The rate in the most polluted area was two times higher than that in the rest of the territory. Regarding HD and STS, the absolute numbers were too small to allow meaningful interarea comparisons.

A large number of roadways, arenas, yards, and other surface sites in Missouri were contaminated with dioxins including TCDD in 1971. Waste by-products from a hexachlorophene and 2,4,5-T production facility in southwestern Missouri were mixed with waste oils and sprayed for dust control throughout the state, including the town of Times Beach. In TCDD-exposed subjects, depressed cell-mediated immunity and altered T-lymphocyte subsets have been reported.[120–122] Thymic hormone levels were examined in a group of 94 persons presumed to be TCDD-exposed from living in contaminated residential areas. Compared with a matched control group of 105 unexposed persons, the exposed group had significantly lower mean α_1-thymosin level.[123] This is in agreement with the finding that the thymus is a target organ in experimental animals exposed to TCDD.[124] There are no reports on the cancer incidence among persons with residential TCDD exposure.

In the early 1970s, three trucking terminals in St. Louis, Missouri were sprayed with TCDD-contaminated waste oil to control dust. Approximately 600 workers were employed at these sites. Among these workers, one self-reported case of porphyria cutanea tarda, a disease involving altered porphyrin metabolism, and STS has been published.[125] In the early 1970s, this person had worked for several years as a truck driver at one of the TCDD-contaminated trucking terminals. He reported that his feet, legs, hands, and arms frequently became covered with oil from the terminal. He had no other history of TCDD exposure. No follow-up data on cancer incidence in the entire cohort of workers are available.

18.8.2 Animal Exposure

A study from New Zealand examined the prevalence of small-intestinal adenocarcinoma in 20,678 female sheep.[126] Exposure to phenoxyacetic acids, picolinic herbicides, or both was associated with increased tumor rates, which were significant for each herbicide. Exposure to recently sprayed feedstuffs was associated with a significantly larger increase in tumor rate than exposure to less recently sprayed food. No additional effect was noted for exposure to TCDD.

A significantly increased risk for seminomas, a cancer of the testis, was found in U.S. military working dogs who had served in Vietnam during the war (OR = 1.9, 95% CI = 1.2 to 3.0).[127] Exposures, or potential exposures, of interest were to tetracycline, malathion, picloram (in Agent White), and 2,4-D and 2,4,5-T (Agent Orange) with contamination of TCDD.

Also of interest is the association between canine malignant lymphoma and the use of 2,4-D on lawns of the dog owners: OR = 1.32 (95% CI = 1.04 to 1.67).[128] The risk increased with the area of the lawns treated with herbicide per year. Several additional analyses of the material were performed without change of the results.[129]

18.9 OTHER ORGANOCHLORINES AND ORGANOBROMINES

During the recent decade, much discussion has focused on the hormonal activity of persistent organic pollutants and the risk for cancer. For TCDD, an antiestrogenic effect has been shown in experimental tests, as may also be reflected in the Seveso data with a decreased incidence of breast cancer in the most contaminated area, and a clear deficit of endometrial cancer in zones B and R.[108]

Regarding PCBs and the risk of breast cancer, data are inconsistent. However, exposure to xenohormones during critical windows of development, including the prenatal and prepubescent periods, has not been studied. Furthermore, hormonally active parent compounds, as well as active metabolites, should be studied.

Most studies have measured total PCBs that cannot distinguish between highly estrogenic and those with antiestrogenic properties.[130] In one Swedish case–control study, both coplanar and noncoplanar congener-specific PCBs were measured.[131] A significantly increased risk was found for coplanar PCBs for patients with estrogen receptor positive breast cancer. This risk increased further in the postmenopausal patient group. Similarly, the highest risk was observed for the sum of noncoplanar PCBs among postmenopausal women with estrogen receptor positive breast cancer: OR = 1.8 (95% CI = 0.4 to 7.3). Of the 34 measured noncoplanar PCBs, no one was significantly different in concentration between cases and controls. More recently, however, a study from Canada reported higher concentrations of certain specific PCB congeners in breast cancer patients.[132] Congener-specific analyses are thus necessary instead of only reporting the sum of PCBs, which has usually been the case in various studies.

In the same Swedish study, octachlorodibenzo-*p*-dioxin (OCDD) on a lipid basis was associated with an increased risk for breast cancer.[133] When the OCDD variable was examined as a continuous risk factor, there was a 1.09 (9%) (95% CI = 0.95 to 1.25) increase in the adjusted excess relative risk for breast cancer per 100-unit (pg/g lipid) increase in OCDD. For other dioxins, including TCDD and dibenzofurans, no significant differences were found between cases and controls.

Certain persistent organic pollutants have immunotoxic properties, and since immunosuppression is a risk factor for NHL, exposure to such chemicals might be of etiologic signficicance.[134] This suggestion gains some support from

the observation that adipose tissue concentrations of PCDDs and PCDFs, measured as TEQ, were increased significantly among potentially exposed patients with malignant lymphoproliferative diseases.[135]

Furthermore, studies have found increased concentrations of PCBs,[136,137] chlordanes,[138] and the flame retardant 2,2′,4,4′-tetrabrominated diphenyl ether[139] in NHL patients. Increased risk for lymphoma (unspecified) was reported in a Michigan cohort exposed to polybrominated biphenyls in 1973.[140] A study focusing directly on the immunotoxic effects from agricultural exposure to commercial 2,4-dichlorophenoxyacetic acid (2,4-D) and 4-chloro-2-methylphenoxyacetic acid (MCPA) formulations showed only short-term immunosuppressive effects, however.[141] The interpretation of this observation in relation to the other findings referred to remains unclear, however.

Regarding NHL, seropositivity for the Epstein–Barr virus early antigen seems to potentiate the effect of chemicals with potential immunotoxic effects (cf. the discussion of EBV and NHL above).[137,142,143] Similar results have also been found for hairy cell leukemia.[144]

18.10 GENERAL CONCLUSIONS

The observations that by now strongly indicate a carcinogenicity of TCDD in humans have rapidly increased during the last decades. The evidence has certainly also generated a considerable debate, which has included economic and even political considerations.[9,18,23,145,146] TCDD has by now been classified as carcinogenic to humans (group 1) by IARC,[147] which is most reasonable in view of the epidemiological findings along with the fact that TCDD is multisite carcinogenic in experimental animals.

Regarding specific types of malignant tumors, STS as well as NHL by now seem to be the cancer forms most clearly associated with exposure to phenoxy herbicides and related chlorinated phenols. STS, especially, seems to be related to dioxins, as judged from the results in studies that have appeared from different countries and research groups.

For NHL, there is epidemiologic evidence for the association with phenoxy herbicides, chlorophenols, but by now also with TCDD specifically. A possibility, supported by recent findings, is that chemicals or conditions that impair the immune system increase the risk for NHL. Interaction with viruses, such as Epstein–Barr virus, might be an important step in the etiology of lymphoma.

Epidemiologic evidence also indicates that TCDD increases the risk for all cancers combined. Higher odds ratios are present in several studies for NHL and STS. It is, as concluded by IARC,[147] unlikely that these findings are due to chance. Studies in experimental animals show that the carcinogenic mechanism involves the aryl hydrocarbon (Ah) receptor. This receptor is highly conserved in an evolutionary sense and functions in the same way in humans as in experimental animals.

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CHAPTER 19

Reproductive and Developmental Epidemiology of Dioxins

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19.1 INTRODUCTION

Dioxin [for purposes of this chapter defined as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD), herein simply TCDD] is a ubiquitous contaminant, produced as an unwanted by-product during the manufacture of many industrial and agricultural chemicals, as well as from incineration of municipal waste. Ingestion of food is the most likely pathway of dioxin exposure for the general population.[1,2] This chapter covers the epidemiologic data on reproductive and developmental endpoints and dioxin exposure. Other reviews have examined data comprehensively from a variety of studies, including exposures estimated using self-report, soil measures, and biomonitoring data.[3–6] Many of the more recent studies have included individual measures of dioxin exposure. This review focuses on these and is not meant to be a comprehensive review of all available data.

The manuscript has been reviewed in accordance with the policies of the U.S. Environmental Protection Agency (EPA) and the National Institute for Occupational Safety and Health (NIOSH/CDC), and approved for publication. Approval does not signify that the contents necessarily reflect the views of the EPA and NIOSH.

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19.1.1 Background

The concerns of a potential link between exposure and adverse developmental outcomes can be traced to early animal studies reporting increased incidence of developmental abnormalities in rats and mice exposed early in gestation to 2,4,5-trichlorophenoxyacetic acid (2,4,5-T).[7,8] This was of grave concern, as the U.S. military's most widely used herbicide during the Vietnam conflict at that time, Agent Orange, was composed of approximately equal proportions, by weight, of the *n*-butyl esters of 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-T. The latter is contaminated during manufacture by TCDD.

Laboratory animal studies, reviewed in Chapter 9, have focused primarily on maternal TCDD exposure and developmental toxicity, while in humans, many studies are of paternal exposures only, or of exposures to both parents (through shared environment). Prenatal exposure to TCDD in laboratory animals has been associated with increased pre- and postnatal mortality, cleft palate and kidney abnormalities, and altered sexual development. Reduced fertility has been observed in studies of maternal exposure in a number of species.

Fewer laboratory animal studies have focused on effects of dioxin on the male reproductive system or on the results of matings in which only the males were exposed to dioxin. Assessment of exposed male animals has most commonly examined effects on spermatogenesis, fertility, and sex organ development,[10] while studies of human males have examined primarily pregnancy outcomes such as congenital malformations and recognized spontaneous abortions.

Laboratory animal research designed to corroborate human investigations may provide the data to fill the gaps in our understanding of the mechanisms through which TCDD exposure may result in adverse reproductive or developmental effects. Standardized study designs are available to screen chemicals for prenatal developmental toxicity, effects on reproduction and fertility over multiple (generally two) generations, and dominant lethal effects. However, these studies cannot exactly mimic the exposure scenario in humans. Generally, dose levels are higher than typically found in humans to increase the power of studies in smaller groups of animals. Developmental toxicity studies typically evaluate maternally mediated toxicity, where dams are exposed to varying doses of TCDD only during gestation and then are sacrificed prior to parturition, whereas in humans, the exposure continues throughout gestation and is typically long term. As a consequence, laboratory animal studies are likely to misrepresent human exposures, because effects resulting from cumulative chronic exposures might not be detected and effects that might result from insult during postnatal development are not examined.

In multigeneration reproduction studies, both parents are exposed continuously. Thus, the pattern of exposure is more similar to humans; however, this design limits the ability to separate effects of male versus female exposure. Alternatively, only male-mediated effects are examined in the typical male dominant lethal study, in which exposed males are mated with unexposed females, but outcomes examined are limited to conceptus viability, as an indi-

cator of germ cell toxicity. However, paternally mediated effects in humans could occur either by "carry-home" exposure to the female through contact with contaminated clothing, direct effects on the sperm, or delivery of the exposure to the conceptus via transport with the sperm or seminal fluid. If the female is exposed from contact with the agent in the seminal fluid, the potential exposure is less in the laboratory since only one mating occurs. With humans, exposure during pregnancy could result through continued sexual activity, although the level of exposure from this source is likely to be low. With either approach, multigenerational studies or dominant lethal studies, strictly parallel information on paternally mediated effects is not available.

Several TCDD-contamination episodes have occurred, resulting in identification of health effects attributed to high-dose exposure to TCDD, including the skin disorders and liver damage.[11] Studies of workers (generally males) exposed to phenoxy acids and chlorophenols have attempted to ascertain health effects at greater than background levels. Two well-known contamination episodes occurred in Japan in 1968 (Yusho) and Taiwan in 1979 (Yucheng), where thousands of persons consumed cooking oil contaminated with polychlorinated biphenyls, polychlorinated dibenzofurans, and polychlorinated quaterphenyls. Adverse reproductive effects observed in these populations included increases in spontaneous abortions, low birth weight, growth retardation, and hypoplastic deformed nails.[12–19] These studies are covered in more detail in Chapters 21 and 22. Perhaps the most widely known investigations of potential dioxin reproductive toxicity in humans are the studies of male Vietnam veterans who were potentially exposed to Agent Orange, and their offspring. For developmental endpoints based on general environmental levels, the most extensively studied groups are two series of studies in the Netherlands. These studies, and others, are described in detail below.

The study of these effects poses several challenges not found in the examination of other health outcomes. Understanding of normal and pathologic reproduction requires knowledge of paternal, maternal, and fetal contributions. Increased interest in male-mediated reproductive toxicity emphasizes the need to consider both parents in environmental studies.[20]

Another challenge is the interrelatedness of reproductive/developmental endpoints for study. Fecundity (joint potential to conceive), fertility (production of live children), and very early pregnancy loss (conceptions not recognized by usual diagnostic methods) have not been evaluated in the same population. Clearly, these early endpoints affect the rates of reproductive outcomes occurring later in the reproductive spectrum.[21]

Another feature is the changing vulnerability of the developing organism throughout gestation. Exposure to a single developmental toxicant throughout pregnancy may result in different effects at various stages of gestation. The window of susceptibility varies; therefore, knowledge of the timing of exposure is critical in these studies.[22]

Since the development of assays in the mid-1980s to quantitate TCDD in serum and adipose tissue, newer studies have used biomarkers of exposure. In this update of an earlier review,[3] the focus is on those studies that have made

use of improved biomonitoring of TCDD and other dioxinlike compounds. The chapter concludes with a summary of the research to date and suggestions for future examinations of this issue.

19.1.2 General Issues in Evaluation of the Studies

As described above, exposure to TCDD and dioxinlike compounds in serum or adipose tissue could be quantified starting in the mid-1980s. This breakthrough helped demonstrate which populations had elevated levels. For some populations with higher exposures (e.g., occupational groups), the estimate reflects continuous exposure over an extended period.

The deposition, metabolism, and excretion of high doses of TCDD in humans have not been fully described. Pirkle and colleagues[23] observed that TCDD decays by one-half in approximately 7.1 years, based on a one-compartment model in which the entire body is considered a homogeneous volume and using a standard half-life equation. If this is true, exposures to trichlorophenol production workers may have been as high as 30,000 pg/g[24] and in excess of 50,000 pg/g in some residents of Seveso[25] (see Chapter 20 for a discussion of the Seveso incident). The data may be limited by incomplete information on human metabolism of TCDD. In a more recent analysis, Michalek et al.[26] estimated the half-life among male veteran Ranch Hands to be 8.7 years (95% CI = 8.0 to 9.5 years). This calculation was based on a mean decay rate of 0.0797 per year. In this analysis half-life increased with increasing body fat, but not age.

In studies of infants, where exposures were both prenatal and postnatal (through breast feeding), exposure times are short, potentially allowing improved classification of exposure. However, the effects of pregnancy and lactation history on current maternal serum TCDD levels have not been fully evaluated.

19.1.3 Biomarkers of Exposure Compared to Exposure Indices

Several efforts have compared surrogate exposure measures with serum levels in Vietnam veterans: (1) a comparison of serum levels (646 ground combat troops and 97 who served elsewhere) with the Centers for Disease Control and Prevention's (CDC) exposure opportunity index and with the subject's self-report of exposure[29] showed that the surrogates were not a good estimate of the measured levels; (2) a rather small comparison of Vietnam veterans (10 reported exposure, 10 reported little or no exposure) and 27 Vietnam-era veterans[31] showed a significant elevation for the exposed men compared to the other two groups; and (3) in the Ranch Hand study, comparisons of serum levels from a sample of men (337 ranch hands) to the exposure index revealed "considerable misclassification."[32] These results support the need to emphasize those studies using biomarkers of exposure.

19.2 REVIEW OF THE LITERATURE

Since the mid-1980s, assays developed to measure TCDD in serum and adipose tissue have been tested and refined. These assays have been used, sometimes only in subsets of the study group, to estimate dioxin exposure and to validate assumptions about exposure. Subsets were generally selected to represent subjects called "high" versus "low" exposure, using self-reports, company or military records, and so on. Tables 19.1 and 19.2 present a summary of exposure analyses in several studies for general comparison.

These data show wide variability in groups presumed to have been exposed to TCDD at levels above background. For example, mean and median serum levels of Vietnam ground combat troops with service in areas heavily sprayed with Agent Orange did not exceed levels found in the general U.S. population. There is evidence for higher exposure to TCDD among subgroups of Vietnam veterans[31,34] and residents of Vietnam,[34] Seveso,[25] and Missouri,[27] and in occupational groups.[24,39]

With the exception of the Ranch Hand study[34] and the Dutch studies,[36–38] subsets were selected to describe TCDD exposure in the total study sample and not to examine the relationship between TCDD and reproductive events. Questions regarding the impact of initial dose, age, gender, and pregnancy and lactation on half-life remain unanswered.

19.2.1 Studies of Development

Environmental Studies

Studies of Infants in The Netherlands In the early 1990s, scientists from several Dutch communities collected data on postnatal developmental outcomes and related them to total polychlorinated biphenyl (PCB)–dioxin–furan toxic equivalents (TEQs) from breast milk, and PCBs in cord blood and maternal blood. These two series of studies are similar in design. One series includes women and infants from Rotterdam, or from both Rotterdam and Groningen[36,37,40–49]; the second series includes women and infants from Amsterdam.[38,50–52]

The outcomes studied in both communities for the first series (Rotterdam and Groningen) included a neurological examination (Prechtl–Touwen) at 10 days and 18 months of age and Obstetrical Optimality score at 10 days of age. In Rotterdam only, mental and psychomotor development (Bayley Scales of Infant Development) were measured at 3, 7, and 18 months of age, and visual recognition memory (Fagan Infant test) at 3 and 7 months of age. At 42 months, these children were assessed for cognitive abilities (Kaufman Assessment Battery) and a subgroup was assessed for verbal comprehension (Reynell Language Developmental Scales). The study in Amsterdam also examined postnatal developmental outcomes and breast milk level; this study is discussed in more detail at the end of this section.

TABLE 19.1 TCDD Levels (pg/g of Lipid) for Selected Populations

Study	Population	Specimen	Range[a]	Mean	Median
Mocarelli et al. (1991)[25]	Seveso, Italy residents:	Serum			
	10 Zone A		828–56,000	19,144	14,000
	10 former Zone A		1,770–10,400	5,240	4,540
	10 non-ABR Zone		nd–137		
Patterson et al. (1986)[27]	39 Missouri residents with history of TCDD exposure	Adipose tissue	2.8–750	79.7	17.0
	57 Missouri residents with no known TCDD exposure		1.4–20.2	7.4	6.4
Smith et al. (1992)[28]	9 New Zealand pesticide applicators	Serum	3.0–131.0	53.3	37.6
	9 controls		2.4–11.3	5.6	9.3
CDC (1988)[29]	646 Vietnam ground combat troops with service in heavily sprayed areas	Serum	nd–45	4.2	3.8
	97 non-Vietnam veterans		nd–15	4.1	3.8
Schecter et al. (1989)[30]	26 Vietnam veterans	Adipose tissue	nd–11	5.8	
Kahn (1988)[31]	10 ''heavily exposed'' Vietnam veterans	Blood (per lipids)		46.3	25.1
		Adipose tissue		41.7	15.4
	10 Vietnam veterans with ''little or no'' exposure	Blood (per lipids)		6.6	5.3
		Adipose tissue		5.1	5.4
	7 non-Vietnam veterans	Blood (per lipids)		4.3	3.9
		Adipose tissue		3.2	3.5
Kang (1991)[33]	36 Vietnam veterans	Adipose tissue		13.4	10.0
	79 non-Vietnam veterans			12.5	11.4
	80 civilians			15.8	11.8
Roegner et al. (1991)[34]	872 Ranch Hands	Serum	0–617.8		12.8
	1,060 controls		0–54.8		4.2
Phuong (1989)[35]	Vietnamese populations: 9 OB/GYN patients from a South Vietnam hospital	Adipose tissue	nd–103	23	11.3

[a]nd, not determined.

TABLE 19.2 Overview of Biologic Measurements in Dutch Studies of Postnatal Developmental Effects

Compound	IUPAC[a]	TEF[b]	Rotterdam/ Groningen Breast Milk[c] [Plasma/Cord Blood][d]	Amsterdam Breast Milk[e]
PCDDs				
2,3,7,8-TCDD	48	1	4.0	3.8
1,2,3,7,8-penta-CDD	54	0.5	10.6	10.6
1,2,3,4,7,8-hexa-CDD	66	0.1	8.7	1.3
1,2,3,6,7,8-hexa-CDD	67	0.1	47.4	49.1
1,2,3,7,8,9-hexa-CDD	70	0.1	6.7	6.5
1,2,3,4,6,7,8-hepta-CDD	73	0.01	63.2	54.3
1,2,3,4,6,7,8,9-octa-CDD	75	0.001	799.6	297.5
PCDFs				
2,3,7,8-TCDF	83	0.1	0.8	2.0
1,2,3,7,8-penta-CDF	94	0.05	0.3	0.2
2,3,4,7,8-penta-CDF	114	0.5	22.7	21.9
1,2,3,4,7,8-hexa-CDF	118	0.1	6.6	7.0
1,2,3,6,7,8-hexa-CDF	121	0.1	5.7	6.2
1,2,3,7,8,9-hexa-CDF	124	0.1	3.6	3.2
2,3,4,6,7,8-hexa-CDF	130	0.1	0.3	BDL
1,2,3,4,6,7,8-hepta-CDF	131	0.01	7.9	6.1
1,2,3,4,7,8,9-hepta-CDF	134	0.01	0.2	BDL
1,2,3,4,6,7,8,9-octa-CDF	135	0.001	2.2	1.3
Planar PCBs				
3,3′,4,4′-PCB	77	0.0005	19.3	
3,3′,4,4′,5-PCB	126	0.1	152.0	
3,3′,4,4′,5,5′-PCB	169	0.01	84.3	
Nonplanar PCBs				
2,4,4′	28		12.1	
2,2′,5,5′	52		2.6	
2,3′,4,4′	66		11.6	
2,3′,4′,5	70		18.5	
2,2′,4,4′,5	99		19.7	
2,2′,4,5,5′	101		1.5	
2,3,3′,4,4′	105[f]	0.0001	9.4	
2,3′,4,4′,5	118[f]	0.0001	35.5 [0.16/0.04]	
2,2′,3,3′,4,4′	128		4.0	
2,2′,3,4,4′,5	137		16.8	
2,2′,3,4,4′,5′	138		129.9 [0.60/0.13]	
2,3,4,5,2′,5′	141		1.1	
2,2′,3,5,5′,6	151		0.9	

(Continued)

TABLE 19.2 *(Continued)*

Compound	IUPAC[a]	TEF[b]	Rotterdam/ Groningen Breast Milk[c] [Plasma/Cord Blood][d]	Amsterdam Breast Milk[e]
2,2′,4,4′,5,5′	153		186.3 [0.91/0.18]	
2,3,3′,4,4′,5	156[f]	0.0005	21.0	
2,2′,3,3′,4,4′,5	170[g]	0.0001	37.1	
2,2′,3,3′,4′,5,6	177		6.3	
2,2′,3,4,4′,5,5′	180[g]	0.00001	76.8 [0.54/0.10]	
2,2′,3,4,4′,5′,6	183		12.2	
2,2′,3,4′,5,5′,6	187		20.0	
2,2′,3,3′,4,4′,5,5′	194		8.6	
2,2′,3,3′,4,4′,5,6	195		2.9	
2,2′,3,3′,5,5′,6,6′	202		0.9	

[a] International Union of Pure and Applied Chemistry.

[b] Toxic equivalence factor (WHO, 1993).

[c] Measurements for this series of studies from Ref. 36.

[d] Mean values: breast milk in pg/g fat, blood in ng/g plasma.

[e] Measurements from Ref. 38.

[f] Mono-ortho PCBs.

[g] Di-ortho PCBs.

STUDIES OF ROTTERDAM, OR ROTTERDAM AND GRONINGEN TOGETHER The reports covering Rotterdam, or Rotterdam and Groningen together, include the same infants. Data collection began in June 1990 and continued longitudinally, depending on the outcome. Women were introduced to the project by their obstetricians or midwives, and details explained to the women at home. The total number of women invited to participate was not presented, so the amount of selection, and possible selection bias, by the physician or the woman, is unknown. First contact took place in the third trimester (32 to 34 weeks), and women were screened for their intention to breast-feed for at least 6 weeks (''exposed'') or for their intention not to breast-feed at all (comparisons). Only women with full-term deliveries to first- or second-born infants, among other characteristics, were selected for inclusion in the study. Originally, 489 women who were willing to participate were identified; 71 (14.5%) of these were lost to the final study when they were not able to breast-feed for the required 6 weeks.

Formula was supplied to those women in the study who did not intend to breast-feed. In each community, the goal was to follow approximately 100 in each group. Response rate and the comparability of respondents to non-respondents were not described. All reports included blood samples from the mother during the last month of pregnancy, cord blood at delivery, and breast milk collected in the second week after delivery. PCB levels (congeners 118,

138, 153, 180) were measured in the blood samples and used to estimate prenatal exposures to the children, and PCB, dioxin, and furan levels were measured in the breast milk [seventeen 2,3,7,8-substituted polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs), 3 coplanar PCBs, and 23 nonplanar PCB congeners] (Table 19.2). These data were used to rank exposure in the women and infants into categories to examine exposure–outcome relationships. The total breast milk values were calculated by multiplying levels by the number of weeks of breast feeding. This approach assumes only small changes in exposures over the duration of breast feeding, or that the relative magnitude of exposure remains consistent. As long as all the women continue breast feeding, for approximately the same length of time, this is a useful approach to determine relative magnitude.

Breast milk samples collected during the second week and about the sixth week after delivery were compared for the following exposure groupings: dioxins–furans (IUPAC 48, 54, 66, 67, 70, 73, 75, 83, 94, 114, 118, 121, 124, 130, 131, 134, 135), coplanar PCBs (IUPAC 77, 126, 169), mono-ortho PCBs (IUPAC 105, 118, 156), diortho PCBs (IUPAC 170, 180), and total PCBs–dioxins–furans, which includes all of the above. This terminology will be used in all the following discussions on this series of studies. With continued breast feeding, a drop in levels would be expected as the body burden decreases. Decreases in levels were observed; not all were statistically significant, in part because of the small number of women evaluated: a decrease in dioxins–furans ($n = 27$, $p = 0.07$), coplanar PCBs ($n = 44$, $p = 0.91$), mono-ortho PCBs ($n = 180$, $p = 0.002$), diortho PCBs ($n = 180$, $p = 0.001$), and total PCBs–dioxins–furans ($n = 19$, $p = 0.10$).[37] It seems likely that the measured levels in breast milk would continue to drop with an extended period of breast feeding. Thus, the studies' assumption of steady-state levels in breast milk, given different lengths of breast feeding, could overestimate the actual exposure levels. Any effects observed might occur at a lower level of exposure than reported.

Effects of exposure in these studies were assessed by analyses of the dioxin–furan TEQ or total PCB–dioxin–furan TEQ (for dioxins, furans, and dioxin-like PCBs) based on levels observed in breast milk, and $\sum \mathrm{PCB}_{\text{cord blood}}$ or $\sum \mathrm{PCB}_{\text{maternal blood}}$ (for IUPAC PCB congeners 118, 138, 153, 180), controlling for such items as sociodemographic indicators and personal habits. Because data from other sources have shown breast milk levels to be well correlated with adipose tissues, the breast milk level during the second week after delivery is a reasonable estimate for prenatal exposures (at least the relative magnitude) to these agents.[53]

About half the 418 mothers and infants were in Rotterdam and half in Groningen. A comparison of a variety of demographic factors, personal habits, and health characteristics for the two communities showed no differences for many of them (e.g., maternal age, weight, smoking status), and significantly ($p < 0.05$) higher parental education, maternal alcohol consumption, and the length of gestation and birth weight of the child in Groningen. Socioeconomic status (SES) indicators, health, and exposure were different in the breast-fed versus formula-fed group[40]; for example, over 60% of both parents in the

breast-fed group had higher education versus 31% or less in the formula-fed group. In addition, breast-feeding mothers were more likely to have consumed alcohol during pregnancy (37% vs. 18%) and less likely to have smoked (16% vs. 35%). The breast-fed infants were also more highly exposed prior to breast feeding: $\sum \text{PCB}_{\text{cord blood}}$ and $\sum \text{PCB}_{\text{maternal blood}}$ were significantly higher in this group. Analysis of breast milk levels in the Rotterdam area and in Groningen[36] showed that the dioxin–furan TEQ and some individual dioxin and congener levels (the dioxins: D 66, D 67; the furans: F 83, F 118, F 121, F 130; and the PCBs: PCB 66, PCB 118, PCB 137, PCB 187) were significantly higher in the more urbanized Rotterdam area. For some of the analyses, the infants were placed into a "low" or "high" group based on the median TEQs, which were 30.8 pg TEQ/g fat for the dioxin–furan TEQ and 72.4 pg TEQ/g fat for the total PCB–dioxin–furan TEQ. They were also grouped into "low," "medium," or "high" based on the PCB–dioxin–furan TEQ times the number of weeks breast-fed. In one report different congeners were analyzed separately. Different analysis strategies make integrating data across reports difficult.

A smaller series of women and their infants were studied by another research team in Amsterdam; pregnant women were identified between June 1990 and May 1991. Only women who intended to breast-feed for 12 weeks were included. The reports from this group[38,51,52] had a different final number of subjects (38 vs. 35), but from the general description, they do appear to be from the same study. Maternal blood samples were collected around the time of delivery, as was cord blood; infant blood samples were collected at 1 and 11 weeks of age. Three weeks after delivery, two breast milk samples were collected. The authors assumed that breast milk levels reflected in utero exposures to the infants. Bottle-fed children were not used as comparisons for the following reasons: first, women who choose to breast-feed tend to be of higher SES than those who choose to bottle-feed; and second, mothers of bottle-fed infants could not give breast milk samples to estimate in utero exposures. The 17 most toxic congeners, 7 dioxins and 10 dibenzofurans, were used to develop a dioxin–furan TEQ (Table 19.2). The final score for consumption for each child used the amount of milk consumed (assumed to be 700 g/day while the child received only breast milk, and half that amount later), amount of milk fat (assumed to be an average of 2.5%), and levels of the 17 congeners. The levels determined were split at the median into "low" and "high" groups (high = 29.2 to 62.7 pg TEQ/g and low was less than 28.0 pg TEQ/g).

The two studies used similar approaches for quantification of exposure. These were reasonable estimates of the general magnitude of exposures. Mean levels of exposure over the total period may be somewhat lower than reported, since the breast milk evaluations occurred early in lactation, while levels were likely to decrease with longer periods of breast feeding. Some adjustment for these reductions over time was made in the Amsterdam study. For both of these study groups, the levels of dioxins, furans, and PCBs were within common/background environmental ranges.

In the following section we discuss these two series of studies by the health

endpoint covered: thyroid function, aspartate aminotransferase and alanine-transferase, immunologic effects, and neurobehavioral effects. Data on growth are covered in Section 19.2.2 because there are data from a number of studies on these issues.

THYROID FUNCTION Both series of studies in the Netherlands examined thyroid function in infants and related this to dioxin (or dioxin–furan) and/or PCB levels in breast milk, cord blood, or third-trimester maternal serum samples. The study in Rotterdam, the Netherlands, examined thyroid function in 105 mother–infant pairs.[42] Exposure was estimated using breast milk collected in the 1 to 2 weeks after delivery. The mother–infant pairs were split into two groups at the median dioxin–furan TEQ based on the congeners listed in Table 19.2. The authors measured total T4, total T3, free T4, and thyroid-stimulating hormone (TSH) levels in the mother during the last month of pregnancy and 9 to 14 days postdelivery, in cord blood, and in infants at 9 to 14 days and 3 months after birth (Tables 19.3 and 19.4). Of those enrolled in the study, 78 mother–infant pairs met all criteria and were included in the final analyses. All the thyroid measures were within normal ranges, with the exception of TSH for one woman. All TEQs (dioxin–furan TEQ, coplanar PCB TEQ, nonplanar PCB TEQ, and total PCB–dioxin–furan TEQ) were significantly correlated with infant plasma levels of TSH at the second week and third month, and inversely correlated with total T3 predelivery and total T3 and total T4 post-delivery for the mothers. The only exception is that the nonplanar PCB TEQ was not significantly correlated with the mothers total T4 after delivery and the infants' third-month TSH. Measures from the infants during their second week of life showed a significant increase in TSH (Table 19.4) and a significant decrease for total T4 and free T4 (Table 19.3) for infants in the "high" group.

The second study, among infants in Amsterdam,[50,51] examined thyroid function among 38 full-term breast-fed infants in relation to the total toxic equivalents per kilogram of breast milk fat (TEQ/kg) of dioxins and furans (Table 19.2). The authors measured total T4, thyroxine-binding globulin (TBG), and TSH levels sequentially in cord blood, infants at 1 week of age, and infants at 11 weeks of age (Tables 19.3 and 19.4). Total T3 was measured in cord blood and at 11 weeks, and free T4 was measured in cord blood. Infants were classified into "high" and "low" groups at the median of the range. At 1 week and 11 weeks postnatally, total T4 and total T4/TBG ratios were significantly higher among infants in the "high" group. At 11 weeks, TSH was also significantly higher for the "high" group. The authors suggest that exposure to high levels of dioxins and furans, either in utero or through breast milk, modulates the hypothalamic–pituitary–thyroid regulatory system of the infant.[50,51]

Comments Two studies of nursing infants suggest that ingestion of breast milk with a higher dioxin–furan TEQ value may alter thyroid function.[42,51] Both studies had similar exposure groupings and some findings in common:

TABLE 19.3 Levels of Thyroxine-Binding Globulin (TBG), Thyroxine (T4), Free Thyroxine (FT4), or T4/TBG in Nursing Infants

		Exposed[a]			Unexposed[b]		
Study	Population	*n*	Mean Level	Standard Deviation	*n*	Mean Level	Standard Deviation
Total T4 (nmol/L)							
Pluim et al. (1992),[50]	Neonates; Amsterdam, The Netherlands						
Pluim et al. (1993)[51]	At birth (cord blood)	15	134.3	4.8[c]	18	122.5	4.1[c]
	1 wk postnatal	19	178.7[d]	5.5	19	154.5	6.3
	11 wks postnatal	16	122.2[d]	3.0	18	111.1	4.0
Koopman-Esseboom et al.	Neonates; Rotterdam, The Netherlands						
(1994)[42]	2nd wk postnatal	39	159.9[d]	31.6	39	177.5	39.2
Free T4 (nmol/L)							
Koopman-Esseboom et al.	Neonates; Rotterdam, The Netherlands						
(1994)[42]	2nd wk postnatal	39	23.0[d]	3.3	39	24.6	3.5
TBG (nmol/L)							
Pluim et al. (1992),[50]	Neonates; Amsterdam, The Netherlands						
Pluim et al. (1993)[51]	At birth (cord blood)	15	589.5	30.5[c]	18	520.1	27.2[c]
	1 wk postnatal	19	546.2	19.1	19	532.6	16.3
	11 wks postnatal	16	500.7	13.0	18	519.0	29.4
T4/TBG							
Pluim et al. (1992),[50]	Neonates; Amsterdam, The Netherlands						
Pluim et al. (1993)[51]	At birth(cord blood)	15	0.232	0.008[c]	18	0.240	0.007[c]
	1 wk postnatal	19	0.332[d]	0.011	19	0.291	0.009
	11 wks postnatal	16	0.247[d]	0.009	18	0.220	0.008

[a] High-exposure group: 29.2–62.7 ng toxic equivalents/kg (dioxin–furan TEQ/kg milk fat)[50,51]; > 30.75–76.43 pg dioxin–furan–TEQ/g fat.[41]

[b] Low-exposure group: 8.7–28 ng TEQ/kg[50,51]; 12.44–30.75 pg dioxin–furan TEQ/g fat.[41]

[c] Standard error of the mean.

[d] $p < 0.05$ compared to the unexposed group.

TABLE 19.4 Levels of Thyroid-Stimulating Hormone (TSH) in Nursing Infants

		Exposed			Unexposed		
Study	Population	*n*	Mean Level (μU/mL)	Standard Deviation	*n*	Mean Level (μU/mL)	Standard Deviation
Pluim et al. (1992),[50] Pluim et al. (1993)[51]	Neonates; Amsterdam, The Netherlands						
	At birth (cord blood)	11[a]	11.9	1.9[b]	14[c]	10.4	1.3[b]
	1 week postnatal	11	2.56	0.41	15	2.93	0.41
	11 weeks postnatal	12	2.50[d]	0.26	18	1.81	0.19
Koopman-Esseboom et al. (1994)[42]	Neonates; Rotterdam, The Netherlands						
	At birth (cord blood)	[e]	11.6[d]	8.0		8.5	6.0
	Week 2 postnatal	39[a]	2.6[d]	1.5	39[c]	1.9	0.8
	3 months	39	2.3[d]	1.0	39	1.6	0.6

[a] High-exposure group: 29.2–62.7 ng toxic equivalents/kg (TEQ/kg milk fat)[50,51]; > 30.75–76.43 pg dioxin–furan–TEQ/g fat.[42]

[b] Standard error of the mean.

[c] Low-exposure group: 8.7–28 ng TEQ/kg[50,51]; 12.44–30.75 pg TEQ/g fat.[42]

[d] $p < 0.05$ compared to low-exposure group.

[e] Total for both high and low = 75.

both had significant increases in TSH at about 3 months of age with higher TEQs, and in one report, significant increases at about 2 weeks of age and in the cord blood. Significant changes were found with high TEQ for total T4, but they were in opposite directions, increased in neonates at 1 and 11 weeks of age[51] and decreased for infants during the second postnatal week.[42] Koopman-Esseboom and her colleagues also noted a significant increase in T4/TBG and a significant decrease in free T4. Both studies covered a short observation period, which limits the examination of persistent or long-term changes in thyroid status, and analyses did not control for other factors that might affect thyroid status. These findings suggest a possible shift in the distribution of thyroid hormones and point out the need for collection of longitudinal data to assess the potential for long-term effects associated with developmental exposures.

These two developmental studies investigated relatively small numbers of infants with thyroid parameters in the normal range. However, the "high" group, at about 3 months of age, had increased TSH levels in comparison to the "low" group. Total T4 levels and total T4 to thyroid binding globulin (TBG) ratio were generally elevated in the high infants.

The exact mechanisms accounting for these observations in humans are unknown, but in perspective of animal responses, the following might apply: Dioxin–furan increases metabolism and excretion of thyroid hormone, mainly T4, in the liver. Reduced T4 levels stimulate the pituitary to secrete more TSH, which enhances thyroid hormone production. Early in the disruption process, the body can overcompensate for the loss of T4, which may result in a small excess of circulating T4 to the increased TSH. In animals, given higher doses of dioxin, the body is unable to maintain homeostasis, and TSH levels remain elevated and T4 levels decrease.

AST AND ALT Abnormal levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) may indicate liver cell damage from a number of causes, including hepatic necrosis, metastatic carcinoma, or obstructive jaundice (AST and ALT) or infectious or toxic hepatitis and cirrhosis (AST). Elevated enzyme levels may also be due to nonhepatic origins, such as myocardial infarction, acute pancreatitis (AST and ALT), or skeletal, cerebral, or renal necrosis (AST).

One report[38] has examined blood measures in 35 babies in Amsterdam. Four blood samples were taken: maternal blood around delivery, cord blood, and the infant's blood at 1 and 11 weeks of age. These samples were used to measure leucocytes (white blood cells), platelets, and differential (white cell count), along with plasma, activity of γ-glutamyltransferase (GGT), AST, and ALT, and levels of cholesterol and bilirubin. Dioxin and furan levels were measured in breast milk collected about 3 weeks postdelivery. None of the maternal blood measurements were outside the normal range. A statistically significant inverse correlation was observed in an uncorrected comparison of the number of polynuclear neutrophils and dioxin–furan levels in breast milk ($r = -0.53,\ p = 0.022$); this disappeared when regression analysis compared

TABLE 19.5 Blood Measures and Cumulative Dioxin–Furan TEQs from 11 Weeks of Breast Feeding

Blood Measure	Correlation Coefficient	*p*-Value
ALT	0.40	0.02
AST	0.44	0.009
Platelets	−0.48	0.011

Source: Data from Ref. 38.

these and controlled for gestational age. None of the other factors compared in the cord blood at 1 week or 11 weeks of age were statistically significant. The next set of analyses used the estimated cumulative dioxin–furan intake from breast feeding at 11 weeks of age; the PCDD/PCDF TEQs ranged from 5.7 to 123.7 pg TEQ/g fat, with a mean of 44.7 pg (significant correlations; Table 19.5). These results are unadjusted, but remain significant after adjusting for maternal age, gestational age, and birth weight (regression coefficients were not presented). The authors proposed that changes in ALT and AST suggest an effect on the liver, associated with cumulative exposure to dioxins–furans, and note that all but three of the children had ALT and AST within normal ranges, but the distribution of some of these findings (e.g., an increase in platelets) did vary. From this study it is not possible to determine the reversibility or the clinical significance of these changes.

Comments The Amsterdam reports are based on small groups of infants with undescribed selection procedures. Thus it is not possible to evaluate selection or volunteer bias. These authors did attempt to estimate the dioxin–furan TEQ more closely, using changes in feeding patterns as well as the measured levels in the early sample, but did not evaluate the potential for decreasing levels with increasing length of breast feeding.

IMMUNOLOGIC EFFECTS One report[48] has examined direct and surrogate measures of immune status in 207 babies in Rotterdam. The surrogate measures were derived from questionnaires given to the mothers, covering incidence of rhinitis, bronchitis, tonsillitis, and otitis (inflammation or infection of the nose, bronchi, tonsils, and ear, respectively) in children up to 18 months of age. Almost all of the children (205) were immunized against measles, rubella, and mumps; the children's antibody levels to these were used to assess humoral antibody production. For the purposes of this report, cord bloods from 48 of these children (the selection criteria were not presented) were analyzed to assess prenatal TEQ levels; at age 3 months, 47/48 bloods were drawn from the original group, with another child, randomly selected, added to this group. At 18 months, 37 of the original children gave blood, and 6 other children, randomly selected, were added to this group. In these samples, the following were measured: monocytes, granulocytes, and lymphocytes in whole blood; lymphocyte

subpopulations were determined using monoclonal antibodies. No relationship was found between pre- and postnatal total TEQ levels and respiratory tract symptoms (i.e., number of periods with rhinitis, bronchitis, tonsillitis, and otitis) or humoral antibody production at 18 months to vaccination against mumps, measles, and rubella at 14 months. A higher prenatal exposure, estimated by cord blood levels, was associated with alterations in T-cell subsets, with an increased number of TcR$\gamma\delta^+$ T-cells; increased total numbers of T-cells, CD8$^+$ cells, and TcR$\gamma\delta^+$ T-cells at 18 months of age were associated with higher TEQ levels (Table 19.6). Higher TEQ levels were also associated with a decreased number of monocytes (total TEQ, dioxin–furan TEQ, mono-ortho PCB TEQ, and diortho PCB TEQ) and granulocytes (total TEQ only) at 3 months. All values were found to be within clinically normal ranges. The authors suggested that the subtle changes in the number of blood leukocytes do

TABLE 19.6 Developmental Immunologic Outcomes in a Study of Dutch Children

Study and Outcome	Exposure	Correlation Coefficient	*p*-Value
Weisglas-Kuperus et al. (1995)[48]			
Monocytes at 3 months	Total TEQ	−0.64	≤ 0.01
	Dioxin–furan TEQ	−0.55	≤ 0.01
	Mono-ortho PCB TEQ	−0.67	≤ 0.01
	Di-ortho PCB TEQ	−0.51	≤ 0.05
Granulocytes at 3 months	Total TEQ	−0.47	≤ 0.05
TCR$\gamma\delta^+$ T-cells at birth	Total TEQ	0.50	≤ 0.05
	Dioxin–furan TEQ	0.57	≤ 0.01
TCR$\alpha\delta^+$ T-cells at 18 months	Total TEQ	0.57	≤ 0.05
	Dioxin–furan TEQ	0.71	≤ 0.01
	Di-ortho PCB TEQ	0.61	≤ 0.05
CD3$^+$ CD8$^+$ cells at 18 months	Total TEQ	0.65	≤ 0.05
	Dioxin–furan TEQ	0.80	≤ 0.01
	Planar PCB TEQ	0.71	≤ 0.01
	Di-ortho PCB TEQ	0.68	≤ 0.05
Weisglas-Kuperus et al. (2000)[49]			
Lymphocytes at 42 months	Total maternal PCB	0.25	0.02
	Total cord PCB	0.22	0.05
CD3$^+$ at 42 months	Total maternal PCB	0.25	0.02
	Total cord PCB	0.21	0.07
CD3$^+$CD8$^+$ at 42 months	Total maternal PCB	0.27	0.01
	Total cord PCB	0.24	0.04
CD4$^+$CD45RO$^+$ at 42 months	Total maternal PCB	0.25	0.02
	Total cord PCB	0.26	0.02
TCR$\alpha\beta^+$ T-cells at 42 months	Total maternal PCB	0.25	0.02
	Total cord PCB	0.20	0.08
CD3$^+$HLA-DR$^+$ at 42 months	Total maternal PCB	0.26	0.02
	Total cord PCB	0.31	0.005

not necessarily mirror alterations in the cell composition of lymphoid and nonlymphoid organs, nor do they necessarily reflect functional defects.[48]

In an update to the report described above,[49] 193 children were examined at 42 months of age. Questionnaires were completed for 175, including questions on infection and allergic disease in the children. Blood samples were collected on a subsample of 85 children (selection process not described), limiting data on concurrent PCB levels (PCBs 118, 138, 153, 180) in plasma and immunologic marker analyses in lymphocytes. An examination of questionnaire reports of infectious diseases and allergies indicated that a reduction in the number of episodes of attacks of shortness of breath with wheeze was associated with prenatal PCB exposure ($n = 175$; OR = 0.44; 95% CI = 0.18 to 0.99); more associations were observed with current PCB levels ($n = 85$): increases in recurrent middle ear infections (OR = 3.05; 95% CI = 1.17 to 7.98); chickenpox (OR = 7.63; 95% CI = 1.21 to 48.54); and reduced allergic reactions (OR = 0.01; 95% CI = 0.01 to 0.37). Dioxin–furan TEQ at birth was associated with increased coughing, chest congestion, and phlegm (OR = 1.06; 95% CI = 1.00 to 1.11). Total PCB levels at 42 months were significantly lower for formula-fed children than for breast-fed children (0.21 μg/L vs. 0.75 μg/L, $p < 0.05$); however, no significant differences were observed for recurrent middle ear infections, chickenpox, or allergic reactions. Interestingly, when these three outcomes were examined by duration of breast feeding, the risk for infections and chickenpox were lower with longer breast feeding and higher for allergic reactions with longer breast feeding (all were borderline nonsignificant at $p = 0.06$ or 0.07). These patterns were observed even though the total PCB levels at 42 months were over 70% higher (not significant). Positive associations were observed between the total PCB levels at birth (cord blood, maternal blood, or both) and lymphocytes, $CD3^+$, $CD3^+CD8^+$, $CD4^+CD45RO^+$, and $CD3^+HLA\text{-}DR^+$ T cells; no associations were observed with the current PCB levels or with the dioxin–furan TEQ at birth. The authors concluded that effects of perinatal exposures persist and are associated with a greater likelihood of infectious disease but less likelihood of allergic conditions. In addition, they concluded that the benefits of longer periods of breast feeding helped to counteract the effects of exposure.

NEUROBEHAVIORAL EFFECTS Five recent reports from the Netherlands have examined neurologic/behavioral outcomes (summarized in Table 19.7). A pair of studies examined the same group of children from Rotterdam (Table 19.7). These infants were tested for (1) psychomotor (PDI) and mental development indices (MDI) based on the Dutch standardized version of Bayley Scales of Infant Development[45] at ages 3, 7, and 18 months; (2) Visual Recognition Memory Scores based on the Fagan Test of Infant Intelligence[44] at ages 3 and 7 months; and (3) use of the Prechtl neonatal neurologic examination[43] to classify infants at about 2 weeks of age as to neurologic normality.

