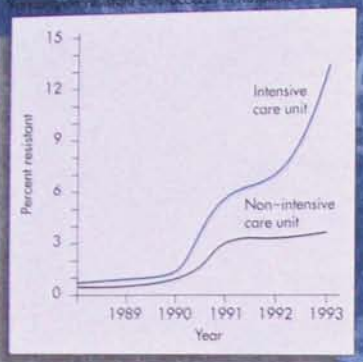
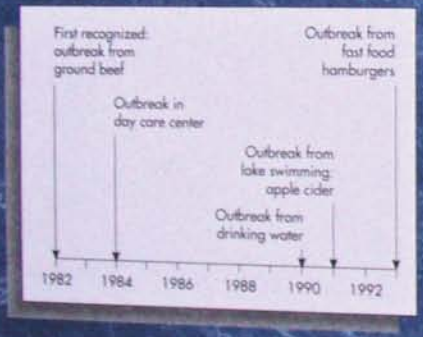
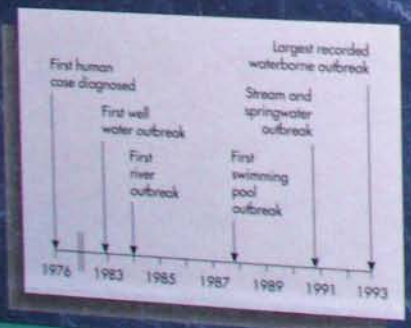
Microorganisms in our World

Ronald M. Atlas

Emerging Infectious Diseases







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Ronald M. Atlas, PhD
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Louisville, Kentucky

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PREFACE

The field of microbiology is fascinating. Most people associate microorganisms only with disease. However, cheese, wine, and sourdough bread, for example, are products of microbial metabolism. The quality of our environment also depends on the activities of microorganisms. *Microorganisms in our World* will introduce the student to the wide world of microbiology. A text written by a microbiologist, *Microorganisms in Our World* gives the perspective of microbiology as a broad relevant field and of microorganisms as important to human health and the functioning of planet Earth.

THE BOOK

The text is written in a manner that is easy to read. Difficult topics are thoroughly explained so that they can be easily understood. Technical terms are explained as they are introduced and a conceptual framework of microbiology is presented. Students will learn how to communicate microbiological information. They will be able to understand the numerous news reports related to microbiology, especially those about relationships between microorganisms and human health. The depth of presentation is limited to the essentials but provides enough information to develop an understanding of a topic. The information presented represents a distillation of endless volumes of scientific studies. Facts are presented that reflect the latest state of knowledge. Accuracy is not sacrificed for brevity or simplicity. The breadth of the field of microbiology is covered at an appropriate level for students.

Topics are organized in a logical yet flexible manner. Coverage of medical microbiology emphasizes microorganisms and how they cause disease. The sense of discovery is used to entice students into wanting to learn more. Microbiology is an exciting field, and students should develop enthusiasm toward scientific discovery. They should never be bored by the information they are learning. The text contains numerous boxes with items that will interest students. The relevance of microbiology in our daily lives and selected careers is presented so that today's pragmatic students know why they are studying a particular topic and want to learn about microorganisms.

The diversity of the microbial world is shown with relevant examples of how microorganisms affect our daily lives. The interactions of microorganisms with humans are highlighted, especially as they relate to human disease. The properties of microorganisms that are responsible for their ability to cause disease and permit prevention, diagnosis, and treatment of disease are emphasized.

ORGANIZATION

Microorganisms in our World is organized into six units. The first three units focus on the principles of microbiology, including cell structure, cellular metabolism, microbial genetics, and growth. Unit Four and Unit Five focus on the medical applications of microbiology—microorganisms as disease-causing entities and the body's defenses against infection. The final unit of the text discusses microbiological applications to industry and the environment.

Microorganisms and Microbiology (Unit One) introduces the student to the field of microbiology and the techniques that are used by microbiologists in research and clinical settings. Chapter 1 (Unity of the Microbial World) introduces the field of microbiology, giving insight into the relevance of microorganisms of human health and how microbiologists work—the scientific method. Chapter 2 (Diversity of the Microbial World) continues by introducing the student to the vast breadth of the microbial world. Chapter 3 (Science of Microbiology: Methods for Studying Microorganisms) discusses the techniques that microbiologists use to do their work. The various techniques of light and electron microscopy are explained in addition to coverage of culturing, aseptic technique, and plating. Unit One concludes with Chapter 4 (Chemistry for the Microbiologist)—an introduction for students who have not had a basic chemistry course and a convenient review for those who have.

The text continues with coverage of **Cellular and Molecular Microbiology** (Unit Two). Chapter 5 (Cell Structure) introduces the student to the general

structure of prokaryotic cells and compares and contrasts them to the structure of eukaryotic cells. Chapter 6 (Cellular Metabolism) explains the energetics of microorganisms—cellular metabolism. Chapter 7 (Microbial Genetics: Replication and Expression of Genetic Information) discusses microbial genetics and is immediately followed by an introduction to biotechnology in Chapter 8 (Genetic Recombination and Recombinant DNA Technology).

Unit Three focuses on **Microbial Growth and Its Control.** The unit begins with a study of growth in viruses in Chapter 9 (Viral Replication), and Chapter 10 (Bacterial Reproduction and Growth of Microorganisms) focuses on the growth of bacteria. Chapter 11 (Control of Microbial Growth and Death) discusses how bacterial growth can be minimized or stopped using techniques such as pasteurization and sterilization and antimicrobial agents, including preservatives, disinfectants, and antibiotics.