The first study[44] demonstrated a significant increase in the visual recognition memory test (Fagan Test of Infant Intelligence) for breast feeding and length of

TABLE 19.7 Studies of Neurologic and Behavioral Effects among Dutch Infants

Study	Exposure	Population	Evaluation	Findings				
Koopman-Essebaum et al. (1995)[44]	Total PCB–dioxin–furan TEQ from breast milk Categories for total TEQ[a]: Low: 3 months = 168–617 7 months = 168–769 Med.: 3 months = 618–810 7 months = 770–1289 High: 3 months = 811–1860 7 months = 1290–4340 $\sum PCB_{plasma}$ – ng/g[e]	*Exposed:* 105 breast-fed infants and their mothers *Not exposed:* 102 bottle-fed infants and their mothers All in Rotterdam	From Fagan Test of Infant Intelligence: Visual Recognition Memory Test 3 months of age 7 months of age	105 61.5 (9.0) 105 59.9 (5.9)		101 62.2 (10.7) 102 57.3 (5.9)		
			Visual Recognition Memory Test at 7 months[b]	Regression Analysis				
				Coefficient		Std. Error		
		$n = 182$	Medium exposure	4.4[c]		1.7		
			High exposure	4.6[d]		2.2		
			Breast feeding-duration	−0.46		1.0		
			or					
		$n = 181$	Ln($\sum PCB_{plasma}$)	−0.50		1.07		
			Breast feeding-duration	1.63[c]		0.56		
Koopman-Essebaum et al. (1995)[43]	PCB–dioxin–furan TEQ from breast milk and thyroid status	*Exposed:* breast-fed infants; 104 in Groningen, 105 in Rotterdam *Comparison:* bottle-fed infants; 107 in Groningen, 102 in Rotterdam	Prechtl neurologic exam (2 wk): 23 neurologically abnormal, 394 neurologically normal	Neurologically Normal			Neurologically Abnormal	
			$\sum PCB_{cord\ blood}$[e]	352	0.5 (0.3)		20	0.4 (0.1)
			$\sum PCB_{maternal\ blood}$	393	2.2 (0.9)		21	2.0 (0.6)
			Dioxin–furan TEQ	189	30.0 (10.2)		12	25.4 (7.9)
			Planar PCB TEQ	194	16.3 (7.4)[d]		12	11.7 (3.8)
			Mono-ortho PCB TEQ	189	14.8 (5.3)		12	12.6 (4.6)
			Di-ortho PCB TEQ	189	4.4 (2.2)		12	3.7 (1.5)
			Total PCB–dioxin–furan TEQ	182	65.3 (21.6)[d]		12	52.5 (15.6)

Huisman et al. (1995)[40]	Dioxins, dibenzofurans, and PCBs in breast milk (no effects observed in maternal or cord blood)	*Exposed:* breast-fed infants; 104 in Groningen, 105 in Rotterdam *Comparison:* bottle-fed infants; 107 in Groningen, 102 in Rotterdam	Prechtl's exam at 10–21 days: neurologic optimality score (NOS) ORs from logistic regression	Odds Ratio	95% Confidence Interval
			Total PCB–dioxin–furan TEQ[d]	3.21	1.37–7.48
			Dioxin–furan TEQ	3.12	1.36–7.18
			D 54 (TEF = 0.5)	2.23	1.10–4.52
			D 66 (TEF = 0.1)	1.64	1.08–2.50
			D 67 (TEF = 0.1)	3.03	1.34–6.81
			D 70 (TEF = 0.1)	1.23	1.03–1.48
			D 73 (TEF = 0.01)	1.61	1.01–2.56
			F 83 (TEF = 0.1)	1.31	1.04–1.66
			F 114 (TEF = 0.5)	2.48	1.16–5.32
			Planar PCB TEQ	1.67	0.97–2.87
			PCB 169 (TEF = 0.01)	2.33	1.13–4.80
			Nonplanar PCB		
			PCB 70	1.88	1.29–2.72
			PCB 99	2.01	1.19–3.40
			PCB 118 (TEF = 0.0001)	2.21	1.24–3.95
			PCB 138	2.73	1.40–5.35
			PCB 153	2.76	1.39–5.48
			PCB 156 (TEF = 0.0005)	2.55	1.31–4.95
			PCB 170 (TEF = 0.0001)	2.33	1.19–4.56
			PCB 177	1.96	1.14–3.39
			PCB 183	2.32	1.21–4.46
			PCB 187	1.87	1.07–3.28
			$\sum \mathrm{PCB}_{\mathrm{milk}}$	2.93	1.45–5.90
			Mono-ortho PCB TEQ	2.78	1.40–5.59
			Di-ortho PCB TEQ	2.38	1.21–4.71

(Continued)

TABLE 19.7 *(Continued)*

Study	Exposure	Population	Evaluation	Findings	
Huisman et al. (1995)[41]	$\sum PCB_{cord\ blood}$ No effects observed with breast milk	*Exposed:* breast-fed infants; 104 in Groningen, 105 in Rotterdam *Comparison:* bottle-fed infants; 107 in Groningen, 102 in Rotterdam	Neurologic development in 18-month-old children	Coefficient (Std. Error)	*p*-Value
			Neurologic optimality score		
			$Log(\sum PCB_{cord}/0.8)$	−0.149 (0.049)	0.003
			Paternal smoking	−0.402 (0.130)	0.002
			$Log(\sum PCB_{cord}/0.8)$ × paternal smoking	0.200 (0.078)	0.011
			Above analysis presented by paternal smoking status		
			Children w/nonsmoking fathers	−0.149	0.003
			Children w/smoking fathers	−0.051	0.402
			Fluency cluster score		
			$Log(\sum PCB_{cord})$	−0.295 (0.175)	0.093
			Breast (0) vs. bottle (1) fed	−0.450 (0.177)	0.012

Koopman-Essebaum et al. (1996)[45]	Total PCB–dioxin–furan TEQ from breast milk	*Exposed:* 105 breast-fed infants and their mothers *Not exposed:* 102 bottle-fed infants and their mothers All in the Rotterdam area	Bayley Scales of Infant Development Psychomotor Development Index (PDI) of infant at age of:	*n*	Mean (SD)	*n*	Mean (SD)
			3 months	99	118 (12)	100	117 (12)
			7 months	105	115 (15)[d]	102	111 (13)
			18 months	105	110 (17)	101	108 (14)
			Mental Development Index (MDI) of infant at age of:				
			3 months	101	128 (31)	100	126 (13)
			7 months	105	115 (11)[d]	102	112 (9)
			18 months	105	113 (18)[c]	102	107 (17)

(Continued)

TABLE 19.7 *(Continued)*

Study	Exposure	Population	Evaluation	Findings	
				Regression Analysis	
				Coefficient	Std. Error
	Categories for total TEQ[a]:		PDI at 7 months[b]		
	Low: 3 months = 168–617		Medium exposure	−9.5[c]	3.9
	7 + 18 months = 168–769	$n = 182$	High exposure	−7.7	4.9
	Med.: 3 months = 618–810		Breast feeding-duration	6.9[c]	2.3
	7 + 18 months = 770–1289	$n = 198$	PDI at 3 months		
	High: 3 months = 811–1860		$\mathrm{Ln}(\sum \mathrm{PCB}_{\mathrm{plasma}})$	−4.8[d]	2.0
	7 + 18 months = 1290–4340	$n = 206$	Breast feeding-duration	0.91	0.91
			MDI at 7 months		
	$\sum \mathrm{PCB}_{\mathrm{maternal\ blood}}$ − ng/g[e]		$\mathrm{Ln}(\sum \mathrm{PCB}_{\mathrm{plasma}})$	2.0	1.7
			Breast feeding-duration	2.0[d]	0.9

[a] pg PCB–dioxin–furan TEQ/g fat times weeks breast feeding.

[b] overall p for high + medium = 0.05.

[c] $p < 0.01$.

[d] $p < 0.05$.

[e] $\sum \mathrm{PCB}_{\mathrm{maternal\ blood}}$ (ng/g), maternal blood level sometime during 36–40 weeks of gestation. This and cord blood are measured in ng/g plasma.

[f] All summary logistic results presented; for individual chemicals, only those with ORs significantly greater than 1 are presented.

breast feeding when examined with maternal serum PCB levels. Neither the prenatal PCBs nor the nondioxinlike PCBs in breast milk were associated with the Fagan outcome at either time period. In this report and another[45] report below, a cumulative score was developed for low, medium, and high exposure which multiplied the pg total PCB–dioxin–furan TEQ/g fat times weeks of breast feeding (see Table 19.7). In the final regression analyses, significant differences were not observed for total PCB–dioxin–furan TEQ with outcomes at 3 months of age. However, at 7 months, there was a dose-related increase in scores with medium and high total PCB–dioxin–furan TEQ (Table 19.7). The authors suggested that these benefits resulted from (1) increased breast feeding and (2) the high total PCB–dioxin–furan TEQ being an artifact of its correlation with the higher level of lipids or lipophilic factors [e.g., hormones, long-chain polyunsaturated fatty acids (LCPUFAs)] that are beneficial to this aspect of development.

The second study[45] observed a beneficial effect on PDI at 7 months of age (PDI-7) for breast feeding versus formula when the total PCB–dioxin–furan TEQ was low (Table 19.7). There were statistically significant deficits observed for PDI-7 in the regression analysis of medium levels of total PCB–dioxin–furan TEQ, and for medium and high levels of total PCB–dioxin–furan TEQ combined. MDI-7 showed a significant increase with duration of breast feeding, but the total PCB–dioxin–furan TEQ did not have a significant effect. The other endpoints (MDI-3, PDI-3, MDI-18, and PDI-18) were not associated significantly with either duration of breast feeding or total PCB–dioxin–furan TEQ. In the analysis of prenatal PCB exposure (using maternal blood levels collected late in pregnancy), PDI scores were lower at 3 months with higher PCB levels. The authors also examined thyroid hormone levels because they are necessary for brain development and found no significant effects of thyroid hormone levels on PDI or MDI.

The third study[43] used the Prechtl neonatal neurologic examination to classify infants (about 2 weeks of age) as to neurologic normality: ''normal,'' ''mildly abnormal'' (e.g., mild hypotonia or tremor), or ''definitely abnormal'' (e.g., hyperexcitability, hypotonia, hypertonia, or a hemisyndrome). Two infants in each location were classified as definitely abnormal, and 20 total were classified as mildly abnormal (11 in Groningen and 9 in Rotterdam). One definitely abnormal child was eliminated from further analyses because of a birth trauma. Because of the small numbers, the remaining 23 children were grouped together and termed neurologically abnormal. These groups were examined for obstetric optimality scores and thyroid levels and no significant findings were observed. The categorization of neurologically normal or abnormal, as expected, was highly correlated with the neurologic optimality scores (postural tone cluster and reflex cluster).[40,41] The levels of coplanar PCB TEQ and total PCB–dioxin–furan TEQ were different in the two groups (Table 19.7). Only free T4 was significantly different in the two groups (total T3, total T4, free T4, and TSH were tested). The authors concluded that there was no signficant

relationship of dioxins, furans, and PCBs with these "clinically relevant" outcomes and recommended follow-up of these children as they aged.

Two more studies examined the Rotterdam children and the Groningen children together (Table 19.7). These studies covered neonatal neurologic development at 10 to 21 days postbirth[41] and neurological condition at 18 months.[40]

In the first report,[41] infants were examined 10 to 21 days after birth, and several evaluations were made: (1) neonatal neurological condition (394 infants were normal, 20 suspect, and 4 abnormal); (2) Prechtl's Neurologic Optimality Scores (NOS), based on 21 items; and (3) these 21 items grouped to develop postural tone cluster scores and reflex cluster scores. The NOS and cluster scores were then dichotomized for use in the statistical analyses: The NOS was divided at the median (a score of 57), the postural tone cluster score [less than or equal to 9 (43% of the children)] versus greater than 9, and the reflex cluster score [less than or equal to 10 (22% of the children)] versus greater than 10. Prenatal PCB measures (maternal blood and cord blood) were not associated with NOS or the clusters. Many individual PCBs, dioxins, and furans in breast milk were associated with NOS (Table 19.7), as were most of the summary measures based on breast milk (total PCB–dioxin–furan TEQ, dioxin–furan TEQ, $\sum \text{PCB}_{\text{breast milk}}$, mono-ortho PCB TEQ, and di-ortho PCB TEQ.) Coplanar PCB TEQ was associated with hypotonia (measured through the postural tone cluster score): OR = 1.64 (95% CI = 1.03 to 2.63). Because the data suggested observer differences in the two communities (by a shift in the distribution between them), analyses controlled for community. However, the scoring of the two observers was not compared for some common subjects.

The next report[40] examined the same groups of infants at 18 months of age. The infants were assessed during an observation of motor functions using techniques described by Touwen and colleagues.[54] Of the 418 children scored, 408 were considered normal and the remainder were mildly abnormal[9] or abnormal.[1] Only the prenatal PCB exposure (estimated by either $\sum \text{PCB}_{\text{cord}}$ or $\sum \text{PCB}_{\text{maternal blood}}$) was associated with abnormality at 18 months (Table 19.7). The authors observed an interaction with paternal smoking, so that the adverse outcome with exposure was observed only in children with nonsmoking fathers. The authors noted that maternal smoking was collected only during pregnancy, so the association of maternal postnatal smoking could not be evaluated. None of the measures of PCB or dioxin–furan were associated with the fluency cluster score, but breast-fed children in general did have higher scores than those of formula-fed children.

These children were again assessed at 42 months of age.[47] In this round, 395 children (94% of the original study group) were assessed for cognitive abilities using the Kaufman Assessment Battery for Children (K-ABC), and a subgroup of 193 (the Rotterdam children) were assessed for verbal comprehension using the Reynell Language Developmental Scales (RDLS). $\sum$PCB was calculated using PCBs (IUPAC 118, 138, 153, 180) from the mother's blood, cord blood, and plasma from the 42-month-old children. PCB and

dioxin–furan levels were available for the breast milk samples collected 2 weeks after delivery from breast-feeding mothers. Exposure metrics included $\sum$PCB, total TEQ (dioxin–furan and PCB), and the sum of 20 nondioxinlike PCBs. Statistically significant deficits were associated with the natural log of the $\sum PCB_{\text{maternal blood}}$ for K-ABC for the entire group, and for those children who were formula-fed. Significant deficits for the RDLS were noted only in the formula-fed children. Analyses of the current body burden in the children were not associated with any cognitive deficits. Statistically significant changes were not observed in the breast-fed children, possibly because of the higher SES status, parental education, and parental verbal IQs. Another possibility is the beneficial effects of breast feeding in general.

Comments One factor supported in this series of studies is the benefit derived from breast feeding. Even though the level of environmental toxicants reaching the child through early dietary exposure may be greater with breast feeding, formula-fed children did not do as well overall on many behavioral and neurological measures in these studies. This may not be true with environmental "accidents," which could result in much higher levels to the child. These differences could also be attributed to the association of breast feeding with socioeconomic status of the households, parental education levels, and so on.

A large number of dioxins, furans, and PCBs were evaluated at different developmental stages. Given the smaller volume in the collection of third-trimester blood from the mother and cord blood at birth, only four PCBs were measured (IUPAC 118, 138, 153, 180). Thus, prenatal dioxin–furan levels can only be approximated in these data. The statistically significant correlations between the different agents and biological sources suggest that it would be difficult to sort out effects of any individual group or class of agents.

In some of these studies, total breast-feeding time and breast milk levels were used to estimate the total exposure via breast feeding. This model is a reasonable relative estimate of broad categories but may be problematic for estimation for women with widely different lengths of breast feeding. The levels in breast milk are likely to decrease over time, and the consumption of breast milk is likely to drop gradually as other food sources are increased. Thus the general levels of the broad groupings are useful, but the individual estimates should be used with caution.

Several of these studies based their results on crude (unadjusted) analyses. Given that there were significant differences between the breast-feeding parents and the bottle-feeding parents as to socioeconomic status (e.g., education, profession) and other lifestyle factors (e.g., smoking and drinking patterns), these results could change with a more in-depth analysis. The observation of hypotonia and prenatal PCB exposures is consistent with another study from the 1980s.[55] This study found effects of prenatal exposure (but not postnatal through breast feeding) on hypotonia, as did one of the Dutch studies.[41] These associations with prenatal exposure have persisted up to 42 months of age.[47] These findings are consistent with findings of cognitive deficits in 11-year-old

children exposed prenatally to PCBs in Michigan; as with the Dutch studies, deficits were not associated with exposures through breast feeding.[56]

Tooth Development in Finnish Children An investigation of dioxin exposure and tooth development was done in Finnish children[57,58] as a result of studies of dental effects in dioxin-exposed rats, mice, and nonhuman primates and in PCB-exposed children.[59] The Finnish investigators examined enamel hypomineralization of permanent first molars in 6- or 7-year-old children. These molars were mineralized during the postnatal period, when the children are exposed through breast feeding. The population was first identified in Helsinki and Kuopio as part of an international effort, coordinated by WHO/EURO, to evaluate possible health effects associated with PCDDs and PCDFs in breast milk. Approximately 150 women, recruited from each area, committed to provide a breast milk sample at 4 weeks postpartum, if still lactating. A total of 167 samples were obtained, with about a 50% response rate in Helsinki (77 women) and about 60% from Kuopio (90 women). Exposure of each child was estimated using the TEQ for PCDDs and PCDFs and their elimination constant, plus the length of breast feeding. At age 6 or 7, 102 children's teeth were examined (61% of those with breast milk samples). Defects were scored as to severity and size, blind to exposure score. Duration of lactation ranged from 1 to 36 months (mean 10.5 ± 5.5 SD) and TEQs from 3.8 to 99.4 pg/g milk fat (mean 19.8 ± 10.9 SD). The length of breast feeding was not associated significantly with mineralization changes, nor was the TEQ alone. However, when the levels and length of breast feeding were combined in an overall score, a statistically significant association was observed ($r = 0.3$, $p = 0.003$, regression analysis). The beta from the analysis was not presented, so the slope of the relationship is unknown. The levels in breast milk might also be a surrogate for in utero exposure; these samples were collected after 4 weeks of breast feeding, so might not be as similar as samples collected earlier in lactation.

Comments These data present interesting findings relating hypomineralization of permanent first molars and TEQ exposure through breast feeding. Unfortunately, the presentation is incomplete, due to limited information on adjustment for other risk factors/confounders; the study has a small number of subjects and consequently low power; and since the beta was not presented for the regression analyses, the potential biological significance cannot be examined. This is an interesting outcome for additional examination.

Seveso, Italy In 1976, an explosion of a trichlorophenol reactor in a 2,4,5-T production facility in Medina, Italy, contaminated the neighboring city of Seveso, Italy with TCDD (see Chapter 20). Several reports[60–67] compared four potentially affected communities (Seveso, Meda, Cesano, and Desio) to nearby unexposed communities. The contaminated area was subdivided into three zones (A, B, and R) of decreasing mean soil levels of TCDD.[68] The mean

TCDD concentration in zone A was 230 μg/m^2; in zone B, 3.0 μg/m^2; and in zone R, 0.9 μg/m^2.

In 1979, Pocchiari et al.[71] reported on initial efforts to screen residents in zones A, B, and R for TCDD-related effects. Since then, a series of studies have evaluated a variety of outcomes, mostly classifying the Seveso population by residence in zones A, B, or R, based on mean soil concentration of TCDD. "This [use of soil contamination] is a rather poor surrogate of exposure, and by no means an indicator of intake, since it does not take into consideration all the possible sources and ignores interindividual variability."[72]

Over time, serum levels of TCDD were evaluated for various groups of potentially exposed residents. Serum levels of Seveso residents were obtained for a small proportion ($n = 20$) of the total number of residents of zone A within 1 year of the reactor release.[25] The levels may be related to a number of factors, including age (younger children were outside at the time of the release), whether the resident was inside or outside, ingestion of local produce, or number of days of residence in the area after the release, to name a few. The potential for substantial exposure was high for people residing in the area. The range of levels in the 20 zone A residents was 820 pg/g to 56,000 pg/g (median = 7400 pg/g). Additionally, mean serum TCDD concentrations in a sample of residents 13 years and older at the time of the explosion were: zone A, 443 pg/g lipid ($n = 177$); zone B, 87 pg/g lipid ($n = 54$); zone R, 15 pg/g ($n = 17$).[69]

Finally, 30 years after the explosion, Landi et al.[70] measured serum TCDD in 62 individuals randomly selected from zones A, B and non-ABR. Geometric mean serum concentrations were 53.2 pg/g lipid in zone A, 11.0 pg/g lipid in zone B, and 4.9 pg/g lipid in zone non-ABR. These data suggest that elevated levels persist but are decreased from the original levels of 1976.

This chapter is limited to those reports using individual biologic measures of TCDD or related congeners. Therefore, the recent study examining sex ratio is the only one included here (see Section 19.2.2).

Studies of Vietnam Experience in Ranch Hands Evidence from earlier studies of Vietnam experience of Agent Orange exposure and the risk of adverse pregnancy outcomes has been described as "sparse, sometimes off the point, sometimes conflicting. . . ."[5] The general dissatisfaction with these studies had a common factor: the lack of a valid measure of dioxin exposure. Once the assays to document individual TCDD exposure became the gold standard for assessing exposure assumptions in studies, this concern for exposure misclassification was justified. Until 1992, when the first study to examine reproductive outcomes among Vietnam veterans based on individual exposure measurements of TCDD was published,[73] this remained the major criticism of the research. Even though TCDD serum levels were available for reanalysis of the 1984 data of the Ranch Hand study, they were not used in that report. A later reanalysis[74] estimated TCDD level at the time of conception. The fol-

lowing discussion focuses on those studies using biomarkers. Earlier data are presented in limited form for historical context.

The epidemiologic studies of U.S. military personnel stationed in Vietnam conducted by the U.S. Air Force has, in recent years, made extensive use of biomarkers of exposure. The study includes Air Force personnel in Operation Ranch Hand employed in the aerial spraying of Agent Orange in Vietnam from 1962 to 1971. Comparisons included Air Force personnel who flew or maintained C-130 aircraft in Southeast Asia during the same time period.

The effort includes cross-sectional medical studies conducted at 5-year intervals, beginning with the baseline study in 1982 ($n = 1045$ exposed, 1224 unexposed). Two follow-up evaluations were conducted in 1985 ($n = 1016$ exposed, 1293 unexposed) and 1987 ($n = 995$ exposed, 1299 unexposed). Each cross-sectional study included comprehensive physical and psychological evaluations. In the 1982 baseline and 1985 and 1987 follow-up studies, ''exposure'' was based on the classification of Ranch Hand group versus comparison group. An additional analysis approximated exposure (low, medium, high) for the Ranch Hand group by using historical military data and herbicide procurement and usage records. The results of these analyses were prepared by Lathrop and colleagues[75,76] and are described in detail elsewhere.[3,6] In 1988, serum TCDD levels were measured for a sample of the 1987 Ranch Hand group ($n = 866$) and the 1987 comparison group ($n = 804$). The 1987 examination data were then reanalyzed using lipid-adjusted serum TCDD levels as the relative measure of exposure. The median serum TCDD level adjusted for lipids for the Ranch Hand group was 12.8 pg/g, ranging to 618 pg/g. For the comparison group, the median level was 4.2, ranging to 54.8 pg/g.[34] For later studies, veterans who refused to give serum samples in 1987 or received a nonquantifiable result were resampled in 1992.

The overall strengths of this study include: (1) it is a large study, with good power to detect many common disorders; (2) follow-up was very good, as is continued participation of Ranch Hand and comparison populations; (3) physical and psychological examinations are extensive, planned to evaluate most, if not all, outcomes potentially associated with TCDD; (4) continued reevaluation of the subjects (every 5 years) permits investigators to monitor the development of chronic diseases and to test for additional outcomes as new biochemical and toxicological data become available; and (5) serum TCDD levels permitted validation of the exposure matrix based on historical records and the subsequent development of disease-specific dose–response models. Repeated measures of serum TCDD over time also provide valuable information on its half-life in humans.

Noteworthy caveats in the study include the fact that the majority of the population had serum levels under the background level of 20 pg/g (median = 12.8 pg/g, range to 600 pg/g in 1987). These data suggest that while some Ranch Hands had very high levels of TCDD, most of the study group had lower exposures, if any at all. In addition, serum TCDD levels indicated

that the exposure matrix used in the analysis of the baseline and 1984 studies did not appropriately describe the potential for exposure. Therefore, analyses described here will refer to only those using serum levels. The adjusted odds ratios for the three categories of serum TCDD selected by Roegner and colleagues[34] are discussed. The categories of TCDD are ≤ 10 pg/g, 15 to ≤ 33.3 pg/g, and > 33.3 pg/g.

In the 1992 follow-up examination findings,[77] results were presented by the current TCDD concentration (current dioxin level) and by the concentration estimated during duty in Southeast Asia (initial level) in the following categories: comparison, current dioxin level ≤ 10 pg/g of lipid; background (Ranch Hand), current dioxin level ≤ 10 pg/g of lipid; low (Ranch Hand), current dioxin > 10 pg/g and 10 pg/g $<$ initial dioxin ≤ 143 pg/g; high (Ranch Hand), current dioxin > 10 pg/g and initial dioxin > 143 pg/g. In subsequent analyses, low and high categories the definitions were changed to the following: low (Ranch Hand), current < 10 and initial < 94 pg/g; high (Ranch Hand), current > 10 and initial > 94 pg/g.[78]

A consequence of this comprehensive study with a large number of statistical tests is an increased possibility of spurious findings. The reader should be aware of this limitation when looking for consistencies with the results of other studies and in the toxicological literature. In addition, the analysis model was developed 15 years ago and has been applied to all outcomes, regardless of the biological plausibility and differing dose–response curves.

Baseline Study, 1984 This initial report of the health of Ranch Hand personnel used cohort status (Ranch Hand vs. comparisons) as the basis for evaluating effects and exposure, and thus only information about the population and response rates are briefly discussed here. This group of exposed veterans included those who served in Vietnam during 1962–1965, when Herbicides Purple, Pink, and Green were sprayed. These herbicides had higher TCDD concentrations (33, 66, and 66 ppm, respectively) than Herbicide Orange, with 2 ppm TCDD.[75]

The protocol consisted of a comprehensive personal and family health questionnaire and a physical examination, including an in-depth laboratory analysis. The response rates for each phase of the protocol were quite different both within and between cohorts. Participation in the questionnaire phase was 97% ($n = 1174$) for the Ranch Hands and 93% ($n = 956$) for controls. In the physical examination phase, participation dropped to 87% ($n = 1045$) for the Ranch Hands and 76% ($n = 773$) for controls.

Nonresponders were ''on the average'' younger than participants. Ranch Hand enlisted personnel had higher participation rates than officers, and black Ranch Hand officers had lower participation rates than nonblack officers. The difference in the response rates for the physical examination phase of the protocol was ascribed partially to the active encouragement of the Ranch Hand Association for participation and the intense media coverage that the study

received. The authors stated that the majority of reasons given for nonparticipation were "no time–no interest" and passive refusal.

The reproductive outcomes evaluated in this phase of the study were ascertained through questionnaires obtained from both the veterans and their spouses or partners. A total of 7399 conceptions were analyzed in this report. There were 3293 conceptions among 1174 Ranch Hands and 4106 among the 1531 controls.

Unadjusted analyses were conducted to examine the relationship between exposure and neonatal death, infant death, physical handicaps, birth defects, and learning disabilities. These analyses were stratified by pre- and post-SEA (Southeast Asia) service periods. The results indicated that Ranch Hands were borderline or statistically significantly more likely to report physical handicaps ($p = 0.07$), birth defects ($p = 0.08$), and neonatal deaths ($p = 0.02$) in the post-SEA analysis. After adjustment for maternal and paternal covariates, the relationship with birth defects achieved statistical significance ($p = 0.04$); the other relationships were not statistically significant.

Twelve of the 76 birth defects reported to have occurred among the Ranch Hands after post-SEA service were skin anomalies (ICD Code 757). When these anomalies are excluded, this relationship is no longer statistically significant ($p = 0.14$), although "still of interest."

Finally, semen samples from Ranch Hands ($n = 560$) and controls ($n = 409$) were analyzed for sperm count and morphology. The response rates for this parameter were 72.5 and 76.5%, respectively, although some of the samples submitted were ineligible for analysis because of prior vasectomies and orchiectomies. Linear regression techniques examined sperm count (as a continuous variable) and percentage of sperm with abnormal morphology as dependent variables. Independent variables were age and exposure to industrial chemicals. No differences in either parameter were identified.

Ranch Hand Study, 1992 The significant association between Ranch Hand status and birth defects found in the baseline study was of sufficient interest to launch a massive project to verify all reported conceptions and pregnancy outcomes through medical record abstraction. In addition, in 1987, serum TCDD levels were obtained from a subset of Ranch Hands and controls. In 1992, the U.S. Air Force released the results of the first study that examined the relationship between direct measure of individual serum TCDD levels and verified reproductive outcomes.[73] A total of 4607 conceptions were examined in this study; 2533 were contributed by 791 Ranch Hands, and 2074 were contributed by 768 controls.

Ranch Hand personnel were shown to have significantly higher TCDD levels compared with the controls in 1987. The median values were 12.8 and 4.2 pg/g, respectively. The 98th percentile for Ranch Hands was 166.4 pg/g; for controls, 10.4 pg/g. These results were used to estimate initial doses received during the veterans' tour in Southeast Asia but not the TCDD level at the time of conception.

TABLE 19.8 Rates of Miscarriage, by Vietnam Tour Status and Time Since Tour of Duty[a]

Time of Conception	Time Since Tour (years)	Miscarriage Rate per 1000 (no./*n*) by Current Dioxin Level			*p*-Value
		10–14.9 pg/g	15–33.3 pg/g	> 33.3 pg/g	
Pretour	≤ 18.6	142.0 (23/162)	146.8 (32/218)	48.8 (2/41)	0.014[b]
	> 18.6	123.9 (14/113)	159.4 (33/207)	166.7 (16/96)	
Posttour	≤ 18.6	92.1 (7/76)	136.6 (22/161)	168.5 (15/89)	
	> 18.6	237.3 (14/59)	198.6 (29/146)	121.5 (13/107)	

Source: Adapted from Ref. 73.

[a]Study of 1475 Ranch Hands with > 10 pg/g serum dioxin.

[b]Comparison of pre- and post-tour data.

There was a significant variation in the association between TCDD and miscarriage with time since SEA tour (≤ 18.6 years or > 18.6 years) and time of conception (pre- or post-SEA tour) among Ranch Hands with current TCDD levels > 10 ppt ($p = 0.01$) (Table 19.8). This was attributed to the low miscarriage rate among the pre-SEA Ranch Hands with current TCDD levels > 33.3 pg/g lipids. In examining post-SEA conceptions only, a linear trend can be seen for spontaneous abortions and increasing TCDD levels among Ranch Hands who had "late tours" in SEA (i.e., less than or equal to 18.6 years had elapsed between their tour of duty and current TCDD levels). The opposite trend is noted in Ranch Hands with "early tours" (i.e., more than 18.6 years had elapsed between the end of duty and the 1987 blood draw). It was concluded that TCDD did not affect the rates of miscarriage because it seemed "implausible that dioxin would act differently in the two groups."

An alternative explanation might be that there is a relationship, but it cannot be detected by this type of analysis. To evaluate the relationship between TCDD level and spontaneous abortion, TCDD level at the time of conception must be considered. Assuming a half-life of 7 years in humans,[23] it would seem reasonable, for example, to assume that the two groups of Ranch Hands with TCDD levels of 10 to 14.9 pg/g lipid with post-SEA conceptions may have had very different TCDD levels at the time their children were conceived. This is possible because the early-tour veterans had more time to decrease their body burden of TCDD before their bloods were drawn in 1987 than did their late-tour counterparts. Paternal TCDD level at the time of conception was estimated in a subsequent analysis of these data.[74]

Table 19.9 illustrates the risk estimates for the Ranch Hand study. Interestingly, the only statistically significant associations between TCDD and adverse

TABLE 19.9 Results of Studies of Paternally-Mediated Associations in the Ranch Hand Study

Study	Characteristics	Outcome	Outcome in Exposed	Outcome in Unexposed	Odds Ratio	95% CI
Wolfe et al. (1992)[73]	*Exposed group:* 2533 conceptions among 791 Ranch Hand (RH) personnel	Total birth defects				
		≤ 10[a]	202.1[b]	208.0	0.96[c,d]	0.69–1.34
		15–≤ 33.3	293.1		1.58	1.10–2.27
	Control group: 2074 conceptions among 768 non-RH personnel	> 33.3	193.8		0.92	0.64–1.32
		Nervous system anomalies				
	Type of exposure: Spraying/handling of Agent Orange	≤ 10[a]	0.0[b]	3.1	—	
		15–≤ 33.3	5.7		1.88[c,e]	0.20–18.3
	Data source: exposure/outcome; serum TCDD levels/hospital and medical records	> 33.3	13.2		4.37[c,e]	0.87–21.8
		Respiratory system anomalies				
		≤ 10[a]	7.1[b]	2.0	3.5[c,e]	0.49–25.0
		15–≤ 33.3	5.7		2.83	0.26–31.4
		> 33.3	4.4		2.17	0.20–24.0
		Digestive system anomalies				
		≤ 10[a]	21.3[b]	24.5[b]	0.83	0.31–2.23
		15–≤ 33.3	34.5		1.30	0.48–3.51
		> 33.3	17.6		0.64	0.21–1.91
		Genital anomalies				
		≤ 10[a]	3.5[b]	18.3[b]	0.19	0.03–1.43
		15–≤ 33.3	51.7		2.92	1.29–6.61
		> 33.3	13.2		0.72	0.21–2.46
		Urinary system anomalies				
		≤ 10[a]	14.2[b]	12.2[b]	1.16	0.37–3.63
		15–≤ 33.3	34.5		2.88	1.07–7.79
		> 33.3	22.0		1.82	0.63–5.22

		Musculoskeletal deformities				
		≤ 10[a]	120.6[b]	134.6[b]	0.88[c,d]	0.59–132
		15–≤ 33.3	143.7		1.08	0.68–1.71
		> 33.3	105.7		0.76	0.48–1.21
		Anomalies of the skin				
		≤ 10[a]	17.7[b]	21.4[b]	0.76	0.25–2.26
		15–≤ 33.3	34.5		1.83	0.72–4.70
		> 33.3	8.8		0.46	0.11–1.95
		Circulatory system and heart anomalies	14.2[b]	16.3	0.85	0.27–2.69
		≤ 10[a]	46.0		2.16	0.81–5.74
		15–≤ 33.3	8.8		0.32	0.04–2.52
		> 33.3				
Wolfe et al. (1995)[74]	*Exposed group:* 1006 conceptions among 454 RH personnel	Spontaneous abortion				
		Comparisons[f]		172	1	
	Control group: 1235 conceptions among 570 non-RH personnel	Background	57		1.1	0.8–1.5
		RH low	56		1.3	1.0–1.7
	Type of exposure: spraying/handling of Agent Orange	RH high	44		1.0	0.7–1.3
		Stillbirth				
	Data source: exposure/outcome; Serum TCDD levels/hospital and medical records	Comparisons[f]		13	1	
		Background	7		1.8	0.7–4.5
		RH low	6		1.8	0.7–4.7
		RH high	1		0.3	0.0–2.3
		Major birth defects				
		Comparisons[f]		56	1	
		Background	17		1.1	0.6–1.8
		RH low	23		1.7	1.1–2.7
		RH high	19		1.2	0.8–2.1

(Continued)

TABLE 19.9 *(Continued)*

Study	Characteristics	Outcome	Outcome in Exposed	Outcome in Unexposed	Odds Ratio	95% CI
		Developmental delays				
		Comparisons[f]		71	1	
		Background	24		1.2	0.8–1.8
		RH low	26		1.5	1.0–2.3
		RH high	21		1.1	0.7–1.7

[a] $\text{Logit}(p) = \beta_0 + \beta_1 d_1 + \beta_2 d_2 + \beta_3 d_3$, where p = probability of an adverse reproductive outcome; $d_1 - d_3$ are indicators for the dioxin categories for Ranch Hands: unknown (< 10 pg/g of lipid current dioxin), low > 15 pg/g and up to 33.3 pg/g), and high (> 33.3 pg/g).

[b] Rate/1000 of abnormals.

[c] Unadjusted.

[d] Adjusted analysis not statistically significant.

[e] No adjusted analysis: total defects < 10.

[f] Comparions: ≤ 10 ppt; Ranch Hand (RH) background ≤ 10; RH low with current level > 10 ppt + estimated initial level ≤ 110 ppt; RH high with current level > 10 ppt and estimated initial level > 110 ppt.

events (total birth defects, genital anomalies, and urinary system anomalies) occurred among Ranch Hands with TCDD levels of 15 to 33.3 pg/g of lipids and not among those in the $>$ 33.3 pg/g group.

The report stated that the "expected dose pattern" for TCDD and total adverse reproductive outcomes (miscarriage, tubal pregnancy, other non-induced abortive pregnancy, or stillbirth) is the "linear one in which the highest anomaly rate occurs at the highest levels of dioxin." This statement raises at least two questions. If a linear response is assumed, might this imply that very early pregnancy losses occur at the highest TCDD levels, so that the conceptus would not survive long enough to be clinically recognized? Or are very early pregnancy losses and clinically recognized spontaneous abortions two separate entities with different thresholds?[21] Such a scenario has been suggested to explain changes in spontaneous abortions observed after exposure to radiation in Hiroshima.[79] These questions are of interest because the rate of each of these endpoints may directly affect the rates of all subsequent outcomes available for examination. Very early losses are unlikely to be identified in a study of this type. No evidence was found to support an association between TCDD and total adverse outcomes. These findings should be viewed with caution in view of the unexplored area of events early in gestation.

Overall, little convincing evidence was presented for an association of birth weight, either as a continuous variable or dichotomized ($<$ 2500 g or $\geq$ 2500 g), and paternal TCDD level. Analyses adjusted for covariates, including parental ages, maternal alcohol use and smoking, and race of the father. No assessment of TCDD and prematurity was reported.

The potential association between cohort status and birth defects was examined for all defects combined and 12 additional categories of malformations. The only categories with sufficient numbers of verified post-SEA cases to detect a relative risk of 2 were total birth defects (229 cases among 1045 Ranch Hands and 289 cases among 1602 controls) and musculoskeletal deformities (132 cases among Ranch Hands and 180 among controls).

Significant differences were observed for total birth defects ($p = 0.03$), defects of the respiratory system ($p = 0.03$), and urinary system abnormalities ($p = 0.04$) by Ranch Hand versus control status with time of conception (pre- or post-SEA). All of these findings were due to a lower rate among Ranch Hands in the pre-SEA conceptions and a higher rate among the post-SEA conceptions for the Ranch Hands.

Analyses of birth defects by TCDD level did not find any "consistent patterns" to support an association. For example, both children of enlisted flying and enlisted ground personnel, and children of Ranch Hands with TCDD levels $\leq$ 10 pg/g lipids had higher birth defect rates (433 per 1000 and 317 per 1000, respectively) than children of controls with background TCDD levels (background: $<$ 10 pg/g lipids; birth defect rate: 229 per 1000). However, rates in children of enlisted ground personnel with TCDD levels $\geq$ 33.3 pg/g lipids were not significantly elevated. If higher TCDD levels were related to early pregnancy loss, these results would make more biological sense, as early losses

associated with high TCDD exposure might have occurred before the pregnancy was recognized.

Neonatal death was associated with TCDD levels (OR = 5.5, 95% CI = 1.5 to 20.7). Insufficient numbers ($n = 13$) precluded the calculation of an adjusted odds ratio for this finding.

Finally, no association was detected between TCDD level and either sperm count or percentage of abnormal sperm in the veterans. These analyses were based on semen samples that had been collected in 1982.

Ranch Hand Study, 1995 This study includes pregnancies to those men described above; but the group has been restricted to confirmed pregnancies occurring after the beginning of service in Vietnam in those men who participated in the 1987 physical, gave blood to evaluate serum dioxin levels, and had usable laboratory measurements.[74] This group potentially included 872 Ranch Hand veterans and 1036 comparison subjects. In fact, 454 Ranch Hand veterans (RH) yielded 1006 recognized conceptions and 419 veterans yielded 792 live births, while 570 comparison subjects yielded 1235 recognized conceptions and 531 controls yielded 981 live births. Paternal dioxin level at the time of conception was used to generate four exposure groupings: (1) comparison with current level $\leq$ 10 ppt, (2) RH with current level $\leq$ 10 ppt, (3) RH with current level $>$ 10 ppt and estimated initial level $\leq$ 110 ppt, and (4) RH with current level $>$ 10 ppt and estimated initial level $>$ 110 ppt. Comparison men with $>$ 10 ppt dioxin were eliminated from the group, as having higher than "background" levels without an understanding of the probable source of the exposure. The children in the second group (RH with $<$ 10 ppt) were considered separately because the fathers' levels could not be used to estimate exposure levels at the time of pregnancy. Levels at conception were estimated using a fixed 7.1-year half-life with a first-order decay rate. As the time between measurement (1987) and conception varied from 15 to 26 years, the number of half-lives ranges from about two to a little over three.

All analyses were adjusted for paternal race, age, and military occupation and maternal age, and smoking and drinking during pregnancy. In addition to these, analyses of spontaneous abortion were adjusted for spontaneous abortions occurring prior to service. The proportion of men who fathered recognized pregnancies or live births were about the same in both groups, Ranch Hand or comparison.

Analyses show modest, borderline significant increases in spontaneous abortion (Table 19.9), defects of the circulatory system and heart (OR = 2.3, 95% CI = 1.0 to 5.1), all anomalies (OR = 1.3, 95% CI = 1.0 to 1.6), major birth defects (OR = 1.7, 95% CI = 1.1 to 2.7), and some developmental delays (OR = 1.5, 95% CI = 1.0 to 2.3), all of these for the low RH group only. Dose–response patterns were not observed, and more detailed analyses were not possible because of the small number of adverse outcomes in each grouping.

Comments The data described above do not present strong evidence for an association of paternal dioxins with developmental effects, occurring preconceptionally, prenatally, or identified around the time of birth. These studies are limited by the ability to define exposure accurately at the critical windows for the events.

19.2.2 Developmental Outcomes Based on Several Studies

Sex Ratio at Birth Sex ratio has been reported to vary with a great number of factors, including race, timing of conception within the cycle, certain parental diseases, and gestational age.[80,81] Sex ratio is defined by demographers as (number of male births)/(number of female births) $\times$ 100. However, many papers covered in this section present the proportion of male births of the total rather than an actual ratio. Although the endpoint will still be the sex ratio, all data will be presented as the proportion, for consistency with the original literature and ease of comparison among the studies.

In response to a report of hormonal variations in men occupationally exposed to dioxin,[82] James[83] (and repeated later[84,85]) proposed that with high gonadotropin and low testosterone levels, sex ratios could be lowered (fewer male births compared to female births). A 1996 letter[86] reported an excess in female births conceived following the Seveso accident. This included births from April 1977 to December 1984, a time period approximating the half-life of dioxin.

Seventy-four births occurred during this time within zone A; 26 (35.1%) were males and 48 females (65.9%), compared to the expected value (51.4% males[80]) used by the investigators. Since 1988, these investigators measured dioxin levels in archived serum samples. Of the 74 births, 17 occurred in families with both parents in zone A. Elevated dioxin level was defined in this report as $>$ 100 ppt (lipid adjusted) and ranged from 104 to 2340 pg/g lipid in fathers and 126 to 1650 pg/g lipid in mothers. Of this group, 100% ($n = 12$) births were female. Eighty percent of those with low dioxin levels were male ($n = 5$). In an unadjusted analysis, the overall sex ratio (0.235) was significantly different from the value expected ($\chi^2 = 12.68$, $p < 0.001$) in an analysis unadjusted for other factors related to variations in sex ratio. After this time period (1985–1994), the sex ratio increased to 0.484 ($n = 124$), a value not significantly different from the value expected. The authors mentioned that the reduced sex ratio in this small series of births could have resulted from excess males in spontaneous abortions (a theory that cannot be assessed with existing data), or that changes in sex ratio could result from the changes in hormonal balance.

The investigators revisited the Seveso cohort, identifying all births for those who lived in zone A, B, or R contaminated at the time of the 1976 explosion.[87] Recently, serum levels collected in 1976–1977 of 239 men and 296 women were evaluated for TCDD in this evaluation of sex ratio. Median level for the

fathers was 96.5 ppt (range 2.8 to 26,400 ppt), and for the mothers, 62.75 ppt (6.45 to 12,500 ppt). Parents with less than or equal to 15 ppt TCDD were compared to those above (all together, or split into categories: $>$ 15 to 80 ppt, $>$ 80 ppt). After comparing levels in mothers and fathers in 1976, for a total of 674 births in 452 families, a pattern of reduced birth ratios was noted for paternal exposure only, or where both parents were exposed, but not for only maternal exposure. More detailed analyses focused on paternal exposure, with the observation that the reduction in sex ratio was greater for those fathers who were less than 19 years old at exposure in 1976 (sex ratio = 0.382, 95% CI = 0.30 to 0.47) versus those who were older (sex ratio = 0.469, 95% CI = 0.41 to 0.53). In addition, the sex ratio in offspring to both groups of exposed fathers were significantly less than unexposed fathers of all ages (sex ratio = 0.557, 95% CI = 0.50 to 0.62). Data on age-specific sex ratio among the unexposed fathers were not presented, thus leaving the comparison incomplete. An additional analysis would have been more informative: the interaction of paternal age by paternal exposure level for sex ratio. Also, details of factors considered and controlled in the multivariable analyses were not presented, limiting the examination of this interesting study. This study presents intriguing data on the possible relationship of sex ratio and age at dioxin exposure.

Michalek and co-workers[88] examined the Operation Ranch Hand study group for differences in sex ratio. Men were grouped in one of four exposure categories [comparison ($n = 1254$) and background ($n = 346$) both less than 10 ppt, low (10 to 79 ppt) ($n = 277$), and high ($n = 280$) with $>$ 79 ppt] based on serum blood levels in 1987 or 1992 extrapolated to the time of conception, and using a fixed 8.7-year half-life for dioxin. Mothers were assumed to have "background" levels. Analyses examined children conceived within 1 month, 1 year, or 5 years, and any time postservice. No significant differences were observed in any analyses. The authors suggest that the findings in Seveso might be associated with maternal exposure (a suggestion not found in the recent study).[86]

Rogan et al.[89] evaluated sex ratio in Taiwanese children whose mothers were affected by dioxinlike compounds (PCBs and PCDFs) after consumption of contaminated cooking oil (Yucheng; see Chapter 22 for a discussion of the Yucheng incident). Health effects observed included developmental delays and ectodermal effects in children born to affected mothers. Overall the proportion of males in live births ($n = 137$) was 0.496 from 1978 to 1985. In an examination of births conceived at the time the oil was first sold (June 1998), the proportion rose to 0.508, similar to the comparison for other analyses.

Examination of 44 (of 59) primiparous mothers in a cotton-growing region in Kazakhstan[90] showed that those living near a reservoir with agricultural runoff (zone A) had higher levels of dioxin in breast milk than those located $>$ 10 miles away (zone B). All live births in the region occurring 2 to 8 weeks before the sampling period in 1997 were grouped by zone. Zone A ($n = 17$) mean breast milk levels were 53 pg/g versus zone B ($n = 24$) with

mean levels of 21 pg/g. No significant differences were observed by zone or by TCDD level ($\geq$ 30 pg/g versus $<$ 30 pg/g). The numbers were small, limiting the power, but in all the subgroups except zone B (45.8%), more males were born (proportions ranging from 54.5 to 70.6%).

More recently, NIOSH[91] has examined its occupational cohort study for altered sex ratio at birth. The study compared births of male workers' mates (292 births with $<$ 20 ppt TCDD; 104 with 20 to 254 ppt TCDD; 88 with 255 to 1119 ppt TCDD; and 60 with 1120+ ppt TCDD) to 647 births to never-exposed referents ($<$ 20 ppt TCDD). The exposed proportions range from 0.51 to 0.55; none were significantly different from the referent pregnancies (proportion = 0.54) either in unadjusted or adjusted analyses (adjusted for maternal education and paternal race). The pregnancies in this occupational setting experienced higher paternal TCDD exposures than in the environmental studies; even the highest exposure group, although limited in size, did not experience a change from the referents (1120+ ppt TCDD: proportion = 0.55, 95% CI = 0.49 to 0.61 vs. $<$ 20 ppt TCDD: proportion = 0.54, 95% CI = 0.52 to 0.56).

Comments Most of these analyses provide limited data due to limited exposure data, no or limited adjustment for other risk factors/confounders, assumption of a gold standard of 51.4% males (and not having comparison groups) or small numbers. The recent NIOSH analysis, of occupationally exposed (adult) men, with a broad range of exposure, an appropriate comparison group and adjustment of the analyses, did not observe any differences.

Sex ratio at birth was significantly depressed in a group of 17 children in zone A of Seveso in the years shortly following the industrial accident. This pattern disappeared a few years later. However, a recent expanded effort suggests that paternal age at the time of exposure may be a key factor. Sex ratio differences were observed only in fathers less than 19 years old in Seveso. The lack of effect elsewhere could be explained by the groups examined: maternal levels of dioxin in community studies or studies of men older than 19. The findings in the most recent Seveso study emphasize the need for more attention on male-mediated developmental effects and the potential importance of exposures prior to and during puberty.

Low Birth Weight, Intrauterine Growth Retardation, and Postnatal Growth In recent years, those investigating developmental outcomes have started looking at a variety of measures of prenatal and postnatal growth. Outcomes have included birth weight and size, intrauterine growth retardation (IUGR), and postnatal measures up to the age of 42 months. IUGR, also known as small-for-gestational-age, basically combines information on birth weight with the length of gestation. Children with low birth weight are not necessarily IUGR because of different expected birth weights at different gestational ages.

New analyses of growth of 38 children in the smaller Dutch study[52] have

been done. Birth weight data at delivery and weight and length data from postnatal examinations (at 10 and 20 weeks) were used to calculate the Quetelet index (weight/length^2). In addition to these measures, the circumference of the head was measured (1, 11, and 26 weeks) and area of the liver determined by ultrasound (10 days and 11 weeks). No differences were found between low and high exposure for any of the growth measures (using Student's *t*-test).

The Rotterdam study also examined birth weight and growth.[46] Birth weight was evaluated only in relation to PCBs and so will not be discussed here. Postnatal growth was examined for TEQs for dioxins, furans, and PCBs in breast milk multiplied by weeks breast fed. Using multivariable regression, and controlling for other factors potentially related to growth, no significant differences were observed at 3 months. A statistically significant decrease in growth in length was observed ($\beta = -0.21$, $p = 0.04$) with TEQ, but not with weight or head circumference between 3 and 7 months of age. No differences were observed between 7 and 18 months or 18 and 42 months.

Another study examined background levels of PCDD and PCDF levels and birth weight in all consecutive births from January through May 1987 in one urban maternity clinic (Helsinki, Finland) and one rural clinic (Kuopio province).[92] Approximately 150 women were recruited from each who were willing to provide a breast milk sample at 4 weeks postpartum, if still lactating. A total of 167 samples resulted in a 50% response rate in Helsinki (77 women/26% of births) and about 60% from Kuopio (90 women/30% of births). TEQ values of breast milk were significantly higher in the urban area (26.3 pg/g TEQ vs. 20.1 pg/g TEQ in Kuopio province). Correlation analyses (Pearson's correlation) were significant for all births and all male births. Regression analyses showed a decline in birth weight with increasing TEQ of milk ($\beta = -0.00228$), primarily in male births ($\beta = -0.00302$; females, $\beta = -0.00107$). Statistical significance was not presented, nor were details on other factors in the analyses. When restricted to examination of first-born children ($n = 84$), no significant relationships were observed.

One report examines placental Ah receptor binding of TCDD in IUGR, preterm birth, and structural malformation.[93] The study group, 86 births, included 21 preterm births, 20 with IUGR (8 of these were preterm), and 7 infants with structural malformations. The B_{max} (concentration of Ah receptor sites for TCDD) and K_d (affinity for binding of TCDD) were not significantly different for the different pregnancy outcomes. Some modest increases were observed for B_{max} (and less so for K_d) with IUGR ($n = 10$) and structural malformations ($n = 5$) over normal deliveries ($n = 23$), but the power was limited by small numbers.

Michalek and colleagues[88] examined IUGR in their study of the veterans of Operation Ranch Hand. Analyses included 2082 liveborn, singleton births during or after the father's service in Southeast Asia for whom paternal serum measures of dioxin were available. Of the 2082, 859 were in the Ranch Hand group and 1223 were comparisons. If serum dioxin levels in 1987 or 1992 were

$>$ 10 pg/g lipid, the investigators modeled the father's level at the time of conception of the child. For those at or under 10 pg/g lipid, levels at conception were considered to be "background." Levels greater than 10 and less than 79 were "low," and above that were "high." Length of gestation and birth weight were obtained from labor and delivery records. Included births occurred between 1959 and 1992; the earliest births were from comparison subjects. No differences were observed in IUGR across the exposure groups. Small, nonsignificant increases were seen in preterm birth ($<$ 37 weeks gestation) for Ranch Hand background and high groups [relative risk (RR) = 1.4, 95% CI = 0.9 to 2.3 and RR = 1.3, 95% CI = 0.8 to 2.3, respectively]. Significant increases were observed in these groups for neonatal death (within the first 28 days of life): RR = 3.2, 95% CI = 1.0 to 10.3 for background, and RR = 4.5, 95% CI = 1.5 to 14.0 for high (for the low group: RR = 1.5, 95% CI = 0.3 to 7.5). Most of the Ranch Hand deaths were due to short gestation and low birth weight, but only a third in the comparison group. Although these numbers were relatively small, the proportions in the background and high groups were much higher: comparison: 3.7% of 54 preterm births; background: 25% of 20; low: 0% of 6; high: 31.3% of 16. An analysis using occupation, so as to include all births, not just those with serum measures, also showed elevated infant deaths in preterm births in the exposed versus the comparison group. However, the proportions did not follow the relative exposures observed among the categories.

Comments Data on growth measures and neonatal death are limited. For example, in one study, decrements in length (but not other measures of growth) were observed early, but disappeared with increasing age.[46] Some changes were observed in the Ranch Hand study for preterm birth and neonatal death, but did not follow an exposure–response relationship.[94] The Finnish data are interesting because birth weight did decrease in males, with increasing TEQ, but lack of detail on analyses makes interpretation difficult.

19.2.3 Studies of the Adult Reproductive System

Hormones In laboratory rats, high doses of TCDD have been related to decreased testosterone levels, with evidence that dioxin decreases testosterone synthesis.[95–98]

NIOSH Chemical Workers The NIOSH cross-sectional medical study included living chemical workers, previously employed for at least 1 day in one of two plants. From 1951 to 1969, 490 workers employed in the New Jersey plant produced sodium TCDD-contaminated 2,4,5-trichlorophenate (NaTCP), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), and 2,4-dichlorophenoxyacetic acid (2,4-D). Many workers had acute symptoms of chloracne and other dermatologic abnormalities.[11,99] At the Missouri plant, NaTCP and 2,4,5-T were

produced intermittently for 4 months in 1968, and NaTCP and hexachlorophene were produced continuously for 22 months between April 1970 and January 1972.

For comparison, unexposed neighborhood referents were recruited.[100] Referents with no prior history of occupational exposure to TCDD were matched to the worker by age (within 5 years), race, and gender. A total of 586 workers were eligible for the study (400 living, 68.3%; 142 deceased, 24.2%; and 44 not located, 7.5%). All 400 living workers were invited to participate in the study; 281 (70%) were examined. Health and exposure status were assessed in 1987–1988 through a medical and occupational history and comprehensive physical and psychological examinations.[100]

As a surrogate for cumulative exposure, serum TCDD levels were measured in 237 workers and a random sample of 79 referents.[101–103] The mean lipid-adjusted serum TCDD level for workers was 220 pg/g (median 80 pg/g, maximum 3400 pg/g). The mean level was significantly greater than for referents (7 pg/g) ($p < 0.001$). No differences were observed in other congeners of dioxins and dibenzofurans.[104]

In this study, reproductive hormone levels were measured and related to serum TCDD levels. In linear regression analyses, serum TCDD was significantly related to serum levels of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) and inversely related to total testosterone after adjustment for potential confounders ($p < 0.05$).[82] The prevalence of abnormally low testosterone was higher among workers with serum TCDD levels of 20 to 75 pg/g (OR = 3.9, 95% CI = 1.3 to 11.3), 76 to 243 pg/g (OR = 2.7, 95% CI = 0.9 to 8.2) or $\geq$ 244 pg/g (OR = 0.1, 95% CI = 0.8 to 5.8) than among unexposed referents (4.8%) (mean serum TCDD = 7 pg/g). More workers in these same TCDD quartiles had abnormally high LH than workers with serum TCDD levels of 244 pg/g to 3400 pg/g (not significant).

U.S. Air Force Ranch Hand Study The Ranch Hand veterans study is the only other one that evaluates serum TCDD and testosterone levels.[34] Ranch Hand veterans with current serum levels exceeding 33.3 pg/g had a lower but not significantly different mean total serum testosterone level (515.0 ng/dL) than the nonexposed comparison group (525.2 ng/dL). No association was observed with FSH and LH. Additional analysis[105] did not find an association between abnormally high or abnormally low testosterone level and dioxin category.

Testicular abnormality was assessed by physician palpation at the 1987 physical examination.[34] Because of this finding, testicular volume was measured by ultrasound at the 1992 physical examination.[77] No association was found between testicular volume measured by ultrasound and dioxin exposure category in 1992.[77] The investigators concluded that the earlier finding (in Ref. 33) may have been statistically spurious.

Comments The human data offer some evidence of alterations in male reproductive hormone levels associated with substantial occupational exposure

to TCDD. Some results support the animal literature, in which dioxin-related effects have been observed on the hypothalamic–pituitary–Leydig cell axis and on testosterone synthesis.

Endometriosis After noting the prevalence and severity of endometriosis in rhesus monkeys chronically exposed to TCDD,[106] investigators examined endometriosis in humans. The first reported effort, a case–control study,[107] compared 79 women, all treated in an infertility clinic during 1991–1995, some with endometriosis ($n = 44$), and the comparisons with tubal infertility ($n = 35$). All women underwent laparoscopic examination for diagnosis and scoring of endometriosis. Altogether, nine women had TCDD above the limits of detection: 2.9% of the controls ($n = 1$ of 35), 12.5% of those women with stage I–II endometriosis ($n = 3$ of 24), and 25% of those with stage III–IV endometriosis ($n = 5$ of 20). Logistic regression was used to control for potential effects of the different racial/ethic compositions of the cases and controls. The results of this analysis, compared to other unadjusted analyses, are not identified explicitly, but are probably OR = 7.6 (95% CI = 0.87 to 169.7). The authors did not present sufficient information on their data analyses to evaluate them (e.g., whether actual levels of TCDD were entered) but did note the limited power of this effort. An exposure–severity relationship was not observed. However, the frequency of exposure increased with increasing severity (not statistically tested).

A recent report[108] examined 101 infertile women treated at a collaborating Center for Reproductive Medicine in Belgium, 1995–1998. Couples were defined as infertile after attempting pregnancy for at least 1 year without success. Using laproscopic examination, 42 women were diagnosed with endometriosis; 25 women had mechanical infertility (e.g., tubal disease), 8 husbands of 20 without diagnosis were found to be infertile. Fourteen women were excluded from analysis because of ovulatory dysfunction. CALUX TEQs were generated using serum ($n = 101$), adipose tissue ($n = 46$), and follicular fluid ($n = 8$) based on major PCB congeners and chlorinated pesticides. In preliminary analyses using a cut point of 100 pg TEQ/g serum lipid weight, proportionately more women with endometriosis had high TEQs (17%) compared to the controls (4%) (OR = 4.0, NS).

Comments The two reports of infertility patients[107,108] raised the potential for an association between endometriosis and TCDD or TEQ exposure. These studies are small and of limited power. Studies currently under way will greatly add to the database on this outcome.

19.3 SUMMARY

The data presented in this chapter describe reproductive and developmental effects in epidemiologic studies of populations with exposure to TCDD and

similar compounds. The purpose of this review is to highlight the salient results of the studies and to assess whether the observed effect was related to exposure. In summary, in adults, altered testosterone level appears to be a long-term consequence of high occupational exposure to TCDD. Other reproductive and developmental outcomes and immunologic disorders require further study before their respective relationships to exposure can be assessed more definitively.

In the best of circumstances when reviewing studies, it would be ideal if all studies examined the same endpoints in the same manner, had sufficient statistical power to detect truly positive findings (and to uphold negative findings), had good estimates of extent of exposure, and had consistent exposure–response relationships. In the absence of ideal situations, epidemiologists examine the evidence of studies using "six tenets of judgment"[5,109] to assess the collective wisdom of the study results. These tenets are temporality (sequence of events); degree of exposure; strength, consistency, and specificity of association; and biological plausibility.

In evaluating many of the studies that examined the relationship between serum TCDD and, in some cases, dioxins, furans, and PCBs, there are several common threads that bear noting. They will be discussed first to avoid repetition throughout the summary.

In terms of temporality, all studies reviewed in this chapter were conducted after the presumed exposure occurred. Some of the studies obtained exposure data at (approximately) relevant time for the outcomes (e.g., Dutch developmental studies of dioxins, furans, and PCBs) or shortly after the exposure, as in Seveso; others were conducted many years after the groups' last exposure to evaluate more chronic health outcomes. One dilemma in assessing the effect of past exposures is ascertaining whether an effect observed many years post-exposure is due to the exposure itself or to another exposure or event occurring during the intervening period. Finally, restricting examination of events to those occurring after exposure does not in and of itself satisfy this time-order criterion. Several factors must be considered, such as the half-life of the contaminant in the body and the concentration at the time of the event. Consistency in the results of similarly designed studies of exposed populations should help strengthen conclusions.

Determination of exposure throughout the studies varied. When the risk of disease increases with the gradient of exposure ("dose"), the evidence for causation is strengthened. There are many possible dose–response patterns, which may result in different threshold levels for different endpoints. Because of the potential for exposure misclassification present in most dioxin research, with the exception of a few studies, it is difficult to assess dose–response relationships. In 1988, the workers in the NIOSH study had the highest serum TCDD concentrations (mean = 220 pg/g) of the studies presented here. Only the Dutch developmental studies examined common environmental exposures, including a variety of dioxins, furans, and PCBs (see Table 19.2).

In terms of the magnitude (or strength) of the association, this criterion refers to the degree to which the measure of association (e.g., odds ratio or

relative risk) exceeds the null value of 1. The stronger the association between exposure and effect, the more convincing is the argument for causation. Other factors, such as the prevalence of the exposure in the population and the study size, affect the significance of the measure.

A critical element that should always accompany the effect measure is a confidence interval. Placement of an interval around the measure enables quantification of the result for a more meaningful interpretation. An odds ratio of 30 is quite impressive, but if the 95% confidence interval is 0.9 to 200, the magnitude of the association is less impressive.

If an association between a factor and a disease is demonstrated across a variety of studies employing different designs and different populations (consistency), the argument for causation is strengthened. Replication of an association under different conditions decreases the likelihood that confounding is responsible for the association observed. Consistency is a powerful criterion for causation, but only when "the variables under test (exposure, outcomes) are similar enough" to justify the comparison of the various studies' findings.[110]

Each study included in the critical evaluation process should adhere to basic epidemiologic principles governing study design and analysis. Deficient studies with suspect results should be excluded. Although this is not to imply that such studies have no worth, as invaluable information has often been derived from those studies that improve on subsequent examinations of the issue, they have no place in the evaluation process. Unfortunately, in studies of TCDD and effects in humans, the probability of exposure misclassification forces exclusion of much of the research to date.

Specificity refers to the uniqueness of the association between a factor and an outcome. If the relationship were absolute, then only factor X would be related to only effect Y. It is indeed rare to encounter this type of association (outside of infectious diseases), which renders this criterion generally less useful.

Finally, according to the criterion of biological plausibility, the association observed between exposure and effect should be consistent with existing theory and information from other scientific disciplines. Certainly, one would feel more secure if the biological basis for an observed association could be explained. However, biological implausibility may simply reflect gaps in existing scientific knowledge that could explain the relationship.

19.3.1 Effects Having a Positive Relationship with Exposure to TCDD

In the following section we describe the endpoint (reproductive hormones), for which there is good evidence from two or more studies suggesting an effect of exposure to TCDD. The criteria for association are examined in detail.

Reproductive Hormones

Strength and Consistency of Association Levels of reproductive hormones have been measured with respect to exposure to TCDD in cross-sectional

medical studies. Testosterone, LH, and FSH were measured in NaTCP and 2,4,5-T production workers[82] and in Ranch Hands.[77,111] The risk of abnormally low testosterone was two to four times higher in exposed workers with serum TCDD levels above 20 pg/g than in unexposed referents.[82] In both the 1987 and 1992 examinations, mean testosterone concentrations were slightly but not significantly higher in Ranch Hands.[34,77] FSH and LH concentrations were no different between the exposed and comparison groups. Only the NIOSH study found an association between serum TCDD level and increases in serum LH.

Specificity The NIOSH study excluded from analysis participants who had conditions that might have influenced gonadotropin and/or testosterone levels: history of prostate cancer, thyroid, or other hormone use, or liver cirrhosis. Similarly, in the Ranch Hand study, individuals with orchiectomies or who were taking testosterone medication were excluded from the analysis of testosterone; no participants were excluded from the analyses of FSH.

Biological Plausibility In rats, TCDD has been shown to decrease testosterone levels[96–98] through a decrease in testosterone synthesis[94] or by decreasing the production of pregnenolone from cholesterol.[112] In addition, TCDD has been shown in rats to reduce the responsiveness of the pituitary to testosterone[113,114] and of the Leydig cells to LH stimulation.[115]

The findings of the NIOSH and Ranch Hand studies are plausible given the pharmacological and toxicological properties of TCDD. A mechanism responsible for the effects may involve the ability of TCDD to influence hormone receptors. The aryl hydrocarbon (Ah) receptor to which TCDD binds can crosstalk with steroid hormone receptors in both structure and mode of action. Studies suggest that TCDD modulates hormone receptors, including estrogens,[116,117] prolactin, and its own Ah receptor.[118,119] However, the effect of TCDD on testosterone receptors has not been evaluated.

In summary, the results from both the NIOSH and Ranch Hand studies are limited by the cross-sectional nature of the data and the type of clinical assessments conducted. However, the available data provide evidence that alterations in human male reproductive hormone levels are associated with serum TCDD.

19.3.2 Possible Effects of TCDD or Mixtures of Dioxins, Furans, and PCBs

In the following section we describe outcomes that may be related to TCDD exposure. Further research would assist in evaluation of the effects of dioxin for the following outcomes.

Possible Postnatal Developmental Effects Given that postnatal developmental effects of dioxin–furans have been studied only in one human population (with the exception of some of the thyroid measures), these studies are being placed in the ''potential'' category. Additional studies in other groups

are recommended, as well as follow-up over time to evaluate whether changes are temporary, with no long-term health effects, or an early indication of chronic effects. All the effects in this section were part of one or both of the Dutch developmental studies. The exposures assessed here are different from the more typical ''dioxin'' study: The first series of studies (in Rotterdam and Groningen) examined dioxins, furans, and PCBs; the second (in Amsterdam) examined dioxins and furans. Thus, any effects observed could be from one agent or some mixture. Even though the studies may have identified certain exposures as statistically significant, this does not mean that other factors not selected are not associated. For example, in the Rotterdam/Groningen studies, only PCBs were evaluated prenatally and at birth, but these values were significantly correlated with dioxins, furans, and PCBs collected about 2 weeks after delivery. Many of the findings in the Rotterdam/Groningen studies were associated with in utero PCB exposures measured in maternal blood (IUPAC 118, 138, 153, 180). However, of these, only 118 is considered dioxin-like. As the technology improves, to measure dioxin levels in smaller samples, direct measurements will help clarify the issues related to surrogate measures (e.g., PCBs).

Neurobehavioral Effects Of the various endpoints covered by the series of reports on the Dutch population, the most interesting findings are related to the neurobehavioral endpoints. Prechtl's Neurologic Optimality Scores (NOS) and the related postural tone cluster scores and reflex cluster scores were collected at 18 months of age[41] and, at 42 months of age, children were assessed for cognitive abilities using the Kaufman Assessment Battery for Children (K-ABC) and for verbal comprehension using the Reynell Language Developmental Scales (RDLS).[47]

The NOS scores were somewhat arbitrarily divided at the median and compared to the individual dioxins, furans, and PCBs, as well as their summary measures.[41] A number of the levels of the foregoing agents in breast milk were associated with the NOS, while the prenatal PCBs were not (Table 19.7). Coplanar PCB TEQ was associated with hypotonia (measured through the posture tone cluster score). This observation of hypotonia and prenatal PCB exposure is consistent with previous findings of PCB exposed children.[55] An evaluation of motor function was associated only with the prenatal PCB levels. Because of the small volume of maternal and cord blood collected, dioxins and furans were not measured during the prenatal period. An interaction observed with paternal smoking suggests that this issue should be examined further by collecting postnatal maternal smoking data.

Statistically significant deficits in K-ABC were associated with $\sum \mathrm{PCB}_{\text{maternal blood}}$ for the entire group, and in RDLS only in the formula-fed children.[47] Importantly, the current body burdens in the 42-month-old children were not associated with any cognitive deficits. Statistically significant changes were not observed in the breast-fed children, possibly because of the higher SES status, parental education, and parental verbal IQs. Another possibility is the beneficial effect of breast feeding in general.

Many of the other outcomes in the Dutch population were "better" in those with exposure: fluency cluster score,[40] mental development index (MDI),[45] and visual recognition memory test at 7 months of age.[44] This may be a result of the inherent benefit of breast feeding (and length of breast feeding) over formula for those measures, or may be due to the way women select to breast feed (e.g., higher-SES women, parents with higher educational levels). As noted above, this later notion is supported in a recent report by Patandin et al.[47]

Transplacental exposures of mice demonstrate neurobehavioral effects of dioxin and dioxinlike compounds. These include effects on postural endpoints, motor function, visual abilities, and learning. Perinatally exposed monkeys showed a deficit in cognitive function. Exposures are presented by dietary levels or dose given, and thus are difficult to compare to the exposure measures used in human studies.

Endpoints varied in the studies described above, as did the components and levels of exposure; despite this, the data suggest a relationship between dioxin and dioxinlike compounds and neurobehavioral outcomes. Examination of other human populations and long-term follow-up of these study groups will greatly benefit this database.

Thyroid Function Two series of studies, both in the Netherlands, have examined thyroid function.[42,51] The two reports did have a finding in common: Both observed higher TSH at 3 months of age with higher TEQs. They both had significant findings for T4, but they were in opposite directions. All these findings, plus other changes found in the second report (an increase in T4/TBG and a decrease in free T4), strongly suggest that more work be done in this area. These findings suggest a possible shift in the distribution of thyroid hormones and point out the need for collection of longitudinal data to assess the potential for long-term effects associated with these changes.

AST and ALT One study looked at blood measures in 35 perinatally exposed children in the Netherlands.[38] AST, ALT, and platelets all varied with exposure (Table 19.5). Even though all but three children had values within "normal" ranges, the distributions shifted (e.g., an increase in platelets), which could have some currently unrecognized short- or long-term health effect.

19.3.3 Effects for Which Further Research Is Needed

In the following section we describe endpoints for which the animal data have demonstrated exposure-related effects, but the human data are inconclusive and require further study.

Adult Reproductive Outcomes

Semen Changes The Ranch Hand study did not find changes when exposure was defined by both cohort status and TCDD levels. However, the data on

alterations in male reproductive hormone levels associated with occupational exposure to TCDD emphasize that further research is needed in this area.

Endometriosis A report of endometriosis in rhesus monkeys following TCDD exposure[106] and two reports of infertility patients[107,108] have raised the potential for an association between endometriosis and TCDD/TEQ exposure. These studies are small and of limited power. Studies of women from Seveso and New York State are currently under way and will add to the database on this outcome.

Developmental Effects

Immunologic Effects More comprehensive evaluations of immunologic function with respect to exposure to TCDD and related compounds are necessary to assess the relationships observed in nonhuman species. Longitudinal studies of the maturing human immunologic system may provide the greatest insight, particularly because animal studies have found significant results in immature animals, and human breast milk is a source of TCDD and related compounds. Data from a longitudinal study of children[48,49] suggest long-term effects. Additional investigations of other populations would be informative.

Other Developmental Effects In this section, we include all developmental effects except those postnatal developmental effects that are covered by outcome (thyroid, neurobehavioral outcomes, and AST and ALT). Outcomes related to fertility are also reported (e.g., time to pregnancy, birth rates, semen changes).

A variety of study designs, including case–control, ecologic, cross-sectional, and historical cohort designs, have addressed the issue of TCDD and reproductive effects in humans. Unfortunately, the different criteria for case definitions across studies make it difficult to compare the results. In addition, the method of case ascertainment for certain endpoints influences the rate of events observed. Rates of birth defects in the Ranch Hand study were similarly reported by the Ranch Hands and controls. Both groups underreported 7% of birth defects in children conceived prior to their SEA tour and 14% after their tour of duty.

No developmental effect measure greater than 2 was noted in any of these investigations. This is not surprising, given the limitations of the studies, particularly with regard to exposure misclassification. Therefore, the trends across these studies carry more import than ''statistically significant'' results.

Although these studies have restricted inclusion of reproductive or developmental events to those that occurred after exposure to TCDD was suspected, no study has evaluated TCDD levels at the time of the outcome. Determination of a TCDD dose–response relationship with adverse reproductive outcomes are not valid unless individual TCDD levels were available, given the misclassification by other methods. The recent Ranch Hand study estimated

the TCDD levels at the time of the developmental outcome, which is an important contribution toward understanding this phenomenon. However, with regard to early losses, this analysis would not be able to address those occurring very early in gestation, before recognition of the pregnancy, or a subsequent effect on adverse outcomes identified later in pregnancy or at birth.

A growing body of animal research described elsewhere lends biological plausibility to the association between dioxin and most of the reproductive endpoints evaluated in these studies, with the notable exception of molar pregnancies. There is growing evidence that dioxin affects testis and accessory gland weight, testicular morphology, spermatogenesis, and fertility in males. A model for a paternally mediated dioxin effect on congenital malformations has not been reported; however, increased interest in this area[20] may yield more information on this topic. Among female animals, the primary reproductive endpoints that have been examined include decreased fertility and pregnancy loss.

The mechanism by which TCDD causes adverse reproductive and developmental effects has not been well described, although considerable insight has been gained from research focusing on the Ah receptor. Although the Ah receptor has been linked with birth defects in several mouse strains, it appears that the mechanism of effect may be dependent on the outcome evaluated, as well as other dioxin congeners to which the population is exposed. Clearly, these relationships in humans have not been adequately investigated.

SPONTANEOUS ABORTIONS Miscarriages were investigated in several studies with different designs and varied patterns of parental exposure. Events were generally ascertained by self- or spousal report. When case ascertainment was through medical records, such as in the Ranch Hand study, the events are by definition restricted to those miscarriages that were clinically recognized.

Research in the area of pregnancy loss indicates that 30 to 50% of all conceptions are lost prior to or during implantation.[120] The rate of loss between implantation and expected first menstrual period ranges from 22 to 31%.[121–123] Thus, it is clear that restriction of the examination of pregnancy loss to those events ascertained through medical records, or even self-reports, results in excluding a large proportion of the outcome of interest. In studies of environmental factors and spontaneous abortion where information is lacking on conception, ''the conflation of different doses with different effects can mislead.''[124] Because of these discrepancies, it is not meaningful to pool results of the research on the association between dioxin exposure and miscarriage to judge the ''consistency'' of the association.

Overall, the data compiled to date are inadequate to address this issue. Simply to enumerate and compare the number of positive versus negative studies to ascertain consistency in the research would be inappropriate. The reasons for this have been described above in detail, with emphasis on exposure misclassification, small sample sizes evaluated, lack of data on dioxin levels at the time of conception, and the unknown impact of early pregnancy loss on identification of birth defects. The animal and human evidence for a TCDD-

pregnancy loss relationship is sufficiently suggestive to warrant further investigation. Several studies of various designs and populations have demonstrated weak but consistent associations. Only two studies, one an analysis of the Ranch Hand developmental data[74] and the other a recent analysis of the NIOSH cohort,[91] have used biological measurements and estimated the TCDD levels present around the time of conception. The first study did note a modest increase in recognized spontaneous abortions and stillbirths but only at the low level. The Ranch Hand study leaves several questions unanswered, including the determination of a dose–response level and the impact of very early pregnancy losses on rates of recognized fetal death and birth defects that survive long enough to be counted. The NIOSH study did not observe increases at any level.

CONGENITAL MALFORMATIONS AND BIRTH DEFECTS The confusing evidence regarding the relationship between dioxin exposure and birth defects suffers not only from the limitations described above for the studies of miscarriage but also from the lack of power to evaluate specific types of malformations. To increase the power to detect a potential relationship, the studies have combined all birth defects together and calculated an odds ratio for total birth defects. Given evidence for etiologic heterogeneity among subgroups of birth defects,[125] it is probable that this approach dilutes the effect measure.

These studies should also more carefully examine type of parental exposure (i.e., paternal, maternal, or both). Timing of exposures and potential biological mechanisms for birth defects are different for maternal and paternal exposures. The field of paternally mediated effects is rather new, but future research may assist in the interpretation of these results.[20] If dioxin exposure is related to malformations among the offspring conceived after paternal service in Vietnam, the effect must occur either premeiotically, or anew with continuing circulating levels of TCDD. Some animal studies have found that spermatogonia and spermatocytes (premeiotic spermatogenic cells) were able to repair DNA after exposure to toxic agents, whereas spermatids and spermatozoa did not have this capability.[126]

A few reports have suggested an association, whereas many studies have failed to find a relationship between dioxin and birth defects. However, these are primarily in studies that do not have biological measures of exposure and thus are not covered here.[3,6] The Ranch Hand study provides modest evidence to support an association with birth defects. Most of the data on grouped birth defects have very small numbers.

DENTAL EFFECTS Finnish investigators examined the association of enamel hypomineralization of permanent first molars in 6- to 7-year-old children and TEQ exposure through breast feeding (these teeth develop during the first 2 years of life).[57,58] These data present interesting findings. Unfortunately, the presentation of the results is incomplete, so the potential biological significance cannot be assessed. This would be an interesting outcome to examine in other populations.

SEX RATIO AT BIRTH Sex ratio at birth was significantly depressed in a group of 17 children in zone A, Seveso, in the years shortly following the industrial accident. This pattern disappeared a few years later. However, a recent expanded effort suggests that paternal age at the time of exposure may be a key factor. Sex ratio differences were not observed in other groups examined. If effects are restricted to offspring to fathers less than 19 years old, as suggested in the new Seveso study, the lack of effect elsewhere could be explained by the groups examined: maternal levels of dioxin in community studies or studies of men older than 19. The findings in the most recent Seveso study emphasize the need for more attention to male-mediated developmental effects and the potential importance of exposures prior to and during puberty. Because this outcome can easily be collected in studies of developmental effects, more thorough examination of this outcome could be useful.

GROWTH MEASURES Growth measures include endpoints such as intrauterine growth retardation (IUGR), low birth weight, and postnatal growth. Available evidence does not support an association between paternal dioxin level and low birth weight.[73–74,94] In the Rotterdam study, decrements in length (but not other measures of growth) were observed early (months 3 to 7 postnatally) and associated with PCB levels, but disappeared with increasing age.[46] The Finnish data[92] are interesting because birth weight did decrease in males with increasing TEQ, but the lack of detail on the statistical analyses makes interpretation difficult.

MISCELLANEOUS ENDPOINTS Additional reproductive outcomes were evaluated in a subset of the studies: neonatal and infant death, and childhood cancer and mortality. Mainly because of small sample sizes, it is difficult to reach conclusions regarding neonatal, infant, and child mortality and childhood cancers. Recently, the increased risk for neonatal death observed in the Ranch Hand study, the only study with individual TCDD levels, was investigated. Some changes were observed in the Ranch Hand study for preterm birth and neonatal death, but these did not follow an exposure–response relationship.[94]

19.4 CONCLUSIONS

In conclusion, the research to date has been successful in resolving some confusion surrounding the evidence for an association of dioxin exposure and various developmental and reproductive endpoints in humans. High occurrence of exposure misclassification, differences in case definitions across studies, and small sample sizes have severely limited the power of these studies to address these questions. Additional research is necessary to better understand the spectrum of outcomes, including a measure of dioxin level at the relevant time in both the father and mother, and in breast milk during nursing for developmental outcomes, and other appropriate time periods for reproductive effects.

Potentially crucial information on individual variation in susceptibility to dioxin toxicity is lacking in all of the published research on TCDD and reproductive/developmental outcomes. The literature describing TCDD and cancer in humans has begun to describe the role of polymorphisms of xenobiotic-metabolizing enzymes and the Ah receptor in explaining differences in risks of various cancer types.[127–130] Researchers now suspect that genetic polymorphisms may also be involved in the pathway, resulting in birth defects as well.[127,130] Several studies are currently under way that may provide valuable genetic susceptibility markers and adverse reproductive effects of TCDD to expand our knowledge.

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CHAPTER 20

Health Consequences of the Seveso, Italy, Accident

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20.1 THE ACCIDENT OF JULY 10, 1976

2,4,5-trichlorophenol (TCP) production at the Givaudan–Hoffmann–LaRoche ICMESA plant in Meda (Milan, Italy) was started in 1969–1970 and brought up to full-scale levels in the years that followed with a big production increase ($> 10^5$ kg/yr) over the 1974–June 1976 period. TCP was obtained by a discontinuous process based on hydrolyzing 1,2,4,5-tetrachlorobenzene to sodium trichlorophenate with sodium hydroxide in the presence of xylene and ethylene glycol and transforming the trichlorophenate to TCP by acidifying it with hydrochloric acid. The hydrolysis reaction was carried out inside Department B by utilizing a 10-m^3 stainless steel reactor in 7000-kg batches of chemicals.[1–4]

At 12:37 P.M. on July 10, 1976, an exothermic reaction raised the temperature and pressure inside the reactor beyond limits, thereby causing a safety device, consisting of a rupture disk set at 3.8 atm (380 kPa), to blow out.[1–5] 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) production was also increased to an unknown extent. The safety device was mounted on an exhaust pipe which was connected directly to the reactor cover and, passing through the roof of Department B, ended up in the open. When the rupture disk collapsed, the overheated fluid mixture burst through the pipe out into the open air propelled by the thrust of the built-up pressure. The chemical cloud that left the reactor entrained nearly 2900 kg of organic matter, including at least 600 kg of sodium trichlorophenate and an amount of TCDD that is still being evaluated.

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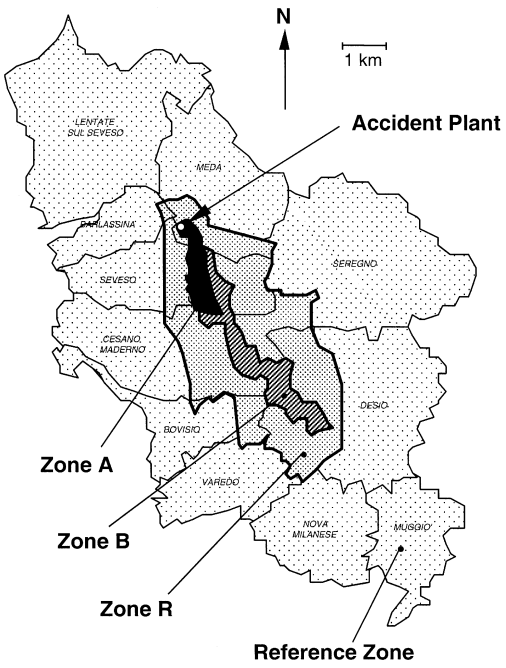

Figure 20.1 Area contaminated by TCDD and surrounding area adopted as reference in the epidemiology studies. Names identify interested municipalities. Zone A fanned out from ICMESA to the south and contained most of the TCDD that had escaped. Zone B was the natural extension of zone A along the main diffusion pathway of the TCDD-containing cloud. Zones A and B were both enclosed by a larger territory coded as zone R. The grid unit cell was a 50-m square for zone A and a 150-m square for zones B and R.

The visible part of the cloud rose up to about 50 m; it subsequently subsided and fell back to Earth but was wind-driven over a wide area of territory (Figure 20.1).[1,6–9]

Emission gradually decreased until it ceased altogether. Within < 2 h of the accident, chemicals settled on the ground as far as 6 km south of ICMESA or were dispersed by wind streams.[2,9,10] Serious environmental contamination followed: The leaves of plants near ICMESA, courtyard animals, and birds were seriously affected, many dying within a few days of the accident. At the same time, dermal lesions among human beings who had been exposed to the toxic alkaline cloud began to appear. About 10 days after the accident, it was found that TCDD was present in various types of samples collected near the plant.

Toward the end of the runaway reaction, reactor temperatures are thought to have increased well above 300°C, thus causing extensive mineralization of

residual organic substances.[1,3,11] After blowout, 2300 kg of residual chemicals was present in the reactor; their approximate composition was composed of sodium chloride (72%), decomposed organic matter, and some 250 to 300 g of TCDD.[3,4] TCDD amount was later reestimated at approximately 600 g.

20.2 DEFINITION OF AREAS AT DIFFERENT CONTAMINATION LEVELS

As a first step, the information available on the location of toxic and pathological events—regarding vegetation, animals, and human beings[12]—and on the airstream pattern at blowout time was used to draw an approximate diagram of the contaminated area. This was further confirmed by chemical monitoring of TCDD in the soil carried out under emergency conditions.[2,9,13,14] Within 5 weeks of the accident, the area hit was subdivided into zones A, B, and R, in descending TCDD contamination levels (Figure 20.1).

The borderline between zones A and B was set at average TCDD concentration levels in the soil of $\leqq 50\ \mu g/m^2$ ($1\ \mu g = 10^{-6}$ g); the boundaries of zones A and B in zone R were fixed where average contamination was $\leqq 5\ \mu g/m^2$. Zone R included the remaining territory where detectable levels of TCDD (formally, $0.75\ \mu g/m^2$) were found. In all cases, borderlines were established following preexisting natural or artificial divisions, in general compliance with the contamination pattern made out.[1,2,4,9,13,15]

On July 26, 1976, approximately 200 people were evacuated from a 15-hectare (ha; 1 hectare = 2.471 acres or 10,000 square meters) area immediately southeast of ICMESA,[1,2,4,9,15–18] the first part of what shortly afterward would be defined as zone A. Following further analytical findings concerning the TCDD contents of soil and vegetation samples, a few days later the entire zone A (more than 730 inhabitants) was evacuated. Zones B and R were subjected to area-specific hygiene regulations, including prohibition of farming, of consuming local agricultural products and of keeping poultry and other animals.

Beginning with the emergency period, soil monitoring was performed repeatedly during the first decade following the Seveso incident (1976–1986) for at least three reasons: for reassessment of TCDD distribution patterns and levels with time, for reassessment and updating of risk estimates and risk management measures, and as a backup tool to determine the effectiveness of remedial actions and reclaiming operations. Thousands of soil samples were collected and assayed according to criteria, techniques, and procedures often developed ad hoc to meet the requirements of a unique and unexpected case for which no reference to a former experience in Italy could be made. In general, the analytical tools and setups grew more sophisticated and reliable as time went by[9,10,16,19,20]; however, studies have proven that the sets of soil data before 1979 may have seriously underestimated TCDD levels.[20,21]

20.3 BIOLOGICAL DATA ON HUMAN EXPOSURE

Seven months after the ICMESA accident, a 55-year-old woman died from pancreatic adenocarcinoma, which had spread to the liver and to the extrahepatic bile ducts and had metastasized in the lungs. The subject had lived in an area of zone A characterized by a mean TCDD level of 200 $\mu g/m^2$. At the moment of the accident, the woman was eating inside her home with the windows and doors open; she also consumed vegetables from her garden in the 4 days following the event, which was before this was prohibited by the authorities. TCDD presence in her tissues was investigated after death occurred. The following results were obtained and reported on a whole or wet weight basis (pg/g)[22]:

• Fat	1840	• Brain	60
• Pancreas	1040	• Lung	60
• Liver	150	• Kidney	40
• Thyroid	85	• Blood	6

Approximately 10 years after the Seveso accident, analytical methods were improved and became available to measure TCDD levels in small blood samples.[23–26] From the 1976–1985 laboratory medical examinations following the Seveso accident, there were some 30,000 1 to 3-mL serum samples that had been kept stored at $-30°C$ since the time they were collected.[27–29] TCDD was measured in several samples of reportedly highly exposed subjects living in zones A, B, and R, and results are shown in Table 20.1.[30] More recently, measurements were performed in the plasma of randomly selected subjects from zones A, B, and the reference area, enrolled in a molecular epidemiology study currently in progress.[31] Results are reported in Table 20.2. It is visible from the tables that the ecological classification of exposure status based on soil levels was not refuted by classification based on available blood dioxin mea-

TABLE 20.1 TCDD Levels in Blood Samples Collected from Highly Exposed Inhabitants in the Contaminated Zones[a] and from a Sample of the Reference Population[b]

Zone	Number of Subjects	Median (ppt)
A	296	447
B	80	94
R	48	48
Reference	52	5.5

[a] Collected in 1976; data from Ref. 30.

[b] Collected in 1993–1994; data from Ref. 31.

TABLE 20.2 TCDD Blood Levels (ppt) in Random Samples of the Inhabitants in the Highly Contaminated Zones and in the Reference Zone[a]

Zone	Gender	GM	Median	Range
A	F	60.5	63.0	45.3–80.7
	M	50.5	73.3	9.8–89.9
B	F	17.6	16.8	1.3–62.6
	M	6.7	6.5	3.5–44.7
Reference	F	6.1	6.6	1.8–18.1
	M	3.7	4.4	1.0–13.8

[a]Collected in 1993–1994; data from Ref. 31.

surements. Blood measurements also strengthen confidence in the nonexposure status of the reference population: Their estimated average blood concentration corresponded to background values measured in industrial areas.[32]

20.4 EARLY HEALTH FINDINGS

Medical examination programs were initiated with the following aims: to ascertain early adverse health consequences in the exposed population, to give guidance for the allocation of services and resources, to identify needs and suggest areas for further surveillance and research.

The earliest sign of adverse effects in humans became apparent when, on the sixth day, 19 children were admitted to local hospitals with skin lesions caused by contact of uncovered parts of the body with caustic chemicals contained in the cloud.

20.4.1 Chloracne

By the end of the year 1976, 34 cases of chloracne (an acne resulting from high-dose exposure to many chlorinated organic chemicals, including dioxins) were diagnosed in persons under 15 years of age. It was then decided to examine all children attending nurseries and infant and primary schools in the contaminated areas. Nearly 90% of them had skin examinations. By April 1977, 187 cases of overt chloracne were diagnosed by an expert panel, and 164 (88%) were in children. Chloracne distribution resembled the TCDD contamination pattern (Table 20.3), however, with exceptions. For example, in a zone R suburb called Polo, located in the top right-hand corner of the accident area, southeast of the plant (Figure 20.1), out of 750 children, 19 (2.5%) were diagnosed as having chloracne. By mid-1978, six additional cases were detected, bringing the total number to 193. No further cases of chloracne were reported after this time.[33–35] The higher frequency of chloracne in children than in

TABLE 20.3 Distributions of 164 Chloracne Cases Diagnosed up to April 1977 in Children Having Their Residence in Different Areas of the Contaminated Territory or Outside

Area	Total Population Aged 3–14 yr	Number of Cases	Percent
Zone A total	214	42	19.6
Zone A max.[a]	54	26	48.1
Zone B	1,468	8	0.5
Zone R	8,680	63	0.7
Zone R, Polo[b]	750	19	2.5
Other	48,263	51	0.1

[a] Includes only the most contaminated part of zone A.

[b] Subzone located near the plant.

adults is possibly explained by the fact that the former had more opportunities to came into contact with the toxic cloud components and to ingest or inhale them through outdoor activities, contact with soil, vegetation, and so on. Other relevant explanatory factors might be the absence of a systematic screening in adults, which possibly left unnoticed other existing cases, or a difference in susceptibility to dioxin effects at different ages.

20.4.2 Subjective Symptoms and Laboratory Tests

The comparison of 146 chloracne cases and 182 controls (age and sex matched to the cases, selected from the area) revealed a significantly higher frequency of nausea, lack of appetite, vomiting, abdominal pain, headache, and eye irritation among the former. In addition, they exhibited a higher frequency of abnormal values for the liver enzymes γ-glutamyltranspeptidase (GGT) and alanine aminotransferase (GPT) as well as for urinary aminolevulynic acid (ALA-U).[33]

Health examination results of 18 subjects over 14 years of age affected by chloracne were also reported (no control group was examined concurrently). There was a high frequency (around 25%) of self-reported symptoms and of signs of liver enlargement. In 1977, biochemical tests showed elevated serum cholesterol values higher than 230 mg per 100 mL in eight subjects and ALA urinary concentration outside the reference range in five subjects.[36]

20.4.3 Peripheral Neuropathy

Peripheral neurological changes were among the signs of TCDD toxicity. Persons evacuated from zone A were invited to undergo neurological examination in 1977 and 1978. A nonexposed population served as reference. Electrophysiological and clinical signs of peripheral neuropathy were nearly three times as frequent among Seveso residents having either raised serum liver

enzyme levels or chloracne (12/55 or 22%) than among controls (13/168 or 8%). When only subjects younger than 20 years with chloracne were considered, the relative frequency rose up to nearly five times.[37]

20.4.4 Enzyme Induction

In the early months after the accident, urinary D-glucaric acid excretion, an indirect index of enzyme induction, was measured in 14 children with and 17 children without chloracne, all from zone A. The former had a significantly higher level of D-glucaric acid in urine.[38]

It is understandable how in the hectic, early postaccident period these observations were often lacking formal design and proper conduct. For example, it was not easy to control biases, such as those linked to interview and reporting, or to assure standardization of diagnostic procedures, proper selection of controls, adequate size of the sample, and so on. Notwithstanding these limitations, the reported early surveys, which concerned primarily chloracne cases, showed that at least a portion of the population had actually been exposed to the powerful toxin 2,3,7,8-TCDD.

20.5 SURVEILLANCE PROGRAMS

Surveillance programs were then designed to continue over time the health monitoring of those subjects exhibiting immediate, acute effects (e.g., chloracne cases) and to identify the possible occurrence of health effects in the short- to midterm period. One of the major problems was the large size of the population in the active surveillance. This difficulty was augmented by the lack of a preexisting validated information system and by the time constraints which led many different teams to be called into the area, with diminished possibilities for quality control and procedure standardization.

20.5.1 Spontaneous Abortion

According to animal data, an increase of spontaneous abortions in exposed pregnant women was to be expected. Ascertainment of spontaneous abortion is, in general, a difficult surveillance task. Specific problems further complicated the picture in our case. One was the absence of a valid ongoing data collection system; another involved the moral and political issues related to legalization of abortion; and finally, a very active birth control campaign was carried out which may have decreased conception rates. In such a context, the completeness, accuracy, and quality of data remained questionable. Nevertheless, several attempts were made to report and interpret the occurrence of spontaneous abortions in the area.

Analysis by trimester (from the accident to early 1978) and by municipality showed some time-related variability with the highest abortion rate seemingly

occurring in the earliest trimester in the contaminated areas. However, it was difficult to determine the possible contribution of TCDD exposure, or of exposure to a ''chemical disaster'' as such, and to exclude a major role of biases related to information recording and data collection. Results were considered not conclusive, if at all valid.[12]

In another report,[35] crude estimates drawn from vital statistics sources were provided. A decrease in the birth rate was observed in 1977–1980. The proportion of abortions compared to live births per year was not considered to depart from the ''generally accepted abortion rate of 10 to 20%.''

In a third analysis, notifications of spontaneous abortions to county medical officers were used. Spontaneous abortion rate in 1977 was higher than in 1976, but not departing from historical rates estimated from 1973 onward. Improved physicians' care in notification was considered a probable explanation for the change in the postaccident period. These data were then supplemented with information provided directly by hospitals in the region (Table 20.4). An increased pregnancy loss percent was seen in late 1976, with a subsequent fall. Zone B rates showed a further increase in mid-1977 and were consistently higher than those of zone R and the reference area; the increase was statistically significant only in the third trimester of 1977. Data quality was considered questionable, and results lacked consistency.[39]

20.5.2 Cytogenetics

The first chromosome analysis was performed in 1976 at the request of hospitals where eight children aged 2 to 10 years and four pregnant women with skin

TABLE 20.4 Pregnancy Loss Rate by Exposure Zone and Trimester[a]

Zone[b]	July–Sept. 1976	Oct.–Dec. 1976	Jan.–Mar. 1977	Apr.–June 1977	July–Sept. 1977	Oct.–Dec. 1977
B						
Number of abortions	3	4	5	8	10	4
Pregnancy loss (%)	11.1	22.2	17.2	28.5	31.2	13.7
R						
Number of abortions	19	17	15	17	16	20
Pregnancy loss (%)	13.7	16.3	12.7	12.5	11.4	13.8
Noncontaminated						
Number of abortions	74	94	119	81	67	99
Pregnancy loss (%)	11.0	14.8	16.6	13.0	10.5	14.3

Source: Special Office for Seveso, Lombardy region.

[a] Abortions/(births + stillbirths + abortions) × 100.

[b] Zone A is not shown because very few pregnancies occurred.