In **Microorganisms and Human Diseases** (Unit Four) the content of the book moves from the principles-based coverage of microbiology in the first three units to more applied topics showing how microorganisms affect our lives. Chapter 12 (Microorganisms and Human Diseases) introduces Koch's postulates, the virulence factors of pathogenic microorganisms, and transmission routes of infectious agents. The body's immune response to infection is explained first in Chapter 13 (Nonspecific Host Defenses against Microbial Infections: The Immune Response), followed by Chapter 14 (Specific Host Defenses against Microbial Infections: The Immune Response). Chapter 14 (Immune Response and Human Disease) discusses topics that include immunization, immunodeficiencies, autoimmunity, hypersensitivity reactions, and transplantation. Chapter 15 (Diagnosis of Human Disease) introduces the student to methodology used in the clinical microbiology laboratory. These methods include skin testing, culturing, immunofluorescence, gene probes, and the polymerase chain reaction. This unit concludes with a discussion of the uses of antimicrobial agents in Chapter 17 (Treatment of Infectious Diseases).

The **Infectious Disease** unit (Unit Five) is comprised of six chapters. Diseases are organized by the causative microorganisms and then divided into the system's of the body that they affect. This allows the student to understand commonalities of viral infection and pathogenesis of viral disease, separate from the treatment of bacterial diseases. This unit begins with two chapters covering viral diseases—Chapter 18 (Viral Diseases of the Respiratory, Gastrointestinal, and Genital Tracts) and Chapter 19 (Viral Diseases of the Central Nervous, Cardiovascular, and Lymphatic Systems). Three chapters follow, focusing on the bacterial diseases—Chapter 20 (Bacterial Diseases of the Respiratory and Gastrointestinal Tracts), Chapter 21 (Bacterial Diseases of the Central Nervous, Cardiovascular, and Lymphatic Systems), and Chapter 22 (Bacterial Diseases of the Urinary Tract, Genital Tract, Skin, Eyes, Ears, and Oral Cavity). The unit concludes with Chapter 23 (Diseases Caused by Eukaryotic Organisms).

The final unit of the text, **Applied and Environmental Microbiology** (Unit Six), explains the importance of microbiology in regard to industrial and environmental applications. Chapter 24 (Industrial Microbiology) covers various topics, including fermentation, production of antibiotics, and the recovery of mineral resources. Chapter 25 (Environmental Microbiology) details the role of microorganisms in solid waste disposal, water treatment, biodegradation of pollutants, and other selected applications.

Because prerequisites for this course vary from school to school and an understanding of the anatomy and physiology of the human body is important to understanding microbiology, an Anatomy Appendix has been added to the text. This illustrated appendix offers a brief overview of the structure and function of each of the eleven body systems, including a section that details the importance of each system from a microbiological perspective.

FEATURES

Many features have been added to *Microorganisms in our World* to make the content accessible and relevant to the student. The strong emphasis on disease-causing microorganisms will be especially interesting to students entering health careers and general education students. The application of microbiological principles to human health is important to the reader.

Boxed Material

Boxed asides have been added to the text to make the microbiological principles and applications more relevant to the students. Four types of boxes appear in the text; each will focus on a specific area as detailed below:

- **Newsbreak:** Features important advances and current events in the field of microbiology.
- **Highlight:** Expands coverage on certain topics that are important in the study of microbiology.
- **Historical Perspective:** Shows the relevance of the past of microbiology.
- **Methodology:** Shows students how microbiologists apply scientific methods and approaches in the study of microorganisms.

Case Studies

Since many students will be entering the health professions and disease applications are interesting to them, clinical case studies have been added to the end of each of the chapters in Unit Five. Written by a medical doctor, these case studies provide the story of a patient's illness from the initial assessment at the health care facility through laboratory findings and diagnosis, culminating with a discussion of treatment and course of the disease. Because most students have not taken a pathophysiology course, italicized remarks in the case studies interpret the health care provider's thought process. The student is able to understand why certain tests have been ordered, what clues the tests offer the physician, and how a final diagnosis was confirmed.

Illustrations

The full-color illustrations are accessible, highlighting key information that is covered in the text. Effort has been made to ensure that the illustrations support the narrative and are easily understood.

A color-coding system is maintained throughout the text whereby specific structures always appear in a specific color. This consistency will enable today's visual learners to readily identify the identical structures in different microorganisms. The key to the color coding is presented below.

In addition, selected colorization of electron micrographs is used to highlight the full extent of structures rather than relying on labels and lead lines that may obstruct important parts of an image. The same color code applies to the micrographs.

In the metabolism chapter, dual panel illustrations present the metabolic pathways in two ways. The actual chemical structures are shown on the left for the instructor who wants detail, while on the right, a conceptual pathway using only words is shown. This allows the instructor the flexibility to use or not use the detail provided based on the objectives of the course.

Finally, the use of real-life photographs throughout the book, in conjunction with an illustration or separately, allows the student to see the relevance of microbiology in everyday life.

Pedagogy

Several learning aids have been added to the text to help students learn the content. They include:

- **Chapter Outlines** Show students the big picture of the topic they are studying and helps them plan study time.
- **Chapter Objectives** Detail essential information that a student should understand from the chapter.
- **Bold-faced Terms** Emphasize key terms within the chapter.
- **Concept Checks** Brief synopses of content at the end of many major sections.
- **Tables** Information is summarized in many tables in each chapter to help the student organize and learn the material.
- **Chapter Summaries** Organize and condense chapter contents for easy access.
- **Review Questions** Allow students to test themselves on key concepts of the chapter.
- **Critical Thinking Questions** Foster critical thinking skills by asking students to answer questions as if they were microbiologists attempting to solve a problem.

KEY TO COLOR CODE OF CHEMICALS AND STRUCTURES

Color	Chemical	Structure	Microorganism
	Protein, lipoprotein	Viral capsid, bacterial pili, flagella	Virus
	Peptidoglycan	Bacterial cell wall	Bacteria
	Carbohydrate glycoprotein, lipopolysaccharide	Bacterial outer membrane, glycocalyx, capsule	
	DNA	Bacterial chromosome, plasmas, chloroplasts	
	RNA, ATP	Ribosomes, nucleus	
	Lipid, phospholipid	Membranes, mitochondria	Eukaryotes

Further Readings A selection of relevant articles and books suggesting sources of further information. Each reference offers a brief synopsis of the article.

ANCILLARIES

In addition to the text, an ancillary package has been assembled to aid in teaching. Selected items will be provided to qualified adopters using *Microorganisms in our World*. The ancillaries include:

Instructor's Manual and Testbank Written by James Parsons of Bloomsburg University, this manual will prove invaluable in planning your course. It features chapter outlines, key terms, lecture outlines, teaching tips, and audiovisual references. The test bank contains over 750 multiple choice questions to aid the instructor in evaluating student progress.

Computerized Instructor's Manual and Testbank The book above in an electronic format that instructors can access through their word processing software. This ancillary is available in IBM and Macintosh versions.

Computerized Testbank The testbank is also available on Diploma IV, a test-generating software program. This ancillary is available in IBM and Macintosh versions.

Transparency Acetates A set of 200 full-color transparency acetates chosen from the illustrations in the text and relabeled with large, bold lettering to enhance projection in the classroom.

Slide Set The same images in the transparency set are also provided in 2 × 2 slides.

Infectious Disease Slide Set A set of fifty 2 × 2 slides of photomicrographs and clinical conditions.

Study Guide Written by William Wellnitz of Augusta College, the Study Guide includes various learning activities to increase students understanding and retention.

Laboratory Manual This comprehensive laboratory manual emphasizes general principles and health science applications of microbiology. An Instructor's Manual accompanies the lab manual.

Microbiology CD-ROM Tutorial This innovative microbiology tutorial supplements lecture and text material.

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Case Studies* and *Anatomy Appendix
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UNIT
ONE

Microorganisms and Microbiology

CHAPTER 1

Unity of the Microbial World

CHAPTER OUTLINE

Microorganisms 3
- What is a Microorganism?
- *Newsbreak:* French Revolution Leads to the Metric System
- Organizational Structure of Microorganisms
  - Cells of Living Organisms
- *Historical Perspective:* Discovery of Microorganisms
  - Unicellular and Multicellular Organisms
  - Prokaryotic and Eukaryotic Cells
  - Acellular Nonliving Viruses
- *Highlight:* Practical Significance of Organizational Differences Among Microorganisms

Importance of Microorganisms to Humankind 14
- *Highlight:* A Practical View of Microbiology
- Microorganisms and Disease
- *Newsbreak:* Origins of Vaccination
- *Newsbreak:* Source of Cholera in London
- Beneficial Uses of Microorganisms
- *Newsbreak:* Biotechnology to Cure Global Warming

PREVIEW TO CHAPTER 1

In this chapter we will:

- Study unifying characteristics of microorganisms.
- Define the scope of the science of microbiology.
- Describe the attributes of microorganisms.
- Distinguish microorganisms from plants and animals.
- Examine the structural organization of microorganisms.
- Identify major characteristics of viruses, prokaryotic eubacteria and archaebacteria, and eukaryotic fungi, algae, and protozoa.
- Gain insight into the relevance of microorganisms to human health and well-being.
- Learn the following key terms and names:

algae
archaebacteria
bacteria
cell
cellular metabolism
DNA (deoxyribonucleic acid)
eubacteria
eukaryotic cells
fungi
microbiology
microorganism
microscope
multicellular
nucleus
organelles
pathogens
plasma membrane
prokaryotic cells
protozoa
tissues
unicellular
viruses

MICROORGANISMS

Many of us equate the terms *microorganisms* and *germs*. This is not surprising since we continuously battle microorganisms to maintain our health. Unseen microorganisms exert a powerful influence on our lives. By learning about microorganisms you will be able to understand why we sometimes suffer infections and disease. You also will be able to see how modern medical practices attempt to control and to eliminate disease-causing microorganisms and to treat infectious diseases when they do occur. At the same time you may be surprised that, while the general layman's view is that all microorganisms are harmful to human health, most microorganisms do not cause disease. Our skin, hair, tongue, intestines, and other body parts are literally swarming with bacteria that do us no harm. In fact, they are advantageous, or even necessary to us. Life on earth depends on microorganisms that have an enormous capacity to degrade and to recycle materials.

WHAT IS A MICROORGANISM?

As you begin your study of **microbiology**—the science that deals with microorganisms—you may ask, exactly what is a microorganism and how do microorganisms differ from other organisms? As implied by the word **microorganism** (from the Greek word *micro*, meaning small), microorganisms are very small life forms—so small that individual microorganisms usually cannot be seen without magnification (FIG. 1-1). These microorganisms include the viruses, bacteria, fungi, algae, and protozoa. They are the organisms that microbiologists study (FIG. 1-2).

To describe size, scientists use the metric system measure of length that is based on a meter (a meter is approximately a yard). Fractions of a meter are described by using prefixes such as milli (one thousandth), micro (one millionth), and nano (one billionth). A millimeter (mm) is a thousandth of a meter