TABLE 20.5 Cytogenetic Study in Seveso, 1977: Frequency of Chromosomal Aberrations in Lymphocytes

Exposure	Number of Subjects	Number of Mitoses	Percent Aberrant Cells	
			Including Gaps	Excluding Gaps
Acute	145	6470	2.49	0.99
Chronic	69	3040	2.53	0.92
Controls	87	3958	1.64	0.48

lesions presumably caused by TCDD exposure had been admitted. For comparison, the earliest available results on 10 unmatched subjects were adopted. The proportion of aberrant cells was higher in the exposed children and women, but the significance of the difference remained uncertain.[40]

A more extensive cytogenetics study included 301 subjects, as indicated in Table 20.5. Those with acute exposure were subjects living in the most contaminated area near the accident plant; workers employed in that plant were considered as having had long-term ("chronic") exposure; age- and gender-matched controls were people living in the surrounding noncontaminated area. The proportion of aberrant cells was higher among the exposed, but statistical analysis showed that the only significant difference was among the scorers of the five laboratories involved in the survey. A further analysis of a larger number of mitoses on samples selected from the three exposure categories was then carried out. Differences between exposure categories did not become significant even after correction for interobserver variability. No consistent evidence of chromosomal effects associated with TCDD exposure was thus provided by this study.[40]

A third set of data was obtained after examining maternal peripheral blood, placenta, and umbilical cord and fetal tissues of induced abortions in 19 women from the Seveso area and in 16 women not known to be exposed to environmental agents known to produce congenital malformations, who had abortions for nonmedical reasons. Within a pattern of marked variability, no significant differences were observed in the frequency of individual types of aberration, average number of aberrations per aberrant cell, and frequency of polyploids in maternal blood and placenta between the two groups. Instead, fetal tissues of exposed pregnancies exhibited aberrant cell frequency significantly higher and a greater number of aberrations per damaged cell than control pregnancies. Several factors might explain these findings, including those related to growth in culture. In addition, the possibility of preexisting chromosome damage in fetal cells could not be ruled out. No differences were seen between those pregnancies started in Seveso before and after the accident. Thus, the extent to which the increased frequency of chromosome aberrations in fetal tissue reflected maternal exposure to TCDD could not be established.[41]

20.5.3 Birth Defects

The first set of data available consisted of 30 cases of induced abortion (of which three were from zone A and five from zone B) and four spontaneous abortions (from zone R), all of which occurred in 1976 after the accident. Embryological and histomorphological investigations were conducted on this material, and no indications of mutagenic, teratogenic, or fetotoxic effects attributable to TCDD were detected. In 23 induced abortions, no anomalies or organic alterations could be found. In six other cases, various morphological alterations were visible; some were probably artifacts, and some were of borderline pathological significance. The four spontaneous and the one remaining induced abortions were probably related to dioxin exposure, but this link could not be proven. Investigations were limited by the fact that in the majority of cases fetal tissues were incomplete.[42]

At the beginning of 1977, a congenital malformation registry was established, which included all live births and stillbirths to women residing in the accident area in July 1976. Data were collected for the period 1977–1982; 742 malformed infants were registered out of a total of 15,291 births (live and still). Out of 26 births in zone A, no cases of major malformations were found. In none of the three exposure zones (A, B, or R) was the frequency of mild, major, or combined defects significantly higher than in the reference population. Table 20.6 shows relative risks for the entire surveillance period and for the first quarter of 1977, when children had probably been exposed to TCDD during the first week of gestation (140 births in all). None of the relative risks was statistically significant. Major information biases were excluded, whereas the small number of exposed pregnancies, especially in zones A and B, might have precluded the identification of low-risk and/or very rare defects.[43]

20.5.4 Follow-up of Special Groups

Long-term effects on the *peripheral nervous system* (PNS) were explored in 152 subjects with chloracne, who agreed to participate in a survey conducted between October 1982 and May 1983, and in 123 subjects without chloracne, frequency-matched by gender and age, who volunteered to serve as a reference

TABLE 20.6 Relative Risk and 90% Confidence Interval for Selected Groups of Malformations in the TCDD-Contaminated Area[a] vs. the Surrounding Noncontaminated Territory

	1977–1982	First Quarter 1977
Total defects	0.97 (0.83–1.13)	1.49 (0.64–3.45)
Major defects	0.83 (0.67–1.04)	0.93 (0.26–3.32)
Mild defects	1.14 (0.92–1.42)	2.50 (0.79–7.94)

[a] Zones A + B + R.

group. None of the subjects had a clear-cut peripheral neuropathy, but clinical and electrophysiological signs of PNS involvement were, significantly, nearly twice as frequent in the chloracne group than in controls. In particular, there were 11 cases vs. 2 controls presenting at least two clinical signs of bilateral PNS involvment, and 25 cases vs. 9 controls exhibiting at least one abnormal electrophysiological function.[44]

Forty-eight children aged 3 to 8 years from zone A underwent repeated examinations from November 1976 to May 1979 for the study of *immunologic effects*. Control subjects were selected from the school population of a nearby noncontaminated town. Total serum complement hemolytic activity had significantly higher values among the exposed subjects at each examination. Exposed children also exhibited higher values for lymphocyte responses to phytohemagglutinin (PHA) and to pokeweed mitogen (PWM) and in the absolute number of lymphocytes of peripheral blood. Results for other tests failed to show clearly diverging values between the exposed and control subjects. Consistently increased values were more evident in children with chloracne.[45]

Induction of microsomal enzymes in the liver was one of the best documented TCDD effects in laboratory animals. An indirect test of enzyme induction, urinary D-glucaric acid, was evaluated between 1976 and 1979 in different groups of the exposed population and controls. Children from zone A with chloracne had, in 1976, significantly higher levels of D-glucaric acid in urine than children without skin lesions. In 1979, children who had left zone A had levels similar to those of controls, whereas in children still living in zone B the urinary excretion was significantly higher. In 1980, however, urine of the zone B children showed significantly lower levels. In 1981, 34 children evacuated in 1976 from zone A had normal values, whereas 61 children from zone B and another 59 children from a zone R sector very close to the plant (Polo, the suburb mentioned earlier) had urinary D-glucaric acid levels almost significantly elevated. Adults living in zone B ($n = 117$) had significantly higher levels of D-glucaric acid excretion in 1978 than those of controls ($n = 127$) from a noncontaminated area.[38]

Children from the three contaminated zones were followed from 1976 to 1982 to examine whether *liver function* and *lipid metabolism* showed alterations as possible consequences of TCDD exposure. In all, nearly 400 children aged 6 to 10 years at the time of the accident were examined on a yearly basis. The only clear-cut difference in test values between exposed and reference children was seen in 1976 and 1977, when boys living in the most contaminated zone A exhibited consistently higher levels of γ-glutamyltransferase and alanine aminotransferase. The increase was slight, perhaps attributable to TCDD exposure, and disappeared with time. No alterations of blood concentrations of cholesterol and triglycerides were found.[46]

Repeated surveys on the group of 193 *chloracne cases* and on unexposed control subjects matched for age and gender were conducted until 1985, with a participation rate between 70 and 80%. No significant differences and temporal

trends in mean values of liver enzymes and lipids were detected. A decrease in cholesterol and triglycerides values was apparent in the chloracne group between 1976 and 1982. At the end of the follow-up in 1984, one subject had persistent chloracne, and five had chloracne scars on face and forearms. Motor and sensory nerve conduction velocity was measured, and neither significant differences between groups nor temporal trends were observed. Apart from skin signs, no clinical or systemic sequelae of chloracne were thus detected eight years after first exposure.[47]

Another special group surveyed was comprised of *workers* employed in *decontamination operations* in the area. These people entered the most contaminated part of zone A under strict personal protection and environmental measures. They underwent preemployment medical examination for eligibility. The values of a set of preemployment test (liver function, lipid and heme metabolism, etc.) were compared with the same values after nine months and with those of an unexposed group. No significant changes were detected.[48] Later analysis of cleanup workers' experience confirmed that on average, the safety measures taken had been effective. No TCDD-related clinical health impairment was found (such as chloracne, liver impairment, peripheral neuropathy, porphyria cutanea tarda), and no significant differences in biochemical outcomes compared to unexposed subjects were detected. Nine subjects left for non-health-related reasons and five for negative job fitness evaluation; for two of them a transient effect of exposure to TCDD could not be excluded completely.[49]

Between 1976 and 1985, laboratory tests were performed periodically on the 30 subjects whose serum was first assayed for TCDD, to detect possible alterations of the liver, kidney, bone marrow, lipid metabolism, and immune system function. However, only modest, transient, small departures from the normal ranges were observed in four children with chloracne, four zone A adults, and one referent subject. None of the alterations observed had pathological significance either with respect to the number of tests involved or with respect to the extent of the alteration.[29]

20.6 LONG-TERM MORTALITY AND CANCER INCIDENCE STUDIES

All surveillance programs were supervised, and their results evaluated periodically by an International Steering Committee which ended its work in 1984. Their conclusion was that chloracne represented the only health outcome clearly attributable to the accidental exposure to TCDD. No conclusion could be reached at that time regarding long-term effects. Surveillance programs were discontinued, but long-term investigations were designed to examine mortality and cancer incidence.

As time passed, it appeared that there might be a migration away from the area by those people who had most suffered physically, emotionally, or economically because of the accident; and they may well have been the most rele-

vant to the determination of late health effects of accident exposure. In addition, a dilution phenomenon related to the moving out of exposed and moving in of nonexposed subjects was to be expected. To avoid these sources of bias, a cohort approach was adopted. The study population was thus comprised of all persons ever resident in one of the 11 towns within the accident scenario, at any time from the date of the accident onward (including newborns and immigrants), irrespective of their current residence. The information about towns and street addresses allowed attributing subjects to one of the three exposure zones or to the surrounding noncontaminated area. Admission into the study cohort was discontinued as of December 31, 1986; no potential for exposure was deemed to exist anymore for newcomers into the area after that date.

The follow-up was based on individual information recorded on vital statistics registries which are maintained by every municipality in Italy. When a person moved outside the study area, the towns concerned were contacted successively, until the person was located. The tracing turned out to be successful for over 99% of study subjects, and for them vital status and, when deceased, cause of death information became available.

Due to the absence of a national registration system of cancer cases, the cancer incidence study had, instead, to be limited to people residing within the Lombardy region (nearly 9,000,000 inhabitants). The linkage of the information on all hospitalization in the region with the records of cohort members allowed the identification of the study subjects admitted/discharged with a diagnosis mentioning cancer. Original medical records were reexamined to ascertain true diagnosis and date of occurrence of cancer. The ascertainment rate for cancer morbidity was close to 95%.

People living in the territory surrounding zone R, not contaminated by TCDD, were the source of reference data. They shared with the index population the main characteristics related to living and occupational environment, personal habits, and social and educational background.

Results for mortality in the 20-year period following the accident have been reported.[50] All-cancer deaths were significantly in excess in the zone A and B merged male population. The magnitude of the excess was similar to that estimated in previous long-term studies of high-exposure male occupational cohorts.[51–54] Also, lung cancer mortality was elevated, the increase being significant in the highest-exposed, zone A male population, after 15 years of latency. Several independent studies found elevated lung cancer risks.[55–60] Rectal cancer was increased in zone A and B males, a finding backed by at least one occupational cohort study.[64] The possible involvement of other digestive sites (stomach in zones A and B, and, less evidently, liver in zone B) was suggested by results of latency analysis among females.

A consistent excess of lymphohemopoietic neoplasm was found in zones A and B. Non-Hodgkin's lymphoma was significantly elevated in the zone A population and had a nonsignificant increase in zone B. In zone B, the increases were significant for Hodgkin's disease and for leukemia, in particular, myeloid leukemia. The mortality increase from multiple myeloma was signifi-

cant among females. Leukemia deaths, instead, showed the highest increase among males. A dose-related increased lymphoma occurrence was seen in mice, both sexes.[61,62] In occupational cohorts with high exposure to TCDD, non-Hodgkin's lymphoma and, less evidently, Hodgkin's disease were found elevated.[55,58,59,63] An association with TCDD exposure was also seen for multiple myeloma[53,59,60] and, possibly, for leukemia.[64]

Mortality from noncancer deaths showed some unusual features, too. The increase in diabetes mellitus was present among females in all exposure zones. The finding, however, should be interpreted with caution given the poor diagnostic accuracy of death certificates for this condition. An elevated prevalence of diabetes and a positive association between TCDD serum levels and fasting serum glucose levels were found in a survey on U.S. chemical workers exposed to dioxin, but confounding by other variables could not be excluded.[65] The follow-up of a large cohort of U.S. chemical male workers failed, instead, to detect any excess mortality from diabetes.[53] In an accidentally exposed German industrial cohort, mean fasting glucose levels increased slightly with current, but not back-extrapolated dioxin levels.[66] Also, highly exposed Vietnam veterans showed a high prevalence of diabetes and a time-to-diabetes onset decrease with dioxin exposure[67] as well as an association of serum dioxin levels with insulin and sex hormone–binding globulin.[68] A merely suggestive increase was also seen in an international cohort of chemical workers exposed to TCDD or higher-chlorinated dioxins.[69]

Circulatory disease mortality (chronic ischemic heart disease, in particular) was elevated in zone A, among males, in the early postaccident period. A possibly differential cause of death certification in the early postaccident period can be hypothesized. That dioxin can adversely affect the cardiovascular system is well documented.[70–80] In an international cohort of pesticide manufacturers and applicators, exposure to TCDD and higher chlorinated dioxins was associated with a significantly increased ischemic heart disease mortality.[69] One German[54] and one Dutch[58] study found a significant excess of ischemic heart disease associated with dioxin exposure, whereas another German study[57] did not. In Seveso, a possibly relevant disease determinant might have also been the heavy psychosocial impact of the accident.[81,82] The burden of disaster-linked psychosocial stressors might have precipitated early deaths from preexisting ill health conditions.

The increased chronic obstructive pulmonary disease mortality was especially apparent among zone A males and also affected zone A and B women. Previous studies in TCDD-exposed populations do not support this association. It is difficult to hypothesize such an extreme and systematic difference in smoking habits between the index and reference populations as to explain a threefold-increased relative risk. The most plausible pathways through which TCDD might have contributed to this finding is its recognized immunotoxic activity.[83,84] Even among people with preexisting chronic obstructive pulmonary disease, the accident-related stressors might have been relevant in precipitating early deaths. Tables 20.7 to 20.10 summarize mortality results, by gen-

TABLE 20.7 Mortality, 1976–1996, from Malignant Causes among Males in the Accident Area

Cause of Death (ICD IX Code)	Zone A			Zone B			Zone R		
	Deaths Observed	Relative Risk	95% CI	Deaths Observed	Relative Risk	95% CI	Deaths Observed	Relative Risk	95% CI
All causes (000–999)	54	1.1	0.8–1.4	384	1.0	0.9–1.1	2696	1.0	1.0–1.1
All cancers (140–208)	14	0.7	0.4–1.2	152	1.2	1.0–1.4	873	1.0	0.9–1.0
Digestive (150–159)	3	0.4	0.1–1.3	50	1.0	0.8–1.3	328	0.9	0.8–1.1
Esophagus (150)	0			2	0.5	0.1–2.1	38	1.4	1.0–2.0
Stomach (151)	1	0.5	0.1–3.2	15	1.0	0.6–1.6	99	0.9	0.7–1.1
Colon (153)	0			10	1.2	0.6–2.2	58	1.0	0.7–1.3
Rectum (154)	1	2.2	0.3–15.6	8	2.4	1.2–4.6	23	1.0	0.6–1.5
Hepatobiliary (155–156)	0			6	0.5	0.2–1.2	61	0.8	0.6–1.0
Liver (155)	0			6	0.6	0.3–1.3	56	0.8	0.6–1.0
Pancreas (157)	1	1.3	0.2–9.5	3	0.6	0.2–1.9	32	0.9	0.6–1.3
Respiratory (160–165)	9	1.3	0.7–2.6	55	1.3	1.0–1.6	300	1.0	0.9–1.1
Lung (162)	9	1.5	0.8–3.0	48	1.3	0.9–1.7	261	1.0	0.9–1.1
Pleura (163)	0			3	3.5	1.1–11.5	4	0.7	0.2–1.9
Bone (170)	0			0			4	0.7	0.3–2.1
Soft-tissue sarcoma (171)	0			0			4	1.3	0.4–3.9
Melanoma (172)	0			1	1.7	0.2–12.5	4	1.0	0.3–2.8
Genitourinary tract (185–189)	1	0.5	0.1–3.7	16	1.1	0.7–1.8	102	1.0	0.8–1.2
Prostate (185)	0			8	1.2	0.6–2.4	50	1.1	0.8–1.5
Bladder (188)	1	1.7	0.2–12	5	1.1	0.5–2.8	30	1.0	0.7–1.4
Brain (191)	0			1	0.5	0.1–3.4	15	1.1	0.6–1.8
Thyroid gland (193)	0			1	4.4	0.6–34.3	0		
Lymphoemopoietic (200–208)	1	0.9	0.1–6.3	14	1.8	1.0–3.0	47	0.9	0.6–1.2
Hodgkin's (201)	0			2	3.0	0.7–12.4	3	0.7	0.2–2.2
Non-Hodgkin's (200, 202)	1	3.2	0.4–23	2	0.9	0.2–3.8	15	1.0	0.6–1.8
Myeloma (203)	0			1	0.7	0.1–5.0	11	1.1	0.6–2.1
Leukemia (204–208)	0			9	2.4	1.2–4.7	18	0.7	0.4–1.2
Lymphatic (204)	0			2	1.9	0.5–7.7	11	1.5	0.8–3.0
Myeloid (205)	0			5	3.8	1.5–9.6	4	0.4	0.2–1.2

TABLE 20.8 Mortality, 1976–1996, from Malignant Causes among Females in the Accident Area

Cause of Death (ICD IX Code)	Zone A			Zone B			Zone R		
	Deaths Observed	Relative Risk	95% CI	Deaths Observed	Relative Risk	95% CI	Deaths Observed	Relative Risk	95% CI
All causes (000–999)	42	1.0	0.7–1.4	265	1.0	0.9–1.1	2241	1.0	1.0–1.1
All cancers (140–208)	13	1.0	0.6–1.8	70	0.9	0.7–1.1	557	0.9	0.8–1.0
Digestive (150–159)	6	1.2	0.5–2.7	25	0.8	0.5–1.2	225	0.9	0.8–1.0
Esophagus (150)	0			0			6	0.1	0.4–2.3
Stomach (151)	2	1.4	0.3–5.5	9	1.0	0.5–1.9	73	1.0	0.8–1.2
Colon (153)	2	1.8	0.4–7.0	3	0.4	0.1–1.3	50	0.9	0.6–1.1
Rectum (154)	0			3	1.3	0.4–4.1	15	0.8	0.5–1.4
Hepatobiliary (155–156)	0			7	1.2	0.6–2.6	39	0.8	0.6–1.1
Liver (155)	0			6	1.5	0.7–3.3	24	0.7	0.5–1.1
Pancreas (157)	0			1	0.3	0–2.4	19	0.8	0.5–1.2
Other digestive (159)	2	5.6	1.4–22.7	2	0.9	0.2–3.6	19	1.0	0.6–1.6
Respiratory (160–165)	0			5	0.8	0.3–2.0	46	0.9	0.7–1.3
Lung (162)	0			4	0.7	0.3–2.0	40	1.0	0.7–1.3
Bone (170)	0			1	1.8	0.2–13.5	6	1.5	0.6–3.6
Soft tissue sarcoma (171)	0			0			0		
Melanoma (172)	1	6.6	0.9–47.7	1	1.0	0.1–7.4	5	0.7	0.3–1.8
Breast (174)	2	0.8	0.2–3.1	12	0.7	0.4–1.3	95	0.8	0.6–0.9
Uterus (179–182)	0			2	0.5	0.1–2.1	32	1.1	0.7–1.6
Ovary (183)	1	1.6	0.2–11.2	2	0.5	0.1–2.0	30	1.0	0.7–1.5
Bladder (188)	0			0			7	0.8	0.4–1.7
Brain (191)	0			3	2.2	0.7–7.0	11	1.1	0.6–2.0
Thyroid gland (193)	0			1	2.2	0.3–16.4	3	0.8	0.3–2.8
Lymphoemopoietic (200–208)	1	1.1	0.1–7.5	12	2.0	1.1–3.5	46	1.0	0.7–1.3
Hodgkin's (201)	0			2	4.3	1.0–18.3	5	1.5	0.6–4.0
Non-Hodgkin's (200, 202)	1	3.3	0.5–23.7	3	1.6	0.5–4.9	14	1.0	0.5–1.7
Myeloma (203)	0			4	3.8	1.4–10.4	9	1.0	0.5–2.1
Leukemia (204–208)	0			3	1.1	0.4–3.5	18	0.9	0.5–1.5

TABLE 20.9 Mortality, 1976–1996, from Nonmalignant Causes among Males in the Accident Area

Cause of Death (ICD IX Code)	Zone A			Zone B			Zone R		
	Deaths Observed	Relative Risk	95% CI	Deaths Observed	Relative Risk	95% CI	Deaths Observed	Relative Risk	95% CI
All causes (1–999)	54	1.1	0.8–1.4	384	1.0	0.9–1.1	2696	1.0	1.0–1.1
Infectious, parasitic disease (1–139)	0			0			13	1.2	0.6–2.1
Diabetes (250)	0			6	0.9	0.4–2.0	56	1.2	0.9–1.6
All circulatory disease (390–459)	24	1.4	1.0–2.2	126	1.0	0.8–1.1	948	1.1	1.0–1.1
Hypertension (400–405)	1	1.7	0.2–11.9	1	0.2	0–1.4	39	1.1	0.8–1.6
Ischemic heart disease (410–414)	9	1.2	0.6–2.3	56	1.0	0.8–1.3	411	1.1	1.0–1.2
Myocardial infarction (410)	4	0.7	0.3–1.9	32	0.8	0.6–1.2	253	1.0	0.4–1.1
Chronic ischemic heart disease (412, 414)	5	2.3	1.0–5.6	23	1.3	0.8–1.9	156	1.3	1.1–1.5
Cerebrovascular disease (430–438)	6	1.4	0.6–3.2	42	1.2	0.9–1.6	250	1.1	0.9–1.2
Respiratory disease (460–519)	6	2.2	1.0–4.9	21	0.9	0.6–1.4	175	1.1	0.9–1.3
Chronic obstructive pulmonary disease (490–493)	5	3.5	1.5–8.5	12	1.0	0.6–1.7	100	1.2	1.0–1.5
Digestive disease (520–579)	2	0.5	0.1–2.1	22	0.9	0.6–1.3	205	1.2	1.0–1.4
Cirrhosis of liver (571)	2	0.7	0.2–3.0	14	0.8	0.5–1.4	140	1.2	1.0–1.4
Accidents (800–999)	4	1.0	0.4–2.8	31	1.1	0.8–1.5	177	1.0	0.8–1.1

TABLE 20.10 Mortality, 1976–1996, from Nonmalignant Causes among Females in the Accident Area

Cause of Death (ICD IX Code)	Zone A			Zone B			Zone R		
	Deaths Observed	Relative Risk	95% CI	Deaths Observed	Relative Risk	95% CI	Deaths Observed	Relative Risk	95% CI
All causes (1–999)	42	1.0	0.7–1.4	265	1.0	0.9–1.1	2241	1.0	1.0–1.1
Infectious, parasitic disease (1–139)	0						6	1.0	0.4–2.4
Diabetes (250)	2	1.3	0.3–5.1	18	1.8	1.1–2.9	112	1.3	1.1–1.6
All circulatory disease (390–459)	13	0.7	0.4–1.3	102	0.9	0.8–1.1	1030	1.1	1.0–1.2
Chronic reumathic heart disease (393–398)	3	11.5	3.6–36.3	0			15	1.2	0.7–2.0
Hypertension (400–405)	3	2.6	0.8–8.0	4	0.6	0.2–1.5	91	1.4	1.1–1.8
Ischemic heart disease (410–414)	1	0.2	0–1.4.0	31	1.0	0.7–1.4	302	1.1	1.0–1.2
Myocardial infarction (410)	1	0.4	0.1–2.8	13	0.8	0.5–1.4	129	1.0	0.8–1.2
Chronic ischemic heart disease (412, 414)	0			18	1.1	0.7–1.8	172	1.2	1.0–1.4
Cerebrovascular disease (430–438)	2	0.4	0.1–1.4	38	1.1	0.8–1.5	350	1.1	1.0–1.3
Respiratory disease (460–519)	3	1.4	0.4–4.3	14	1.0	0.6–1.7	113	0.9	0.8–1.2
Chronic obstructive pulmonary disease (490–493)	2	2.7	0.7–10.8	10	2.1	1.1–4.0	46	1.1	0.8–1.6
Digestive disease (520–579)	3	1.4	0.4–4.3	16	1.2	0.7–1.9	113	1.0	0.8–1.2
Cirrhosis of liver (571)	0			5	0.7	0.3–1.6	53	0.9	0.7–1.2
Accidents (800–999)	3	1.6	0.5–4.9	14	1.1	0.7–1.9	97	1.0	0.8–1.3

TABLE 20.11 Cancer Incidence, 1977–1986, in the Population Aged 20–74 Living in the TCDD-Contaminated Area[a]

	Zone B			Zone R		
Cancer Site	Cases Observed	Relative Risk	95% CI	Cases Observed	Relative Risk	95% CI
All	115	1.0	0.8–1.2	790	0.9	0.9–1.0
Digestive system	30	1.0	0.7–1.4	211	0.9	0.8–1.0
Hepatobiliary tract	10	2.3	1.2–4.4	23	0.7	0.5–1.1
Respiratory system	24	1.0	0.7–1.5	163	1.0	0.8–1.1
Soft-tissue sarcoma	0			8	2.3	1.0–5.1
Skin	5	0.9	0.4–2.1	41	1.0	0.7–1.4
Breast[b]	10	0.7	0.4–1.3	113	1.1	0.9–1.3
Genitourinary	18	0.9	0.5–1.4	133	0.9	0.7–1.1
Uterus	2	0.4	0.1–1.5	23	0.6	0.4–0.9
Lymphatic hemopoietic tissue	15	2.1	1.2–3.5	45	0.9	0.6–1.2
Hodgkin's disease	3	2.6	0.9–9.0	7	0.9	0.4–2.0
Non-Hodgkin lymphoma	4	1.6	0.6–4.3	23	1.3	0.8–2.0
Multiple myeloma	4	3.9	1.4–1.7	4	0.5	0.2–1.4
Myeloid leukemia	3	2.8	0.9–9.0	7	0.9	0.4–2.1

[a] Results for cancers selected among males and females combined.

[b] Females only.

der, in the three exposure zones, separately by malignant and nonmalignant causes.

Results of the mortality study do not permit conclusively associating any of the unusual cancer mortality findings with exposure to TCDD in 1976. Other limiting factors were the still relatively short time period elapsed since first exposure, the small number of deaths from certain causes, and exposure definition based on soil TCDD levels rather than on individual biological indicators.

Cancer incidence findings for the first postaccident decade are available and are summarized in Table 20.11. Cancer cases in zone A were too few (seven cases among males and seven among females) to elicit any meaningful conclusion. In zone B the relative risk for all cancer was 1.0. The noted hepatobiliary increase was sustained mainly by primary liver cancer in men (four cases, RR = 2.1; confidence interval 95%, CI_{95} = 0.8 to 5.7), and by extrahepatic bile ducts and gallbladder cancer in women (four cases, RR = 4.8, CI_{95} = 1.7 to 13.5, statistically significant). Lymphoemopoietic tissue neoplasms were significantly increased in zone B consistently among males and females (RR = 2.3 and RR = 1.9, respectively). In particular, lymphoreticulosarcoma among males (three cases, RR = 5.3, CI_{95} = 1.6 to 17.5) and multiple myeloma among females (two cases, RR = 5.1, CI_{95} = 1.2 to 21.6) showed statistically signifi-

cant increases.[85] Thus, the population of zone B exhibited the clearest suggestions of a possibly increased cancer occurrence, a finding that might be consistent with their postaccident experience (they remained in the contaminated area, and their compliance to restrictive regulations was never evaluated). The suggestively lower incidence of estrogen-dependent cancers (breast and uterus) was also considered of interest since TCDD is known to exert a powerful antiestrogenic action.[86]

The relevant result in zone R was, instead, an elevated risk of soft tissue sarcomas. The increase noted in zone R was twofold and of borderline statistical significance. A significantly lowered risk of uterine cancer was also noted.

The results above concern people 20 to 74 years of age. Mortality and cancer incidence of the young members of the cohort (1 to 20 years of age) were analyzed separately. Mortality data showed an increase of leukemia deaths above expectations, although statistically nonsignificant, in both males and females. A suggestive increase of congenital anomalies was also noted; however, five out of the seven anomalies observed in the contaminated area turned out to have occurred in children born before the accident.[87] Cancer incidence was only slightly above expectations.[88] Given the small number of events, results are presented in Table 20.12 for the entire contaminated area (A + B + R). A statistically nonsignificant increased risk was observed for thyroid cancer and myeloid leukemia. Again, results of this analysis should be viewed with caution, especially because of the very limited number of events involved and the absence of information on individual exposure.

20.7 OVERALL INTERPRETATION

Results of short- and midterm surveillance programs clearly documented that the accident caused toxic damage to the population. Chloracne was the only

TABLE 20.12 Cancer Incidence, 1977–1986, in the Population Aged 0–19 Living in the TCDD-Contaminated Area: Results for Selected Cancers

Cancer Site	Cases Observed	Cases Expected	Relative Risk	95% CI
All	23	18.2	1.26	0.8–2.0
Ovary and uterine adnexa	2	0.0		
Nervous system	5	3.4	1.45	0.5–3.9
Brain	4	3.0	1.32	0.4–4.0
Thyroid[a]	2	0.4	4.66	0.7–33.1
Non-Hodgkin's lymphoma	2	1.3	1.54	0.3–7.6
Hodgkin's lymphoma	3	1.9	1.54	0.4–5.7
Lymphatic leukemia	3	2.8	1.07	0.3–3.7
Myeloid leukemia	2	0.9	2.30	0.6–9.2

[a] Cases are restricted to females.

health effect established consistently. Other health outcomes known to be possibly associated with dioxin exposure were investigated. An unusual pattern, either for the frequency or for the type of outcome concerned, attributable to TCDD was not firmly established for any of them. These investigations, however, suffered from all the constraints related to a postdisaster scenario, which often precluded proper design (e.g., lack of referent subjects), efficient conduct (e.g., compliance to the scheduled examinations), and valid conclusion (e.g., information and selection bias).

The follow-up of small, selected groups, mainly chloracne children, failed to show health impairment in these heavily exposed subjects up to 8 to 9 years after the accident. In addition, they showed that chloracne is reversible and not necessarily associated, at least in the time span considered, with other systemic effects. Longer follow-up and larger sample size are necessary to corroborate this conclusion.

The results of long-term investigations revealed the departure from the background occurrence of certain tumors, which was consistent with previous experimental and human data and plausible from a biological perspective. The increase in cardiovascular and respiratory mortality, early after the accident, was possibly explainable in terms of precipitation of preexisting diseases caused by the stressful experience following the chemical disaster. The suggestive increase in diabetes is consistent with previous human data and knowledge of dioxin toxic effects. The small number of events for certain causes and the short observation period for cancer incidence prevent definite conclusions. An additional limitation is exposure classification, which was based largely on soil contamination data, which do not actually indicate individual exposure and body burden.

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CHAPTER 21

The Yusho Rice Oil Poisoning Incident

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21.1 INTRODUCTION

A mass poisoning, called Yusho, occurred in western Japan, mainly in Fukuoka and Nagasaki prefectures, in 1968. Yusho was caused by ingestion of rice oil contaminated with Kanechlor-400, a commercial brand of Japanese polychlorinated biphenyls (PCBs).[1] It was later found that the rice oil had been contaminated not only with PCBs but also with polychlorinated dibenzofurans (PCDFs),[2] polychlorinated quaterphenyls (PCQs),[3] and others. Consequently, Yusho was a poisoning by a mixture of PCBs, PCDFs, PCQs, and others. A very similar mass poisoning, called Yucheng (see Chapter 22), occurred in central Taiwan in 1979, 11 years after the Japanese Yusho incident.[4,5] These two incidents of food poisonings are very valuable as a source of information concerning the toxic effects of these chemicals on humans. Several books and reviews on the broad fields of poisoning have been published.[6–10] Kuratsune reviewed the Yusho PCB poisoning incident in English with some members of the Study Group for the Therapy of Yusho.[11] In this chapter we update the rice oil poisonings, focusing primarily on Yusho.

21.2 EPIDEMIOLOGICAL STUDY

An epidemic of a strange skin disease similar to chloracne was announced to the public in Fukuoka, Japan, in October 1968. Kuratsune et al.[12] examined the outbreak conditions of this epidemic disease, called Yusho or oil disease. The most common initial symptoms were increased eye discharge and swelling of eyelids, acneiform eruption and follicular accentuation, and pigmentation. Most of the patients (99%) were affected during 1968, 55% of the occurrences

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being concentrated in the three months from June to August. All of the patients had used Kanemi brand rice oil, and the oil was produced or shipped by the Kanemi company on February 5 and 6, 1968, or soon thereafter. Gas chromatographic and x-ray fluorescence analyses of the rice oil revealed that only the sample produced or shipped at the beginning of February was contaminated by a large amount of chlorine (maximum 462 ppm); none of the oils shipped in the other months was contaminated with more than a trace amount of chlorine. Kanechlor had been used at the company in its equipment for heating the processed oil at a reduced pressure to remove odors from the oil. It is believed that it must have leaked into the rice oil through a hole formed in the heating pipe by a welding mistake.

21.3 TOXIC AGENTS IN RICE OIL

When the Yusho incident was disclosed in 1968, the rice oils were analyzed for PCBs by gas chromatography and x-ray fluorometry, estimated at 2000 to 3000 ppm of Kanechlor-400 in the rice oil based on the organic chlorine content,[1] since no specific method for PCBs was available at that time. Samples of the rice oil produced on February 5 or 6 were analyzed for PCBs using the standard analytical method, yielding approximately 1000 ppm of PCBs in the rice oil.[13] PCBs in the rice oil showed a somewhat different gas chromatographic pattern from that of Kanechlor-400, indicating that Kanechlor-400–contaminated rice oil was heated under reduced pressure, which eliminated some amounts of lower-chlorinated PCBs, which have shorter retention times on the gas chromatogram, as shown in Figure 21.1.[14]

The oil was found to contain 5 ppm of PCDFs, about 250 times the concentration (0.02 ppm) expected from the concentration of PCDFs in other unused Kanechlor-400.[2] This finding was confirmed by Miyata et al.[15] The marked increase of PCDFs in the oil could have occurred in the following way. The Kanechlor-400 used as a heat transfer medium for deodorizing rice oil was heated to higher than 200°C for a long time, and PCBs were gradually converted to PCDFs. The PCBs with increased PCDF concentrations leaked to the rice oil through a hole in the heating pipe. The conversion of PCBs to PCDFs by heating at higher temperatures was confirmed by Miyata and Kashimoto[16] and Nagayama et al.[17] Concentrations of PCBs, PCDFs, and PCQs in the rice oil and Kanechlor-400 and their ratios were reported by Kashimoto and Miyata[18] as shown in Table 21.1. PCDFs in the rice oil were composed of more than 40 congeners, including toxic congeners of 2,3,7,8-tetra-, 1,2,3,7,8-penta-, 2,3,4,7,8-penta-, 1,2,3,4,7,8-hexa-, and 1,2,3,6,7,8-hexa-CDFs.[19,20] A total of 74 PCBs and 47 congeners of tetra- through octa-PCDFs were quantitatively determined in the rice oil.[21] The concentrations of major PCDF congeners are given in Table 21.2. It was later discovered that high-chlorinated dibenzofurans decompose during the alkaline treatment of samples, a common analytic cleanup process for PCB and PCDF analyses. By an improved ana-

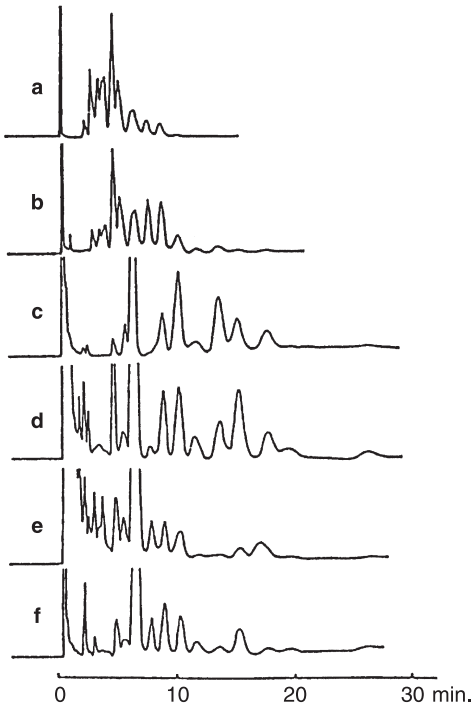

Figure 21.1 Gas chromatograms of PCB fractions on the 5% SE-30 glass column (3 mm × 2 m): a, Kanechlor-400; b, Kanemi rice oil; c, d, blood of typical Yusho patients; e, blood from a type C Yusho patient; f, blood from a normal control. (Adapted from Ref. 14.)

TABLE 21.1 Concentrations of PCBs, PCDFs, and PCQs in Yusho Oil and Kanechlor-400

	Concentration (ppm)			Ratio (%)	
	PCBs	PCDFs	PCQs	PCDFs/ PCBs	PCQs/ PCBs
Yusho oil produced					
Feb. 5, 1968	968	7.4	866	0.76	89
Feb. 9, 1968	151	1.9	490	1.3	320
Feb. 10, 1968	155	2.3	536	1.4	350
Feb. 11, 1968	43.7	0.48		1.1	
Feb. 15, 1968	12.3	0.085		0.69	
Feb. 18, 1968	1.8	0.012		0.67	
Unused KC-400	999,800	33	209	0.003	0.021
Used KC-400	968,400	510	31,000	0.052	3.2
	999,000	277	690	0.028	0.069
	971,900	20	28,000	0.002	2.9

Source: Data from Ref. 18.

TABLE 21.2 Concentrations of PCDD, PCDF, and PCB Congeners and TCDD Toxic Equivalents (TEQs) in the Rice Oil[a]

	Concentration (ppb)	TEQ Factor Used	TEQs (ppb)
2,3,7,8-Tetra-CDD	nd	1	0
Other tetra-CDDs	3		0
1,2,3,7,8-Penta-CDD	7	1	7.0
Other penta-CDDs	77		0
2,3,7,8-Hexa-CDDs	71	0.1	7.1
Other hexa-CDDs	203		0
1,2,3,4,6,7,8-Hepta-CDD	185	0.01	1.9
Other hepta-CDDs	160		0
Octa-CDD	120	0.0001	0.01
Total PCDDs	826		16.0
2,3,7,8-Tetra-CDF	660	0.1	66.0
Other tetra-CDFs	2,570		0
1,2,3,7,8-Penta-CDF	525	0.05	26.3
2,3,4,7,8-Penta-CDF	1,350	0.5	675.0
Other penta-CDFs	3,580		0
2,3,7,8-Hexa-CDFs	1,225	0.1	122.5
Other hexa-CDFs	1,259		0
2,3,7,8-Hepta-CDFs	267	0.01	2.7
Other hepta-CDFs	42		0
Octa-CDF	76	0.0001	0.01
Total PCDFs	11,600		892.4
3,3′,4,4′-Tetra-CB	11,500	0.0001	1.2
3,3′,4,4′,5-Penta-CB	630	0.1	63.0
3,3′,4,4′,5,5′-Hexa-CB	27	0.01	0.3
2,3′,4,4′,5-Penta-CB	32,000	0.0001	3.2
2,3,3′,4,4′-Penta-CB	28,000	0.0001	2.8
2,3,3′,4,4′,5-Hexa-CB	2,950	0.0005	1.5
Other mono-ortho PCBs	91,800		0
Di-ortho PCBs	135,100		0
Tri-ortho PCBs	7,580		0
Other PCBs	71,000		0
Total PCBs	380,000		71.9
Total	392,400		980.3

[a]Concentrations are average of two samples and data from Ref. 21. TEQ factors from Ref. 30. nd, Not detected.

lytical process, hepta-CDFs were found to be major components in the rice oil, which contained 160 ppm of PCBs.[22]

At an early stage after the Yusho incident, 2000 to 3000 ppm of PCBs was thought to be contained in the rice oil by x-ray fluorometry. Actual levels of PCBs were about 1000 ppm, one-half to one-third of the PCB levels above.

The rice oil was expected to contain a large amount of chlorinated compounds other than PCBs and PCDFs. Miyata et al.[3,23] detected 866 ppm of PCQs consisting of penta- through deca-chlorinated congeners in the rice oil, as shown in Table 21.1. The presence of PCQs in the rice oil was confirmed by Kamps et al.[24] and Yamaguchi and Masuda.[25]

Polychlorinated quaterphenyl ethers (PCQEs) (i.e., dipolychlorobiphenyl ethers) and polychlorinated terphenyls were also identified as minor components in the fraction of PCQs from the rice oil.[3] Formation of PCQEs from PCBs was accompanied by the formation of PCQs in the heating process of Kanechlor-400 at high temperatures.[23–26] Polychlorinated sexiphenyls, namely trimers of PCBs, 70 ppm, were separated from the PCQ fraction from the rice oil by gel permeation chromatography.[25] In the PCDF fraction from the rice oil, polychlorinated naphthalene[19] and polychlorinated phenyldibenzofurans[27] were identified in small quantities. Kashimoto et al.[22] found 0.13 ppm of PCDDs and 1.41 ppm of three coplanar PCBs in a sample of the rice oil that contained 169 ppm of PCBs. Tanabe et al.[21] also quantified the congeners of PCDDs and coplanar PCBs. These concentrations are shown in Table 21.2.

Toxicities of individual congeners of PCDDs, PCDFs, and PCBs were evaluated relative to 2,3,7,8-tetra-CDD (TCDD) toxicity.[28,29] Using the TCDD toxic equivalent (TEQ) factors established by the World Health Organization (WHO) in 1997,[30] concentrations of TEQ in the rice oil were calculated as shown in Table 21.2. Total TEQ in the rice oil was calculated to be 0.98 ppm, of which the amount contributed from PCDFs was 91%, from PCBs 7.1%, and from PCDDs 1.6%.

21.4 INTAKE OF THE CONTAMINATED RICE OIL

A survey of 141 Yusho patients who consumed the rice oil containing 920, 866, and 5 ppm of PCBs, PCQs, and PCDFs, respectively, showed that the average consumption of the rice oil was 688 mL in total and 506 mL during the latent period before illness was apparent. Therefore, the total amounts of PCBs, PCQs, and PCDFs ingested by a patient were 633, 596, and 3.4 mg, respectively, on average, and the amounts ingested during the latent period were 466, 439, and 2.5 mg, respectively.[31] The smallest amounts ingested by a patient during the latent period were estimated to be 111, 105, and 0.6 mg, respectively. The average concentration of TCDD toxic equivalents in the rice oil was determined to be 0.98 ppm (Table 21.2), and the intakes of TCDD equivalents by patients were calculated. Table 21.3 lists the intake of rice oil and TCDD equivalents by Yusho patients. The clinical severity of illness and the blood PCB levels showed a close positive correlation with the total amount of oil consumed but not with the amount of oil consumed per kilogram body weight per day.[31,32] This may indicate that during exposure to these highly persistent toxic substances, the level of toxic substances in the body increased to the level needed for development of the toxic symptoms of Yusho.

TABLE 21.3 Mean Estimated Intakes of Rice Oil and TCDD Toxic Equivalents (TEQs) by Yusho Patients (Range in Parentheses)

	Rice Oil	TCDD TEQ[a]
Average total intake per capita	688 mL (195–3375)	0.62 mg (0.18–3.04)
Average intake during latent period	506 mL (121–1934)	0.456 mg (0.11–1.74)
Average daily intake	0.171 mL/kg (0.031–0.923)	154 ng/kg (28–832)
Smallest intake during latent period	121 mL	0.11 mg
Smallest daily intake during latent period	0.031 mL/kg	28 ng/kg

Source: Data from Ref. 31.

[a]TCDD TEQs are calculated by 0.98 ppm in Yusho oil and 0.92 of oil density.

21.5 TOXIC AGENTS IN TISSUES AND BLOOD OF YUSHO PATIENTS

21.5.1 PCBs

Table 21.4 summarizes the concentrations of PCBs in adipose tissue and liver.[33–35] Soon after the onset of Yusho in 1968, PCB concentrations in the adipose tissue were very high compared with the later levels of Yusho patients and controls. The level of PCBs in the adipose tissue decreased rapidly to several parts per million in the next year, 1969. However, these levels, which were only slightly higher than those in the controls,[36] were maintained until recent years. The level of PCBs in the liver was considerably lower than that of the adipose tissue in the same patient. In 1973, five years after the onset of Yusho, average blood levels of PCBs in Yusho patients ($n = 41$) and controls ($n = 37$) were determined to be 7 and 3 ppb, respectively.[37] After that time, average blood PCB levels of Yusho patients were found to be 6.1 ppb in 1979 ($n = 64$),[38] 3.08 ppb (seven PCB congeners) in 1983 ($n = 18$),[39] and 7.9 ppb in 1991 ($n = 9$). These levels were always only two to three times higher than the control levels. Recently, using an improved analytical method of high-separation gas chromatography/low-resolution mass spectrometry, 49 PCB congeners were identified and quantified in the blood of Yusho patients in 1998. Average total PCB concentration of 5.0 ppb in whole blood of 13 Yusho patients (sampled in 1998) was 3.6 times higher than those of control persons.[40] The same PCB congeners were identified in the blood of Yusho patients and persons from the general population. However, the PCB congeners in Yusho patients differed quantitatively from those found in nonexposed persons. Table 21.5 shows that the composition of PCB congeners present in blood obtained from Yusho patients in 1983 is still quite different from those of control persons, being characterized by low concentrations of 2,3′,4,4′,5-penta-CB and

TABLE 21.4 Mean Concentrations of PCBs in Tissues of Yusho Patients and Controls

Case	Gender	Age	Date of Death or Surgical Operation	PCB Concentration (ppm, wet basis)	
				Adipose Tissue	Liver
1[a]	F	Stillborn	Oct. 1968	0.02	0.07
2[b]	M	About 17	Nov. 1968	76 (face) 13 (abdomen)	
3[b]	?	Adult	Nov. 1968	46	
4[a]	M	17	July 1969	1.3	0.14
5[a]	M	25	July 1969	2.8	0.2
6[a]	M	46	May 1972	4.3	0.08
7[a]	M	59	Mar. 1977	1.2	0.006
8–14[c]	M, F	43–55	Feb. 1986	1.0–5.7	
Controls					
A ($n = 31$)[a]	M, F	0–61	1981	1.39[d] (0.09–13)	0.05[d] (0.01–0.2)
B ($n = 11$)[c]	M, F	29–61	Feb. 1986	0.44–1.3	

[a]Data from Ref. 33.

[b]Data from Ref. 34.

[c]Data from Ref. 35.

[d]Range in parentheses.

TABLE 21.5 Concentrations of PCB Congeners in the Blood of Yusho Patients Sampled in 1983

	Concentration (ppt)		
	Yusho Patients ($n = 18$), Mean ± SD	Control ($n = 27$), Mean ± SD	Yusho/ Control Ratio
2,3′,4,4′,5-penta-CB	55 ± 44	71 ± 95	0.77
2,2′,4,4′,5,5′-hexa-CB	1086 ± 678	348 ± 430	3.12
2,3,3′,4,4′-penta-CB	38 ± 26	27 ± 35	1.41
2,2′,3,4,4′,5′-hexa-CB	522 ± 294	139 ± 160	4.02
2,3,3′,4,4′,5-hexa-CB	836 ± 343	50 ± 33	16.7
2,2′,3,4,4′,5,5′-hepta-CB	311 ± 239	91 ± 124	3.42
2,2′,3,3′,4,4′,5-hepta-CB	198 ± 142	45 ± 55	4.4
Total	3080 ± 1440	763 ± 922	4.04

Source: Data from Ref. 39.

much higher concentrations of 2,3,3′,4,4′,5-hexa-CB.[37,39] This characteristic difference has been adopted as one of the criteria for diagnosis of Yusho.[41] The characteristic type is classified into three, as follows: type A, peculiar to Yusho, with a gas chromatographic pattern quite different from that observed in normal persons; type B, an intermediate pattern between types A and C; and type C, commonly observed in the blood PCBs of the general population. Biological half-lives of these PCB congeners were determined in three Yucheng patients who had very high blood PCB levels of 156 to 397 ppb.[42] The half-life of 2,3′,4,4′,5-penta-CB, 1.16 years, was much shorter than those of 2,2′,4,4′,5,5′-hexa-CB, 4.28 years, and 2,3,3′,4,4′,5-hexa-CB, 4.21 years. The shorter half-life of the penta congener may partly cause the peculiar pattern in Yusho patients. Among the PCB congeners identified in Yusho patients, 2,3,3′,4,4′,5-hexa-CB showed strong enzyme-inducing activity in the liver and marked atrophy of the thymus in rats.[43] Therefore, 2,3,3′,4,4′,5-hexa-CB was considered to be one of the PCB congeners most causally related to the symptoms of Yusho. Recently, Kashimoto et at.[22] and Tanabe et al.[21] reported the presence of highly toxic coplanar PCBs in the tissues of Yusho patients, 3,3′,4,4′,5-penta-CB, a coplanar PCB, being measured at 330 and 410 ppt in intestines and 720 ppt in adipose tissue, respectively. The levels of three coplanar PCBs were very low in the tissues relative to other PCBs, being 0.06 to 0.6% in Yusho patients and 0.03 to 0.08% in controls.[22] However, dioxinlike toxicity of 3,3′,4,4′,5-penta-CB is the highest among PCBs in controls, as shown in Table 21.6, since its TCDD toxic equivalent factor is much higher than those of other PCBs. Hirakawa et al.[44] analyzed coplanar PCBs in the subcutaneous adipose tissue resected from Yusho patients and found the concentration of 3,3′,4,4′,5-penta-CB in Yusho patients ($n = 7$, 70 ppt) was lower than that of the controls ($n = 8$, 135 ppt). This relatively low level of the toxic PCB congener was also observed in the blood sampled from Yusho patients in 1990–1991.[45]

Only several selected congeners of PCBs in the rice oil were retained in the body of patients as described above, and most of the PCB congeners had disappeared from the body within a year by excretion or by being metabolized to hydroxy and methylsulfone PCBs. The methylsulfone PCBs, which were probably derived from the PCBs ingested with the rice oil, were identified in the tissues of Yusho patients.[46] The concentration (fat basis) of methylsulfone PCBs was higher in the lung (0.67 ppm) than in the adipose tissue (0.07 ppm), contrasting with the concentrations of PCBs in the tissues, 0.8 and 1.3 ppm, respectively.[47] Some congeners of methylsulfone PCBs either induced or changed the enzyme condition in the human body. One of the metabolites, 3-methylsulfone-3′,4,4′,5-tetra-CB, was found to demonstrate a strong inhibition on the aromatic hydrocarbon hydroxylase (AHH) activity which was either previously or simultaneously induced by TCDD in a human lymphoblastoid cell culture.[48,49] The same methylsulfone PCB inhibited methylcholanthrene-induced AHH activity in mouse liver microsomes in aryl hydrocarbon (Ah) responsive strains of mouse, whereas it greatly enhanced the same enzymes in Ah nonresponsive strains.[50] Some 3-methylsulfone PCBs had

TABLE 21.6 Concentrations of TCDD Toxic Equivalents (TEQs) in the Tissues and Blood of Yusho Patients

		TCDD TEQs (ppt)			
		Yusho Patient			Control
	WHO TEQ Factor	Adipose,[a] 1977, Wet Basis	Liver,[a] 1977, Wet Basis	Blood,[b] 1990–1991, Fat Basis	Serum,[b] 1991–1992, Fat Basis
2,3,7,8-tetra-CDD	1	0.95	0.35	2.25	3.10
1,2,3,7,8-penta-CDD	1	18.00	1.00	7.20	9.16
1,2,3,4,7,8-hexa-CDD	0.1	0.08	0.02	0.29	0.43
1,2,3,6,7,8-hexa-CDD	0.1	16.00	2.20	3.57	3.88
1,2,3,7,8,9-hexa-CDD	0.1	0.08	0.02	0.54	0.83
1,2,3,4,6,7,8-hepta-CDD	0.01	0.06	0.02	0.17	0.46
Octa-CDD	0.0001	0.02	1.30	0.53	1.14
Total PCDDs		35.2	4.9	14.6	19.0
2,3,7,8-tetra-CDF	0.1	4.40	4.70	0.25	0.47
1,2,3,7,8-penta-CDF	0.05	1.45	4.95	0.09	0.04
2,3,4,7,8-penta-CDF	0.5	850.00	1150.00	120.75	8.70
1,2,3,4,7,8-hexa-CDF	0.1	130.00	840.00	15.25	1.19
1,2,3,6,7,8-hexa-CDF	0.1	14.00	223.00	3.44	0.83
2,3,4,6,7,8-hexa-CDF	0.1	0.08	0.23	0.07	0.83
1,2,3,7,8,9-hexa-CDF	0.1			0.42	0.34
1,2,3,4,6,7,8-hepta-CDF	0.01	0.95	15.00	0.17	0.09
1,2,3,4,7,8,9-hepta-CDF	0.01			0.03	0.01
Octa-CDF	0.0001				
Total PCDFs		1001	2238	140	12.5
3,4,4′,5-tetra-CB	0.0001				
3,3′,4,4′-tetra-CB	0.0001	0.07	0.01	0.00	0.00
3,3′,4,4′,5-penta-CB	0.1	72.00	5.40	4.50	14.15
3,3′,4,4′,5,5′-hexa-CB	0.01	3.80	0.50	1.26	0.92
2,3,3′,4,4′-penta-CB	0.0001			0.35	1.00
2,3,4,4′,5-penta-CB	0.0005			1.56	1.38
2,3′,4,4′,5-penta-CB	0.0001			1.41	4.23
2′,3,4,4′,5-penta-CB	0.0001				0.08
2,3,3′,4,4′,5-hexa-CB	0.0005			16.69	8.05
2,3,3′,4,4′,5′-hexa-CB	0.0005			4.38	1.81
2,3′,4,4′,5,5′-hexa-CB	0.00001			0.05	0.08
2,3,3′,4,4′,5,5′-hepta-CB	0.0001			0.24	0.09
Total PCBs		76	5.9	30.4	31.8
Total TEQ		1112	2249	185	63.3

[a] Data from Ref. 21.