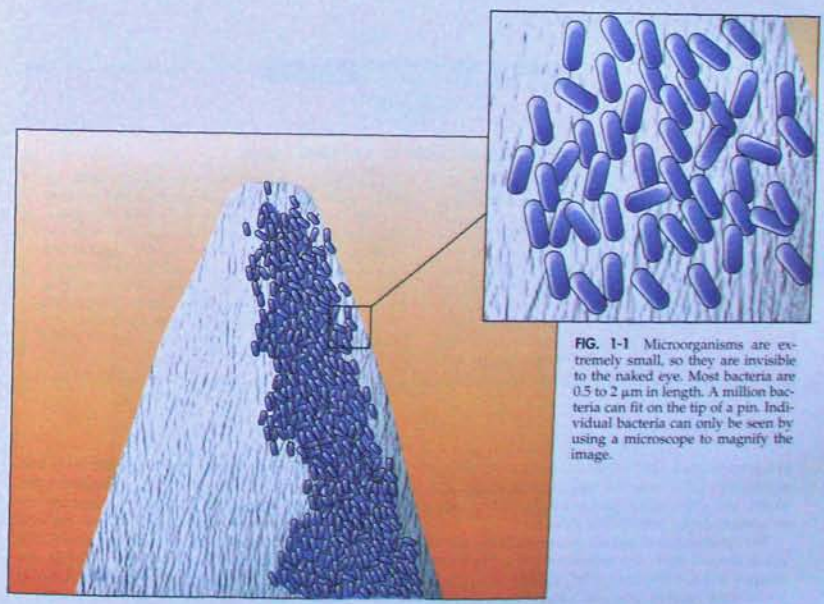


FIG. 1-1 Microorganisms are extremely small, so they are invisible to the naked eye. Most bacteria are 0.5 to 2 μm in length. A million bacteria can fit on the tip of a pin. Individual bacteria can only be seen by using a microscope to magnify the image.

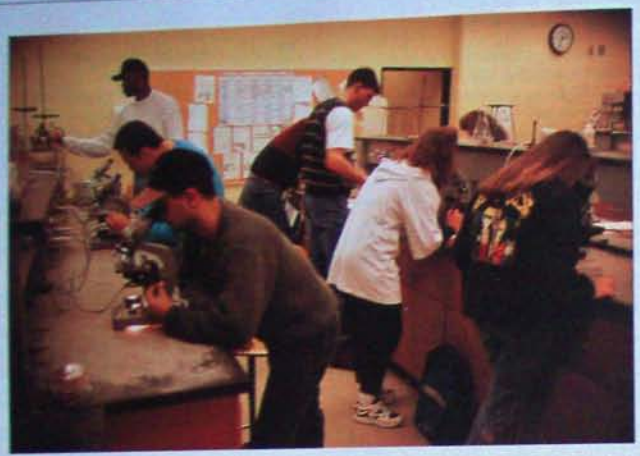


FIG. 1-2 Students in an introductory microbiology laboratory class get their first view of the microbial world while peering through their microscopes.

NEWSBREAK

French Revolution Leads to the Metric System

The metric system was introduced during the French Revolution, in part because of concerns with differences in the measuring systems used in various parts of France but equally to demonstrate that a new governmental regime was in charge. The fundamental unit of measuring length is the metric system, the meter (m), was defined as one ten-millionth the distance from the earth's north pole to the equator. The official measurement was made in 1799 geometrically between Dunkirk and Barcelona. A platinum meter was deposited in the Archives of the Republic as the official standard measure of length. Although French proponents of the reform in weights and measures sought a system based on some natural universal unit to be used throughout the world, England rejected the metric system as impractical. Jefferson, although enthusiastic about such a change for the United States, rejected the French system and tried to develop his own system of measures. Thus, while the rest of the world uses the metric system, the United States and Britain do not generally use this system of measure, except for scientists—who, the world over, use the metric system.

or approximately 0.004 inches; a micrometer (μm) is a millionth of a meter or approximately 0.000004 inches, and a nanometer (nm) is a billionth of a meter or approximately 0.000000004 inches.

The smallest object that can be seen with the naked eye is about a tenth of a millimeter (0.1 mm), which is equal to 100 micrometers (100 μm). Most microorganisms are smaller than this. Largest bacterial cells, for example, are only 5 μm in length (0.005 mm), and small bacterial cells are about 0.1 μm in length (much less than a millionth of a meter). Therefore we generally must magnify the images of most bacteria about 1,000 times just to be able to see them. The viruses are smaller yet. The largest viruses are almost 0.1 μm and the smallest viruses are 0.01 μm (less than a millionth of a meter). Thus the images of viruses must be magnified 10,000 to 100,000 times to be seen.

Microorganisms are defined by their small size, since they generally are invisible to the naked eye and can only be seen with a microscope.

Organizational Structure of Microorganisms

Cells of Living Organisms

The **cell** is the fundamental unit of all living systems, including all living microorganisms. Cells have a boundary layer, called the **plasma membrane**, that separates the living cell from the external surroundings. The plasma membrane controls the flow of materials into and out of the cell. It permits the cell to maintain the highly organized state that is a major characteristic of living systems. If the plasma membrane is damaged and fails to regulate the flow of materials, the cell dies and life ceases. To protect this essential structure, many cells have a rigid cell wall surrounding the plasma membrane.

Cells are the fundamental functional units of living organisms.

All cells have a plasma membrane that separates the living system from the nonliving surroundings.

Cells carry out the essential functions of life, including processing of energy and materials for growth and replication of hereditary information for reproduction. Cells contain the molecule **DNA (deoxyribonucleic acid)**, which is the universal substance of living cells that passes hereditary information to offspring cells. The DNA of a cell specifies the potential characteristics of that cell and is often called the "master molecule of life" because it directs the activities of the cell. In all living cells there is a flow of information from the DNA through molecules of RNA (ribonucleic acid) to direct the synthesis of proteins. The synthesis of proteins occurs at numerous ribosomes within the cell. Proteins are the action molecules of life, catalyzing chemical reactions within living cells. By specifying the proteins that a cell makes, the information contained in the DNA directs **cellular metabolism**, the process in which living cells utilize energy and transform materials into the structures necessary for growth and reproduction. All living cells must carry out metabolism to sustain life, one of the essential functions of which is to generate ATP (adenosine triphosphate) as a central currency of cellular energy. Thus cells uniformly have structures that regulate the flow of materials, the storage and expression of genetic information, and the ability to carry out metabolism (FIG. 1-3).