[b] Data from Ref. 45.

stronger inductive effects on aminopyrin *N*-demethylase, 7-ethoxycoumarin *O*-deethylase and benzo[*a*]pyrene hydroxylase than corresponding parent PCBs did, while 4-methylsulfone PCBs had little effect.[51] Therefore, the health conditions of the patients are possibly altered by the accumulation of methylsulfone PCBs in the tissues. Human serum was found to contain 4-hydroxy-3,5,-chlorinated PCBs at a concentration of 0.6 ppb, which corresponded to one-fourth of the PCB level.[52] According to animal experiments in mice and rats, the hydroxy PCBs bound to transthyretin and interfered with the thyroxine transport in plasma[53]; thus the human thyroxin levels are presumed to be disturbed in both the plasma and tissue. In fact, significantly elevated thyroxin levels were actually observed in the plasma of Yusho patients in 1984, 16 years after onset.[54]

21.5.2 PCDFs

Although Yusho patients ingested more than 40 different PCDF congeners with rice oil,[19] only several particular PCDF congeners have been retained in the tissues of patients.[20] Most of the retained PCDF congeners had all lateral (2, 3, 7, and 8) positions chlorinated, and all of the congeners apparently excreted had two vicinal hydrogenated C atoms (no chlorine-substituted C atoms) in at least one of the two rings. Concentrations of major PCDF congeners identified in the tissues and blood of Yusho patients are shown in Table 21.7. High concentrations of 2,3,4,7,8-penta-CDF up to 25 ppb were observed in the liver and adipose tissues in 1969, a year after the incident. Subsequently, such high concentrations of PCDF congeners were not found in the tissues of Yusho patients, except 5 ppb of hexa-CDF in the liver in 1977. Higher than control levels of PCDF congeners continued up to 1986, when the levels of PCDF congeners were up to 40 times higher than control levels, while PCB levels in the patients were only two to three times higher than those in the controls. It is noteworthy that the PCDF concentrations in the liver were almost as high as those in adipose tissue, while PCB concentrations were much lower in the former than in the latter. This relative abundance of PCDFs to PCBs in the liver and adipose tissue was also seen in normal persons.[15] In 1995, twenty-seven years after the onset, an average concentration of the most toxic congener, 2,3,4,7,8-penta-CDF, in the blood of Yusho patients was only 16 times higher than that of control blood (Table 21.7). The concentrations of this toxic congener in Yusho patients are gradually approaching concentrations in controls, but the difference in one order of magnitude is still maintained for 30 years after the exposure.

In Yucheng patients, the same PCDF congeners were identified in various tissues and blood,[62,63] with the concentrations of 2,3,4,7,8-penta-CDF in some patients being comparable in adipose tissue and liver with Yusho patients and being 10 to 40 times higher in blood relative to Yusho patients. Biological half-lives of 2,3,4,7,8-penta-CDF and 1,2,3,4,7,8-hexa-CDF were determined to be 2.1 and 2.6 years, respectively, in the blood of three Yucheng patients, whereas

in the blood of Yusho patients, their half-lives were estimated to be about 10 years.[64] This discrepancy may be caused by the difference in PCDF concentrations in blood at the time of first samplings, being 13 to 42 ppb in Yucheng early after exposure and 1 to 5 ppb in Yusho later after exposure. Adding later determined data, biological half-lives of PCDF and PCB congeners were calculated (Table 21.8) in Yucheng patients during 0.6 to 15.6 years after onset and in Yusho patients during 14.0 to 29.1 years after onset.[65] Data in Table 21.8 explain that half-lives of PCDF and PCB congeners in Yucheng patients early after exposure were shorter than those of corresponding congeners in Yusho patients later after exposure, and half-lives of PCDF congeners were shorter than those of the five PCB congeners except 2,3′,4,4′,5-penta-CB in the blood of both Yucheng and Yusho patients. From the elimination curves in Yucheng and Yusho patients, concentrations of 2,3,4,7,8-penta-CDF in the Yusho patients just after the exposure were calculated to be about 60 ppb in fat base.

Among the PCDF congeners retained in the tissues of Yusho patients, 2,3,4,7,8-penta-CDF showed the highest enzyme-inducing activities and acute toxicity in rats[43,66] and was the most highly accumulative in the livers of rats and monkeys.[67] Therefore, 2,3,4,7,8-penta-CDF is considered to be the most important etiologic agent for the Yusho symptoms. Relative toxicities of PCDD, PCDF, and PCB congeners to 2,3,7,8-tetra-CDD (TEQ factor) have been estimated at many research organizations throughout the world.[28–30,68,69] Using WHO TEQ factors, toxic contributions of the PCDDs, PCDFs, and PCBs retained in Yusho patients have been calculated and are shown in Table 21.6. 2,3,4,7,8-Penta-CDF showed highest toxicity in the adipose tissue (76%), liver (51%), and blood (65%) of patients. However, in the blood of a control subject, it contributed 14% of total TEQ, and 3,3′,4,4′,5-penta-CB contributed the highest dioxinlike toxicity (22%) among PCDDs, PCDFs, and PCBs.

Iida et al.[70,71] recently found that combined administration of rice bran fiber and cholestyramine for 2 weeks increased fecal excretion of 2,3,4,7,8-penta-CDF in four Yusho patients 31 to 69%, and in six Yucheng patients 60 to 160%.

21.5.3 PCQs

Table 21.9 shows the concentrations of PCQs and PCBs in adipose tissue, liver, blood, buccal mucosa, and hair of Yusho patients and normal controls. In typical Yusho patients who have type A PCBs, PCQ concentrations in the tissues and blood were approximately the same or two to four times lower than the PCB levels. The concentrations of PCQs seemed to decrease with time, as the high concentration of PCQs (2400 ppb) in adipose tissue in 1969 was not observed in the same tissue in 1984 when the level of PCQs in adipose tissue was on average 207 ppb. However, the PCQ concentrations in the tissues and blood of patients were always much higher than the corresponding concen-

TABLE 21.7 Concentrations of PCDF Congeners in the Tissues and Blood of Yusho Patients[a]

Sample[b]	Tissue	Year of Sampling	PCB (ppm)	PCDF Congeners (ppb, wet basis)			
				2,3,7,8-	2,3,4,7,8-	1,2,3,4,7,8-/ 1,2,3,6,7,8-	1,2,3,4,6,7,8-
1	Liver	1969	1.4	0.3	6.9	2.6	
2	Liver	1969	0.2	0.02	1.2	0.3	
	Adipose		2.8	0.3	5.7	1.7	
3	Liver	1969		1.6	16.4	21.8	9.8
	Adipose			0.5	8.7	7.4	0.5
4	Liver	1969	0.22	0.11	25	72	140
	Intestine		3.6	0.13	5.2	7.2	15
5	Liver	1972	0.03	< 0.01	0.3	0.03	
	Adipose		4.3	nd	0.8	0.2	
6	Adipose	1975	0.2	nd	0.1	0.5	
7	Liver	1977	0.06	nd	1.49	5.31	1.39
	Adipose		3	0.002	1.45	1.99	0.22
	Lung		0.016	0.002	0.365	0.41	0.05
8	Liver	1977	0.036	0.047	2.3	8.4	1.5
	Adipose		1.8	0.044	1.7	1.3	0.095
9	Abscess	1977	2.44	nd	2.76	1.86	0.17
10	Comedo	1982	0.2	nd	0.36	0.39	0.1
11	Uterus	1985	0.005	nd	0.026	0.031	nd
12	Adipose	1986	1.0	nd	0.16	0.066	
	($n = 7$)		~5.7	~0.018	~3.0	~1.22	
13	Adipose	1986	1.2	0.018	0.1	0.11	nd
	($n = 6$)		~5.7	~0.034	~1.74	~1.44	~0.11
	Blood		0.003		0.0002	0.0002	0.0002
	($n = 6$)		~0.022		~0.0066	~0.0061	~0.0006

14	Breast milk	1988		0.0047	0.67	0.347	0.0179
				~0.024[c]	~0.793[c]	~0.598[c]	~0.063[c]
15	Breast milk	1990		0.0023	0.212	0.1076	0.0023
				~0.0182[c]	~0.429[c]	~0.177[c]	~0.020[c]
16	Blood	1995	0.789[c]	0.003[c]	0.230[c]	0.128[c]	0.010[c]
	($n = 83$)		±0.663	±0.003	±0.297	±0.171	±0.007
Controls							
17	Adipose	1981		0.0027	0.00423	0.0216	0.0043
18	Adipose	1986	0.44	nd	nd	nd	
	($n = 11$)		~1.3	~0.019	~0.039		
19	Adipose	1986	0.071	0.004	0.013	0.009	nd
	($n = 3$)		~1.3	~0.012	~0.027	~0.035	~0.019
	Blood		0.002		0.00005	0.00004	0.00008
	($n = 3$)		~0.004		~0.00009	~0.0001	~0.0001
20	Breast milk	1991		0.0008	0.0037	0.0034	0.001
	($n = 9$)			~0.0029[c]	~0.0123[c]	~0.0085[c]	~0.0025[c]
21	Blood	1995	0.339[c]	0.001[c]	0.014[c]	0.013[c]	0.007[c]
	($n = 39$)		±0.223	±0.001	±0.006	±0.007	±0.002

[a] nd, Not detected.

[b] Data for samples 1, 2, 5, and 6 from Ref. 55; 3 and 17, Ref. 56; 4, Ref. 22; 7 and 11, Ref. 57; 8, Ref. 21; 9 and 10, Ref. 58; 12 and 18, Ref. 35; 13 and 19, Ref. 59; 14, 15, and 20, Ref. 60; 16 and 21, Ref. 61.

[c] Fat basis.

TABLE 21.8 Biological Half-Life of PCDF and PCB Congeners in Yusho and Yucheng Patients

	Half-Life (yr)									
	Yucheng Patients 0.6 to 15.6 yr after Onset				Yusho Patients 14.0 to 29.1 yr after Onset					
	BS[a]	SS[a]	RK[a]	Median	KK[a]	TS[a]	YUM[a]	TH[a]	HH[a]	Median
2,3,4,7,8-penta-CDF	2.7	3.6	2.9	2.9	14.3	7.7	6.1	5.2	11.4	7.7
1,2,3,4,7,8-hexa-CDF	2.7	3.6	3.5	3.5	6.5	4.5	3.9	5.1	6.9	5.1
1,2,3,4,6,7,8-hepta-CDF	2.6	2.5	2.2	2.5	6.6	2.6	3.5	3.5	3.4	3.5
Average	2.7	3.2	2.9	3.0	9.1	4.9	4.5	4.6	7.2	5.4
2,3′,4,4′,5-penta-CB	1.6	1.9	1.5	1.6	19.5	6.9	33.7	17.6	10.4	17.6
2,2′,4,4′,5,5′-hexa-CB	3.4	4.2	4.2	4.2	9.1	7.4	16.0	12.9	7.4	9.1
2,2′,3,4,4′,5′-hexa-CB	4.4	4.5	5.5	4.5	12.8	8.9	13.7	31.0	9.5	12.8
2,3,3′,4,4′,5-hexa-CB	3.8	5.6	5.3	5.3	9.4	8.5	21.5	13.2	14.4	13.2
2,2′,3,3′,4,4′,5-hepta-CB	4.7	6.0	5.9	5.9	18.4	12.3	−237.5	13.3	443.7	18.4
2,2′,3,4,4′,5,5′-hepta-CB	4.3	6.0	6.0	6.0	16.7	12.2	20.4	10.3	224.6	16.7
Average	3.7	4.7	4.7	4.6	14.3	9.4	21.1 (Except −237.5)	16.4	118.3	14.6

Source: Data from Ref. 65.
[a]Personal code.

TABLE 21.9 Concentrations of PCBs and PCQs in the Tissues and Blood of Yusho Patients

Sample[a]	Tissue/Blood	Year of Sampling	Type of PCBs	Concentration (ppb, whole basis)	
				PCB	PCQ
1	Adipose	1969	A	5091	2400
	Lung		A	226	218
	Adipose	1972	A	6091	1444
	Liver		A	69	144
	Intestine	1975	A	3472	1770
	Liver		A	114	52
	Intestine	1977	A	3630	1125
	Liver		A	68	27
	Intestine	1977	B	1273	25
	Liver		C	18	1
2	Blood ($n = 29$)	1979	A	7.3 ± 4.5	3.04 ± 2.11
	Blood ($n = 15$)		B	5.4 ± 3.6	1.39 ± 1.34
	Blood ($n = 8$)		C	2.7 ± 1.2	0.28 ± 0.19
3	Blood ($n = 56$)	1979		5.6 ± 4.4	2.0 ± 2.0
4	Blood ($n = 11$)	1979	A	6.2 ± 4.9	$0.09 \sim 5.85$
	Blood ($n = 20$)		B/C	2.7 ± 1.4	$< 0.02 \sim 0.42$
5	Blood ($n = 31$)	1979	A	9.6 ± 6.4	2.9 ± 2.3
	Blood ($n = 4$)		B	4.7 ± 5.2	2.0 ± 3.4
	Blood ($n = 29$)		C	2.6 ± 1.1	0.02 ± 0.03
6	Blood ($n = 10$)	1979		5.3 ± 3.4	3.9 ± 2.7
7	Blood ($n = 91$)	1979		$0.6 \sim 18$	$< 0.02 \sim 3.2$
8	Blood ($n = 194$)			5.2	0.50
9	Buccal mucosa ($n = 27$)	1983		279 ± 41	66 ± 13
	Blood ($n = 25$)			7.2 ± 0.82	0.79 ± 0.13
10	Blood ($n = 230$)	1983		5.1 ± 3.9	0.65 ± 0.98
	Blood ($n = 199$)	1984		4.3 ± 3.0	0.57 ± 0.83
11	Adipose ($n = 11$)	1984		1579 ± 657	207 ± 112
	Blood			6.45 ± 2.38	1.39 ± 0.64
12	Adipose ($n = 11$)	1986		1579 ± 627	207 ± 106
	Blood ($n = 32$)			5.36 ± 2.51	1.34 ± 1.11
	Hair ($n = 13$)			28.9 ± 18.1	0.53 ± 0.36
13	Blood ($n = 27$)	1988/1989		6.41 ± 3.17	0.61 ± 0.52
	Hair			25.9 ± 19.3	0.44 ± 0.38
14	Blood ($n = 124$)	1988		5.4 ± 5.0	0.34 ± 0.46
	Blood ($n = 135$)	1989		4.6 ± 3.3	0.54 ± 0.62
	Blood ($n = 150$)	1990		4.5 ± 2.8	0.47 ± 0.52
15	Blood ($n = 22$)	1990		5.4 ± 2.5	0.65 ± 0.55
	Skin surface lipid			581 ± 325	29 ± 12.9
	Blood ($n = 16$)	1991		6.6 ± 2.8	1.31 ± 0.86
	Skin surface lipid			676 ± 309	25.9 ± 11.7
	Blood ($n = 23$)	1992		4.8 ± 2.5	0.57 ± 0.36
	Skin surface lipid			863 ± 463	53.4 ± 23.7

(Continued)

TABLE 21.9 *(Continued)*

Sample[a]	Tissue/Blood	Year of Sampling	Type of PCBs	Concentration (ppb, whole basis)	
				PCB	PCQ
Controls					
1	Adipose ($n = 3$)	1978		248 ~ 1478	1.3 ~ 2.7
	Liver ($n = 3$)			18 ~ 71	0.6 ~ 0.8
2	Blood ($n = 29$)	1979		2.3 ± 1.5	< 0.02
3	Blood ($n = 60$)	1979		2.0 ± 1.3	< 0.02
4	Blood ($n = 18$)	1979		3.3 ± 1.2	< 0.02
5	Blood ($n = 23$)	1979		2.9 ± 1.0	0.02 ± 0.03
6	Blood ($n = 10$)	1979		3.4 ± 1.3	< 0.02
9	Buccal mucosa ($n = 7$)	1983		64.9 ± 16	< 4
	Blood ($n = 7$)			1.86 ± 0.13	< 0.02
11	Adipose ($n = 10$)	1984		410 ± 280	1.74 ± 1.27
	Blood			1.2 ± 0.63	< 0.02
12	Adipose ($n = 40$)	1986		778 ± 670	1.4 ± 0.96
	Blood ($n = 32$)			2.43 ± 1.74	< 0.02
	Hair ($n = 19$)			8.06 ± 5.60	< 0.1
13	Blood ($n = 22$)	1988/1989		2.25 ± 0.92	< 0.02
	Hair			9.41 ± 5.55	< 0.10
15	Blood ($n = 20$)	1990/1991		2.1 ± 0.7	< 0.02
	Skin surface lipid			324 ± 104	< 10

[a] Data for samples 1 and 2 from Ref. 72; 3, Ref. 73; 4, Ref. 74; 5, Ref. 38; 6, Ref. 75; 7, Ref. 76; 8, Ref. 77; 9, Ref. 78; 10, Ref. 79; 11, Ref. 80; 12, Ref. 81; 13, Ref. 82; 14, Ref. 83; 15, Ref. 84.

trations in normal controls. In 1984, 16 years after the onset, PCQ levels in the adipose tissue and blood of patients were still more than 100 times higher than the corresponding levels in controls. In contrast, the PCB levels in Yusho patients were only two to three times higher than those of controls. In the officially certified Yusho patients, as the type of PCBs alters from A (typical Yusho) to B and C (control type), the PCQ concentrations in blood decrease in parallel with the PCB concentrations. Japanese workers who had been occupationally exposed to PCBs did not show any detectable levels of PCQs in blood, although their PCB levels were as high as 33 ppb.[73] Since the PCQ concentrations were reflective of the amount of rice oil intake, blood PCQ levels have been adopted as one of the criteria for diagnosis of Yusho,[41] together with the type of PCBs. As the skin surface lipid showed relatively high levels of PCBs and PCQs, the cutaneous sebaceous system is one of the excretory systems of PCBs and PCQs from the body of patients.[84] Although PCQs were actually ingested by the patients with the rice oil and retained in the body for years, the major toxicities for Yusho were considered to be caused by

PCDFs and not by PCQs, as PCQs have been found to be much less toxic in rats and monkeys.[85]

21.6 CLINICAL FEATURES

Most patients were affected within the 9 months from February 1968, when the contaminated rice oil was shipped to the market from Kanemi company, to October 1968, when the epidemic of Yusho was reported to the public. The subjective symptoms of Yusho as reported by patients are summarized in Table 21.10. Pigmentation of nails, skin, and mucous membranes, distinctive hair follicles, acneiform eruptions, increased eye discharge, increased sweating of the palms, and a feeling of weakness were the most notable symptoms.[12] Yucheng patients in Taiwan who ingested the rice oil contaminated with PCBs, PCDFs, and PCQs in almost the same manner as Yusho[86] showed symptoms very similar to those of Yusho patients.[87]

TABLE 21.10 Percent Distribution of Symptoms of Yusho Patients Examined before October 31, 1968

Symptoms	Males ($n = 89$)	Females ($n = 100$)
Dark brown pigmentation of nails	83.1	75.0
Distinctive hair follicles	64.0	56.0
Increased sweating of palms	50.6	55.0
Acnelike skin eruptions	87.6	82.0
Red plaques on limbs	20.2	16.0
Itching	42.7	52.0
Pigmentation of skin	75.3	72.0
Swelling of limbs	20.2	41.0
Stiffened soles of feet and palms of hands	24.7	29.0
Pigmented mucous membrane	56.2	47.0
Increased eye discharge	88.8	83.0
Hyperemia of conjunctiva	70.8	71.0
Transient visual disturbance	56.2	55.0
Jaundice	11.2	11.0
Swelling of upper eyelids	71.9	74.0
Feeling of weakness	58.4	52.0
Numbness in limbs	32.6	39.0
Fever	16.9	19.0
Hearing difficulties	18.0	19.0
Spasm of limbs	7.9	8.0
Headache	30.3	39.0
Vomiting	23.6	28.0
Diarrhea	19.1	17.0

Source: Data from Ref. 12.

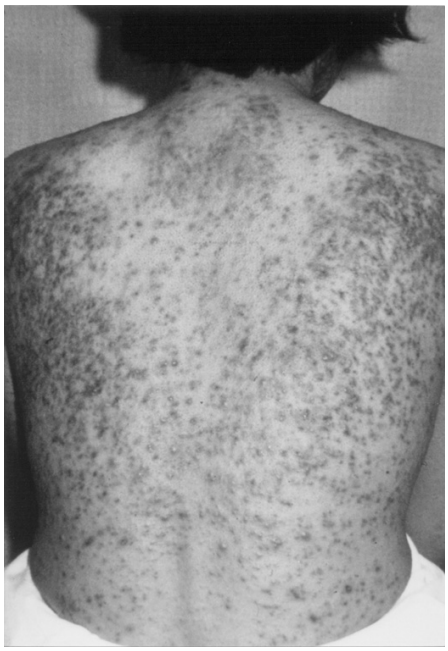

Figure 21.2 Acneiform eruptions on the back of a Yusho patient (female, age 33; photographed December 1986).

21.6.1 Dermal Signs

The most notable symptoms of Yusho are dermal lesions such as follicular keratosis, dry skin, marked enlargement and elevation of the follicular orifice, comedo or blackhead formation, and acneiform eruption (Figure 21.2).[88,89] The acneiform eruptions develop in the face, cheek, jaw, back, axilla, trunk, external genitalia, and elsewhere. Dark-colored pigmentation of the corneal limbus, conjunctivae, gingivae, lips, oral mucosa, and nails is a specific finding of Yusho. Clinical symptoms of 72 patients were examined in relation to the types and concentrations of blood PCBs in 1973 and 1974, about 5 years after the onset. As shown in Table 21.11, dermatological symptoms were observed mostly for the type A group of blood PCBs. The type C group showed few such symptoms. General signs such as fatigue and headache were complaints of patients with all Yusho types of blood PCBs.[90] The skin symptoms have diminished gradually in the 10 years since the onset, while continual subcutaneous cyst formation with secondary infection was still occurring in a relatively small number of the severely affected patients.[91] According to the annual physical examination of 109 Yusho patients in 1986, the proportion of patients with dermal signs decreased to 59% from 72% in 1976.[92]

TABLE 21.11 Types and Concentrations of Blood PCBs and Incidence of Clinical Symptoms among 72 Yusho Patients from April 1973 to March 1974

	Percent of Incidence			
Clinical Symptoms	Type A ($n = 43$)	Type B ($n = 26$)	Type C ($n = 3$)	Total ($n = 72$)
Pigmentation				
Skin	51	0	0	31
Palpebra	72	19	0	50
Gingiva	95	58	67	81
Nail	76	35	0	57
Acneiform eruption	35	0	0	21
Comedo	35	23	0	29
Infection of skin	33	12	0	24
Deformation of nails	56	38	0	53
Alopecia	0	4	0	1
Tooth disorders	19	8	0	14
Meibomian gland hypersecretion	93	81	100	89
Fatigue	49	58	33	51
Fever	2	4	0	3
Phymata in articular region	9	8	0	8
Cough and sputum	65	46	33	57
Digestive disorder	35	46	67	40
Headache	47	35	33	42
Numbness of extremities	52	31	33	33
Menstrual disturbance	(5/17)29	(2/9)22		(7/26)27
Blood PCBs (ppb)	7.2 ± 4.9	4.3 ± 3.1	1.7 ± 0.2	5.9 ± 4.8

Source: Data from Ref. 90.

21.6.2 Ocular Signs

The main ocular signs right after onset were hypersecretion of the meibomian glands and abnormal pigmentation of the conjunctiva. Cystic swelling of the meibomian glands filled with yellow infarctlike contents was observed in the typical cases.[93] These signs have markedly subsided in the 10 years after the onset of Yusho, but 84% of the 75 patients showed somewhat abnormal changes of the tarsal glands, such as irregularity or disappearance of the gland pattern, infarction (tissue death due to loss of blood flow), and lithiasis (stone formation), and 43% showed pigmentation of the eyelids and conjunctivae in 1978. Eye discharge was still a complaint of 64% of the patients in 1978.[94] The contents of the tarsal glands were found to contain PCBs in 1984. Type A PCBs were found in the contents of tarsal glands from some severely ill Yusho patients.[95] The ocular signs of both abnormal pigmentation of conjunctivae and hypersecretion of the meibomian glands were closely related to PCB concentrations and patterns in blood.[96] In an examination of the meibomian

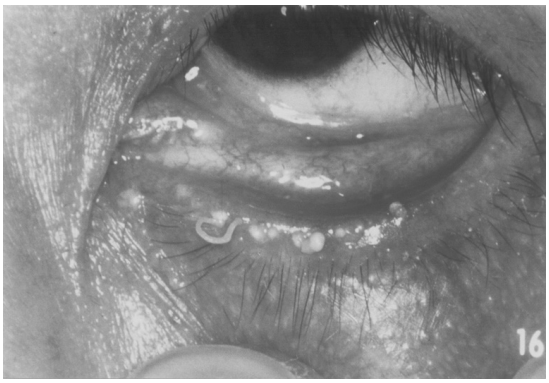

Figure 21.3 Lower eyelid of a 64-year-old Yusho patient. Thirteen years after the onset of Yusho, white cheesy secretions were noted from the ducts of the meibomian glands when the eyelid was manually squeezed. (From Ref. 98.)

glands by transillumination and infrared photography in 1986, more than half of the eyes examined in 52 patients demonstrated loss of the meibomian glands, suggesting meibomian cyst formation, while only 10% of unaffected eyes in controls showed such abnormalities.[97] It is notable that the cheeselike material can be squeezed out from the meibomian glands (Figure 21.3) in some patients for 25 years after onset.

21.6.3 Neurological Signs

Most Yusho patients complained of various neurological symptoms, such as headache, numbness of the limbs, hypoesthesia (reduced sensitivity to stimulation), and neuralgic limbs. However, reduced sensory nerve conduction velocities of radial and/or sural nerves were observed in 9 of 23 cases examined soon after the poisoning, while reduced motor nerve conduction velocities of the ulnar and tibial nerves were seen in only two cases.[99] Electroencephalography (EEG) examination did not reveal any significant changes. It was thought, therefore, that central nervous damage was not clinically prominent or significant in Yusho patients.[100] Frequency of headache was 59.6% in 208 patients in 1973, being comparable to the frequency in controls. But no relationship was observed between blood PCB concentrations and patients with or without headache. Headache was reported by 12 of 41 patients having type A blood PCBs and 19 of 46 patients having type C blood PCBs. Frequency of headache was not related to the type of PCBs, in contrast to the dermal signs.[101]

21.6.4 Respiratory Signs

About 40% of 203 patients complained of persistent cough with expectoration, suffering from chronic bronchitis at an early stage after onset. Second-

ary bacterial infections were often observed in the respiratory system of patients. Immunity was examined in relation to the infection. The serum IgA and IgM levels of the patients were significantly lower than those of controls, while IgG was higher.[102] PCBs were identified in the sputa of patients at concentrations several times lower than those in blood.[103,104] Frequency and severity of the respiratory symptoms correlated well with the PCB concentrations in blood but not well with the type of PCBs.[105] Thereafter, the respiratory distress occurring in these patients improved gradually in the 10 years following onset. However, over the next 5 years, little or no improvement of respiratory symptoms was observed in most cases.[106]

21.6.5 Endocrine Signs

Rapid adrenocorticotropic hormone (ACTH) tests, for detecting a disturbance in adrenocortical functions by examining serum 17-hydroxycorticosteroids after injections of ACTH performed in 86 patients in the second year after onset showed no evidence of severe abnormalities of adrenocortical functions.[107] About 40% of 95 patients exhibited an elevated urinary excretion of 17-ketosteroids and 17-hydroxycorticosteroids. Androsterone, etiocholanolone, and dehydroepiandrosterone tended to increase in male patients and to decrease in females. The elevated excretion of these steroids was possibly related to the poisoning.[108] Irregular menstrual cycles were observed in 58% of 81 female patients in 1970. Frequency of the irregular cycles was not related to the severity of Yusho. Urinary excretion of estrogens, pregnanediol, and pregnantriol tended to be low in Yusho patients.[109] Thyroid function was investigated in 123 patients in 1984, 16 years after onset. The serum triiodothyronine and thyroxine levels were significantly higher than those of normal controls, while thyroid-stimulating hormone levels were normal. There was no correlation between PCB levels and levels of these thyroid hormones.[110] According to the examination of serum thyroid hormone levels of 81 patients with Yusho in 1996, thyroid-stimmulating hormone was elevated in seven cases (8.6%), and all of them showed normal triiodothyronine, thyroxine, and free thyroxin levels.[111]

21.6.6 Liver Signs and Functional Changes

Abnormalities of the liver in gross appearance were rarely observed in Yusho patients. However, an electron microscopic study on the liver biopsy specimens from one male Yusho patient revealed a reduction of the rough-surfaced endoplasmic reticulum and hypertrophy of the smooth-surfaced endoplasmic reticulum,[112] suggesting that drug-metabolizing enzymes were induced in the liver of Yusho patients. The mitochondria showed morphological heterogeneity, and inclusion bodies and giant mitochondria were frequently observed in the hepatic cells.[113] Basic liver functional tests such as glutamate oxaloacetic transaminase and glutamine pyruvic transaminase were within normal levels.

However, accelerated erythrocyte sedimentation rate, high titer in thymol turbidity, increased M fraction of lactate dehydrogenase, and elevated alkaline phosphatase were observed in severe cases, suggesting possible liver damage.[114,115] Serum ribonuclease levels in 101 patients were significantly higher than those in healthy controls in 1974.[116] The increased serum ribonuclease activity decreased gradually with time by 1978.[117] The serum bilirubin concentration, 0.48 mg per 100 mL mean of 121 patients, was lower than the level of healthy adults and correlated inversely with the blood PCB levels and serum triglyceride concentrations.[118] The levels of urinary porphyrin in 16 and 71 Yusho patients were determined to be within the range of control levels except for one patient, who was suspected of porphyria cutanea tarda.[119,120] Marked elevated serum triglyceride was one of the abnormal laboratory findings peculiar to Yusho in its early stages. High serum triglyceride levels ranging from 200 to 600 mg per 100 mL (normal 60 to 107 mg per 100 mL) were observed in 12 of 24 cases, while total serum cholesterol remained unchanged and phospholipids tended to be somewhat lowered, ranging from 94 to 172 mg per 100 mL (normal 156 to 219 mg per 100 mL).[121] A significant positive correlation was observed between serum triglyceride levels and blood PCB concentrations of 42 patients in 1973. The high level of serum triglyceride was also observed in typical patients with type A blood PCBs.[122] For the 10 years following onset, follow-up study on serum triglyceride levels in 24 patients indicated that persistent hyperglyceridemia decreased to the normal range since 1973 for females and 1975 for males.[123] Subsequent chemical analysis of the sera of 110 patients in 1979 revealed that elevated serum triglyceride levels persisted in 26 of the patients.[124] A weak but significant association between blood PCB and serum triglyceride was observed in 259 Yusho patients 20 years after exposure, although their blood PCB and serum triglyceride were relatively close to the normal level.[125] Among 265 Yusho patients examined in 1993, serum levels of triglyceride and total cholesterol were associated with the blood concentration of PCB.[126] Aryl hydrocarbon hydroxylase (AHH) activity in lymphocytes from 42 patients in 1985 was compared with the corresponding activity in 128 healthy nonsmokers. Both 3-methylcholanthrene–induced and noninduced AHH activities in the patients were significantly higher than those of controls, indicating that minute amounts of remaining PCDFs and PCBs are influencing the enzymatic effects in Yusho patients.[127]

21.6.7 Oral and Dental Signs

Pigmentation of the oral mucosa was one of the characteristic signs of Yusho. A high incidence (62.9%) of pigmentation of the gingivae and lips was observed in 70 Yusho patients in 1968 and 1969.[128] This pigmentation persisted for a long time and was still observed in 74 of 99 patients examined in 1982.[129] When the affected gingiva of one patient was surgically removed, the pigmentation recurred within a year.[130] The concentrations and type (A, B, C) of blood PCBs corresponded to the degree of oral pigmentation in 1973.[131]

However, Yusho patients with type C blood PCBs also showed a somewhat high incidence of oral pigmentation.[130] Radiographic examination of the mouth of Yusho patients demonstrated anomalies in the number of teeth and in the shape of the roots and the condition of the marginal bone resorption. In the examination of 118 patients in 1984, 24.5% of the teeth examined had periodental pockets on the mesial surface. This frequency was much higher than that (16%) in teeth of 54 control persons in Fukuoka.[132] These changes were considered to have been caused by the remaining PCBs and PCDFs, which had been influencing endocrine factors in the body.

21.6.8 Babies Born to Patients and Infants

Eleven women who were officially certified Yusho patients delivered nine living and two stillborn babies in Fukuoka prefecture in 1968. Ten of them had shown the characteristic grayish dark brown skin at birth. Most of the babies also had dark-colored gingivae and nails and increased eye discharge.[133–135] The majority were small-for-date and their postnatal growth curves for body weight were similar in shape to the national standard curves, but evidently lower for some of the babies. The skin pigmentation faded 2 or 3 months after birth, but the increased eye discharge and nail pigmentation remained for more than a year in some of the babies. Even babies born to Yusho mothers 1 to 3 years after onset showed some pigmentation in the skin and gingivae at birth.[136] One Yusho mother gave birth to three babies with dark brown skin, the pigmentation being most serious in the first baby and diminishing steadily in the second and third babies. Two of seven babies fed the milk of Yusho mothers were diagnosed as Yusho; they are termed *trans-milk Yusho babies*, as they had been born before the mothers ingested the contaminated rice oil. The gains in height and body weight of 42 schoolchildren with Yusho were compared with those of controls before and after the poisoning. Both height and body weight gains of the boys with Yusho significantly decreased after the poisoning. The same tendencies were also observed in some of the girls with Yusho.[137] These temporal growth suppressions were reversed and thereafter tended to be close to the average values for the controls.[138] Taiwan Yucheng children prenatally exposed to PCBs and PCDFs had poorer cognitive development at age of 4 to 7 years.[139] The body height and penis length of Yucheng children were lower than those of controls at age of 11 to 14 years.[140] These changes might be caused by the estrogenic or antiestrogenic effects of the PCBs/PCDFs in Yucheng children, which were 10- to 30-fold higher than in the controls.[141]

21.7 DEATHS AMONG YUSHO PATIENTS

Total number of patients officially registered as suffering from Yusho by March 1990 was 1870. Ikeda and Yoshimura[142] compared the number of deaths

TABLE 21.12 Observed and Expected Number of Deaths and Standardized Mortality Ratio (O/E) by Cause of Death

	Males ($n = 887$)			Females ($n = 874$)		
Cause of death[a]	Observed	Expected	O/E	Observed	Expected	O/E
Total	127	107.29	1.18	73	81.52	0.90
Tuberculosis	1	1.54	0.65	0	0.58	0.00
Malignant neoplasms	45	29.03	1.55[b]	13	19.18	0.68
Esophagus	2	1.40	1.43	1	0.30	3.29
Stomach	10	8.97	1.12	1	5.12	0.20
Rectum, sigmoid colon, and anus	2	1.20	1.67	0	0.82	0.00
Liver	12	3.58	3.36[b]	3	1.33	2.26
Pancreas	2	1.47	1.36	1	1.01	0.99
Lung, trachea, and bronchus	9	4.96	1.81	0	1.69	0.00
Breast				1	1.30	0.77
Uterus				2	1.53	1.31
Leukemia	2	0.78	2.57	0	0.56	0.00
Diabetes	1	1.22	0.82	0	1.18	0.00
Heart disease	20	17.44	1.15	16	14.51	1.10
Hypertensive disease	1	1.57	0.64	1	1.91	0.52
Cerebrovascular disease	14	20.50	0.68	7	17.82	0.39[b]
Pneumonia and bronchitis	6	6.57	0.91	1	4.60	0.22
Gastric and duodenal ulcer	0	0.93	0.00	1	0.50	2.02
Chronic liver disease and cirrhosis	6	3.61	1.66	3	1.30	2.31
Nephritis, nephrose syndrome and nephrose	1	1.58	0.63	3	1.45	2.07
Accidents	10	6.86	1.07	2	1.32	0.94

Source: Data from Ref. 142.

[a] Unknown cause of death: male, 9; female, 12.

[b] $p < 0.01$.

among 1815 patients registered as Yusho by the end of March 1990 with the expected number of deaths calculated on the basis of the national death rates. The results are shown in Table 21.12. A significant excess mortality was recognized for malignant neoplasms at all sites and cancer of the liver in males. However, excess mortality for liver cancer was not significant in females. The excess deaths from liver cancer were seen mainly in Fukuoka prefecture, while no such excess was seen in Nagasaki prefecture, where 550 patients were registered. Although these findings suggest that the poisoning might have caused

liver cancer in males in Fukuoka, it is still too early to draw any conclusions from this cancer mortality study. In Table 21.12, excess mortality tendency was observed for cancer of the lung, trachea, and bronchus in males, and in a related study of 5172 workers exposed to TCDD, excess mortality from all cancers combined, cancer of the respiratory tract, and soft tissue sarcoma appear to be related to TCDD exposure.[143] Excess mortality from cancer of the respiratory tract was found both in studies of TCDD and in the Yusho study involving the closely related PCDFs and PCBs.

21.8 RISK ASSESSMENT OF PCDDs, PCDFs, AND PCBs IN HUMANS

As shown in Table 21.3, the patients began to show manifestations of Yusho when their body burden of PCDFs and PCBs reached 456 μg of TEQ on average with a range of 110 to 1740 μg. The latency period for these symptoms was 71 days on average, with a range of 20 to 190 days, and average daily intake during this period was estimated to be 154 ng/kg of TEQ. One patient showed some manifestations of Yusho when his body burden reached 210 μg of TEQ during the 135-day latency period. Daily intake during the latency period was calculated to be 28 ng/kg of TEQ, which was the smallest daily intake among the Yusho patients. Ryan et al.[144] calculated the body burden of TEQ associated with nausea and anorexia to be 2.2 μg/kg and that associated with chloracne to be 3 μg/kg, using the data for rice oil ingestion by Yusho patients. These values are equivalent to 132 and 180 μg for an adult patient and quite close to the smallest body burden of 210 μg TEQ for a patient.

The general population ingests small amounts of PCDDs, PCDFs, and PCBs from contaminated meat, fish, and dairy products; daily intakes were calculated to be 1 to 3 pg/kg of TEQ in Canada,[145] Germany,[146] Japan,[147] the Netherlands,[148] the United States,[149] and other countries (levels are shown in Figure 21.4). PCDDs, PCDFs, and PCBs accumulate in the body, and their equilibrated concentrations were estimated to be 10 to 50 ppt (fat basis) of TEQ in human tissues.[150] Breast milk was contaminated with PCDDs, PCDFs, and PCBs at about the same level of TEQ as for human tissues.[151] Babies feeding breast milk were estimated to ingest 20 to 160 pg/kg per day of TEQ for several months, assuming that the baby consumes about 150 ml/kg body weight of breast milk which contains about 3% fatty materials.

Figure 21.4 illustrates[152,153] tolerable daily intake (TDI) issued from various countries, Yusho intake of TEQ, and ingestion of TEQ from foods and breast milk. When the daily intakes of TEQ by Yusho patients (28 and 154 ng/kg) are compared with those of the general population (1 to 3 pg/kg), there is a difference of more than four orders of magnitude. However, as the periods of ingestion differ greatly for the two groups, 71 and 135 days for Yusho patients and lifelong for the general population, the toxicity levels of PCDDs, PCDFs, and PCBs remaining in Yusho patients were presently only about 20 times

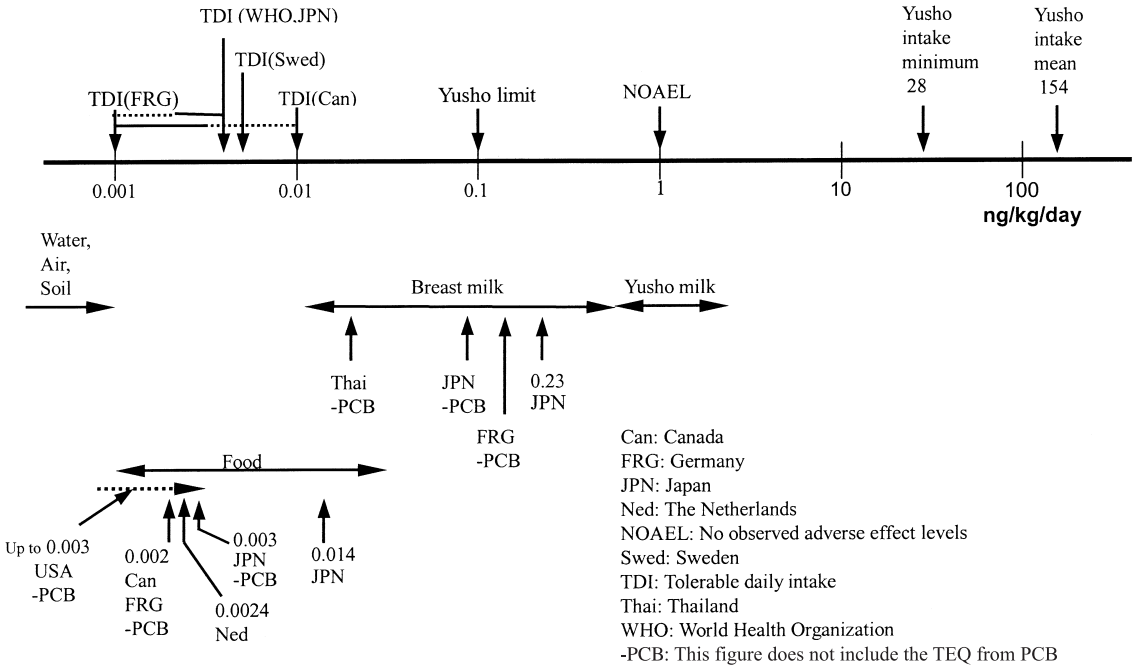

Figure 21.4 Yusho intakes, regulations, and personal intakes of TEQ.

higher than those of controls, about 600 ppt of TEQ in adipose tissue of Yusho patients versus 20 to 50 ppt for the general population. When the intake of these chemicals by nursing babies is compared in the two populations, the intake of TEQ by breast-fed babies of the general population, a maximum of 160 pg/kg per day, is only two orders of magnitude lower than that of Yusho patients, a minimum of 28 ng/kg per day. Moreover, feeding periods for the toxic chemicals are very similar in the two groups, several months for babies of the general population and from 1 to 5 months for Yusho patients. Two babies were actually certified as Yusho patients after ingesting PCDFs and PCBs through breast-feeding from mothers with Yusho for 4 months in Nagasaki prefecture.[136] The Yusho research group at Kyushu University advised a Yusho mother not to breast-feed her baby in 1990, as her breast milk contained 2 ng/kg per day of TEQ. In the general population, Pluim et al.[154] found that exposure to high levels of PCDDs and PCDFs, both intrauterine and via breast milk, modulates the hypothalamic–pituitary–thyroid regulatory system in human newborns. Jacobson and Jacobson[155] examined 212 children born to women who had eaten Lake Michigan fish contaminated with PCBs and found that highly exposed children were likely to have low intelligence quotient scores.

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CHAPTER 22

The Yucheng Rice Oil Poisoning Incident

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22.1 INTRODUCTION

Eleven years after the Japanese Yusho incident (see Chapter 21), a similar tragedy happened in Taiwan in 1979. A Japanese-produced polychlorinated biphenyl (PCB) mixture (Kanechlor-400 and 500) was used as the heat transfer medium in the process of deodorization and decolorization of rice oil by a rice oil company in central Taiwan. PCBs and their heat-degraded by-products, polychlorinated dibenzofurans (PCDFs) and ter- and quaterphenyls (PCTs and PCQs), leaked into the rice oil and poisoned 2000 people who had consumed the oil. The initial clinical symptoms consisted of acne, pigmentation of the nails and skin, and hypersecretion of the Meibomian glands. Because the disease was caused by ingestion of rice oil, the syndrome was then referred to as *Yucheng* (pronounced "Yo-Jun"), which translates to *oil disease*, and the exposed subjects were referred to as the *Yucheng cohort*. In this chapter we describe the discovery and epidemiologic findings of the outbreak, the chemical and toxicological data, and the clinical findings of the original Yucheng cohort and their children exposed to heat-degraded PCBs prenatally and by lactation and include updated data not available when the experience of this cohort was last reviewed.[1]

22.2 DISCOVERY AND EPIDEMIOLOGIC FINDING

The outbreak and the discovery of the cause of Yucheng have been reviewed and published by Hsu et al.[2–4] On May 21, 1979, a local health bureau in Tai-

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chung County, central Taiwan, was notified of an outbreak of skin disease among staff and students boarded at the Hwei-Ming School for Blind Children. The initial clinical symptoms consisted of acne, pigmentation of the nails and skin, and hypersecretion of sebaceous glands. Routine microbiological and toxicologic tests were done on various foodstuffs and water but failed to yield any helpful result. When similar cases involving 85 of 150 workers from a nearby plastic shoe factory were reported in early September 1979, an epidemiologic investigation was conducted and a common exposure, a rice bran cooking oil (C-rice bran oil) manufactured by a rice oil company in Changhua and purchased from an edible-oil store in Taichung, was identified. More cases from other working groups and local households in both Changhua and Taichung Counties were reported, and all were proven to have consumed the C-rice bran oil.

Local clinicians did not recognize the acne shown on the victims' faces and bodies as chloracne, a persistent form of acne characterized by hyperplasticity, hyperkeratinization, and alterations in pigmentation, so analyses directed specifically toward acnegenic halogenated hydrocarbons were not done at first. After the cause of the outbreak, consumption of C-rice bran oil was identified, and since both the etiology and the symptoms resembled the Japanese Yusho incident (see Chapter 21), the Taiwan Department of Health consulted Japanese scientists who had been involved in the 1968 Yusho investigation. Samples of C-rice bran oil from the Hwei-Ming School and the oil store in Taichung and blood from victims were analyzed, and PCBs resembling Kanechlor-400 and 500 were detected on October 6, 1979.

The etiology of the outbreak was announced by the government on October 8, 1979, and distribution of all C-rice bran oil was then prohibited. Local residents who had consumed the oil and physicians who had treated the victims started reporting cases to local health bureaus. As of February 1983, 2061 Yucheng subjects (including 39 children born to Yucheng women) were reported and included in the Yucheng registry that was maintained by the Taiwan Provincial Health Department. Figure 22.1 shows the distribution of

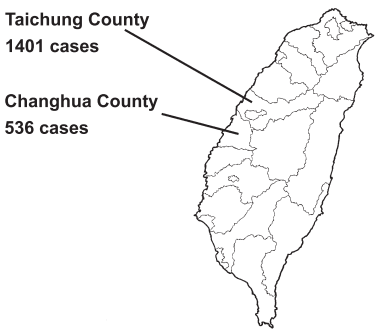

Figure 22.1 Geographic distribution of PCB-poisoned patients.

Yucheng individuals in central Taiwan. Sixty-eight percent of the victims were from Taichung and 26% from Changhua County. Victims from other counties were mainly from two sources: monks and nuns in Shih-Tou Mountain temples who had purchased the contaminated oil in Taichung and students from Miaoli County who had come to Taichung for training courses. There were similar numbers of female and male Yucheng subjects, and more than 50% of the victims were less than 25 years of age. In Taichung, 60% of the victims were factory workers and 15% were students; in Changhua, the majority of the victims were students (39%), and 19, 16, and 14% were factory workers, housewives, and farmers, respectively. Generally speaking, the Yucheng cohort is a young cohort of low socioeconomic status.

22.3 ANALYTIC CHEMISTRY, EXPOSURE, AND LEVELS

22.3.1 Oil Contamination

The only oil samples that were positive for PCBs/PCDFs were those from the Taichung oil store, the School for Blind Children, and victims' homes. No PCBs/PCDFs were detected in oil samples from Changhua oil stores and the oil company; perhaps the owners removed all contaminated oil after the etiology of the illness had been released. Even though no machine containing PCBs was found at the oil company, the high PCB levels from both soil samples at the site and blood samples from plant workers suggested that PCBs had been used in the plant recently. In addition, the detection of PCQs and PCDFs in the toxic oil further suggested that PCBs had been subjected to heating at high temperature. It was suggested that pipes filled with PCBs, presumably Japanese-manufactured Kanechlor-400 and 500, were used as the heat transfer medium in the process of deodorization and decolorization of the C-rice bran oil. If the pipes developed leaks, the PCBs would then mix with the oil. Since PCB mixtures at room temperature are clear, colorless, and tasteless, a large amount of mixing could go unnoticed.