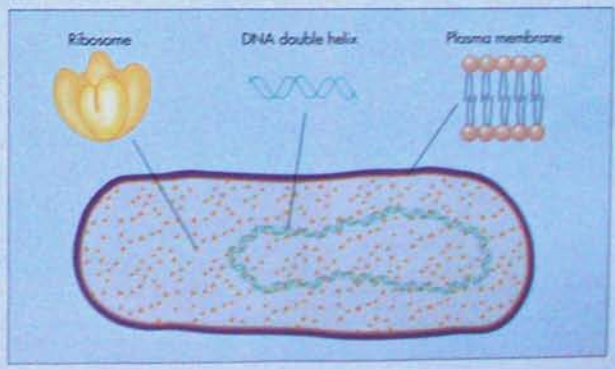


FIG. 1-3 The cell is the fundamental unit of all living organisms. It is separated from its surroundings by a plasma membrane that regulates the flow of materials into and out of the cell. The organism's hereditary information is contained within the cell in molecules of DNA. Proteins that catalyze the metabolism of the living organism are synthesized at ribosomes within the cell.

HISTORICAL PERSPECTIVE

Discovery of Microorganisms

Considering their small size, it is no wonder that the existence of microorganisms was not recognized until only a few centuries ago. The advent of the microscope, which is an instrument used to enlarge objects and images, permitted us to see them. Nor is it surprising that we still have a lot to learn about microorganisms. The invention of the microscope occurred at the end of the sixteenth century, essentially at the same time as the introduction of the telescope. As some scientists looked upward and outward toward the stars with telescopes, others began to search inward with microscopes.

The first microscopes were simple ground glass lenses that magnified images of previously unseen objects. By the late seventeenth century, microscopes permitted magnifications of several hundred times, making it possible to discover the microbial world. Among the first to observe the previously invisible microbial world was Robert Hooke, an English scientist. His detailed drawings of fungi, made in 1667, reflect hours of tedious observations (FIG. A).

Antonie van Leeuwenhoek, an amateur scientist and official winetaster of Delft, Holland, made the first recorded observations of bacteria in 1670 (FIG. B). Van Leeuwenhoek's interest in microscopes was probably related to the use of magnifying glasses by drapers to examine fabrics. His hobby was microscopy and he made over 100 microscopes, each consisting of a simple glass lens (FIG. C). These microscopes were little more than magnifying glasses, each capable of magnifying an image about 300 times, so that bacteria could barely be seen as fuzzy images. Leeuwenhoek must have had great patience and persistence to squint through the lens of his handheld microscope at dimly lighted specimens and record drawings of microorganisms as he did. He thought the bacteria he observed were little animals because they moved, therefore he called them "animalcules." Van Leeuwenhoek's observation of bacteria (animalcules) set the stage for the development of the field of microbiology (FIG. D).

Although not a professional scientist, van Leeuwenhoek asked questions and performed experiments. For example, after observing animalcules in rainwater from his garden, van Leeuwenhoek decided to test whether microorganisms came from heaven or whether they came from earthly sources. He washed a porcelain bowl in fresh rainwater, set it out in his garden during a storm, and observed no microorganisms in the freshly collected rainwater sample. After allowing the water to sit for a few days, he observed numerous microorganisms in the sample. He concluded that a few microorganisms in the sample had multiplied and that "life begets life—even for animalcules."

Although his observations stimulated much controversy, no one at that time made a serious attempt to repeat or to extend them. Van Leeuwenhoek's animalcules remained mere oddities of nature to the scientists of the day. It was not until two centuries later that the significance of the observations made by van Leeuwenhoek became evident, when the science of microbiology began to flourish and to develop into the vibrant field of scientific study it is today (FIG. E).

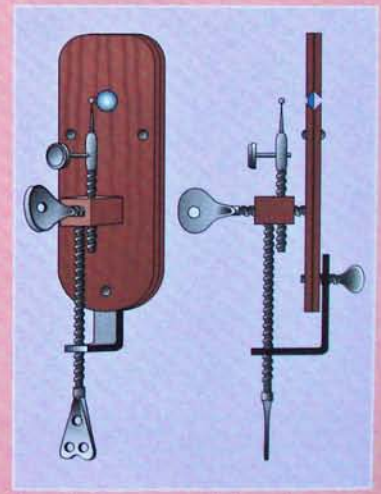
A

Robert Hooke made microscopic observations and provided the earliest descriptions of many fungi. Various species of fungi can clearly be identified in his drawings made from 1635 to 1703 and recorded in his book, *Micrographia*.

B

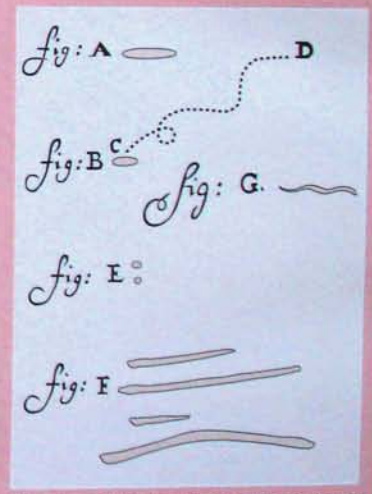
Antonie van Leeuwenhoek (1632-1723), here seen holding one of his microscopes, opened the door to the hidden world of microorganisms when he described bacteria. Although he was only an amateur scientist, Leeuwenhoek's keen interest in optics and his diligence allowed him to make this important discovery.

C



Leeuwenhoek's microscopes were little more than magnifying glasses.

D



Colorized reproduction of Leeuwenhoek's sketches of bacteria from the human mouth illustrate several common types of bacteria, including rods and cocci. *A*, A motile *Bacillus*; *B*, *Selenomonas sputigena*; *C* and *D*, the path of *S. sputigena*'s motion; *E*, micrococci; *F*, *Leptothrix buccalis*; *G*, a spirochete.

Continued

HISTORICAL PERSPECTIVE

THE DISCOVERY OF MICROORGANISMS—CONT'D

E

Time line of some scientific discoveries important to the development of microbiology. The study of microorganisms, which began in the late 1600s with the observations of Leeuwenhoek, developed in the mid-nineteenth century into a true science through the studies of Robert Koch and Louis Pasteur. Major developments have been made in the twentieth century, including the discovery of antibiotics and the molecular basis of heredity. Today microbiology is a flourishing field of science.

Cells have genetic information in the form of DNA that directs the form and function of the cell and passes hereditary information from one generation to the next.

Cells carry out metabolism that transforms energy and materials for growth and reproduction.

Unicellular and Multicellular Organisms

Living organisms are composed of one or more cells. The human body, for example, is composed of trillions of cells. Many microorganisms are **unicellular,** meaning that the entire organism is composed of a single cell. Most bacteria, for example, are unicellular. Each bacterial cell comprises the entire organism and is capable of independently carrying out metabolism for growth and reproduction to form progeny (offspring). Some microorganisms are **multicellular,** meaning that they are composed of many cells. Most multicellular microorganisms—such as the fungi that can often be seen as fuzzy filaments growing on bread—can be viewed as aggregates of cells with each individual cell having the properties of the entire organism. Each individual cell retains the capacity of reproducing to form the entire organism.

Microorganisms do not exhibit the advanced structural organization of plants and animals that form specialized groups of cells called **tissues**. Microorganisms lack tissues. The cells of a plant or animal tissue function together as integrated units. In animals, for example, we find epithelial tissue covering the external body surface, connective tissue binding together and supporting other tissues, muscle tissue moving parts of the animal's body, and nerve tissue transmitting messages from one part of the animal to another. The cells of these differentiated tissues usually lose the ability to generate the entire organism.

Microorganisms are more simply organized than plants and animals.

Microorganisms lack differentiated tissues.

Prokaryotic and Eukaryotic Cells

Among all living organisms there are two fundamentally different types of cells: **prokaryotic cells** (from the Greek word meaning before a nucleus) and **eukaryotic cells** (from the Greek word meaning having a nucleus) (FIG. 1-4). Both prokaryotic cells and eukaryotic cells carry out all the essential life functions: exchange of materials with the environment, energy processing, and reproduction. However, the structures of these types of cells, discussed in detail in Chapter 5, are quite different (Table 1-1).

A prokaryotic cell has a much simpler internal structure than a eukaryotic cell. Eukaryotic cells have numerous membrane-bound compartments, called **organelles,** that perform specialized functions. Prokaryotic cells do not contain membrane-bound organelles. Of prime importance is the fact that the DNA (the substance that encodes the hereditary information of the cell) is not separated within a specialized organelle from the rest of the cell contents in a prokaryotic cell. The DNA of a eukaryotic cell, in contrast, is contained within an organelle called the **nucleus**.

TABLE 1-1

Comparison of Prokaryotic and Eukaryotic Cells

	PROKARYOTIC CELL	EUKARYOTIC CELL
Plasma membrane	Present	Present
Internal organelles (membrane bound)	Absent	Present
Genetic (hereditary) molecule	DNA as a single circular bacterial chromosome not enclosed in a nucleus	DNA as multiple linear chromosomes enclosed within a nucleus
Site of energy (ATP) generation	Cytoplasm and, in some cases, plasma membrane or photosynthetic membranes	Cytoplasm and mitochondria or chloroplasts

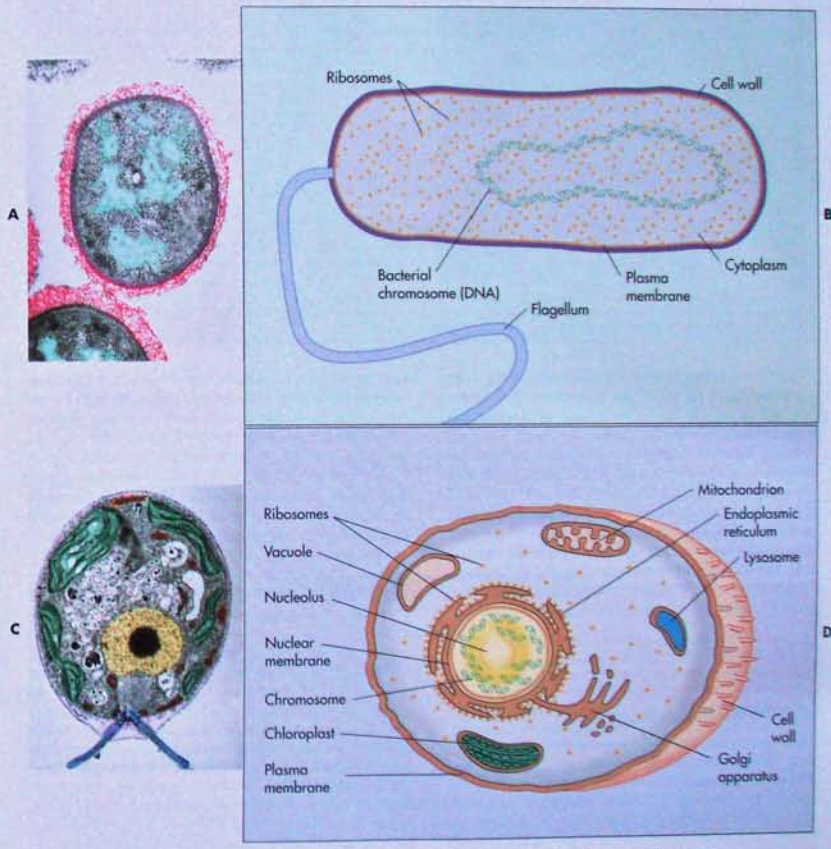


FIG. 1-4 A comparison of structural organization reveals that the eukaryotic cell has far more internal organization than the prokaryotic cell; the membrane-bound organelles found in eukaryotic cells do not occur in prokaryotic cells. **A,** Colorized micrograph of a prokaryotic cell of the bacterium *Pseudomonas aeruginosa* (32,400×). **B,** Drawing of a prokaryotic cell. **C,** Colorized micrograph of a eukaryotic cell of the green alga *Chlamydomonas reinhardtii* (6,750×). **D,** Drawing of an algal eukaryotic cell.