The chemicals from eight oil samples were measured with gas chromatography/mass spectrometry (GC/MS) by Chen et al.,[2,5–8] Kashimoto et al.,[9] and Masuda et al.[10] Seven samples contained about 53 to 100 ppm total PCBs, one-tenth of the level in the Japanese Yusho incident, while one sample contained 405 ppm. The PCB peaks present in the oil were those with higher number of chlorine atoms and with relatively longer retention times; the percentages of tetra-, penta-, hexa-, and heptachlorobiphenyls were 33.8, 47.1, 12.4, and 4.5, respectively, a pattern closely resembling Kanechlor-400 and 500; and the most prominant congeners were 2,4,5,3′,4′- (12.4%) and 2,3,4,3′,4′-pentaCBs (11.5%).[2,11] There were about 0.01 to 1.68 ppm total PCDFs in the oil samples, with a constant PCDF/PCB ratio of about 0.1 to 0.3%. Thus, it appears that the PCBs were contaminated with a relatively constant amount of PCDFs, but the amount of PCBs/PCDFs that got into the rice oil varied.

There were PCDFs with four, five, and six chlorines in the oil, and 2,3,4,8-tetra- and 2,3,4,7,8-penta-CDFs were the major congeners.[2,11] The PCQs/PCBs ratio varies in the reports on the Taiwan oil from about 39%[9] to 180%.[10] This may represent analytic differences rather than real ones, since the PCQs are quantitated by a prechlorination procedure rather than by summing the values of individual isomers.

22.3.2 Exposure

Ninety-eight patients were interviewed by Lan et al.[12] on food and oil consumption patterns prior to and during the outbreak. It was estimated that patients consumed the contaminated oil on average at a rate of 1.4 kg/month (range from 1.0 to 1.6 kg/month) for two to three (average = 2.7) months before they became symptomatic, and then for another 6 months before the oil was withdrawn. The PCB and PCDF concentrations in the oil samples were about 67 to 99 ppm and 0.21 to 0.40 ppm, respectively. Thus, the patients consumed on average 302 mg (range = 196 to 457) of PCBs and 1.3 mg (range = 0.5 to 1.9) of PCDFs before they developed symptoms, and about 1 g (range = 0.7 to 1.4) of PCBs and 3.8 mg (range = 1.8 to 5.6) of PCDFs total. Because the rice oil in Taiwan was withdrawn at a later time, the Taiwanese patients consumed about 10 times as much contaminated oil as the Japanese patients; however, since the PCB/PCDF concentration in the Japanese oil was 10 times that of the Taiwanese oil, patients from both countries consumed about the same amount of PCBs and PCDFs.

22.3.3 Levels

Blood Levels Total blood PCB/PCDF levels have been measured by several research groups at different times. Kashimoto et al.[9] and Masuda et al.[10] analyzed blood samples from two small groups of patients within the first year of exposure. Chen et al.[2,7,8] analyzed 165 blood samples from Yucheng patients attending a dermatology clinic within 18 months of exposure. Between 1979 and 1983, the Taiwan Provincial Department of Health collected 2378 serial blood samples from 1246 Yucheng subjects, with 1 to 8 samples per subject. Except for 11 persons in the Department of Health collection, all persons tested in these studies had detectable PCB levels, and the mean levels ranged from 38 to 99 ppb (Table 22.1). These levels were higher than those of 92 Taiwanese blood donors, which had a mean PCB level of 9.8 ppb (range = 0 to 25.3 ppb).[13] Serum PCDFs and PCQ levels were measured by Kashimoto et al.[9] and Chen et al.[2,11] (Table 22.1). Chen et al. also identified 2,3,4,7,8-penta- and 1,2,3,4,7,8-hexa-CDFs as the major detectable congeners in 10 samples. Lundgren et al.[16] identified the same congeners in 12 samples collected from different subjects in 1985, but at a lower concentration, suggesting some metabolism or excretion over time (Table 22.1). The PCB levels in the Yucheng subjects were found to be higher than those of the Japanese Yusho subjects (mean

PCB value of 72 Yusho patients was 5.9 ppb),[14] probably because the Japanese samples were drawn much longer after the exposure incident. Chen et al.[15] studied the comparative elimination rates of individual PCB congeners from the blood of patients. The results indicate that the concentrations of those congeners with adjacent unsubstituted carbons at the meta and para positions, such as 2,5,3′,4′-tetra-, 2,3,2′,4′,5′-, 2,5,2′,3′,4′-, and 2,3,6,3′,4′-penta-CBs, declined faster than those of other congeners. Mean total serum PCB concentration of 32 Yucheng samples drawn in 1985 was found to have declined to about 15 ppb (range = 0.6 to 86.8), and the major persistent congeners detected then were the hexachlorinated biphenyls, such as the 2,2′,3,4,4′,5- and the 2,2′,4,4′,5,5′-[16] (Table 22.1). In 1985, Rogan and colleagues collected blood samples from 21 children born to Yucheng women during or after the outbreak and from 15 age-, gender-, and neighborhood-matched control children. Fourteen of the 21 exposed children and 6 of the 15 controls had detectable PCBs in blood. The mean PCB levels were 8.45 ppb (range = 0.12 to 77.8 ppb) in the in utero exposed children and 3.49 ppb (range = 1.06 to 6.99 ppb) in the controls.[17] In February 1991, 13 years after the Yucheng incident, blood levels were determined in 45 in utero exposed children and their age-, gender-, neighborhood-, maternal age–, and socioeconomic status–matched controls; age ranged from 6.5 to 12.5 years.[18] Twenty (44%) of the 45 exposed children had detectable PCBs (sum of 16 congeners) in the blood, with a mean of the positive 7.6 ng/kg whole base (range = 0.9 to 36). Twenty-two (49%) had detectable 2,3,4,7,8-pentachlorodibenzofuran, with a mean of the positive 300 ng/kg lipid (range = 89 to 1230). Twenty-four (53%) had detectable 1,2,3,4,7,8-hexachlorodibenzofuran, with a mean of the positive 620 ng/kg lipid (range = 120 to 3040). The corresponding levels in the pooled control sample of the chemicals cited above were 0.56 ng/kg whole base for PCBs, 19 ng/kg lipid for 2,3,4,7,8-penta-CDF, and 25 ng/kg lipid for 1,2,3,4,7,8-hexa-CDF, respectively. The serum levels of the dibenzofurans in Yucheng children were highly correlated with duration of breast-feeding, indicating that the main source of these chemicals in exposed child was lactation. In 1992, 14 years after the incident, blood levels were determined in 56 Yucheng women and their age-, gender-, neighborhood-matched controls.[19] Serum samples from all subjects had detectable PCBs, 2,3,4,7,8-pentachlorodibenzofuran, and 1,2,3,4,7,8-hexachlorodibenzofuran. Mean serum levels of PCBs were 2820 ± 295 ng/g lipid base (range = 602 to 13,420), or $11{,}590 \pm 1500$ ng/kg whole base (range = 2220 to 67,800). The corresponding levels in the pooled control sample were 386 ng/g lipid, or 1670 ng/kg whole base. Mean serum levels of 2,3,4,7,8-penta- and 1,2,3,4,7,8-hexachlorodibenzofurans were 1090 ± 63 ng/kg lipid (range = 390 to 2300) and 2560 ± 167 ng/kg lipid (range = 870 to 6920). The corresponding levels in the pooled control sample of the chemicals cited above were 28 and 20 ng/kg lipid, respectively. The serum levels of these chemicals in Yucheng women were correlated negatively with duration of their breast-feeding their children, indicating that breast-feeding was an effective way of eliminating these toxins from the body.

TABLE 22.1 Concentrations of PCBs and Heat-Degraded By-Products in Blood of Yucheng Subjects

Study	Year Blood Samples Drawn	*n*	PCBs	PCDFs	PCQs	PCDF Congeners					
						2,3,7,8 Tetra	1,2,3,7,8 Penta	2,3,4,7,8 Penta	2,3,4,6,7 Penta	1,2,3,4,7,8 Hexa	1,2,3,4,6,7,8 Hepta
Kashimoto et al. (1981)[9]	1979–1980	15 Directly exposed	Mean, 60 ± 39 ppb Median, 54 Range, 4–188	Mean, 0.14 ± 0.07 ppb Median, 0.09 Range, < 0.005–0.27	Mean, 19.3 ± 13.0 ppb Median, 16.9 Range, 0.9–63.8						
Masuda et al. (1982)[10]	1979–1980	23 Directly exposed	Mean, 99 ± 163 ppb								
Chen et al. (1984),[8] Chen and Hsu (1987)[2]	1980–1981	165 Directly exposed	Mean, 38 ppb Median, 28 Range, 10–720	Range, 0.02–0.2 ppb ($n = 10$)			Trace	Detectable		Detectable	Trace
Yucheng Registry	1979–1983	2378[a] Directly exposed	Mean, 53.5 ppb Median, 60 Range, 0–853								
Lundgren et al. (1988)[16]	1985	32	Mean, 15.4 ± 19.0 ppb Range, 0.6–86.8			< 0.3 ppt	< 0.3 ppt	2.7 ± 1.8 ppt (0.4–5.5) ($n = 12$)	< 0.3 ppt	10.8 ± 4.9 ppt (0.7–18.6)	

Yu et al. (1991)[17]	1985	21 Children of Yucheng mothers	Mean, 8.45 ppb Range, 0.12–77.8				
Ryan et al. (1994)[18]	1991	45 Children of Yucheng mothers	44% of children had detectable serum levels	More than 50% of children had detectable serum levels		49% of children had detectable serum levels	Median, 143 ng/kg lipid
Guo et al. (1997)[19]	1992	56 Directly exposed women	Mean, 2820 ± 295 ng/g lipid Median, 2250 Range, 602–13,420	All subjects had detectable levels		Mean, 1090 ± 63 ng/kg lipid Median, 1030 Range, 390–2300	Mean 2560 ± 167 ng/kg lipid Median, 2220 Range, 870–6920

[a] 2378 serial measurements of 1246 subjects, 1–8 samples per subject.

Tissue Levels One patient had his blood and adipose tissue sampled 10 months after the incident and the total PCB level in his blood and adipose tissue were 39 ppb and 12.8 ppm (wet weight basis).[8] Three autopsy studies have been reported, two of adults and one of a 22-month-old child.[2,4,8,20,21] In general, highest PCB levels were in fat or fatty tissues, and there was some evidence for elevated PCDF concentration in liver. The concentrations of total PCBs in intestinal fat and total PCDFs in the liver of a patient who died 2 years after the outbreak were 10.8 ppm and 35.1 ppb (wet weight basis), respectively.[2,20] The major PCDFs retained in the liver and other tissues were 1,2,3,4,7,8-hexa-, 2,3,4,7,8-penta-, and 1,2,4,7,8-penta-CDFs. In the adult reported by Hsu et al.,[4] the PCB concentrations in bladder, colon, blood vessels, and heart, were 10,208, 5889, 5320, and 3317 ppm (wet weight basis), but an adipose sample was not mentioned separately. The PCB concentrations in blood, liver, and adipose tissue of the 22-month-old child, who was exposed in utero, were 4, 17, and 814 ppb, respectively.[21] These levels were relatively low, but it is not clear that the analytical methods were comparable.

Schecter et al.[22] measured dioxin and dibenzofuran levels in six placentas of Yucheng women obtained in 1984 and 1985 and one general population placenta from an American woman. The Yucheng placentas were found to contain elevated levels of two congeners; 2,3,4,7,8-penta-CDF ranged from 820 to 12,560 ppt lipid compared to 6.8 ppt lipid in the nonexposed placenta; and 1,2,3,4,7,8-hexa-CDF ranged from 2345 to 26,540 ppt lipid compared to 8.7 ppt lipid in the nonexposed placenta.

22.4 CLINICAL FINDINGS OF THE YUCHENG COHORT

22.4.1 General Symptoms

Subjective complaints of the Yucheng subjects varied as the course of the illness changed. Ocular symptoms such as increased eye discharge, swelling of eyelids, and disturbance of vision were the major complaints at early stages, and as time went by, constitutional symptoms such as general malaise, numbness of limbs, pruritus (''itchiness''), and headache and dizziness became more obvious and serious.[23–25] Ten percent of the female victims also were found to have abnormal menstruation. Lan and Yen[26] reported decreased growth in both height and weight of 30 elementary school students in Changhua who had ingested the contaminated oil.

In 1993, the prevalence of medical conditions in the Yucheng people 30 years of age or older and a neighborhood control group matched for age, gender, and neighborhood in 1979 was studied and compared.[27] Lifetime prevalence of chloracne, abnormal nails, hyperkeratosis, skin allergy, goiter, headache, gum pigmentation, and broken teeth are more frequent in the exposed men and women. The exposed women reported anemia 2.3 times more frequently than did controls. The exposed men reported arthritis 4.1 times and herniated intervertebral disk 2.9 times more frequently than did their controls.

22.4.2 Dermatology

Skin symptoms of the Yucheng subjects have been studied in detail by two groups of dermatologists (Table 22.2). Both groups reported that mucocutaneous pigmentation was the most common symptom, occurring in at least 90% of the patients; it usually occurred at conjunctiva (lining of eye), gingiva (gum) and buccal mucosa (cheek), nasal apex and ala, and finger and toenails, and the hue varied from brown to brownish gray to gray.[23–25,28,29] The next most common symptom was acneform eruptions, predominantly open comedones (blackheads), papules, and pustules. It differs from acne vulgaris in that it locates not only on the classical sites for acne but also on extremities, axilla (armpit), and external genital areas. Deformity of fingernails and toenails were found in more than 68% of Wong et al.'s patients and in 38% of Lü's patients. Lü et al. also found follicular accentuation and horny plugs (accentuated and elevated hair follicles with blackish keratinous material plugs in the enlarged orifice) to be prominent (at least 21%) in their patients, especially in the age group 11 to 20. Other findings included keratotic plaques, horny growth such as warts or callosity, of palms and soles, dry skin, and itching. When grouping the patients according to the Japanese Goto and Higuchi grading system (ocular signs alone for grade 0, pigmentation of nails and skin for grade I, comedo formation and follicular accentuation for grade II, localized acneform lesions and cysts for grade III, and widespread and extensive distribution of the foregoing lesions for grade IV[30]), 79.5% of Wong's patients and 61.6% of Lü's patients were in grades 0, I, or II.[24,25,28,29]

Both Lü et al. and Wong et al. tried to relate the total blood PCB level to the severity of skin symptoms, but neither group showed any consistent association. The lack of associations plus the fact that neither report had any control or background rates for the skin lesions made the interpretation difficult. Nevertheless, the very high rates of mucocutaneous pigmentation, the unique distribution pattern of acneform eruptions, and the unusual picture of hyperkeratotic plaques make a strong case that these symptoms are related to the ingestion of PCB- and PCDF-contaminated rice bran oil. Histologic examination of 21 skin biopsies showed hyperkeratosis, increased pigmentation of epidermis, and cystic dilatation of hair follicles.[31] Except for the highly pigmented epidermis, the eruptions were indistinguishable from acne vulgaris. Fu studied the ocular manifestations of 117 patients and found a positive correlation between total blood PCB level and severity of conjunctival pigmentation.[32,33]

22.4.3 Neurology

Two groups of exposed subjects have been evaluated for neurological dysfunction (Table 22.2). One hundred and fifty-five Yucheng subjects from the Hwei-Ming School for Blind Children, including students, staff, and their families, were seen by neurologists at National Taiwan University Hospital in early 1980[34–36]; all of them had neurologic examinations and nerve conduction velocity (NCV) tests. The most common neurologic complaints were

TABLE 22.2 Selective Physical and Laboratory Findings among Directly Exposed Yucheng Cohort Subjects (%)

Findings	Wong et al. (1982)[29] $n = 122$	Lü et al. (1984, 1985)[24,25] $n = 358$	Chang et al. (1980)[41] $n = 143$	Chen et al. (1981, 1983, 1985)[34–36] $n = 155$	Chia et al. (1981, 1984)[38,40] $n = 39$	Lu et al. (1980)[50] $n = 69$
Hyperpigmentation	92	90				
Acne	75	51				
Nail dysplasia	68	38				
Enlarged meibomian glands		17				
Peripheral sensory neuropathy[a]				36	64	
Headache and/or dizziness				35	38	
Slowed nerve conduction				52	46 ($n = 35$)	
Abnormal EEG					22 ($n = 27$)	
		$n = 133$				
Hematocrit		↓	↓			
SGOT		↑	↑			
SGPT		↑	↑			
Triglycerides		↑	↑			
Bilirubin			↓			
White blood cells		↑	↑			
T-cells			↓			
Active T-cells			↓			
Helper T-cells			↓			
Suppressor T-cells			↑			
γ-Globulin			↓			
α_2-Globulin			↑			
Increased urinary porphyrins						↑Mean
Uro/coproporphyrin > 1						52

[a]Paresthesia, numbness in the distal part of the extremities.

numbness and paresthesia of extremities (36.1%) and headache and/or dizziness (34.8%). Neurologic examination showed that 7.7% of the patients had decreased vibration sensation in lower limbs, and 5.8% had hearing impairment. A few patients had hyperalgesia, absent or decreased ankle jerks. On NCV testing, 28.4% had sensory nerve slowing, 7.8% had motor nerve slowing, and an additional 16.1% had both deficits. However, the prevalence rates of the above-mentioned symptoms/signs in general population were not reported. When compared with values of 63 to 150 nonexposed normal subjects, the exposed subjects were on average 4 m/s slower than the controls in both sensory and motor NCV. Patients with blood PCB levels of 24 ppb or greater had significantly slower peroneal nerve motor NCV than those with blood PCB levels below 24 ppb. For 65 patients with known blood PCQ levels, the blood PCQ level was negatively associated with median nerve sensory NCV. Chen et al.[34] suggested that these deficits might be due to a PCB-induced porphyria (abnormal porphyrin metabolism), and they did porphyrin analyses on 24-h urine samples from 48 patients. Although a higher percentage of patients with abnormal NCV had abnormal urinary porphyrins (63% vs. 50%), the difference was not statistically significant. Ogawa[37] found motor paresis of the hind limbs and reduced motor NCV on experimentally induced PCB-poisoned rats, and histologic examination revealed segmental demyelination (loss of a nerve's myelin covering) with loss of large nerve fibers in peripheral nerves; this may explain how PCB exposure affects NCV.

In 1980, 39 patients admitted to the Veterans General Hospital for treatment of skin lesions were examined by Chia and colleagues for neurological function.[38–40] Thirty-five of them (with 44 age-matched controls) had NCV tests, 27 had electroencephalography (EEG), and four had cerebrospinal fluid (CSF) samples drawn. Paresthesia and numbness of extremities (64%), pain over the back, limbs or orbits (41%), and headache (38%) were the most common neurological symptoms. Thirty-one percent of the patients had slower sensory nerve conduction, and 29% had slower motor nerve conduction. The patients had both sensory and motor NCV 5 to 6 m/s slower than that of 44 nonexposed healthy subjects. Twenty-two percent of the 27 patients receiving EEG had mild abnormal EEG pattern of paroxysmal bilateral slow waves, occasionally mixed with spikes or sharp waves in the frontotemporal region, a pattern not specific to any defect. PCB levels in CSF were normal for all four patients. Twenty-eight of the 39 patients were reexamined 2 years later, and it was found that all symptoms had diminished except for dizziness and absent and sluggish deep tendon reflex. Although all NCV had improved, the values were still 4 to 10% slower than that of the controls.

22.4.4 Immunology

There is a strong impression among physicians who care for the Yucheng victims that they suffer more frequent and more severe skin and respiratory infections. There is also a general belief among many of the victims that they have lowered resistance to infectious diseases. In 1979, Chang et al.[41] studied

immune function on 143 patients from the Hwei-Ming School. When compared to the normal Taiwan laboratory values, total leukocyte (white cell) count of the patients was elevated (9650 ± 2800 vs. $7053\ mm^{-3} \pm 1205$) but with a normal differential, α_2-globulin was slightly increased, and γ-globulin was slightly decreased (Table 22.2). A delayed-type skin hypersensitivity test using streptokinase/streptodornase solution was positive in 36% of patients compared to 79% of Taiwan's general population; this result suggests suppression of cellular immunity in the Yucheng patients.

Serum immunoglobulin tests on a subset of 30 patients who had blood PCB levels above 15 ppb and on 23 age- and gender-matched controls showed significant decreases in IgA and IgM in the exposed patients (185 ± 88 vs. 245 ± 70 for IgA, and 105 ± 58 vs. $173\ mg\% \pm 48$ for IgM), suggesting suppression of humoral immunity.[42] The 30 patients had two-thirds the percentage of T cells (42% vs. 63%) of controls, and the percentages of active T cells (11% vs. 22%) and helper T cells (22% vs. 37%) were also decreased, while the percentages of B cells and suppressor T cells were not affected. This suggests that different types of lymphocytes may have different sensitivity toward PCB toxicity. Since helper T cells help the proliferation, differentiation, and immunoglobulin secretion of B cells, when the percentage of helper T cells decreases, serum immunoglobulin, such as IgA and IgM, also decreases.[42] There were rough correlations among serum PCB concentration, clinical grade of the severity of the skin lesions, decreasing percentages of active T cells, and decreased size of induration on skin hypersensitivity testing.[43]

A significantly lower percentage of phagocytes, monocytes, and polymorphonuclear leukocytes of the same patients bear immunoglobulin and complement receptors.[44] Phagocytes are responsible for the elimination of the infectious microorganisms, and the decrease of phagocyte complement and immunoglobulin receptors may be associated with the lowered resistance to infectious disease of the Yucheng patients. Three years later, using a newly developed monoclonal antibody technique, the percentage of T cells and active T cells of the same patients recovered to normal, yet the percentage of helper T cells was still low.[45] Wu et al.[46] observed enhanced lymphocyte proliferation in vitro in response to phytohemagglutinin, pokeweed mitogen and purified protein derivative (PPD) in 83 patients in the first year after the outbreak, and they still found increases in both spontaneous and mitogen-induced in vitro lymphocyte proliferation in a subset of 30 patients studied 3 years after the exposure.[45] This suggests that the effect of heat-degraded PCBs on lymphocyte function still exists 3 years after the exposure.

22.4.5 Liver Function, Urinary Porphyrin, and Laboratory Findings

Blood chemistry was studied on 143 patients from Hwei-Ming School for Blind Children in late 1979 by Chang et al.[41] and 133 patients in a special clinic by Lü et al.[24] The profiles of the two groups were relatively similar (Table 22.2): mild anemia of a normocytic type, leukocytosis, and elevations of the liver enzymes, SGOT, SGPT, and alkaline phosphatase but not of BUN or LDH.

Serum triglyceride and conjugated bilirubin were elevated, but total bilirubin was reduced. The elevated triglyceride may be due to abnormal lipid metabolism caused by liver damage. The low bilirubin is probably due to induction of bilirubin UDP-glucuronyl transferase in liver by PCBs and enhancement of conjugation of bilirubin to glucuronic acid, which results in rapid biliary excretion of bilirubin.[41] Chen and Shen[47] studied the association between blood PCB level and serum triglyceride on 104 patients admitted to Veterans General Hospital for skin lesions, and no association was found. The serum triglycerides pose an unsolved analytical problem, since they might lead to an artifactual increase in serum PCBs, which are largely present in fat. There is no consensus on whether or how to adjust for the fat content of serum when reporting PCB concentrations. With adipose tissue measurement, their problem would not exist.

Fifteen percent of body's heme production occurs in the liver. When there are enzyme deficiencies along the biosynthesis pathway, abnormal and excessive porphyrins, by-products of the biosynthesis, and other heme precursors appear in body fluid. Hepatic porphyria is caused by damage to the cell membrane of the hepatocytes and is characterized of abnormal urinary porphyrin excretion.[48] Type B hepatic porphyria, greater excretion of uroporphyrin than coproporphyrin, is the first or mildest form of porphyria induced by PCBs in laboratory animals[49] and is relatively specific to exposures to PCBs and hexachlorobenzene. F.-J. Lu and colleagues studied urinary excretion of porphyrins and heme precursors on 69 blind students from the Hwei-Ming School and 20 healthy volunteers.[50,51] The mean 24-h excretion of uroporphyrin for the exposed students was 41.2 mg $\pm$ 24.6 compared to 13.6 $\pm$ 11.8 for the controls; excretion of δ-aminolevulinic acid, a heme precursor, for the exposed and the controls was 1.0 $\pm$ 0.6 and 0.7 $\pm$ 0.3, respectively. Excretion of coproporphyrin and porphobilinogen was not affected. The mean uro/copro ratio was 1.4 $\pm$ 1.3 in the exposed patients and 0.5 $\pm$ 0.3 in the controls. Thirty-six (52%) of the 69 exposed students had ratios above 1, whereas none of the 20 controls had a ratio that high. Red blood cell δ-aminolevulinic acid dehydratase activities of 23 exposed subjects from Changhwa County were depressed at a solution of pH 6.0 compared with those of 20 healthy volunteers. This depressed activity may cause an impaired hematopoietic process, which results in decreased red blood cells and serum hemoglobin concentration.[52]

22.4.6 Menstruation and Reproduction

In 1993–1994, 356 Yucheng women aged 30 to 59 years and 312 neighborhood controls were identified and interviewed for their reproductive experience.[53] Of the Yucheng women, 16% reported abnormal menstrual bleeding compared to 8% ($p < 0.05$) of control women; 4.2% vs 1.7% reported a stillbirth since 1979 ($p = 0.068$). Other characteristics of the menses, fertility, frequency of intercourse, and age at menopause appeared unaffected. More of the Yucheng women reported that one of their offspring had died during childhood (10.2% vs 6.1%, $p < 0.05$) and that they had decided to limit childbearing because of

health problems (6% vs 2%, $p = 0.01$). These findings suggest that high-level PCB/PCDF exposure has some effect on female endocrine and reproductive function.

22.4.7 Chromosomes

PCBs and PCDFs are not considered to be mutagens. However, their substantial metabolic effects on systems responsible for metabolism of potentially mutagenic xenobiotics makes their long-term in vivo action somewhat unpredictable. Wuu and Wong[54] studied chromosomes in lymphocytes of 36 exposed patients and 10 unexposed healthy controls in 1982. Nineteen (53%) of the 36 patients, compared to none of the controls, had chromosome or chromatid aberrations; the aberrations included gaps, breaks, exchanges, and acentric fragments. Lundgren et al.[16] assessed sister chromatid exchange (SCE) levels in 35 Yucheng women and 24 gender- and neighborhood-matched controls in 1985 and found little difference using the conventional assay; the mean frequencies were 7.3 in Yucheng women and 7.6 in controls. However, after adding α-naphthoflavone (ANF), which may be metabolized by PCB/PCDF-induced lymphocytes into more reactive SCE-causing genotoxic metabolites, into the assay, the SCE frequency in Yucheng women became greater than that of the controls (mean frequencies 10.8 and 8.9). Significant dose–response relationships were observed between ANF-induced SCEs and serum concentrations of total PCBs and of several PCB congeners: 2,2′,4,4′,5′-penta, 2,2′,3,4,4′,5-, 2,2′,4,4′,5,5′-, 2,3,3′,4,4′,5-hexa-, 2,2′,3,3′,4,4′,5-, and 2,2′,3,4,4′,5,5′-hepta-PCBs.

22.4.8 Placenta Studies

One of the most consistent effects of PCBs and related chemicals is induction of activities of monooxygenase enzymes which are involved in the metabolism of chemical carcinogenes, such as benzo[*a*]pyrene. Wong and colleagues[55] studied placental tissues from four Yucheng women who delivered their babies in 1983, 12 Taiwanese hospital controls, and smoking and nonsmoking volunteers from the obstetric service at the University of North Carolina Hospital. Placental homogenates of the exposed women showed large increases in monooxygenase enzymes, including aryl hydrocarbon hydroxylase, 7-ethoxycoumarin-*O*-deethylase, and diol, quinone, and phenolic metabolites of benzo[*a*]pyrene.

22.5 CLINICAL FINDINGS OF THE IN UTERO-EXPOSED YUCHENG CHILDREN

22.5.1 Teratology and Sex Ratio

Infants born to Yucheng women have been studied in some depth. Nine infants born between October 1979 and December 1980 were evaluated for birth

defects at birth by three groups of researchers.[21,56,57] All were noticed at birth to have had hyperpigmentation of their skin, especially on the lip, gingiva, and nails, and hypersecretion of the Meibomian gland. Wong and Hwang[56] also reported skin desquamation (flaking of skin), black color of the nose, and deformed nails. Law et al.[57] examined twins and Lan et al.[21] reported on another infant that had swelling of the upper eyelid, respiratory distress, and pneumonia. Six of these nine infants were small for gestational age. Eight of these infants have been reviewed in Miller's[58] and Rogan's papers.[59] The Yucheng children's growth profile, bone mineral density and soft-tissue composition, and joint laxity were examined in 1991 and compared with those of matched controls.[60] The Yucheng children were 3.1 cm smaller and had less total lean mass and soft-tissue mass than did the control children. Bone mineral density and joint laxity were not different between exposed and control children.

To assess whether exposure to PCBs/PCDFs alter the sex ratio at birth, women who were registered at the Yucheng registry, who had at least one child born during or after the incident, and who had at least one live child in spring 1985 were interviewed in summer 1985.[61] Seventy-four women reported 137 births, including 68 boys and 69 girls, occurring between June 1978 and spring 1985. The report suggests that sex ratio in the second generation is unlikely to be a sensitive indicator of women's exposures to chemicals of persistent polychlorinated double-ring structures, such as PCBs, PCDFs, and dioxins. Whether exposed men give birth to offspring with changed sex ratio in the Yucheng cohort is under active investigation.

22.5.2 General Symptoms and Porphyrins

Lan et al.[62] reported the birth weight of 49 children born to Yucheng women between 1979 and 1985. The gestational age-adjusted birth weights for female and male exposed babies were 83 and 87% of those of the "normal" babies, and the deficits were significant in the first and second child born after the outbreak, but not in the third.

In April 1985, Rogan et al.[63] identified 132 in utero-exposed children born between June 1978 and March 1985 and 190 age-, gender-, and neighborhood-matched control children. Parents of 128 exposed and 115 control children were interviewed, and 117 in utero-exposed and 108 control children attended physical examinations. The exposed children had lower reported birth weights (mean $\pm$ SE; 2749 g $\pm$ 46 in exposed vs. 3228 g $\pm$ 40 in control babies) and were 7% lighter and 3% shorter at examination. One hundred and seven pairs of children were reexamined in February 1992, 7 years after the initial examination; the Yucheng children were still 2% shorter than their controls.[64]

Spot urines, one-time voided urine specimens, were collected in 1985 for 75 exposed children, 74 controls, and 12 siblings of the exposed children.[65] Four of the in utero-exposed children (5%), two controls, and one sibling had a type B hepatic porphyrin (i.e., a uroporphyrins/coporphyrins ratio of greater than

1). Total porphyrin excretion was elevated in the exposed children as a group (95 vs. 81 μg/L), and 11% of the exposed children compared to 3% of the control children had total urinary porphyrin concentrations greater than 200 μg/L. This suggests that PCBs and their heat-degraded by-products exposed in utero does store in liver and cause a mild disturbance in porphyrin metabolism; the effect was weaker than that of the original cohort.

22.5.3 Dermatology

Information on birth and medical history of the 128 in utero-exposed and 115 control children identified in 1985 by Rogan et al.[63] was derived from questionnaires filled out by parents. A significantly higher percentage of exposed children were reported to have hyperpigmentation, conjunctivitis, swelling of the eyelid, eye discharge, deformed or small nails, natal teeth, and swollen gums at birth; they also had more deformed finger- and toenails and acne scars in their lifetime history.[63,66] Children born longer after the exposure did not differ from those born earlier.[66] On examination, the exposed children had a much higher rate of dystrophic fingernails and dystrophic or pigmented toenails than controls; dystrophic fingernails were more specific, occurring only in the exposed children. They also had increased rates of hyperpigmentation, acne, and scaly or keratotic disorders than the controls. The dermatologic symptoms of these children were similar but weaker than those of the original cohort subjects. In addition, the exposed children had more generalized itching, localized skin infections, and hair loss.[66] The effects in the children are more apparent in nails, hair, teeth, gums, skin hyperpigmentation, and growth and development; the characteristic defect was described by Rogan et al. as a type of acquired ectodermal dysplasia, which means that the defects were formed on the ectodermal layer during the embryonic stage.[63] The dermatological manifestations of Yucheng children were followed up in 1991.[67] Most children recovered from chloracne, and chloracne scars were found in only one child. However, nail changes were found in about one-third of the exposed children. Transverse grooves, irregular depressions, and koilonychias/nail flattening were found significantly more frequently in Yucheng children compared to their controls. The author suggested these findings to indicate developmental retardation of the fetal nail matrix.

22.5.4 Immunology

When the Yucheng children and their controls were first examined and interviewed in 1985, 24% of the parents of Yucheng children compared to 4% of the parents of control children reported a medical history of bronchitis for their study children in the first 6 months after birth, and the Yucheng children were more likely to have abnormalities of pulmonary auscultation at examination. Higher frequencies of upper respiratory infection and otitis media were repeatedly reported by the Yucheng parents during the 6-year follow-up.[63] In

1993, two otolaryngologists examined the middle ears of 103 Yucheng and 96 control children with pneumatic otoscope and measured the middle ear pressure by tympanometry with a Rion RS 20 impedance audiometer.[68] Forty-three percent of the Yucheng children versus 19% of the control children had abnormal tympanic membranes ($p = 0.0003$). The effect is most significant in Yucheng children born within the first 4 years after the Yucheng incident. For the 30 Yucheng children whose serum PCB and PCDF levels were measured in 1991, those who had middle ear diseases had significantly higher serum levels of 2,3,4,7,8-pentachloro- and 1,2,3,4,7,8-hexachlorodibenzofurans than those of the Yucheng children with normal ears. Serum PCB levels were not related to middle ear diseases. In fall 1995, immune function was evaluated for 105 Yucheng and 101 control children.[69] Serum levels of IgA, IgG, and IgM were found similar between the two groups. Cell-mediated immunologic analysis was done for 51 children, 29 Yucheng and 21 control. The percentage of various T cell markers, CD3, CD4, CD8, and B- and NK-cell markers, were not different between the two groups.

22.5.5 Neurology, Cognitive, and Behavioral Development

In 1985, the 117 in utero-exposed children and their controls examined by Rogan et al.[63] were also given neurologic tests. Ten percent of the exposed compared to 3% of the control children were considered by the neurologists to have developmental or psychomotor delay. This group of controls was originally selected to provide background rates for physical findings, and they were thought to be too loose for developmental assessment, so a new set of controls who matched 118 in utero-exposed children on age, gender, maternal age, parents' combined educational level and occupation, and neighborhood were selected 6 months after the initial survey. These 118 pair of children were given age-appropriate cognitive and behavioral assessments. The exposed children scored 4 to 7 points lower than the control children on all cognitive tests except for verbal IQ, on which they scored the same, on the Chinese version of the Wechsler Intelligence Scale for Children, revised version (WISC-R); and they had worse scores on Rutter's Behavioral Scale. The same group of children have been followed by Hsu and his research team with cognitive and behavioral tests biannually since fall 1985. The yearly reports published by this team[70–76] continued for 6 years to show a mild, yet consistent deficit of the in utero PCB-exposed children on all scales of all cognitive tests. On behavioral assessment, the exposed children were more intense in reaction and more negative in quality of mood on temperament assessment, had more psychosomatic, habit, and behavioral problems than the controls on Rutter's Behavioral Scale, and were hyperactive on a modified Werry–Weiss–Peters Activity Scale. Chen et al.[77] merged data from 12 rounds of assessment and examined the hypothesis that the time elapsed between the exposure and the child's birth does affect the degree of development delay, and that the effect diminishes as the children grow. Their results showed that the exposed children scored approximately 5

points lower on all scales of all IQ tests for the ages of 4 through 7 years. Children born up to 5 or 6 years after their mothers' exposure continued to be affected, and the effect of in utero PCB and PCDF exposure on children's cognitive development persisted in the children up to the age of 7 years. By analyzing the same data set, Lai et al.[78] expanded the findings to the ages of 6 months through 12 years. Yucheng children scored 4 to 11 points lower on Bayley Scales for Infant Development for the ages 6 to 30 months, 3 to 8 points lower on Stanford–Binet IQs for ages 2 and 3, and 3 to 9 points lower on WISC-R for ages 8 through 11. The children were also evaluated for spatial and deductive ability with Raven's Colored Progressive Matrices (CPM) and Standardized Progressive Matrices (SPM).[79] Yucheng boys scored significantly lower than their controls in CPM at ages 6, 7, and 8, and borderline lower in SPM at age 9. The effect was not found for Yucheng girls. Cognitive function was also evaluated in the youngest elder siblings of 15 in utero-exposed children, and WISC-R IQ scores of the in utero-exposed children were 9 to 19 points lower than that of their youngest elder siblings at each year from 1985 to 1990.[80] To examine whether the time lapse between the exposure and the child's birth affects the degree of behavior and activity level, behavior and activity data from the same 12 rounds were analyzed.[81] The Yucheng children scored 11 to 63% worse on the mean Rutter scale for ages 3 through 12. The effect for children born later is the same as that for those born earlier. There was no improvement as the children aged. The finding for the activity score is similar but weaker. The later-born Yucheng children, those born 7 to 12 years after their mothers' exposure, were examined for developmental and behavioral problems in 1991–1992 with the Chinese Child Development Inventory (CCDI), an instrument adopted and modified from the Minnesota Child Development Inventory (MCDI).[82] The children born to Yucheng mothers had significantly lowered scores on the combined general development and several CCDI subscales; girls were more affected than boys. The children born of Yucheng fathers did not score differently from their controls.

Yu et al.[17] examined the relationship between developmental findings and physical and historical findings on the in utero-exposed children. They found that the exposed children who were shorter or who were reported to have exhibited neonatal symptoms of intoxication, such as eye discharge, eyelid swelling, and hyperpigmentation, or who had ever had deformed nails, had greater developmental delay. However, there was little relationship between other physical findings of the 1985 examination or measures of maternal exposure based on either serum PCB levels or degree of clinical symptoms and developmental scores. In another paper,[81] behavioral findings were not related to physical or cognitive findings or to serum PCB levels.

22.5.6 Reproductive System

In 1998, the reproductive system of 12 young men (sexually mature, aged 16 to 20 years) born to Yucheng mothers was examined.[83] Semen analysis showed

that the sperm in Yucheng men have increased abnormal morphology (37.6% vs. 25.9%), reduced motility (35.1% vs. 57.1%), and reduced capacity to penetrate hamster oocytes (65.8% vs. 73.5%) compared to that of 25 control young men. The semen volume and sperm count were not different between Yucheng young men and their controls.

22.6 MORTALITY

Twenty-four deaths among 2022 Yucheng cohort subjects had been reported between October 1979 and February 1983. Half of them died of hepatoma, liver cirrhosis, or liver diseases with hepatomegaly.[4] It should be noted that Taiwan has an extraordinary prevalence of hepatitis B (15%), and liver cirrhosis and liver cancer are common. The cohort was traced through December 31, 1991, and mortality was compared with age-, gender-, and calendar time-specific mortality rates for the Taiwan general population.[84] Even though the overall standardized mortality ratio (SMR) was 0.8 (95% confidence limit 0.7 to 1.0), there was a substantial elevation in the mortality rate for chronic liver disease and cirrhosis (SMR = 2.7, 95% CI = 1.3 to 4.9). Eight of the first 39 babies born to Yucheng women died of pneumonia, bronchitis, sepsis, and prematurity between October 1979 and February 1983, causing a very high infant mortality rate (20.5% vs. 1% in the general population).[4] There were three more deaths among the Yucheng offspring noted by 1985,[63] and one of the 118 in utero-exposed children followed by Hsu's team died in a car accident during the 6-year developmental follow-up.[85]

22.7 MANAGEMENT AND TREATMENT OF THE VICTIMS

Cholestyramine, a basic anion-exchange resin shown in an animal study[86] to bind chlordecone in rat intestine, to increase its excretion into the feces, and to decrease its content in the tissues by 30 to 52% after 2 weeks of treatment, was suggested as a promising therapy for treating chronic poisoning with chlordecone and possibly with other lipophilic toxins. Cholestyramine was used in a clinical trial at Veterans General Hospital in Taipei.[87] After 2 to 8 months of treatment, the blood PCB levels of 20 patients receiving cholestyramine did not decline more than that of the placebo group; however, 45% of the treatment group compared to 3% of the control group were reported to have greater relief of their clinical symptoms. Sixteen patients with severe clinical symptoms voluntarily participated in a fasting trial in mid-1981 and/or early 1982.[88] After fasting for 7 or 10 days, all patients experienced improvement of symptoms such as headache, lumbago, arthralgia, cough, sputa, and acneform eruptions. Chinese medicine and acupuncture have been used by the China Medical College to treat the victims; however, no document or publication has been released on the treatment effect so far.

22.8 SUMMARY AND FUTURE RESEARCH PLAN

It has been 24 years since the Yucheng outbreak; and although the painful memory of the outbreak may have faded somewhat, among those involved, some of the chemicals persist in the body and continue to affect the original Yucheng cohort and their offspring. Most information regarding the outbreak and the effects of direct exposure to the chemicals came from case reports and clinical observations of subgroups of the cohort during the first 6 years after the incident. Most of the observations were done on small populations with no or poorly defined controls. Since April 1985, more carefully designed epidemiologic studies of children born to Yucheng mothers during or after the outbreak and proper controls have provided thorough information on physical, cognitive, and behavioral effects of in utero heat-degraded PCB exposure.

To understand the effect of PCBs and heat-degraded by-products on various organs or systems of the body, several research projects are ongoing or in the planning stage. To investigate the long-term health effects of direct exposure to these chemicals, the original Yucheng cohort who had ingested the contaminated oil and proper controls have been identified, and the mortality rate, cancer and other chronic disease morbidity rates, and reproductive functions of the two groups have been and will continue to be studied and compared. More PCB and PCDF congener levels will be measured, and the relations of blood chemical levels to physical findings will be evaluated. The caffeine breath test[89] will be given to test the PCB/PCDF effect on cytochrome P450 IA2 activity in liver and the effect of the altered enzyme activity on the metabolism of sexual hormones and sexual maturation of Yucheng children. A second in utero PCB-exposed child cohort, children born to either exposed mothers or exposed fathers between June 1985 and July 1992, has been identified, and the research questions of whether PCBs continue to affect offspring through transplacental transfer and nursing 6 to 13 years after the exposure and of whether PCB/PCDF affects the offspring through paternal reproductive pathways will be studied. Gender ratio in the second generation will be examined for both exposed women and exposed men. Reproductive systems in both exposed men and women will be evaluated further.

The findings of these studies will provide dose-related etiologic information not usually available in most occupational or environmental exposure settings. There may be a unique opportunity to test a specific hypothesis about virus-chemical interaction with respect to hepatocarcinogenesis. Taiwan has a high prevalence of hepatitis B infection (15%) and a consequent high rate of hepatoma. In Japan, preliminary data from the Yusho cohort with almost identical exposure suggested a sixfold excess of liver cancer mortality in men and a threefold excess in women[90]; hepatoma has been shown in animal studies to be caused by PCB exposure.[91–95] Also, as immunosuppressants, the PCBs and PCDFs may contribute to a decreased ability to fight against cancers or infectious diseases. Thus, it is plausible to study the possible interaction between the two factors.

It is very unlikely that there will be another similar incident, but the potential for exposure to PCBs that have not been so heat-degraded or to dibenzofurans and dioxins is much more widespread, through accidents, improper disposal practices and transport of PCB-containing transformers and capacitors, and the now usually contaminated food chain. The findings from the investigations of the Yucheng incident surely provide clues about the toxicities that background PCB exposure may cause, and because dibenzofurans and dioxins are thought to have a similar mechanism of action, the information found in this incident may contribute to the understanding of human health response to these compounds.

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INDEX

Abatement strategies, 165
Abiotic environment, 7
Absorption, in pharmacokinetics:
  following dermal exposure, 199–201
  following inhalation exposure, 201–202
  following oral exposure, 192–199
Acceptable daily dose, 15–16, 27
Accumulation/bioaccumulation, 67, 603–604
Accuracy/precision, in data analysis, 92–93
Acetaldehyde, 340
Acetone, 340
2-acetylaminofluorene (AAF), 9
Activation-induced cell death (AICD), 543
Acute lethality, 142
Acute toxicity, 11
Adaptive immune response, 301–302
Adaptive response pathway, 560, 616–617
Adaptor proteins, 539
Additive effects, 656–657
Adenosine monophosphate (AMP), 435
Adenosine triphosphatase (ATP), 282
Adipose tissue:
  exposure assessment:
    environmental exposure, 651–652
    general population, 656
    occupational exposure, 649
    partitioning, 663
  implications of, 202–206, 208, 211, 217, 223, 226, 233
  rice oil poisoning effects, 905
ADME (administered chemical, its tissue distribution, metabolism, and elimination), dose-response modeling, 276
Adverse effects, 171–172
AEA Technology, 98
African-American population, PCB effects, 704
Agent Orange, *see* Vietnam veterans
  characteristics of, generally, 1, 3, 20, 56, 64
  developmental epidemiology, 792
  developmental toxicology, 345, 767
  epidemiological studies, 735, 740, 742, 752
  exposure assessment, 630–631, 637, 639–642, 651–654, 670
  health risks, 167
Agent Orange Scientific Taskforce, 21
Agent White, 752
AhR interacting protein (AIP), 569, 610, 612–613
AhR-null studies, 147–148, 507–508
AhR1, 575, 579
AhR repressor (AhRR), 503–504, 511, 535, 575–577, 579, 610, 613
AhR response element (AhRE), 9, 566
AhR2, 575, 579
AIB-1, 169
AINt-1, 169
AIP1, 145
Air emissions, 6–7, 72–75
Alanine aminotransferase (ALT), 778–779, 812–813, 832
Albumin, 148, 203
Aldehyde dehydrogenases, 172
Aldehyde-3-dehydrogenase, 535
Alkaline phosphatase, 904
Alleles, 146, 168, 497, 501, 507–508, 510
Allergies, 299, 900
Allogeneic cells, 319
Altered hepatocellular foci (AHF):
  carcinogenesis, 465–466
  dose-response modeling, 281–283
  liver tumor promotion, 470–471, 474
Aluminum smelting, as contamination source, 73
Ameloblasts, 29, 355
Amino acids, 11, 494, 569, 611–612
γ-aminobutyric acid (GABA), 386, 437
Aminolevulynic acid (ALA-U), 832
Aminopyrin *N*-demethylase, 7-ethoxycoumarin *O*-deethylase, 864
Amitrol, 743
Analytical chemists, functions of, 124
Analytical methodology:
  cleanup, 75
  improvement strategies, 81–883
  measurement methods, 75–81
  preconcentration, 75
  problems and limitations of, 81

Dioxins and Health, Second Edition, Edited by Arnold Schecter and Thomas A. Gasiewicz
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Androgen concentration, 175, 357–359
Angiogenesis, 508
Animal feed, contaminated, 56, 74–75, 122–123
Animal products, contaminated:
  eggs, 115–116
  implications of, generally, 8
  meat, 113–115
  milk/milk products, 110–113
Animal studies:
  developmental epidemiology, 814
  dioxin effects, generally:
    laboratory, 25–26
    livestock, 23–25
    wildlife, 23–25, 30
  pharmacokinetics:
    distribution, 203–204
    excretion, 216–217
Anorexia, 142, 879
Antagonism, 447–448
Anti-IgM antibody, 308
Antibody/antibodies:
  PCB effects, 700
  production, *see specific types of antibodies*
  response, 173, 176, 308
Antiestrogenic effects, 394–399, 877
Antigenic peptides, 301
Antigen-presenting cells (APC), 309–310
Antigens, *see specific types of antigens*; T cells
Antipyrine, 711
AP-1, 500, 535, 540, 542
Apomorphine, 385–386
Apoptosis, 170, 173, 317, 469, 471, 543–544, 615–616
Appendicitis, 534
Appetite suppression, 142–143
Aquatic systems, 8
Aqueous samples, workup procedures, 82
Ara9 protein, 145, 170, 569, 610
Arachidonic acid, 537, 564
Arachidonic acid metabolites, 307, 507
Aroclor, 388, 396, 436, 441, 448, 463, 539, 546
Aromatic amines, 9
Aromatic hydrocarbons, *see* Aryl aromatic hydrocarbons receptor (AhR); Halogenated aromatic hydrocarbons (HAHs); Polyhalogenated aromatic hydrocarbons (PHAHs)
Arsenic, 538, 729
Arthropods, AhR signaling, 573
Aryl hydrocarbon hydroxylase (AHH), 201, 491, 537–538, 862, 876, 906
Aryl hydrocarbon receptor (AhR):
  adaptive functions, 560, 579–580
  agonists, 543, 562, 564–566
  antagonists of, 504–505
  apoptosis, role in, 543–544
  biochemical response to, 535, 537
  carcinogenesis, 467, 469, 473, 477–478
  characteristics of, 2, 8–11, 18–19, 31, 89, 175, 180
  CYP1A coevolution, 577–578
  developmental toxicity, 341–344, 351–352, 354–355
  dose-response modeling, 279
  evolutionary characteristics, 335–336
  expression of, 535–536
  fish toxicity, 610–617
  future research directions, 510–512
  as gene regulatory protein:
    CYP1A1 induction, 492–494
    functional features, 494–495, 541
    structural features, 494–497, 501–503
  invertebrate homologs, 569–574
  ligands, 162, 564–566
  modes of action, 168–170
  molecular mechanisms, 145
  normal functions of, 506–509
  null studies, 147–148
  oxidative stress, 445
  pharmacokinetics, 192
  physiological functions:
    AhR-deficient mice and cells, 563
    genes regulated by, 562–563
    ligands, 564–566
    overview, 560–562
    PAS gene family, 567–568
    phylogenic studies, 568
    toxic endpoints, 562
  prenatal expression, 336–337
  regulation, 500–501, 503–504
  reproductive epidemiology, 810
  reproductive toxicity, 394–395
  sensitivity to dioxin, 146, 168–169
  signaling, comparative and evolutionary biology:
    invertebrate homologs of AhR and ARNT, 569, 571, 573–574
    ligand binding in vertebrate AhRs, 574–578
  signaling pathway:
    characteristics of, 170, 306–307, 507–509
    in fish, 610–614
    signal transduction, 337–338
  structure-activity relationships, 145
  TCDD dependence in immunotoxicology, 304–307, 319–320
  toxicity mechanisms, 337–338, 578–579

toxic responses, involvement in:
ligand efficacy, 504
mediator of toxicity, 497–500
overview, 491–492
oxidation-reduction processes, 496, 505–506
phosphorylation, 496, 500, 505–506, 511
potential susceptibility to chemical contaminants and human disease processes, 509–510
regulation of gene expression and tissue levels, 500–501, 503–504
Aryl hydrocarbon nuclear translocator (ARNT):
biochemical response to, 535, 537
developmental toxicity, 335–340, 342–345, 349–351, 353, 359, 361, 376–377
fish, 611–612
functions of, generally, 9, 145–146, 169–170, 175, 304, 492–499, 503, 505–506, 508, 511
invertebrate homologs, 569, 571, 573–574
PAS gene family and, 567
phylogenetic research, 577
toxicity mechanisms, 578–579
Aryl hydrocarbon-responsive elements (AhREs), 492
Asbestos, 629, 729
Aspartate, 494
Aspartate aminotransferase (AST), developmental epidemiology, 778–779, 812–813
Association reactions, 65
Astaxanthin, 564
Asthma, 299
Atmospheric photo degradation, 67, 71
Australia:
air emissions, 6
animal feedstuffs, 122
epidemiological studies:
malignant lymphoma, 741
soft-tissue sarcoma, 737
food contamination research, 108
Austria, intake assessment research, 98
Autoinduction, 215–216
Automated chromatography, 82
Automated sample workup procedures, 82
Azo-benzenes, 160
Azoxybenzenes, 160

bab, 573
Background exposures, 166
Backyard burning, as contamination source, 55–56, 72–73
Ball clay contamination, 74–75, 167
Banbury Center, 18–19
BaP gene, 546
Barbecued foods, 120
Barrel burning, as contamination source, 163
BASF spill:
case study, 636–637
cohort dose-response study, 272–273, 275
effects of, generally, 533–534
BAX gene, 543–544
Bayley Scales of Infant Development (BSID), 691, 694, 696, 769
B cells:
functions of, generally, 14
rice oil poisoning effects, 904
TCDD immunotoxicology effects, 307–309, 318
BCL-1 cells, 309
Beef:
consumption research, 100–105
contaminated, 167
contamination research, 113–115
Beery Test of Visual-Motor Integration (VMI), 691
Behavioral development, rice oil poisoning effects, 909–910
Belgium:
animal feedstuffs, contaminated, 123
contamination source research, 176
intake assessment research, 98–99
Benchmark Dose Software (BMDS), 258–259
Bender Gestalt Test, 697
Benzo[α]pyrene (BaP), 537
Benzo[α]pyrene hydroxylase, 864
bHLH-PAS family, 170, 336, 494–496, 508, 535, 561, 563, 568–569, 574, 577–578
Bilirubin levels, 162, 564, 568, 778
Bilirubin UDP-glucuronyl transferase, 905
Biliverdin, 564, 568
Binding affinity, 504
Binghamton State office building fire, 631–636
Bioassay tests, 92
Biochemical effects:
dose-response modeling, 259, 262, 264, 266
health risks, generally, 171–173, 180, 182
toxicology, 147–149, 171–172
Biochemical responses:
apoptosis, 543–544
cell cycle regulation, 541–543
dioxin-related genes and metabolism, 536–539
global gene expression array research, 544–547
overview, 533–536

Biochemical effects: *(Continued)*
signal transduction pathways activation, 539–541
Biochemistry:
biological persistence, 8–10
growth factors, 10–11, 31
hormones, 10–11, 31
Biocide production, 182
Bioconcentration factor (BCF), 205
Biologically-based dose-response (BBDR) models, 469
Biotransformation enzymes, 578
Biphenylols, 445
Birds:
developmental toxicity, 338
environmental contamination, 8
Birth defects:
cleft palate, 11
developmental epidemiology, 814–815
future research directions, 815
health risks, 171
mouse studies, 11
Operation Ranch Hand, 799–800
rice oil poisonings, 906–907
Sevoso, Italy accident, 836
sources of, generally, 1
toxicity and, 11–12
Birth weight:
developmental epidemiology, 803–804
PCB exposure, 683–684
significance of, 28
Bladder cancer, 744
Bleaching, implications of, 163, 182, 630
B-lineage specific activator protein (BSAP), 306
Blood, generally:
distribution, pharmacokinetics, 202–203
levels, contamination research, *see* Exposure assessment
Bluegill, toxicity in, 604
B lymphocytes, 173, 300, 306, 739
BMMT, 547
Body burden, significance of, 27, 30, 138, 150, 168, 178–182, 194, 227–228, 233, 251–252, 399–403, 435, 534, 630–631, 706
Body mass index (BMI), 229
Body weight, significance of, 161, 166, 181, 194, 210, 227, 464. *See also* Body burden
Boilers, as contamination source, 73–74
Bone marrow, 301, 303, 317, 562
Bony fish, 577–578
Boston Naming Test, 697
Bottom-feeding fish, 116
Bovine serum albumin (BSA), 318
B[α]P, 537, 539
Brain, AhR expression, 537. *See also* Neurobehavioral development; Neurology
Brain stem auditory evoked potentials (BAEPs), 697
Breast cancer:
cohort studies, 751
epidemiological studies, 746
estrogen receptor positive, 753
health risks, 169, 178
PCB effects:
associations in subgroups, 704, 706
large case-control studies, 701, 703
nested case-control studies, 704
small case-control studies, 701–702
reproductive toxicology, 375, 395
toxicology, 148
Breastfeeding, *see* Breast milk; Lactation
characteristics of, generally, 8, 27–29
developmental toxicity, 395–396
health risks, generally, 166
immunotoxicology, 320
PCB effects, 683–684, 688–699, 772–779, 781–790
pharmacokinetics, 199, 230–234
Breast milk:
contamination, 30–31, 647, 649–650, 772–779, 781–790
exposure assessment, 658–661
PCB exposure research, 683–684, 688–699, 781–786
Brominated congeners, 160
Brominated dibenzo-*p*-dioxins (BDDs), 334
Brominated dibenzofurans (BDFs), 334, 351
5-bromo-2′-deoxyuridine (BrdU), 470
Brownfields, 56
Buspirone, 386
Butter:
consumption research, 102
contamination research, 93, 108, 113
By-products, as contamination source, 3, 7, 55, 533, 905

CAAT box, 535
Cadmium, 538–539
Caenorhabditis elegans, 169, 567, 569, 571, 574
Calcium:
extracellular, 540
intracellular, 540
neuronal, PCB effects, 435–440
signaling pathways, 170
Calcium-induced calcium release, 438

California Verbal Learning Test, 697
Calmodulin-binding proteins, 546
cAMP, 535
Canada:
  average daily intake, 16
  air emissions, 6
  arctic, 7
  environmental trends, 30
  epidemiological studies:
    breast cancer, 753
    farmers, 749
  milk contamination, 112
Canadian Total Diet Programme, 105
Cancer, *see specific types of cancer*
  development of, *see* Carcinogenesis
  health risks, 180
  human effects:
    overview, 20
    qualitative evidence, 21–22
    quantitative evidence, 22–23
  incidence, Seveso, Italy accident, 839, 845–846
  linearized multistage (LMS) model, 16–18
  mechanisms, 1, 12–14
  occupational exposure, 171
  PCB exposure, 706–710
  reassessment, 14, 16–19
  risk assessment, 14–17, 31
  slope factor, 181
  as source of, 12
Canthaxanthin, 564
Capacitors, as contamination source, 3, 164
Capillary gas chromatography, 82
Capsule, defined, 143
Carbohydrates, 142, 148
Carcinogenesis:
  animal studies, carcinogenic potential, 457–458
  cancer bioassay:
    spontaneous tumor incidence, 463–464
    tumor incidence sites, 460–463
  dose-response modeling, 253–254
  mechanism of action:
    liver tumor promotion, 469–475
    lungs/respiratory system, 475
    overview, 468–469
    thyroid, 476
  mode of action:
    implications of, generally, 464–465
    liver tumor promotion, two-stage models, 465–467
    lung tumor promotion, two-stage models, 468
    skin tumor promotion, two-stage models, 467–468
  multistage, 458–459
  TCDD carcinogencity, relevance to humans, 477–479
Carcinogenicity, 138, 144, 178, 181
Carcinogens, as contamination source, 1, 173
Cardiomyopathy, 534
Cardiovascular disease, 173–174, 534, 840, 710
Carotenoids, 507, 564
Case-control studies:
  colon cancer, 742
  developmental epidemiology, 813
  gastric cancer, 742
  hematological malignancies, 741
  liver cancer, 742
  malignant lymphoma, 731, 737–741
  nasal and nasopharyngeal cancer, 731, 741–742
  soft-tissue sarcoma, 731–737
Case studies:
  environmental exposures, 649–655
  occupational exposures, 632–649
Caspase-3, 544
Catabolism pathways, 253
Catechol estrogens, 472
Catecholamines, 443, 447
CBP/p300, 496
Cdc2 p34, 542
CD8 cells, 300, 318
$CD8^+$ T cells, 302–303, 313, 318, 700
$CD11B^+$ cells, TCDD immunotoxicology effects, 310
CD4 cells, 300, 318
$CD4^+$ T cells, 301–303, 314–317
CD40 gene, 309–310
cdk4, 542
cDNA, 559, 567, 575, 610–612
CD19 gene, 306, 309
$CD3^+$ cells, 700
CeAhR protein, 569, 571, 573
Cell(s), *see specific types of cells*
  cycle, *see* Cell cycle
  death, 445, 447, 469–470. *See also* Apoptosis; Necrosis
  development, 1
  differentiation, 1, 11, 148, 300, 339, 562, 566
  growth, 148
  proliferation, 13–14, 17, 19, 398, 562
  regulation, 1, 11, 148
  signaling pathways, 473
Cell cycle:
  arrest, 542
  characteristics of, 11, 14, 170, 508, 511, 542

Cell cycle: *(Continued)*
gene expression, 5420543
kinetics, 474
regulation, 541–543, 545
Cement kilns, as contamination source, 69–70, 73
Centers for Disease Control, 768
Central nervous system:
developmental toxicity:
AhR and ARNT expression, 376–377
characteristics of, 332, 375–376, 347
deficits, generally, 347, 441
neurobehavior research, 381–390
neurobehavior research:
drugs, behavioral responses, 385–386
methodology, 381–382
night vision, 391
open-field locomotor activity, 387
operant responding, 384–385
passive avoidance behavior, 386–387
spatial learning, 382–384
thermoregulation, 389–390
transitional behavior, 385
visual discrimination reversal learning, 384
PCBs, effects on, 440–443
sexual differentiation, 377–381
toxic effects on, 26, 173, 177
Ceramic products, as contamination source, 75
Cereal/cereal products, 96
Cerebrospinal fluid (CSF), 903
Cervix, reproductive toxicity, 373
c-*Fos*, 541
Chemical Abstract Services (CAS), 58
Chemical-by-chemical regulation, 33
Chemical carcinogenesis, 288
Chemical waste, incineration, 137
Chemical workers, health risks, *see specific case studies*
Chemophobia, 1
Chick edema disease, 23
Chickenpox, 28, 176
Chicken research:
biochemical effects, 148
consumption research, 98, 102
contamination research, 93, 108, 114
short-term toxicity, 140
Child Behavior Record, 691–692
Child Health and Development Study, 687
Children, *see* Infants
cognitive effects, 28, 877, 909–910
developmental neurotoxicity, 180
hormonal effects, 180
immunotoxicity, 180
mortality rates, 816
noncancer effects, 28
risk assessment, 182
China:
contamination research, 108
pentachlorophenol dioxin exposure, 647, 649
Chinese Child Development Inventory (CCDI), 910
Chloracne:
implications of, 20, 28, 143–144, 171–173, 224, 272, 630, 646, 712, 714, 744, 831–832, 837–838, 847, 900
TCDD immunotoxicology, 319–320
Chlorella, 225
Chlorinated benzene, 66
Chlorinated dibenzofurans (CDFs), 164–166, 332–335, 347–349, 354, 375
Chlorinated dibenzo-*p*-dioxins (CDDs), 164, 166, 221, 334–335, 347–349, 354
Chlorinated naphthalenes, 23
Chlorinated phenols, 75, 630, 754
Chlorine, generally:
age, 64
chemistry, 3, 5–7
health effects, 55, 63
Chlorine Institute, 18
Chlorobromo congeners, 160
4-chloro-2-methylphenoxyacetic acid (MCPA), 731, 741–742, 746, 754
Chlorophenol:
characteristics of, generally, 21
cohort studies, manufacturers and users, 748–749
Swedish ban, 738
Cholesterol levels, 711, 713, 778, 838
Cholestyramine, 911
Choline chloride, 123
Chromatins, 495–496
Chromatographic mass spectrometry, 63
Chromosomes, rice oil poisoning effects, 906
Chronic liver disease, 534
Chylomicrons, 202–203
Cigarette smoke, health effects, 74, 539, 788. *See also* Tobacco smoke
Cipro, 546
Circadian feeding rhythm, 142
Circadian rhythms, 494
Cleaning agents/solvents, 66
Cleanup procedures/protocols, 75, 82, 92
Cleft palate, developmental toxicity:
AhR mechanism evidence, 351–352
characteristics of, 11, 172, 341, 350
critical periods, 345–346
maternal toxicity, 350–351
species and strain differences, 352–353

Cleft phallus, 371
CLOCK protein, 568
Cloning, 146, 567
Clophen, 463
Clustering, 545
c-MYC gene, 541
Coalite chemical plants, 111
Cobalt, 546
Cognitive development, 28, 877, 909–910
Cohort studies:
  BASF dose-response study, 272–273, 275
  developmental epidemiology, 813
  dose-response modeling, 271–274
  epidemiology:
    general public after accidents, 750–751
    producers and users of dioxin-contaminated chemicals, 742–749
    pulp and paper mill workers, 730, 751
  rice oil poisoning, 177
Collaborative Perinatal Project, 687
Collagen, 354
Colon cancer, 742, 745
Colored Progressive Matrices (PCM), 910
Combustion sources, 7, 32, 56, 62, 65, 71–72, 163, 533, 539
Commercial food supply, 167
Comparative biology, AhR signaling, 569–578
Confidence interval, 809
Confounding, 734
Congeners, *see specific types of congeners*
  characterization of, 62–63, 68–69, 75, 160–161
  food contamination, 89
  half-life of, implications of, 70, 223
  tissue distribution, 208–209
Congenital malformations, 815
Congestive heart failure, 535
Constitutive androstane receptor (CAR), 566, 579
Contamination studies, 7
Cooking techniques, 119–120
Coplanar HAHs, 434, 440, 447
Coplanar PCBs:
  carcinogenesis, 462
  developmental toxicity, 332, 382, 385–386
  epidemiological studies, 753
  exposure assessment, 662–664
  food contamination, 91–92, 114
  health risks, 180
  neuronal signaling, 445
  pharmacokinetics, 191–192, 206, 232, 234
  rice oil poisoning, 859, 862
Copper, primary, 74
Copper smelters, as contamination source, 69, 72–74
COS-7 cells, 612
Cot kinase, 546
Covalent bonding, 214
Cow's milk, *see* Milk
Cox regression analysis, 270–271
Crematoria, 73
Cross-sectional studies, 813
c-src:
  kinase, 542
  protein, 145, 170, 306, 473, 540
Cumulative exposure, 32
Cu/Zn superoxide dismutase (SOD1), 445
CV-1 cells, 566
Cyclohexamide, 546
Cyclooxygenase, 537–538
Cyclooxygenase-1 (Cox-1), 546
Cyclooxygenase-2 (Cox-2) induction, 339–340, 537–538, 546
Cyclosporin A, 306
CYP:
  expression, 561
  isozymes, 507
CYP1A:
  AhR signaling, 509, 577–578, 613–615
  dose-response modeling, 264
  pharmacokinetics, 214
  proteins, 264, 284
  toxicity mechanisms, 578
CYP1A1:
  AhR response, 492–496, 498, 502–504, 506–508, 560, 564
  biochemical response, 535–538, 540, 546–547
  carcinogenesis, 466–467, 472, 475, 477
  characteristics of, generally, 9–10, 13–14, 18–19
  developmental toxicity, 336, 340, 395
  dose-response modeling, 252–253, 256, 278–279
  health risks, 172, 174, 178
  human health effects, 704, 706
  immunotoxicology, 318–319
  pharmacokinetics, 215, 226
  toxicology, 145, 147, 150
CYP1A2:
  biochemical responses, 535–536, 547
  carcinogenesis, 464, 472, 475
  characteristics of, generally, 9, 13–14, 19
  dose-response modeling, 251, 277–279, 284
  health risks, 168, 170, 172, 174, 178
  pharmacokinetics, 212, 215, 226
  toxicology, 147

CYP1B1:
AhR response, 503–504
biochemical responses, 535–537
carcinogenesis, 474–475, 477
characteristics of, generally, 9
health risks, 172
pharmacokinetics, 215
toxicology, 147
CYP2B10, 547
CYP2C29, 547
CYP450, 616
CYP4501A, 616
CYP4A10, 547
CYP4A14, 547
Cysteine sulfhydryl, 506
Cytochrome *c*, 446
Cytochrome P450:
AhR responses, 561
biochemical responses, 536
carcinogenesis, 472, 475
characteristics of, 9, 14
developmental toxicology, 336, 339
dose response modeling, 277
health risks, 174
immunotoxicology, 304, 306
neuronal signaling, 445
pharmacokinetics, 191
toxicology, 145, 147
Cytogenetics, 834–835
Cytokines:
characteristics of, 11
health risks, 172
immunotoxicology, 301–302, 305–306, 313, 316
toxicology, 142
Cytosol, 145, 491
Cytosolic signaling proteins, 542
Cytotoxic T lymphocytes (CTL), 302, 305, 309–313, 316

Dairy products, as exposure source, 630. *See also specific types of dairy products*
DDE, 30
DDT, 700, 713
Dean-Stark modification, 75
Death, dioxin-induced, 11, 172. *See also* Apoptosis; Mortality rates
Decomposition mechanism, 72
Decontamination operations, 63, 838
Degradation mechanisms, 67, 71–72, 164
Delayed-type hypersensitivity (DTH), 26, 318
Dendritic cells (Dcs), 300–302, 309
Denmark:
epidemiological studies:
farmers, 749
pesticide manufacturers, 743
intake assessment research, 99
Dental defects:
developmental epidemiology, 815
developmental toxicology, 347, 355–357
hypomineralization, 29
rice oil poisoning effects, 876–877, 900, 908
tooth development, 355–356
types of, generally, 29
Dephosphorylation, 500, 506
Dermal exposure, pharmacokinetics, 199–201
Dermal lesions, 872
Dermal toxicity, 143–144
Dermatologic abnormalities:
PCB exposure, 712
rice oil poisoning effects, 901, 907–908
Desmasculinization, 378–380
Detoxification, 167, 214, 538, 541
Developmental deficits, neurological, 253
Developmental effects, 138
Developmental epidemiology:
birth weight, 803–804
background, 765–767
biomarkers, 768
characteristics of, 768
environmental studies, 769–801
future research directions, 813–816
gender ratio at birth, 801–803
intrauterine growth retardation (IUGR), 803–805
literature review, 768–769
postnatal growth and development, 803–805, 810–812
Developmental neurotoxicity, 180
Developmental toxicity:
Agent Orange research, 642
AhR-mediated, 339
biochemical responses, 534
cell proliferation and differentiation, 339
death, growth, and clinical signs:
critical development periods, 345–346
in humans, 346–348
maternal toxicity, 343–345
mouse studies, 331, 341–346
prenatal mortality, 342–343
primate studies, 331
rat studies, 331, 348–349
sex ratio, 348–349
structure-activity relationships, 346
in fish and birds, 338
functional alterations:
central nervous system, 375–390

female reproductive system, 369–375
male reproductive system, 357–369
sensitivity endpoints:
human susceptibility, 402
maternal and fetal TCDD body burdens, 402–403
TCDD LOAELs, 399–402
structural malformations:
cleft palate, 349–353
eye opening, 356
hydronephrosis, 346, 349, 353–355
overview, 349–350
tooth development, 355–356
TCDD:
components of, 330–333
lipid peroxidation, 339–341
overview, 329–330
TCDD-like chemicals:
in birds, 338
in fish, 338
Diabetes, 175, 180, 710, 840
Diazomethane, 213
Dibenzofurans, 5
2,4-dichlorophenoxyacetic acid (2,4-D), 637, 731, 740–743, 746, 754, 766
2,2′-dichlorobiphenyl (2,2′-DCB), 435–436, 439
Diesel trucks, as contamination source, 69, 73
Dietary exposure, *see* Breastfeeding; Breast milk; Food contamination
estimating, 96–97
PCBs, 684
types of, generally, 108–110
Diethylnitrosamine (DEN), 13–14, 282, 466, 471
Diethylstilbestrol (DES), 371
5α-dihydrotestosterone (DHT), 357
7,12-dimethylbenz[α]anthracene (DMBA), 375
Diol, 906
Diortho PCBs, 91, 657
Dioxinlike compounds:
characteristics of, 2
complex mixtures of, 160–163
effects of, 171–178
environmental fate, 164–165
exposure process, 167–168
mode of action, 168–171
sources of, 163–164
transport, 164–165
Dioxin-related genes, metabolism, 536–539
Dioxin response element (DRE):
AhR response, 492, 494–496, 498–499, 508
developmental toxicity, 395
dose-response modeling, 279
health risks, 169
immunotoxicology, 304–305, 307
toxicology, 145
Dioxins and Health, 90
Dioxins, generally:
characteristics of, 2, 55
defined, 1, 55, 160
historical perspective, 2–4
production of, 32
sources of, generally, 2–7, 72, 74, 163–164
Diphenyl ethers, 2
Diruone, 743
DLX genes, 573
DmAhR protein, 571, 573
DNA:
carcinogenesis, 473
fish toxicity, 610
gene transcription, 145–146
metabolite toxicity, 214
oxidative stress, 445
synthesis, 172
DO11.10 transgenic T-cell model, 314–315
Dogfish, AhR functions, 610
Dogs, toxicity research, 149
Dopamine, 436, 441–444, 447–448
Dose metric, 248–252
Dose-response assessment, 159, 469
Dose-response characterization, 160
Dose response modeling:
biological responses, 252–254
characteristics of, 247–248, 255–256, 286–291
data gaps in assessment, 285–286
dose metric choice, 248–252
empirical:
carcinogenic effects in experimental animals, 267–269
individual human data sets, 269–276
noncancer effects in experimental animals, 256–267
overview, 256
mode-of-action-based, 276–285
Dose-response relationship:
epidemiological studies, 746
health risks and, 160, 178–180
human disease process, 509
neuronal calcium, PCB effects, 439
PCB exposure, 439, 708, 712
significance of, 15–19, 26
Dow Chemical, 460
Dreissena polymorpha, 573
Drinking water, exposure through, 89
Drosophila, 169, 496, 567
Drosophila melanogaster, 569, 571, 573
Drum reclamation, 74

DT-diaphorase, 172
Dust, as contamination source, 164
dUTP, 543
Dysplasia, 148–149

Ear infection, implications of, 28, 176
Eating disorders, 148
E-box sites, 535
Ecologic studies, 813
Ectodermal dysplasia, 173–174, 347
ED_{01} values, dose-response modeling, 179, 181, 254, 257–263, 265–268, 273, 276, 284, 286, 288–289, 291
EDC/vinyl chloride, 73–74
EGAS, 169
EGFR tyrosine kinase, 539–540
Eggs:
  consumption research, 100, 103
  contaminated, 115–116
Eicosanoid metabolism, 537–538
Electroencephalography (EEG), 874, 903
Eleventh International Symposium on Chlorinated Dioxins and Related Compounds, 19
Elimination mechanisms, 67, 71–72, 167
ELK-1, 541
Ellagic acid, 341
Embryo, developmental toxicity, 336–337, 339, 341, 345–346, 544
Emission sources, 70, 163–164, 166
Emotionality Activity Sociability (EAS) Temperament Survey for Children, 692
Empirical dose-response modeling:
  carcinogenic effects in experimental animals, 267–269
  individual human data sets, 269–276
  noncancer effects in experimental animals, 256–267
  overview, 256
Emulphor:ethanol, 193, 201
Endocrine-related disorders, 175–176
Endocrine system:
  contamination effects, 172
  rice oil poisoning effects, 875
Endogenous ligands, AhR, 564–566
Endogenous response pathway, 560, 578
Endometriosis:
  developmental toxicity, 395–398
  health risk, 176
  reproductive epidemiology, 807, 813
  sources of, 11–12, 26, 28
Endonucleases, 447
Endoplasmic reticulum, 436
Environmental exposure:
  animal effects, 752–753
  case studies:
    Agent Orange in Vietnam, 630–631, 637, 639–642, 649, 651–654
    rice oil poisonings, 654, 750–751, 802, 855–881, 893–911
  human effects, 751–753
  implications of, generally, 7–8
Environmental fate, 164–165
Environmental hormones, 11
Environmental legislation, 30
Environmental scientists, functions of, 124
Environmental trends, 30–31
Enzyme induction, 833. *See also specific enzymes*
E1A oncoprotein, 536
Epidemiologists, functions of, 124
Epidemiology:
  case-control studies:
    colon cancer, 742
    gastric cancer, 742
    hematological malignancies, 741
    liver cancer, 742
    malignant lymphoma, 731, 737–741
    nasal and nasopharyngeal cancer, 731, 741–742
    soft-tissue sarcoma, 731–737
  clinical observations, 730–731
  cohort studies:
    general public after accidents, 750–751
    producers and users of dioxin-contaminated chemicals, 742–749
    pulp and paper mill workers, 730, 751
  dioxin-exposed populations, 730
  environmental exposure, through pollution:
    animals, 752–753
    human, 751–752
  health risks, 177–178
  organochlorines/organobromines, 730, 753–754
  scope of, 729–730
Epidemiology studies, TCDD immunotoxicology, 319–320
Epidermal changes, 11
Epidermal growth factors (EGFs), 352, 354, 356
Epidermal growth factor receptors (EGFRs):
  carcinogenesis, 473, 478
  developmental toxicity, 354, 356, 395
  dose-response modeling, 278
  functions of, generally, 10–11, 14, 19, 29
  health risks, 172, 174, 178
  signaling pathway, 539, 544

Epididymis, developmental toxicity, 364–365
Epithelial hyperplasia, 534
Epstein-Barr virus (EBV), 14, 739, 754
ERAP140 protein, 496
ERK MAP kinases, 541
ERK protein, 540
ERK2, 542
EROD induction, 253
Erythropoietin gene, 508
Estradiol, 14
17β-estradiol, 357
Estrogenic hormones, 13
Estrogen, 371–373, 394–395, 472
Ethanol, 194
7-ethoxycoumarin-*O*-deethylase, 906
Ethoxy-resorufin-*O*-deethylase (EROD), 265, 352, 537
Ethylene bromide, 5
Ethylene dichloride (EDC), 5–6
E2F, 542
European research studies, 6, 98–103. *See also specific countries*
European Union (EU), 97, 119
Evolutionary biology, AhR signaling, 569–578
Excretion, in pharmacokinetics:
  in animals, 216–217
  fecal, 223–225
  in humans, 217–223
  lactation, 225
Experimental toxicology, carcinogenesis, *see* Carcinogenesis
Exposure:
  assessment, *see* Exposure assessment
  to dose, 167–168
  dose-response relationship, 178–180
  process overview, 165–167
Exposure assessment:
  background, 631–632
  case studies:
    environmental exposures, 649–655
    occupational exposures, 632–649
  general population and dibenzofuran levels:
    partitioning of dioxinlike compounds, 662–663
    PCBs in human tissue, 656–657
    special populations, 657, 662
    trends in, 657–661
  health risks, 159
  methodology, 632
  significance of, 629–631
Exposure indices, 768
Exposure-response relationship, 175
Extraction methods, 75, 92
Eye opening, developmental toxicity, 356
Fagan Test of Infant Intelligence (FTII), 691, 781
Farmed fish, characteristics of, 117–118
Farmers, epidemiological studies, 749–750
Fas-FasL signaling, 314
FasL, 317
Fasting behavior, 147
Fat-flush theory, 195
Fats:
  consumption research, 105
  contamination research, 96, 108, 119
  cooking techniques and, 120
Fatty acids, 564
Fat weight, in data analysis, 96
Fecundity, 767
Female reproductive system:
  breastfeeding, *see* Breastfeeding
  developmental toxicity:
    aging reproductive tract, 373
    cleft phallus, 371
    estrous cyclicity, 372–373
    mammary gland, 373–375
    mild hypospadias, 371
    ovaries, 372
    reproductive performance, 372–373
    vaginal thread malformation, 369–371
  menstrual cycle, 900, 905–906
  pregnancy, *see* Intrauterine exposure
  spontaneous abortion, 683, 795, 814–815, 833–834
  uterine cancer, 464, 537
Feminization, 378–379
Fertility:
  reduced, 11, 25
  reproductive toxicity:
    female, 392–394
    male, 390–391
Fetotoxicity, 172
Fetus:
  developmental toxicity, 336–337, 339, 341, 345–346
  environmental contamination, 8
  TCDD exposure, laboratory animal research, 8
FGL-2, 546
Fibronectin, 354
Fingernail deformity, 900–901, 908
Finland:
  developmental epidemiology, 805, 816
  fish, contaminated, 117
  food contamination research, 114, 117, 120
  intake assessment research, 95, 99
  pollution effects, 751

First-order, generally:
decay kinetics, 202
elimination, 227
kinetics, 199, 207, 221, 270–271
Fish, *see specific types of fish*
adult, toxicity in, 604–605
adverse effects to dioxins, 171
AhR:
adaptive responses, 616–617
characteristics of, 574–577, 610–611
direct toxicity mechanisms, 615–616
indirect toxicity mechanisms, 616
interacting protein, 612–713
nuclear translocator, 611–612
repressor, 610, 613
tissue-specific expression, 613–614
toxicity mechanisms, 614–617
toxic responses, 616–617
consumption research, 18, 99–100, 102, 105, 107
contamination research, 93, 96, 108, 116–119, 166–167, 347–348
developmental toxicity, 338
embryonic, toxicity in, 605–609, 613–614
environmental contamination, 6, 8
as exposure source, 630
future research directions, 617
juvenile, toxicity in, 604–605
larval, toxicity in, 605–609, 613–614
PCB:
contamination, 24, 28, 679–680, 881
exposure research, 690–693
toxicity:
acute toxicity, 604
comparative, 609
exposure and bioaccumulation, 603–604
histopathology, 604–605
mechanisms of, 614–617
Fish oil dietary supplements, 102–103
FixL, 568
FK506, 306
FKBP12, 438
Flavones, 9
Flounder, AhR functions, 610
Fly-ash-catalyzed chlorination, 3, 66
FMN, 568
FMO5, 547
Follicle stimulating hormone (FSH), 364, 394, 534, 806, 810
Food and Drug Administration (FDA), 18, 303
Food chain, 7–8, 56, 110, 164, 191, 913
Food consumption, exposure through, 165–166
Food contamination:
animal feedstuffs, 122–123
animal products:
eggs, 115–116
meat, 113–115
milk/milk products, 110–113
overview, 110
cooking techniques and, 119–120
data analysis:
accuracy/precision, 92–93
detection limitations, 93–94
fat weight, 96
representativeness, 94–96
whole weight reporting, 96
dietary exposure:
estimating, 96–97
reduction strategies, 123–124
types of, generally, 108–110
fats, 96, 119
fish, 96, 116–119
food processing, 119–120
fruits, 96, 108–110
grains, 108–110
legislation, 97–98
oils, 119
overview, 1, 3, 89–90
pulses, 108–110
regional studies/intake assessments:
Europe, 98–103
Japan, 105, 107
Korea, 107
New Zealand, 95, 103
North America, 105
overview, 95, 107–108
time trends, 120–112
tolerable intakes, 97
toxic equivalency factors (TEFs):
implications of, 90–91
PCBs, 91–92
PCDDs, 91
PCDFs, 91
vegetables, 96, 108–109
Food diaries, 96–97, 100
Food inspection programs, 99
Food processing methods, 119–120
Food production industry, 56
Food supply, contaminated, 171
Forest fires, as contamination source, 533
Formaldehyde, 340
Fossil fuel combustion, 533, 539
France:
food contamination research, 109
intake assessment research, 99
milk contamination, 112

Free fatty acids, 148
Free radicals, 446–447
Free thyroxine (FT4), 776
Fried foods, 119–120
Fruits:
  consumption research, 105
  contaminated, 96, 108–110
Fundulus heteroclitus, 575
Fungicides, 630
Furan, 76
Furnaces, as contamination source, 74
FXR, 566

$GABA_A$, 437, 446
Gallbladder cancer, 750
Gallium oxide, 201
Gas chromatography, applications, 93, 856
Gas chromatography/mass spectroscopy (GC-MS), 208, 629, 632, 634, 895
Gasoline:
  as contamination source, 5–6
  leaded, 74, 137, 163, 165
  unleaded, 73
Gastric cancer, 742–743, 750
Gastrointestinal absorption, 192, 195
Gastrointestinal hemorrhage, 331
Gastrointestinal tract, toxic effects, 178
Gavage, 192–193
Gel permeation chromatography, 859
Gene(s), *see specific types of genes*
  expression, 149
  transcription, 174
Genetic polymorphism, 173
Genetic studies, 168
Genotoxic carcinogens, 465
Genotoxicity, 472
Germany:
  animal feedstuffs, contaminated, 122–123
  BASF cohort dose-reponse study, 272–273, 275
  contamination source research, 176–177
  environmental trends, 30
  epidemiological studies, pesticide manufacturers, 744–746
  fish, contaminated, 117, 119
  food contamination research, 109, 112–113, 115–116, 120, 122–123
  Hamburg cohort dose-response modeling study, 271–274
  intake assessment research, 99–100
  neurodevelopment, PCB contamination research, 29, 685, 688–689, 698–699
  pentachlorophenol-exposed workers, case study, 637
  pharmacokinetic studies, excretion, 222–223
  toxicity research, 176–177
Gestation index, 366
Global gene expression array research, 544–547
Glucocorticoid hormone receptor, 11, 172
Glucocorticoids, 307
Gluconeogenesis, 142, 147
Glucose:
  metabolism, 710
  tolerance, 180
Glucose-6-phosphatase (G6P), 147, 282
Glucuronic acid, 10, 905
β-glucuronidase, 213
Glucuronides, 214
Glucuronosyltransferase (UGTs), 264, 464, 476–477
Glucuronyl transferase, 172, 175
Glutamate, 446, 494
γ-glutamyl transferase, 534
γ-glutamyl transpeptidase (GGT), 465, 710, 713, 778, 832
Glutathione, 445
Glutathione-*s*-transferase (GST), 464
Goiter, 900
G1/S phase, 508, 543
G protein-coupled receptors (GPCRs), 540
GR-1^+ cells, TCDD immunotoxicology effects, 310–311
Graft versus host (GVH) response, 311–313
Grains, contaminated, 108–110
Granulocytes, 300
GRB2 protein, 530
Great Lakes embryo mortality, edema and deformities syndrome (GLEMEDS), 24, 30
Greenland shark, 610
Grilled foods, 120
Growth, PCB exposure, 684–685
Growth factor receptor signaling, 542
Growth factors, 10–11, 31, 172–173, 148
GTPase p21Ras, 539
G2/M regulatory kinases, 546
Guinea pig studies:
  absorption, 193–194
  biochemical effects, 148
  excretion, 217
  short-term toxicity, 141
  toxicity, 144, 149

Hairy cell leukemia, 739
Halogenated aromatic hydrocarbons (HAHs), 303–304, 351, 434, 440, 445, 448
Halogenated biphenyls, 564

Halogenated dioxins, 564
Haloperidol, 385
Halowaxes, 20, 164
Hamburgers, 119–120
Hamster studies:
  absorption, 193
  AhR responses, 502–503
  carcinogenesis, 460–462
  dose-response modeling, 263
  short-term toxicity, 140
  toxicity studies, 149
Hazard assessment, 303
Hazard characterization, 160
Hazardous waste, 56–57, 70, 539
Hazardous waste incinerators, 73, 163
Headache, implications of, 900
Health risk characterization, defined, 160
Heart, AhR expression, 537
Heat transfer fluids, as contamination source, 3
HEF-1, 546
Helix-loop-helix proteins, 169, 494
Hematological malignancies, 741
Hematopoietic cells, 317
Hematopoietic stem cells, 301
Hepa-1 cells, 537–538, 566
Hepatic sequestration, 167
Hepatocarcinogenesis, 458–466, 473
Hepatocellular carcinoma, 269
Hepatoma, 912
Hepatomegaly, 144
HepG2 cells, 540, 545, 547
Hepta-CDD (heptachlorodibenzodioxin), 204, 647
Hepta-CDFs, 644, 647, 858
Herbicide(s):
  characteristics of, generally, 1, 3, 20–21, 56
  exposure, 143
  pharmacokinetics, 222–223
  production, 64, 66, 182
Herbicide Orange, 64
Hexa-CDD congeners, 62–63, 75, 647
1,2,3,6,7,8-hexa-CDFs, 856, 897
Hexachlorobenzene (HCB), 194–195
Hexachlorophene, 165
Hexaclorodibenzofuran (hexa-CDF), 634, 744, 647
1,2,3,4,7,8-hexachlorodibenzofurans (hexa-CDFs), 856, 897, 909
12-HHT, 538
HIF proteins, 563
HIFα, 499, 508
HIF1α, 612
HIF2α, 612
High-density lipoprotein (HDL), 203, 534
High-dose exposure, 138, 142
High-performance liquid chromatography, 213
High-resolution gas chromatography (HRGC) analysis, 75
High-resolution mass spectrometry, 92
Hill coefficient, 257, 261
Hill kinetics, 277–279
Hill models, 256
Hippocampus, 382, 440
Histone deacetylase (HDAC), 536
HM74, 546
Hodgkin's disease (HD), 143, 751–752, 839
Hodgkin's lymphoma, 839
Hormonal effects, 175–177, 180. *See also specific types of hormones*
Hormones:
  characteristics of, 10–11, 31
  developmental toxicity research, 391–392
  ovarian, 472
  reproductive toxicity:
    female, 394–395
    male, 391–392
  synthetic, 1
  toxicology, 148
Hormone-dependent cancers, 464
Houk, U.S. Assistant Surgeon General Vernon, 1–2
HRGC with low-resolution mass spectrometry (HRGC/LRMS) analysis, 75–76
HRGC with high-resolution mass spectrometry (HRGC/HRMS) analysis, 75–76, 80–82
Hsp90 (heat shock protein 90), 145, 306, 492–494, 500, 503, 540, 610, 616
Human cancer, dose-response modeling, 275–276
Human chorionic gonadotropin, 392
Human effects, 5, 20–23
Human epidemiology, 28–30
Human exposure:
  daily, 15–16
  dermal toxicity, 144
  milk, 7–8
  risk assessment of, 15–17
Human studies:
  AhR, in disease process, 509–510
  developmental toxicity, 388–389
  dose-response modeling, 280–281
  pharmacokinetics:
    distribution, 205–206
    excretion, 217–223

Hydraulic fluids, as contamination source, 3
Hydrogen peroxide, 445
Hydrolysis, GTP, 493
Hydronephrosis:
  AhR mechanism evidence, 354–355
  characteristics of, 172, 346, 349, 353–354
  species and strain differences, 355
Hydrophilic groups, 9
Hydroquinone, 445
2-hydroxydiphenyl ether, 214
4-hydroxyestradiol (4-OH-E2), 472
2-hydroxyestradiol (2-OH-E2), 472
Hydroxyl radicals, 7
Hyperalgesia, 903
Hyperkeratosis, 23, 373, 900–901
Hyperlipidemia, 713
Hyperpigmentation, 712, 908
Hyperplasia, 148–149, 172, 373
Hypertriglyceridemia, 538
Hypertrophy:
  hepatic, 471
  implications of, 173
Hypoplasia, 148–149
Hyporeflexia, 333
Hypospadias, 371
Hypothalamic-pituitary axis, 142
Hypothalamic-pituitary-thyroid regulatory system, 881
Hypothalamus, 10, 148, 177, 380
Hypothyroidism, 685
Hypotonia, 694
Hypoxia-inducible factor (HIF), 175, 577
Hypoxia-inducible factor (hIF)-1β, 494
Hypoxia response pathway, 175

IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, 90
IFNy, 313, 316
IgA, 316, 700, 904, 909
IGF-1R, 172
IgG, 316, 700, 909
IgG2a antibody, 313
IgM, 313, 316, 700, 904, 909
IL-18, 547
IL-4, 313
IL-1, 172
Il-1β, 11, 546
IL-6, 313, 546
IL-10, 303
IL-2, 305, 308, 314–315, 319
Immune suppression, 24, 143, 176, 299. *See also* Immunosuppression
Immune system:
  characteristics of, 28, 300–303
  developmental epidemiology, 779–781, 813
  dysfunction, 534
  PCB effects:
    antibodies, 700
    infections, 700
    overview, 700–701, 711, 779–781
    T cells, 700
  toxic effects, 173, 176–177
Immunity, cell-mediated, 26
Immunoassay, 75–76
Immunoglobulin, 308
Immunology, rice oil poisoning effects, 903–904, 908–909
Immunophilin proteins, 306
Immunosuppressants, 912
Immunosuppression, 173
Immunosuppressive drugs, 306
Immunotoxicity:
  characteristics of, 11–12
  health risks, 180
  PCB effects, 680
Immunotoxicology:
  assessment, 303
  growth of, 300
  immune system basics, 300–303
  overview, 138, 299–300
  TCDD:
    AhR dependence, 304–307, 319–320
    antigen-presenting cells, effects on, 309–310
    B cells, effects on, 308–309
    $CD11B^+$ cells, effects on, 310
    epidemiology studies, 319–320
    $GR\text{-}1^+$ cells, effect on, 310–311
    indirect mechanisms, 307
    overview, 303–304
    perinatal effects, 317–319
    T-cell development, effects on, 317
    T-cell responses in vivo, functional effects, 313–319
    T cells, direct effects on, 311–313
Incineration, as contamination source, 182, 533, 539, 630
India, food contamination research, 108
Indigo, 507, 564
Indirubin, 507, 564
Indole acetic acid, 564
Indole derivatives, 162, 576
Indole-3-carbinol (I-3-C), 162, 564
Industrial accidents, 167, 181. *See also* Case studies
Industrialized countries, 15, 27, 165–166

Industrial waste, as contamination source, 533. *See also* Hazardous waste
Industrial wood combustion, 69
Infant(s):
  birth weight/size, 28, 803–804
  breast-feeding, *see* Breastfeeding
  death, 683
  formulas, 102
  milk contamination, 8
  noncancer effects, 28
  PCB exposure research, 688–699
  postnatal growth, 803–805, 810–812
Infection:
  immune system response, 301–303
  PCB effects, 700
Infectious disease, 534, 912
Infertility, 807, 813
Influenza A virus infection model, 315–317
Inhalation exposure:
  characteristics of, 7, 89
  pharmacokinetics, 201–202
Inhibitory dioxin-response elements (iDREs), 473, 499, 504
Initiating agent, 12
Initiation, in chemical carcinogenesis, 458–459, 465
Innate immune response, 300–302
Inositol 1,4,5-triphosphate-gated intracellular calcium stores, 540
Inositol phosphate (IP_3), 435–437, 439
Insulin, 148, 175, 180, 840
International Agency for Research on Cancer (IARC), 21–22, 145, 178, 248, 269, 288, 459, 743, 746–747, 754
International Joint Commission, 32–33
International Statistical Classification of Diseases and Other Related Health Problems (ICD), 744
International TCDD equivalents (I-TEQs), 102, 658–661
International toxic equivalency factors (I-TEFs), 91, 99–100, 107, 633
Intrauterine exposure:
  biochemical response, 534
  developmental toxicity, 357–392
  implications of, 654, 663, 665, 767
  rice oil poisoning effects, 906–911
Intrauterine growth retardation (IUGR), 803–805, 816
Inuit population, PCB effects:
  immune system, 700
  neurodevelopment, 687
Invertebrates:
  AhR adaptive functions, 561
  homologs of AhR and ARNT, 569, 571, 573–574
In vitro techniques, 434–435, 499, 580
In vivo techniques, 434–435
Ionomycin, 308
Ireland, intake assessment research, 100
Iron ore sintering, 73
Iron sintering, 163
Isotope dilution techniques, 76
Israel, contamination source research, 176
Italy:
  epidemiological studies:
    chlorophenol manufacturers, 749
    farmers, 749
    malignant lymphoma, 740
    soft-tissue sarcoma, 737
  intake assessment research, 100
  Seveso accident, *see* LaRoche ICMESO plant (Seveso) accident

Japan:
  air emissions, 6
  developmental toxicity studies, 332, 767
  food contamination research, 110
  intake assessment research, 105, 107
  PCB effects, 177
  rice oil poisoning in Yusho, 20, 654, 750–751, 802, 855–881
Jawless fish, 577
Johnson, U.S. Assistant Surgeon General Barry, 2
Joint Expert Committee on Food Additives (JECFA), 97
JUN gene, 540
Jun-N-terminal kinases (JNKs), 540

Kanechlor-400, 856–857, 859, 895
Karolinska Institute, 60
Kaufman Assessment Battery for Children (K-ABC), 696, 788–789, 811
38-kDA protein, 493
Keratinocytes, 144, 566
Keratosis punctata plamaris et plantaris (KPPP), 144
Keratotic disorders, 908
7-ketocholesterol, 507, 566
Killfish:
  AhR, functions of, 575–576, 610
  toxicity in, 605
Kilns:
  cement, 69–70, 73
  lightweight aggregate, 73
Korea, intake assessment research, 107
K-RAS protein, 539, 706

Laboratory animals, *see* Animal studies; Primate studies; Rodent studies
Lactational exposure:
  developmental toxicity, 330–331, 357–392
  implications of, 8, 27–29
  PCB contamination, 772–775
  pharmacokinetics, 217, 225, 231–233
  TCDD exposure, 342
Laminin, 354
Landfill fires, as contamination source, 74
LaRoche ICMESA plant, Seveso (Italy) accident:
  contamination levels, 829
  description of, 827–892
  human exposure, biological data, 830–831
  impact of:
    cancer incidence, 838–846
    developmental epidemiology, 791
    early health findings, 831–833
    generally, 1, 20, 28, 175, 222, 231, 330, 345, 533–534, 636–637, 750, 753, 768
    long-term mortality, 838–846
    sex ratio differences, 801–803, 812
    surveillance programs, 833–838
Larynx cancer, 744
Lawn herbicides, 753
Lead smelting, as contamination source, 74
Learning ability, 382–386
Learning deficits, 178
Leblanc process, 5
Legislation:
  food contamination, 97–98
  environmental, 30
Lesions:
  dermal, 144, 872
  development of, 536–537
  endometriosislike, 26
  in juvenile fish, 604–605
  liver, 144, 463
  neoplastic, 466
  precancerous, 12
  preneoplastic, 13, 281–284, 465–466
  skin, 200–201, 905
Lethal pathology, 138
Lethality, 161
Leukemia, 741, 750–751, 839–840
Leukocytes, 566, 778
Leydig cells, 391–392
Ligand(s):
  binding:
    AhR response, 493–494, 503–505, 507, 564–565, 568, 579–580
    developmental toxicity, 337–338
    immunotoxicology, 306
    in vertebrate AhRs, 574–578
  efficacy, 504
  functions of, 9, 11, 564–566
Lignin, 6
Limited pleiotropic response, 560
Limit of quantitation (LOQ), 93–94
Limits of detection (LOD), food contamination research, 93–94, 114
Linear regression, 256
Lipid(s):
  biochemical response, 537–540
  glucose metabolism, 710
  implications of, *see* Exposure assessment
  metabolism:
    generally, 173–175
    Seveso, Italy accident, 837
  PCB effects, 682, 711
  peroxidation, 339–340, 445
  tissue distribution, 211–212
Lipophilic chemicals, 138
Lipophilicity, 167
Lipopolysaccharide, 546
Lipoprotein lipase (LPL), 147–148
Lipoproteins, absorption process, 202–205
Lipoxin A4, 564
Liquid-liquid extraction methodology, 75, 82
Liver:
  absorption process, 202–204
  AhR expression, 537
  cancer, 22, 742, 878–879
  cirrhosis, 710
  damage, 11
  fatty, 173
  function, Seveso, Italy accident, 837
  hemangiosarcoma, 729
  necrosis, 144
  PCB exposure, 710
  rice oil poisoning effects, 875–876, 904–905
  tissue distribution, 203–206, 208, 210–211
  toxicity research studies, 144–145
  tumor promotion:
    mechanism of action, 469–475
    two-stage models, 465–467
  tumors, 179, 751
Livestock:
  contaminated, 167
  dermal toxicity, 144
  exposure, 110
  sensitive noncancer effects, 23–25
Localized contamination, 95, 166
Locomotor activity, developmental toxicity, 387
Log-linear models, 256