TABLE 1-2

Cellular Structures of Different Microorganisms

ORGANISM	INTERNAL ORGANIZATION	SITE OF ATP GENERATION	CELL WALL
Prokaryotic Cells			
Eubacteria	No internal organelles*	Plasma membrane or in cytoplasm	Plasma membrane protected by a surrounding cell wall with unique chemical structure
Archaebacteria	No internal organelles	Plasma membrane or in cytoplasm	Plasma membrane protected by a surrounding cell wall with chemical structure different than eubacterial cell walls
Eukaryotic Cells			
Fungi	Numerous internal organelles	Mitochondria and cytoplasm	Plasma membrane generally protected by surrounding cell wall
Algae	Numerous internal organelles	Chloroplasts and in mitochondria and cytoplasm	Plasma membrane generally protected by surrounding cell wall
Protozoa	Numerous internal organelles	Mitochondria and cytoplasm	Plasma membrane generally not protected by surrounding cell wall

*Although prokaryotic cells lack membrane-bound organelles, a few specialized eubacteria (for example photosynthetic bacteria) have internal membranes; these membranes are derived from and often continuous with the plasma membrane.

Some microorganisms are prokaryotic and others are eukaryotic (Table 1-2). All **eubacteria** (commonly referred to simply as bacteria) and **archaebacteria** (often called Archaea) are prokaryotic cells. (The differences between eubacteria and archaebacteria are discussed in Chapter 3.) All other living organisms are composed of eukaryotic cells. Three of the major groups of microorganisms—**fungi, algae,** and **protozoa**—are composed of eukaryotic cells. Plant cells and animal cells, including human cells, are eukaryotic. The difference between prokaryotic and eukaryotic cells sets the eubacteria and archaebacteria apart from all other organisms in a fundamental way.

All eubacteria and archaebacteria are prokaryotic cells that lack membrane-bound organelles; all other living organisms are composed of eukaryotic cells that contain membrane-bound organelles.

Acellular Nonliving Viruses

So far we have considered organisms that have cells and clearly meet all the criteria of living systems. These criteria include a high degree of organization, ability to exchange materials with their surroundings, ability to transform energy, ability to grow, and ability to reproduce independent progeny. Some microorganisms, however, do not have cells and do not meet all these criteria. Viruses are acellular (noncellular) microorganisms, meaning that they have neither prokaryotic nor eukaryotic cells. By some criteria, these organisms give the appearance of being alive, but by many other criteria they clearly are nonliving entities.

Viruses lack the fundamental structure of living systems. Viruses have a genetic molecule—which may be either DNA or RNA (ribonucleic acid)—surrounded by a protein coat (FIG. 1-5). Although the viral genetic molecule is capable of directing viral reproduction, viruses do not have the cellular support structures and metabolic machinery necessary to perform other life functions. They depend on living host cells to produce the materials necessary for their replication. Although some viruses are surrounded by an envelope composed of a membrane that they obtain from a host cell in which they replicate, viruses do not have functional plasma membranes separating them from their surroundings. They have no means of carrying out independent metabolism or replication.

Viruses rely entirely on the metabolic activities of living cells to provide energy and materials for their replication. On their own, viruses are inanimate objects. They passively interact with their environment and are unable to reproduce themselves. They do not transform energy, carry out metabolism, or actively respond to their environment. All of these are essential characteristics of living systems. Therefore viruses can be considered as nonliving. However, when viruses are able to enter (infect) living cells, the viral nucleic acid molecule has the capability of directing the replication of the complete virus. Within the confines of a living cell, the genetic information of the viral nucleic acid takes over control of the metabolic activities of that cell. Many microbiologists, therefore, view viruses as genetic extensions of the host cells in which they replicate.

Viruses can carry out the functions characteristic of living organisms only within living cells.

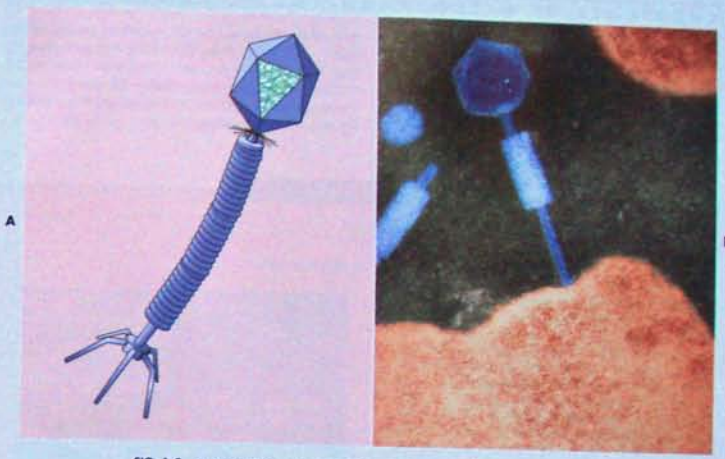


FIG. 1-5 **A,** A virus has a protein coat (capsid) surrounding a nucleic acid hereditary molecule (RNA or DNA *[green]*). **B,** Colorized micrograph of a bacteriophage *(blue)* that infects and replicates within cells of the bacterium *Chromatium violaceum (tan)*.