3T3-L1 cells, 501
Long-Evans (L-E) rats, 146
Long-term potentiation (LTP), 447
Love Canal, 1, 30, 56
Low-affinity ligands, 566
Low density lipoprotein (LDL), 202, 538
Low-dose exposure, 138, 177
Lower-bound estimates, defined, 94
Lowest observed adverse effect level (LOAEL), 16, 25, 179, 399–402
Lung(s):
  AhR expression, 535
  cancer, 269–273, 475, 729, 745, 747, 751, 839
  tumors, 468, 751
Lupus, 299
Luteinizing hormone (LH), 10, 28, 377, 379–380, 391–392, 394, 534, 806, 810
LXR, 566
Lymph distribution, pharmacokinetics, 202–203
Lymph node cancer of the prostate (LNCaP), 537
Lymphocytes, 13, 143, 150, 259, 563
Lymphoid cells, 307
Lymphoma:
  Hodgkin's, 737–739, 839
  malignant, 730–731, 737–741, 751
  non-Hodgkin's, 143, 178, 714, 731, 737–739, 744, 746–754, 839–840
Lymphopoietic malignancies, 748

Macrophages, 300, 302, 307, 309
Major histocompatibility complex (MHC), 301, 309IL-12, 309
Malathion, 752
Male reproductive system, developmental toxicity:
  androgenic status, 357–359
  epididymis, 364–365
  prostate, 359–361
  reproductive capability, 366–368
  seminal vesicle, 362
  sperm numbers, 365–366
  testis, 362–364
  tract effects mechanisms, 368–369
Malignant fibrohistiocytic sarcoma, 736
Malignant lymphoma, 731, 737–741, 751
Malondialdehyde, 340
Mammary carcinoma, 463–464, 537, 541
Mammary gland:
  developmental toxicity, 338, 373–375
  neoplasms, 463
  reproductive toxicity, 398–399
Mammary tumors, 375, 464
Manmade pollutants, 74
Manufacturing processes, as contamination source, 66
Margin of exposure (MOE), 180, 182
Margin of safety, 27
Marine fish, 117–118
Masculinization, 379
Mass balance model, 227
Mass spectrometry, 56, 63, 82, 93
Matching Familiar Figures Test, 692
Maternal exposure, *see* Intrauterine dioxin exposure; Maternal toxicity
  developmental toxicity and, 330–331
  pharmacokinetics, 225
Maternal toxicity:
  cleft palate and, 350–351
  developmental toxicity, 330–332, 343–345
  health risks, 172, 178
  reproductive toxicity, 392–399
Maximum tolerated dose (MTD), 458
McCarthy Scales of Children's Abilities, 691, 694
McCarthy Verbal and Memory Scales, 692, 694
MCF-7 cells, 541–542
MCF-10A cells, 544
Measurement methods:
  problems and limitations of, 81
  types of, 56, 75–81
Meat:
  consumption research, 100, 103, 107
  contaminated, 113–115, 167
  as exposure source, 630
Medaka:
  AhR functions, 610
  carcinogenesis, 462–463
  toxicity in, 606, 608–609, 617
Median bound estimates, 94
Mediastinal lymph nodes (MLNs), 316
Medical waste incinerator, as contamination source, 69, 70, 73–74
Meibomian glands, 873
Melanoma, 710
Melatonin, 172
Menstruation, rice oil poisoning effects, 900, 905–906
Mental Developmental Index (MDI), 691, 694, 781, 787, 812
Mesenchyme, developmental toxicity, 339, 353–354
Mesothelioma, 729
Metabolic degradation, 8–9
Metabolic disorders, 144
Metabolism, health risks, 167

Metabolites, pharmacokinetics:
  autoinduction of, 215–216
  structure, 213–214
  toxicity of, 214–215
Metal(s), *see specific types of metals*
  as contamination source, 3
  coexposure, 538–539
  refining, 163
Metalloid coexposure, 538
Metallothionein, 546
Metamyelocytes, 344
Metaplasia, 148–149, 172
Metastasis, 534
Methoxyresorufin *O*-deethylase (MROD), 212
3-methylcholanthrene (3-MC), 8, 475, 491
Methylmercury (MgHg), 29, 447–448
Methyl-sulfone PCBs, 862
Mg-adenosine triphosphate, 446
Microarray technology, applications, 338
Microwave cooking, 120
Milk:
  analytical accuracy/precision, 92
  breast, *see* Breast milk; Lactational exposure
  consumption research, 98–103, 105
  contaminated, 109–113, 122
  exposure through, 7–8
Milk products:
  consumption research, 102–103, 105
  contaminated, 110–113
Minker Stout, 194
Mink studies, short-term toxicity, 141
Minnesota Child Development Inventory (MCDI), 910
Miscarriage, 683, 795. *See also* Spontaneous abortion
Mitogen-activated protein (MAP), 539–540
Mode of action (MOA):
  carcinogenesis, 464–468
  dioxinlike compounds, 168–171
Mode-of-action-based dose-response modeling:
  knowledge and data gaps, 284–285
  overview, 276
  PBPK model:
    applications, 281–284
    structures, 276–281
Molecular elimination reaction, 65
Molecular mechanisms:
  AhR-null studies, 147–148, 507–508
  health risks, 174
  overview, 145–146
  sensitivity, 146
Mollusks, AhR signaling, 573
Monkey research studies, *see* Primate studies
Mono-ortho PCBs, 657
Monsanto, exposure research, 21–22
Monurone, 743
MOPS, 169
Mortality rates:
  cancer, 21–23
  prenatal, 342–346
  rice oil poisoning, 911
  Seveso, Italy accident, 838–845
  spontaneous abortion, 814–815, 833–834
  stillbirth, 392, 683
Motor reinforcement, 384–385
Mouse studies:
  absorption, 200
  AhR:
    functions, 506–507, 575
    as mediator, 497–498
  AhR-deficient, 563
  AhR-null studies, 147–148, 507–508
  cancer development, 12, 17–18
  dermal toxicity, 144, 173
  gene expression arrays, 546–547
  developmental epidemiology, 812
  developmental toxicity, 341–346, 350–375
  dose-response modeling, 263, 265, 267, 279
  reproductive toxicity, 397–398
  sensitivity to dioxin, 146, 169
  short-term toxicity, 139–140
  skin tumor promotion, 467–468
  TCDD immunotoxicology, 304–305, 308–319
  tissue distribution, 207–208, 212–213
  toxicity, 149, 172–173, 609
mRNA:
  AhR response, 501
  biochemical responses, 537–538, 544
  developmental toxicity, 377, 395
  dose-response modeling, 259, 265
  health risks and, 178
  immunotoxicology, 317–319
  pharmacokinetics, 212
  synthesis, 542
Müllerian ducts, 370
Multiple-dose studies, dose-response modeling, 260–261
Multiple myeloma, 741, 743–744, 750, 839–840
Multiple sclerosis, 299
Municipal incinerator workers, case study, 642–644
Municipal solid waste incinerator (MSWI), as contamination source, 66–68, 70, 73–74, 137, 163, 630
Municipal wastewater sludge treatment, as contamination source, 73
Mutagens, 13

Mya arenaria, 573
Myeloid cells, 307
Myeloid leukemia, 750
Mytilus edulis, 573

Na-2,4,5-TCP, 64
NAD(P)H:
  menadione oxidoreductase, 535
  quinone oxidoreductase-1 (NQO-1), 537
Naphthalenes, 2
α-naphthoflavone (ANF), 13, 906
β-naphthoflavone (β-NF), 543, 571
Nasal/nasopharyngeal cancer, 731, 741–742, 749
NaTCP, 810
National Center for Health Statistics, 273
National Food Survey, 95
National Health and Nutrition Examination Survey (NHANES), 30
National Human Adipose Tissue Survey (NHATS), 30
National Institute of Occupational Safety and Health (NIOSH):
  developmental epidemiology, 805–806, 808, 810
  dose-response modeling study, 270–271, 273, 275
  functions of, generally, 21–22, 175, 229, 273, 275, 743–744
National Institutes of Health (NIH), 248, 288
National Research Council, 159
National Toxicology Program (NTP), 145, 267, 303, 459, 463–464, 475
Natural killer (NK) cells, 300
Nausea, implications of, 143
Necrosis, 173, 469–470, 604, 606
Negative selection model, 283
Nematodes, AhR signaling, 573
Neonatal Behavioral Assessment Scale (NBAS), 690–691, 694–695
Neoplastic development, 458
Neoplastic lesions, 466
Nerve conduction velocity (NCV) tests, 901, 903
Netherlands:
  developmental epidemiology, PCB effects, 769–791, 803–804, 811–812
  developmental toxicity studies, 332–333
  epidemiological studies, pesticide manufacturers, 746
  fish, contaminated, 118–119
  food contamination research, 109, 111, 113–115, 118–120, 123
  intake assessment research, 95, 100–101
  neurodevelopment, PCB contamination research, 688–690, 694–696
  PCB effects, 177, 700–701, 769–791
Neural pathways, 697
Neurobehavioral development:
  characteristics of, 28, 30
  epidemiology, 813
  PCB effects, 781–790, 811–812
  TCDD toxicity, 332
Neurobehavioral Evaluation System (NES 2), 697
Neurodegeneration, 443–445
Neurodevelopment, PCB contamination research studies:
  Dusseldorf birth cohort, 688–689, 698–699
  Dutch birth cohort, 688–690, 694–696
  Faroe Islands birth cohorts, 685, 688, 690, 696–697
  Michigan birth cohort, 685, 688–693
  North Carolina birth cohort, 685, 688–690, 693–694
  Oswego birth cohort, 688, 698
  research results, 699
Neurodevelopmental effects, generally, 28–29
Neurologic abnormalities, PCB exposure, 712
Neurologic Optimality Scores (NOS), 788, 811
Neurological defects, rice oil poisonings, 874, 901, 903, 909–910
Neurospora, 169
Neurotoxic damage, 28
Neurotoxicants, environmental interaction, 447–448
Neurotoxicity, 180
Neurotoxins, 443–444
Neurotransmitters, 441, 443–445
Neutrophils, 310–311, 344, 445
New Zealand:
  environmental exposure, animal effects, 752–753
  epidemiological studies:
    malignant lymphoma, 740–741
    soft-tissue sarcoma, 736–737
  food contamination research, 112–113
  intake assessment research, 95, 103
α-NF, 544
NF-κB, 306, 499, 541, 616
NIEHS, 226
Night vision, 391
Nitric oxide, 446
N-methyl-D-aspartate (NMDA), 441
N-nitrosodimethylamine (NDMA), 468
Nonadditive effects, 32
Noncancer effects:
  dioxinlike compounds, generally, 177–178

dose-response relationship, 179
Noncoplanar PCBs:
gene expression research, 546
intracellular calcium:
elevated by IP_3 mechanism, 435–438
ryanodine receptor activity effects, 438–440
neurotransmitter functions, effects on, 442
Non-Hodgkin's lymphoma (NHL), 14, 143, 178, 708, 714, 731, 744, 746–754, 839–840
Non-PVC polymer production, 66
Nonverbal Analogue Profile of Mood States, 697
No observed adverse effect level (NOAEL), 16–17
North American research studies, 105. *See also specific countries*
Northern blot analysis, 546
Northern pike, toxicity in, 606–607
Norway, intake assessment research, 101–102
Notch gene expression, 318
NPAS 1,2, 169
NPAS2 protein, 568
Nqo-1, 538
NRC protein, 578
Nuclear export sequence (NES), 501
Nuclear localization sequence (NLS), 492–493
Nuclear morphology, 544
Nucleosomal fragmentation, 544
Nutritionists, functions of, 124

Occupational exposure:
case studies:
Agent Orange in Vietnam vets, 630–631, 637, 639–642
BASF factory, 636–637
Binghamton State office building fire, 631–636
chemical workers in Russia, 646–648
German pentachlorophenol-exposed workers, 637
municipal incinerator workers, 642–644
pentachlorophenol dioxin exposure in China, 647, 649
secondary exposure of worker spouses, 644–645
Vienna TCDD poisoning incident, 645–646
developmental epidemiology, 806–807
implications of, 171, 181, 275
PCB effects, 708–713
OCDF, 91, 632
Octa (O)-CDD, 91
Octa-CDD, 223, 644
Octa-CDF, 637, 647
Octachlorinated dioxin, 6
Octachlorodibenzo-*p*-dioxin (OCDD), 193–195, 203, 208, 224, 632, 753
Octa-PCDFs, 856
Ocular defects, rice oil poisonings, 873–874
Odonotoblasts, 355
Oils, contaminated, 119
Olestra, 138, 224–225, 646
Oocytes:
developmental toxicity, 336–337
fish contamination, 603
Operant conditioning, 384–385
Operation Ranch Hand:
cancer, epidemiological studies, 748, 768, 791–802, 804–805, 810, 813–815
hormonal studies, 806–807
pharmacokinetics, 221, 226, 229
Oral exposure, pharmacokinetics, 192–199
Organic chlorine, 65
Organobromines, 753–754
Organochlorines, 24, 56, 65, 71, 730, 753–754
Organogenesis, 609
Organ transplantation, 299
Orphan receptors, 11
OVA, 315
Ovaries/ovarian:
developmental toxicity, 372
hormones, 11, 472
reproductive toxicity, 394
Ovulation, 394
Oxidation-reduction processes, 496, 505–506
Oxidative stress, 14, 307, 445–447, 472, 615

Pain, epigastric, 143
PAI-2, 546
Pancreatic adenoma, 463
Pancreatic cancer, 706, 708, 714, 751
Paper mill workers, cancer research, 730, 751
Paper production, as contamination source, 3, 17, 55, 73–74, 163, 630
Paresthesia, 903
Parkinson's disease (PD), 444
Partitioning, 226, 662–663, 711
Parts per quadrillion (ppq), 641
Parts per trillion (ppt), 630, 640, 642–643, 645, 647, 649–650
PAS domain, 9, 503, 505, 507
PAS superfamily, 169–170, 252, 335, 494, 535, 567–568

Passive avoidance behavior, 386–387
PB-PK model, in pharmacokinetics, 226–227
PCBs, *see* Polychlorinated biphenyls (PCBs)
PCDDs, *see* Polychlorinated dibenzo-*p*-dioxins (PCDDs)
PCDFs, *see* Polychlorinated dibenzofurans (PCDFs)
Peabody Picture Vocabulary Test–Revised (PPVT-R), 691
P815 tumor allograph model, 313–314
1,2,3,7,8-penta-CDD, 647
4-penta-CDF, 213
1,2,3,7,8-penta-CDF, 195, 900
Penta-CDF, 637
2,3,4,7,8-penta-CDF, 193, 212, 217, 634, 856, 864–865, 900
3,4,5,3′,4′-pentachlorobiphenyl, 435–436
2,3,6,2′,5′-pentachlorobiphenyl, 438, 440
2,3,4,7,8-pentachlorodibenzofurans, 193, 897, 909
Pentachlorophenol (PCP):
  defined, 6
  dose-response modeling, 270
  exposure, 637–638
  health risks, 165, 167
Per-ARNT-Sim, 494
Percent body fat (PBF), 221–222
Perch, toxicity in, 604
Peripheral nerve system disorders, 534
Peripheral nervous system (PNS), 836–838
Peripheral neuropathy, 832–833
Peroxisome proliferators, 546
Persistent organic pollutants (POPs), 33
Pesticides:
  characteristics of, 55–56
  cohort studies, manufacturers and users, 742–747
  exposure to, generally, 143
  production of, as contamination source, 66
Petroleum refining catalyst regeneration, 73
p50, 170
p53, 542
p43, 170
P450 system, 561, 711
P4501A1, 174, 473, 478, 610
P4501A2, 212
Pharmaceutical drugs, 9
Pharmacodynamics, 287
Pharmacokinetic (PBPK) model, dose-response modeling:
  applications, 281–284
  disposition modeling of TCDD in humans, 279–280
  implications of, 280–281
  structures, 276–281
Pharmacokinetics:
  absorption:
    following dermal exposure, 199–201
    following inhalation exposure, 201–202
    following oral exposure, 192–199
  bioavailability, 194–195, 199–201
  characteristics of, 149, 168, 174
  distribution:
    in blood and lymph, 202–203
    tissue, 203–213
  excretion:
    in animals, 216–217
    fecal, 223–225
    in humans, 217–223
    lactation, 225
  metabolites:
    autoinduction of, 215–216
    structure, 213–214
    toxicity of, 214–215
  models, physiologically-based:
    overview, 225–227
    TCDD, estimating daily intake, 227–229
  special populations, prenatal/postnatal exposure, 230–234
Pharyngeal cancer, 745, 751
Phenobarbital, 536, 546
Phenoxyacetic acids, 730–731, 743
Phenoxyherbicides, 630, 637, 731
Phenylhydrazine, 546
Pheochromocytoma (PC12) cells, 436, 442, 463
Philippines, food contamination research, 113
Phorbol 12-myristate 13-acetate (PMA), 308, 310
Phosphoenolpyruvate carboxykinase (PEPCK), 147
Phospholipase A, 447
Phospholipase C, 447
Phosphorylation, 11, 170, 496, 500, 505–506, 511, 541–543
Photolysis, 7
Photosensitivity, implications of, 144
Phthalates, 56
Phycocyanobilin, 568
Phylogenic studies, AhR, 560, 568
Physiological ligands, AhR, 564
Phytochromobilin, 568
Phytoheagglutinin (PHA), 837
Picloram, 752
Pituitary adenoma, 463
Pituitary gland, 10, 476, 810
Placenta:
  AhR expression, 537
  developmental toxicity, 336–337, 340

rice oil poisoning, 906
Placental glutathione-*s*-transferase (PGST), 281, 466, 472
Planar configuration, implications of, 59–60
Plasminogen activator inhibitor, 11
Plasticizers, as contamination source, 3, 6, 55
PLC-γ, 540
Poisson regression, 256
Pokeweed mitogen (PWM), 837
Pollution abatement, 165
Polyaromatic hydrocarbons (PAHs), 8–9, 13, 491, 543–544, 559, 564
Polybrominated biphenyls, 23
Polybrominated diphenyl ethers (PBDEs), 164
Polybrominated naphthalenes (PBNs), 160, 164
Polychlorinated aromatics, 66
Polychlorinated azobenzenes (PCABs), 164
Polychlorinated azoxybenzenes (PCAOBs), 164
Polychlorinated biphenls (PCBs):
  accumulation, 67
  adult exposure, 443–448
  AhR expression and, 576
  animal exposure, 24
  Belgium dioxin poisoning, 171, 395
  case studies:
    environmental exposure, 654
    occupational exposure, 632–633, 635–637, 639–640
  characteristics of, generally, 2–3, 8, 14, 58–63, 138
  congeners, 57–58
  defined, 2
  degradation, 164
  developmental epidemiology, 773–775, 780–790, 804, 811, 816
  developmental toxicology, 334–335, 347–348, 351–352, 354–355, 357, 360, 381–390
  dose-response modeling, 269
  elimination mechanisms, 71
  environmental trends, 30
  exposure assessment:
    in general population, in human tissue, 656–657
    significance of, 629–631
  fish contamination, 603–604, 613, 617
  food contamination, 89–124
  formation of, 65
  half-life, 62, 220
  health risks, 160–163
  human health effects:
    birth weight, 683–684
    breast cancer, 701–706
    cancers, generally, 706–710
    cardiovascular disease, 710
    dermatologic abnormalities, 712
    diabetes, 710
    growth, 684–685
    immune system, 700–701, 711
    liver function, 710
    methodologies, 681–683
    neurodevelopment, 687–699
    neurologic abnormalities, 712
    overview, 20, 28–29, 679–681
    P450 system, 711
    reproductive outcomes, 683, 712
    serum lipid levels, 711
    thyroid axis, 685–687, 711
  in human tissue, 656–657
  isomers, 58
  neuronal signaling, effects on:
    central nervous system functions, 440–443
    characteristics of, 433–434, 448–449
    intracellular neuronal calcium, 435–440
    in vitro *vs.* in vivo studies, 434–435
    neurodegeneration, 443–445
    neurotransmitters, 443–445
    oxidative stress, 445–447
  nomenclature, 60
  noncancer effects, 28–29
  occupational exposure, 708–713
  perinatal exposure, 320
  pharmacokinetics:
    absorption, 194–199
    bioavailability, 194–195, 199
    excretion, 224
    lactation, 225
    metabolism, 214–215
    models, 227
    overview, 191–192
    postnatal exposure, 233–234
    tissue distribution, 206, 213
  reproductive toxicity, 357, 397–398
  in rice oil poisoning, 177, 855–864, 873–874, 877, 879–881, 895–900, 903–907, 911–912
  risk assessment, 879, 881
  sample analysis methodology, 78–79, 81–83
  sources of, 72, 164
  toxic equivalent factors (TEF), 60, 91–92
  toxicity, 177
  transport, 67
  zero discharge, 33
Polychlorinated dibenzo-*p*-dioxins (PCDDs):
  accumulation, 67
  in animal feed, 74
  biological persistence, 8
  case studies:
    environmental exposure, 651, 654–655

Polychlorinated dibenzo-*p*-dioxins (PCDDs): *(Continued)*
occupational exposure, 632–635, 645, 647–648
characteristics of, 58–63, 137–138
chemical considerations, 57–58
chlorine age production, 62
decomposition, during incineration, 72
defined, 3
developmental epidemiology, 779
dose-response modeling, 269
environmental effects, 7–8, 56
environmental trends, 30
epidemiological studies, 730, 754
exposure assessment, 629–631, 656–657, 662–663
fish contamination, 603–604
food contamination, 89–124
formation of, 5–6, 65–67
half-life, 8, 57–58, 62, 70, 218
health risks, 160–163, 180
human exposure, 21–22
isomers, 67
nomenclature, 59
pharmacokinetics:
absorption, 194–199
bioavailability, 194–195, 225
excretion, 222–224
lactation, 225
metabolism, 213–214, 216
models, 227
overview, 191–192
postnatal exposure, 230–234
prenatal exposure, 230–231
tissue distribution, 204–206, 208–209, 212
physical considerations, 57–61
production of, 66–69
potential releases, 75
rice oil poisoning, 879–881
risk assessment, 879, 881
sample analysis methodology, 75–77, 82–83
sources of, 72–74, 163–164
toxic equivalent factors (TEFs), 59, 91
transport, 67
tumor promotion studies, 466–467
Polychlorinated dibenzofurans (PCDFs):
accumulation, 67
case studies:
environmental exposure, 651, 654–655
occupational exposure, 632–635, 645, 647–648
characteristics of, 58–63, 137–138
characterization of, 160–163, 180
defined, 3
developmental epidemiology, 779
dose-response modeling, 269
environmental effects, 7–8, 56
environmental trends, 30
epidemiological studies, 754
exposure assessment, 629–631, 656–657, 662–663
food contamination, 89–124, 603–604
formation of, 5–6
half-life, 57, 62, 218–220
health effects, neurodevelopment, 687
human exposure, 20–22, 28
nomenclature, 59
pharmacokinetics:
absorption, 194–199
bioavailability, 194–195
excretion, 222–224
lactation, 225
metabolism, 213–214, 216
models, 227
overview, 191–192
postnatal exposure, 230–234
prenatal exposure, 230–231
tissue distribution, 204–206, 208–209, 212
physical considerations, 57–61
rice oil poisoning, 855–870, 877, 879–881, 895–896, 900, 906–907, 912
risk assessment, 879, 881
sources of, 2, 72, 163–164
toxic equivalent factors (TEFs), 59, 91
transport, 67
tumor promotion studies, 466–467
Polychlorinated naphthalenes (PCNs), 20, 160, 164
Polychlorinated phenols, 66
Polychlorinated quarterphenyls (PCQs), 333, 346, 375, 855–857, 859, 869–871, 896, 903
Polychlorinated quaterphenyl ethers (PCQEs), 859
Polycyclic aromatic hydrocarbons, 56
Polyethylene glycol (PEG), 63
Polyhalogenated aromatic hydrocarbons (PHAHs), 137–138, 144, 161–162, 164–166, 171, 559–560, 562, 578
Polymorphisms, AhR, 502–503, 510–511
Polyvinyl chloride (PVC), 6, 66
Pores, defined, 144
Pork:
consumption research, 102, 105, 107
contamination research, 98, 113–115
Porphyria:
characteristics of, 174
hepatic, 173, 905

Porphyria cutanea tarda, 144, 752
Porphyrin, 876, 903–905, 907–908
Postnatal exposure, 230–234, 320. *See* Breastfeeding; Infant(s); Lactation
Potassium alkoxide, 63
Potassium chlorate, 743
Potassium hydroxide, 63
Potency, 16
Poultry/poultry products:
  consumption research, 100, 105
  contaminated, 167
  as exposure source, 56, 74–75, 630
PPAR, 566
Preconcentration, 75
Pregnancy, *see* Intrauterine exposure; Intrauterine growth retardation; Prenatal exposure
Prenatal exposure:
  health risks, 177
  implications of, 334–335. *See also* Developmental toxicity
  noncancer effects, 28
  pharmacokinetics, 230–234
  TCDD, 8
Prenatal mortality, developmental toxicity, 342–346
Preneoplastic lesions, 281–284, 465–466
Pressure cooking, 120
Primate studies:
  biochemical effects, 148
  dermal toxicity, 144
  developmental toxicity, 344, 347, 387–388
  dose-response relationships, 178
  neurobehavior effects, 387–388
  PCB effects, 443–444
  reproductive toxicity, 392–393, 396–397
  short-term toxicity, 140
  TCDD effects, 26, 176
  tissue distribution, 205, 208–209
Production process, as contamination source, 64–67
Progression, in chemical carcinogenesis, 458–459
Proliferating cell nuclear antigen (PCNA), 362, 398–399
Prolines, 494
Promoting agent, 12
Promotion, in chemical carcinogenesis, 458–459
Proportional mortality ratio (PMR), 749
Prostaglandin E_2, 307
Prostaglandins (Pgs), 507, 537
Prostate:
  developmental toxicity, 339, 359–362
  lymph node cancer of (LNCaP), 537
Proteases, 447
Protein kinase C (PKC), 11, 436, 439, 505
Protein kinases, 11, 435
Protein-protein interaction, 169–170, 561
Proteins, functions of, 9, 142. *See also specific types of proteins*
Prothymoctye stem cells, 317
Protooncogenes, 14, 458
Provisional tolerable monthly intake (PTMI), 97
Psychomotor Developmental Index (PDI), 691, 696, 781, 787
p21 protein, 542
p23 enzyme, 145
p27 protein, 542
p27Kip1 protein, 508
p38, 540
p37, 170
p300 protein, 508, 578, 616
Pulp mill workers, exposure case study, 730, 751
Pulp mills, as contamination source, 17, 55, 68, 73–74, 163, 630
Pulses, contaminated, 108–110
Purified protein derivative (PPD), 904
Purkinje cells, 438
PXR/SXR, 579

Quatraphenyls, 160
Quinone, 447, 472, 906

Rabbit studies:
  biochemical effects, 148, 538
  dermal toxicity, 144
  developmental toxicity, 336, 340
  short-term toxicity, 139
  toxicity, 149
Radical-molecular pathways, 65
RAS, 543
RAS mitogen-activated protein (MAP) kinase pathway, 539–540, 545
RAS-RAF-MEK signaling pathway, 540
Rat studies:
  absorption, 192–193, 201
  AhR regulation, 501–502
  biological persistence, 10
  carcinogenesis, 12–13, 17–18, 22, 460–464
  developmental epidemiology, 766, 805–806
  developmental toxicity, 341, 348–349, 353, 355, 357–390
  dose-response relationship, 179, 261–268, 278

Rat studies: *(Continued)*
excretion, 216
hepatocarcinogenesis, 458–466
hormone levels, 10, 26
noncancer effects, 25–27
pharmacokinetics, 225–227
reproductive toxicity, 393–394, 397–398
sensitivity to dioxin, 146, 176–177
short-term toxicity, 140–141
TCDD immunotoxicology, 318
testosterone levels, 26
tissue distribution, 203–204, 206–208, 210–211
toxicity studies, 149
tumor incidence, 463–464
tumor progression, 12–14
wasting syndrome, 142
Receptor response, *see specific types of receptors*
Recombination reactions, 65
Rectal cancer, 745, 750, 839
Reference dose (RfD/RfC), 182
Regulatory agencies, 32–33
Reilly, William, 1
rel, 170
RelA, 541
Relative potency factors (REPs), 161–162
Reporter genes, 617
Representativeness, in data analysis, 94–96
Reproductive effects, *see* Reproductive toxicity
dose-response modeling, 263
health risks, 172–173, 177
rice oil poisoning effects, 905–906, 910–911
toxicology, 138
Reproductive epidemiology:
endometriosis, 807
future research directions, 812–813
hormones, 805–807, 809–810
Reproductive system, effects on, 172–173
Reproductive toxicity:
Agent Orange research, 642
biochemical response, 534
female:
antiestrogenic action, 394–395
endometriosis, 395–398
mammary gland, 398–399
ovarian function, 394
reproductive function and fertility, 392–394
health risks, generally, 180
male:
hormone level alterations, 391–392
reproductive function and fertility, 390–391
PCB exposure, 683, 712
sensitivity endpoints:
human susceptibility, 402
maternal and fetal TCDD body burdens, 402–403
TCDD LOAELs, 399–402
TCDD, generally, 333
Reservoirs, as contamination source, 166
Resin manufacturing, 55
Respiratory cancer, 744–745
Respiratory disease, 28, 176
Respiratory system:
carcinogenesis, 475
rice oil poisoning effetcs, 874–875
Respiratory tract infection, 316
Restricted pleiotropic response, 560
Retinoblastima, 508
Retinoblastoma protein (RB), 170, 499, 508, 541–542
Retinoblastoma tumor suppressor protein, 306
Retinoids, 172
Reynell Development Language Scale (RDLS), 696, 788, 811
Rice oil poisonings:
case studies:
Yucheng, Taiwan, 20, 28, 177, 893–911
Yusho, Japan, 20, 871–881
cancer, epidemiological studies, 750–751
developmental toxicity, 330, 333, 344–348
dose response modeling, 273
future research directions, 912–913
health effects, generally, 177, 767, 900
pharmacokinetics, 223
treatment, 911
RIP-140, 395, 496
Risk assessment:
cancer, 14–18, 31
defined, 159
Risk assessors, functions of, 124
Risk characterization, defined, 159–160
Risk management:
decision-making process, 160
defined, 159
Rodent studies, *see specific types of rodents*
absorption, 192–193
acute toxicity, 604
AhR regulation, 501–502
biochemical effects, 147–148
carcinogenesis, 458–479
developmental toxicity, 343, 355, 359
dose-response modeling, 263, 278–279
excretion, 216–217
pharmacokinetics, 225–227
reproductive toxicity, 397–398, 400–402

rice oil poisoning effects, 911
sensitivity, 174, 176–177
short-term toxicity, 140–142
toxic endpoints, 144–145
toxicity research, 149–150, 174
RT-PCR, 546
RTG-2 cells, 611
Russia:
chemical workers case study, 631, 646–648
contamination research, 7
intake assessment research, 102
milk contamination, 112
PCB production, 164
Rutter's Behavioral Scale, 909
Ryanodine receptor (RyR), 438–442, 446–448

SAA112, 547
Saccharin, 379
Sample extract analysis, *see* Analytical methodology
Sample workup procedures, 56, 81–82. *See also* Analytical methodology
Saos-2 cells, 542
Sarcoplasmic/endoplasmic reticulum (SR/ER), 438, 440
Schistosomiasis, 108
Scientific Co-operation on Questions relating to Food (SCOOP) Task, 98–99, 109, 111, 119
Scientific Committee on Food (SCF), 97
Sebaceous gland, 144
Sebum, 217
Secondary exposure, worker spouses case study, 644–645
Sediment contamination, 7–8, 30, 56, 67, 70, 164–165, 654
Selected Cancer Cooperative Study Group, 741
Seminal vesicle, developmental toxicity, 362
Semiquinone, 472
Sensitive noncancer effects:
laboratory animals, 25–26
livestock, 23–25
near background levels:
animal evidence, 26–27
human epidemiology, 28–30
wildlife, 23–25
Sensitivity:
in analytical methodology, 56, 82
health risks, 171–172
modes of action and, 168–169
toxicity research, 146, 150
in toxicology, 146
Serine, 494, 540
Serotonin, 148
Serum response element (SRE), 541
Sewage sludge:
commercially marketed, 73
as contamination source, 6, 69
fertilization, 110
sludge incineration, 73
Sex ratio differences:
developmental epidemiology, 801–803, 812, 816
developmental toxicity and, 348–349
rice oil poisoning effects, 906–907
Sexual behavior, male, 377–381
Sexual differentiation, 378–380
SGOT, 904
SGPT, 904
SHC protein, 530
Sheep red blood cells, 318
Shellfish, consumption research, 100, 105
Short-term toxicity, 139–141
Signal transduction pathways:
activation, 539–541
biochemical responses, 535
Signal-to-noise (S/N) ratio, 93
Silica, 66
Single-dose studies, dose-response modeling:
adult animals, 261–262
developmental studies, 262–264
Sister chromatid exchange (SCE), 13, 906
SK-Hep-1, 503
Skate, AhR functions, 610
Skeletal muscle, AhR expression, 537
Skin, *see* Dermal defects; Dermatology
allergy, 900
disorders, PCB effects, 712
tumor promotion, two-stage models, 467–468
Slope factor, 16–17
Smoking-related cancer, 270
SMRT protein, 496
Sodium pentachlorophenate, 108
Sodium pentachlorophenol (Na-PCP), 647
Soft tissue sarcoma (STS), 21, 178, 730–737, 751, 754
Soil contamination, 7–8, 56, 67, 70, 194
Solid-phase sorption, 61
Sonication, 75
SOS protein, 530
Sources, generally, 2–7, 72, 74, 163–164
Soxhlet extraction, 75, 82
Spain:
environmental trends, 30
food contamination research, 109, 113–115
intake assessment research, 102
Spatial learning ability, 382–384

Specificity:
in analytical methodology, 56, 82
defined, 809
in toxicity, 173–174
Sperm numbers, developmental toxicity:
ejaculated, 366
epididymal, 365–366
Spermatocytes, 390, 815
Spermatogonia, 815
Spermatozoa, 390
Spleen:
AhR expression, 537
TCDD immunotoxicology, 305, 310, 318–319
Sp1 protein, 496, 500, 535
Spontaneous abortion, 814–815, 833–834
Sprague-Dawley rats, 192–193
Squamous cell carcinoma, 462
SRC kinase, 540
SRC-1, 169, 395, 616
Standard incidence ratio (SIR), 743, 749, 751
Standardized Progressive Matrices (SPM), 910
Standard mortality ratios (SMRs), 270–272, 710, 743–751, 911
Stanford-Binet IQ, 910
Steady-state approximation, 228
Sternberg Memory Test, 692
Steroid hormones, 8–9, 11, 170
Stillbirth, 392, 683
Stomach cancer, 745, 751, 839
Stroma, developmental toxicity, 339–340
Structure-activity relationship (SAR), 145, 346, 436, 497–498
Subcutaneous edema, 331, 334, 347
Subtractive hybridization, 563
Sudden infant death syndrome, 233
Superoxide anion (SOA), 445, 447
Surface-catalyzed synthesis, 65
Sweden:
developmental toxicity research, 347
epidemiological studies:
breast cancer, 753
malignant lymphoma, 731, 737–739
soft-tissue sarcoma, 731–735
environmental trends, 31
fish, contaminated, 117
intake assessment research, 103
PCB effects, immune system, 700
postnatal exposure, 232–233
Synergism, 447–448, 494
Synthetic chemicals, 630

Taiwan, Yucheng rice oil poisoning, 20, 28, 108, 177, 654, 767, 893–913
TATA box, 535
TBP (TATA-box binding protein), 495
TCDD, *see* 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD)
TCDD equivalents (TEQs):
absorption, 195
developmental epidemiology, 805, 807, 812
exposure assessment:
case studies, 644, 647, 649–651
general population, 656
trends, 657
food contamination research, 91–94, 97–98, 101, 107, 120
implications of, generally, 3, 8, 15, 28–29, 72, 74, 161–162
PCBs, health effects research, 695
pharmacokinetics, 234
reproductive toxicity, 396–398
rice oil poisonings, 865, 879, 881
tissue distribution, 211
TCDD-like chemicals, characteristics of, 334–335
2,3,7,8-TCDF, 647
TCF, 541
T cells:
developmental epidemiology, 779
PCB effects, 700
rice oil poisoning effects, 904, 909
TCDD immunotoxicology:
direct effects, 311–313
indirect mechanisms, 307
perinatal effects, 317–319
responses in vivo, functional effects, 313–319
TEB cells, 398–399
Teeth, *see* Dental defects
Teratogenesis, 138, 254, 340, 534
Teratology, rice oil poisoning effects, 907
Terphenyls, 160
Testis, developmental toxicity, 362–364
Testosterone levels, implications of, 10, 26, 175, 357, 377, 380, 391–392, 534, 810
2,2′,4,4′-tetrabrominated diphenyl ether, 754
2,3,7,8-tetra-CDD, 865
3,3′,4,4′-tetrachloroazoxybenzene (TCAOB), 351, 390–391
1,2,4,5-tetrachlorobenzene, 64
2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD), *see* TCDD
AhR functions, *see* Ah receptor (AhR)
antiestrogenic effects, 394–399
biological persistence, 8–9
as carcinogen, 12–14, 16–17, 22, 31, 55, 457–479

characteristics of, generally, 138
chemical considerations, 57
daily doses, acceptable or tolerable, 15–16
defined, 2
development epidemiology, 765–769, 791–817
dose-response modeling:
  biological responses, 252–254
  characteristics of, 247–248, 255–256, 286–291
  data gaps in assessment, 285–286
  dose metric choice, 248–252
  empirical, 256–276
  mode-of-action-based, 276–285
as environmental hormone, 11
environmental exposure, effects:
  animals, 752–753
  humans, 751–752
environmental trends, 30
estimating daily intake, 227–229
exposure assessment, 630–632, 634–637, 639, 642, 645–647, 649–654, 663
fish contamination, 6, 603–617
food contamination, 89–91
formation, 3
growth factors, influences on, 11
half-life, 7–8, 27, 57–58, 62–63, 67, 71, 138, 218–225, 229, 233
health risks, generally, 160–163
hormones, influences on, 10–11
immunotoxicology:
  AhR dependence, 304–307
  antigen-presenting cells, effects on, 309–310
  B cells, effects on, 308–309
  $CD11B^+$ cells, effects on, 310
  epidemiology studies, 319–320
  $GR\text{-}1^+$ cells, effect on, 310–311
  indirect mechanisms, 307
  overview, 303–304
  perinatal effects, 317–318
  T-cell responses in vivo, functional effects, 313–319
  T cells, direct effects on, 311–313
industrial accidents, *see* LaRoche ICMESA plant
maternal and fetal body burdens, 402–403
noncancer effects, 274–275
LOAELs, 399–402
modes of action, 169–170
PB-PK model, 226–227
pharmacokinetics:
  absorption, 192–194, 199–202
  blood and lymph distribution, 202–203
  bioavailability, 194, 200–201
  daily intake estimates, 227–229
  excretion, 216–217, 221–225
  lactation, 225
  metabolism, 213–216
  models, 226–229
  overview, 191–192
  postnatal exposure, 230–233
  prenatal exposure, 230–231
  tissue distribution, 203–208, 210–213
physical considerations, 57–58
production process, 64
rice oil poisonings, 859–860, 862–863
sample analysis methodology, 76, 80–81
sources of, generally, 533
thymic atrophy and, 143
toxicity, 176
tumor progression, 12, 149, 275
water quality standards, 18–19
2,3,7,8-tetrachlorodibenzofuran (TCDF), 193–195, 222–223
1,3,7,8-tetrachloro-2-hydroxydibenzo-*p*-dioxin, 214
Tetracycline, 752
12-*O*-tetradecanoylphorbol-13-acetate (TPA), 504
Tetrapyrroles, 507, 568, 576
TFIIF/TFIIB, 495
T4 cells, 14, 175–176
TGFα, 544
TGFβ, 504, 507
TGFβ1, 543
TGFβ2, 543
Thermoregulation, 389–390
Threonine kinases, 540
Threonines, 494
Thymic atrophy, 143–144, 161, 172, 317
Thymic hypoplasia, 334, 349
Thymic involution, 534
Thymocytes, 317, 543
Thymus:
  AhR expression, 537, 562
  atrophy of, 11
  TCDD immunotoxicology, 303, 305–306
Thyroid:
  carcinogenesis, 476
  developmental epidemiology, 812
  hormone levels, 10, 28, 680
  PCB effects, 680, 775
Thyroid axis, PCB effects, 685–687, 711
Thyroid disease, 534
Thyroid-stimulating hormone (TSH), 10, 176, 264–265, 476, 478, 685–687, 775, 777–778

Thyroxin, 253, 259
Thyroxin glucuronidation (T4UGT), 264–265
Thyroxine (T4), 476, 685–686, 776
Thyroxine-binding globulin (TBG), 776, 778
Time-of-flight mass spectrometry (TOF-MS), 82
Times Beach accident, 1–2, 23, 171, 194, 345, 752
Tire combustion, as contamination source, 74
Tissue distribution:
  dose-response modeling, 276
  pharmacokinetics:
    disproportional dose-dependent, 212–213
    dose-dependent, 210–212
    humans, 205–206
    laboratory animals, 203–213
    time-dependent, 206–210
T lymphocytes, 143, 173, 300
TNFα, 546
Tobacco smoke, impact of, 510, 706
Toenail deformity, 900–901, 908
Tolerable daily intake (TDI), 29–30, 97, 123, 257. *See also* Tolerable intakes
Tolerable intakes, food contamination and, 97
Tolerable weekly intake (TWI), 97
Tomcod, AhR functions, 610
Total diet study (TDS), 95, 107, 116, 121
Toxic endpoints, 144–145, 149
Toxic equivalence factor (TEFs):
  carcinogenesis, 467
  exposure assessment, case studies, 632–633, 639
  food contamination, 90–91
  health risks, 161–162, 180
  implications of, generally, 57–60
  PCBs, 91–92
  PCDDs, 91
  PCDFs, 91
Toxicity:
  dermal, 143–144
  implications of, 11–12, 18, 138
  short-term, 139–141
  thymic atrophy, 143–144
  toxic endpoints, 144–145
  wasting syndrome, 142–144
Toxic mimicry, 11
Toxicologists, functions of, 124
Toxicology, generally:
  biochemical effects, 147–149
  implications of, generally, 137–138
  molecular mechanisms:
    AhR-null studies, 147–148, 507–508
    overview, 145–146
    sensitivity, 146
  species differences, 149–150
  toxicity, 138–145
Toxic response pathway, 560, 616–617
Trace chemistry of fire hypothesis, 5
Transcription activity domain (TAD), 494–495
Transcription factors:
  AhR response, 494
  biochemical response, 541–542, 545
  developmental toxicity, 337–338
  in toxicology, 145
Transcription inhibitory domain (TI), 497
Transformers, as contamination source, 3, 164
Transgenic mice, *see* Mouse studies
Transport, 67, 164–165. *See also specific types of dioxins*
Transpulmonary absorption, 201
Trash burning, as contamination source, 163
Trend analysis, 72, 74
Trichlorophenols, 64, 143–144, 534
2,4,5-trichlorophenol, 744
2,4,5-trichlorophenoxyacetic acid (2,4,5-T), 64, 66, 637, 646, 731, 740–743, 752, 766
2,4,5-trichlorophenoxyacetic ester, 534
Trichlorophenyls, 3
Triglycerides, 838, 905
Triiodothyronine (TTE), 685, 687
Trinitrophenyl lipopolysaccharide (TNP-LPS), 308
Troponin, 546
Trout, toxicity in:
  brook, 606
  bull, 606
  lake, 605–607
  rainbow, 604–607, 610–611
Tryptamine, 564
Tryptophan, 148, 507, 564
Tumor growth, 149
Tumor growth factor (TGF), 172
Tumor necrosis factor, 142
Tumor progression, 12
Tumor(s), *see specific types of tumors*; Tumor promotion
  generally, 10
  increased sites, 460–463
  spontaneous, 463–464
  suppressors, 542
Tumor promotion:
  biochemical responses, 534–535
  health risks, 173
  mathematical models, 474–475
  PCB effects, 680
  research studies, 162
  spontaneous, 463–464

Tumor suppressor genes (TSGs), 14, 458
TUNEL, 317, 543
Tunisia, food contamination research, 113
Tyrosine hydroxylase (TH), 436, 444
Tyrosine kinases, 170, 306, 539, 543

UDP-glucuronosyltransferase (UDPGT), 10, 148, 535
UDPGT1*6, 253
Ultraviolet radiation, 67
Umbilical cord, PCB exposure research, 698, 772–773
Umbilical cords, 28
United Kingdom:
  epidemiological studies, chlorophenol manufacturers, 748–749
  fish, contaminated, 117–118
  food contamination research, 109, 111–113, 115–118, 120, 122
  intake assessment research, 95, 103
United States:
  air emissions, 6
  epidemiological studies:
    malignant lymphoma, 739–740
    pesticide manufacturers, 743–744
    soft-tissue sarcoma, 735–736
  exposure sources, 165–167
  food contamination research, 113–114, 116
  intake assessment research, 106
  PCB effects research:
    health risks, 177
    immune system, 700
    neurodevelopment, 685, 688–694, 698
U.S. Clean Air Act, 30
U.S. Department of Agriculture (USDA), 74–75, 105
U.S. Department of Health and Human Services (DHHS), 178, 269, 459
U.S. Environmental Protection Agency (USEPA), 1, 12, 14–19, 21, 23, 31–32, 57, 64, 74–76, 80–81, 161, 165, 178–179, 181–182, 256, 258, 269, 273, 287–288, 303, 435, 439, 478, 632
U.S. National Cancer Institute, 735
Upper-bound estimates, defined, 94
Uroporphyrin, 905
Uroporphyrinogen decarboxylase (UROD), 144, 147, 174
Uterus/uterine:
  cancer, 464, 537
  developmental toxicity, 339–340
  tumors, 463
Utilities, as contamination source, 73

Vagina, developmental toxicity, 369–371
Vapor, as contamination source, 7
Vascular smooth muscle cells (vSMCs), 537–538, 543
Vasculature endothelial cells (vECs), 537
Vegans, exposure of, 657, 662
Vegetables:
  consumption research, 101, 105
  contaminated, 96, 108–110
VEGF, 546
Vertebrates:
  AhR responses, 574–578, 616
  comparative toxicity, 609
Very low density lipoprotein (VLDL), 202–203
Vesicular monoamine transporter (VMAT2), 442
Vienna, TCDD poisoning incident case study, 645–646
Vietnam, environmental exposure, 649, 651–654. *See also* Agent Orange; Vietnam veterans
Vietnam veterans:
  Agent Orange, 735, 740, 742
  developmental epidemiology, 767, 792
  epidemiological studies, 58, 747–748
  liver cancer, 742
  malignant lymphomas, 740
  soft-tissue sarcoma, 735–736
Vinyl chloride, 729
Viruses, 14
Visual discrimination reversal learning, 384
Visual evoked potentials (VEPs), 697
Vitamin A:
  deficiency, 142
  importance of, 148, 172
Vitamin E, 341
Vitellogenesis, 603
Volatile organic compounds (VOCs), 138, 629
Volatilization, 164
Voltage-sensitive calcium channels (VSCCs), 437–438
Vomiting, implications of, 143

Washing procedures, 82
Waste incineration, as contamination source, 1, 3, 6–7, 55, 65–66
Wastewater sludge treatment, 73
Wasting syndrome, 11, 142–144, 147, 172, 534, 706
Water quality standards, 18
Webb-McCall method, 690, 713
Wechsler Intelligence Scales for Children–Revised (WISC-R), 693, 697, 909–910
Weibull model, 258, 261

Weight changes, implications of, 142, 161, 471–472
Werry-Weiss-Peters Activity Scale, 909
WHO/FAO, Joint Expert Committee on Food Additives (JECFA), 97
WHO-TEF, 91, 467
WHO-TEQ, 101, 109
Whole weight reporting, in data analysis, 96
Wide Range Achievement Test–Revised, 693
Wildlife studies, 23–25
Wolffian ducts, 370
Women:
  breast cancer research, *see* Breast cancer
  breastfeeding, *see* Breastfeeding; Lactation
  intrauterine exposure, 654, 663, 665
  menstrual cycle, 900, 905–906
  ovulation, 394
  reproductive toxicity, *see* Female reproductive system
Wood:
  burning, as contamination source, 73
  pentachlorphenol-treated, 167
  preservatives, 3, 630
Woodcock Reading Mastery Test–Revised, 693
World Health Organization (WHO), 21, 29–30, 55, 57–58, 60, 91, 162, 182, 630, 632, 859, 865
World War I, 2, 20
Wy-16,463, 546

X disease, 23
X-ray fluorometry, 856, 858
XAP2, 145, 170, 569, 610
Xenobiotics, generally:
  AhR response, 504, 509, 564, 616
  biochemical response, 535, 541
  characteristics of, 9–10, 28
  developmental toxicity, 337
  dose-response modeling, 276
  health risks, 170, 172, 174
  PCB effects, 711
Xenobiotic response element (XRE), 145–146, 169
XMP, 546

y-glutamyl transpeptidase (GCT), 281–282, 472
YAC-1 tumor cells, 311
Yeast, 571

Zebrafish:
  AhR, 576, 610–616
  toxicity in, 606–608, 613–615, 617
Zebrafish liver epithelial (ZLE) cells, 612