HIGHLIGHT

Practical Significance of Organizational Differences Among Microorganisms

Organizational differences between the acellular viruses, prokaryotic eubacteria and archaebacteria, and eukaryotic fungi, algae, and protozoa are of more than just scientific interest. They also have great practical importance. The use of many antibiotics (drugs that adversely affect microorganisms) to treat human diseases caused by bacteria, for example, depends on the ability of the antibiotic to act selectively against the prokaryotic cells of the invading bacteria without killing the eukaryotic human cells at the same time. Antibiotics, such as penicillin, are widely used to treat bacterial infections, such as syphilis, because they specifically target prokaryotic cells. If you were to develop pneumonia caused by a particular species of bacterium, a physician could prescribe any number of antibiotics that could kill the infecting bacteria and help cure you of the disease. It is more difficult to use antibiotics against disease-causing fungi and protozoa, such as malaria, because fungi and protozoa, like humans, are composed of eukaryotic cells, so that what is toxic to them is also often toxic to the human cells.

Likewise, it is hard to find antibiotics that can be used to treat viral diseases because viruses replicate within the confines of the living cells of the organism they infect. Chemicals that inhibit viral replication generally do so by killing the host cell within which the virus replicates. This prevents the therapeutic use of these chemicals. No one wants to use a drug that kills the patient at the same time it kills the disease-causing microorganism. So, when you have the common cold—a disease caused by a virus—a physician generally can only tell you to rest, stay warm, and drink plenty of fluids. This prescription enables your body's own defenses to fight the invading viruses.

IMPORTANCE OF MICROORGANISMS TO HUMANKIND

Without question we have learned a great deal about microorganisms in the last century. This knowledge now enables us to control microorganisms for human good—at least to some extent. We have developed methods for preventing microbial attack on foods and other materials. Refrigeration, freezing, canning, and other methods prevent microbial growth and, thereby, preserve foods. We paint wood surfaces and keep fabrics dry to prevent deterioration due to microbial growth. Microorganisms are used for the treatment of sewage and municipal wastes, ridding the world of waste materials.

HIGHLIGHT

A Practical View of Microbiology

Microbiological researchers at university, government, and industrial laboratories carry out studies that reveal the fundamental nature of microorganisms and the practical applications of that knowledge. Understanding the genetics and metabolism of microorganisms facilitates the beneficial uses of microorganisms.

Health care workers treat individuals with diseases caused by microorganisms and try to prevent the spread of infectious diseases. Vaccination is used to prevent many once deadly diseases. Antiseptics are used to preclude microorganisms from infecting wounds. Antibiotics are administered to cure patients of infecting microorganisms.

The clinical microbiology laboratory helps physicians diagnose diseases and determine the most effective treatments (FIG. *A*). The clinical microbiologist and staff receive samples from physicians and run various tests to identify the underlying causes of disease in patients. The clinical microbiology laboratory also tests the effectiveness of various drugs against disease-causing microorganisms.

The food industry maintains strict quality control to preserve foods and to protect against the foodborne spread of disease. Technicians in the food industry frequently test batches of foods to ensure safe levels of microorganisms. Canning, refrigeration, freezing, and other methods are routinely used to prevent food spoilage.

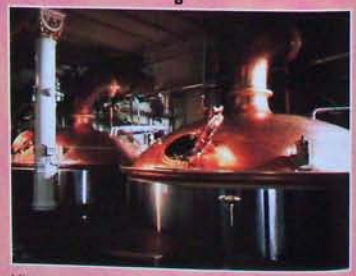
Many foods and beverages are produced using microorganisms, including bread (yeasts are used for leavening to cause the dough to rise), cheese (bacteria are used to convert milk into various cheeses), wine (yeasts are used to convert grapes into this alcoholic beverage), beer (yeasts are used to convert grains into this alcoholic beverage), and numerous other products. We all have enjoyed eating or drinking the foods produced by microorganisms (FIG. *B*).

Farmers use agricultural practices that control the spread of plant pathogens (microorganisms that cause diseases of plants). Agricultural extension service workers help farmers plan appropriate control measures to ensure maximal crop production. Fungicides are often used to prevent fungal diseases that destroy crops.

A

The clinical microbiology laboratory helps diagnose the causes of infectious diseases and aids the physician in determining appropriate therapies.

B



Microorganisms are cultivated to produce alcoholic beverages such as beer. Copper kettles often are used for preparing the substrate for yeast fermentation (mash production). The fermentation often is carried out in open fermentors. Bubbles of carbon dioxide in the fermentor accompany ethanol production.

Microorganisms and Disease

Our knowledge of how disease-causing microorganisms (pathogens) spread has permitted us to reduce the incidence of many diseases. Improved sanitation practices, based on our understanding of how disease causing microorganisms are transmitted to humans, have greatly reduced the incidence of diseases such as plague and gastrointestinal infections. Disinfection of water and proper cooking and handling of foods has greatly reduced the incidence of disease such as typhoid that were once widespread. Sanitary practices in medicine introduced in the latter half of the nineteenth century are now routinely used to protect patients and medical staff against infections (FIG. 1-6).

Some microorganisms can also be used as biological control agents to replace chemical pesticides.

Microorganisms are used to degrade wastes and pollutants (FIG. *C*). Sewage treatment plants, compost piles, and septic tanks use microorganisms to decompose wastes into products that can be accommodated by the environment. Sanitary engineers are important in seeing that these microbial processes run effectively. Microorganisms are also used to bioremediate polluted sites, such as shorelines contaminated by oil spills.

Microorganisms are used in industry to produce antibiotics, vaccines, solvents, and numerous other products of economic value (FIG. *D*). Numerous technicians oversee the daily production operation and ensure the quality of the final product. The biotechnology industry, which uses microorganisms for the production of these products, has been rapidly growing. The ability to genetically modify microorganisms now permits the production of many new products and undoubtedly will lead to the production of many more.

C

Microorganisms are used to degrade wastes and pollutants to maintain and restore environmental quality. An activated sludge treatment facility has tanks in which microorganisms degrade wastes. Extensive aeration and agitation maintain aerobic conditions that favor complete degradation of organic compounds by microorganisms.

D

Microorganisms are grown in fermentors to produce pharmaceuticals. Small-scale fermentors are used in the research and development phase of pharmaceutical development.

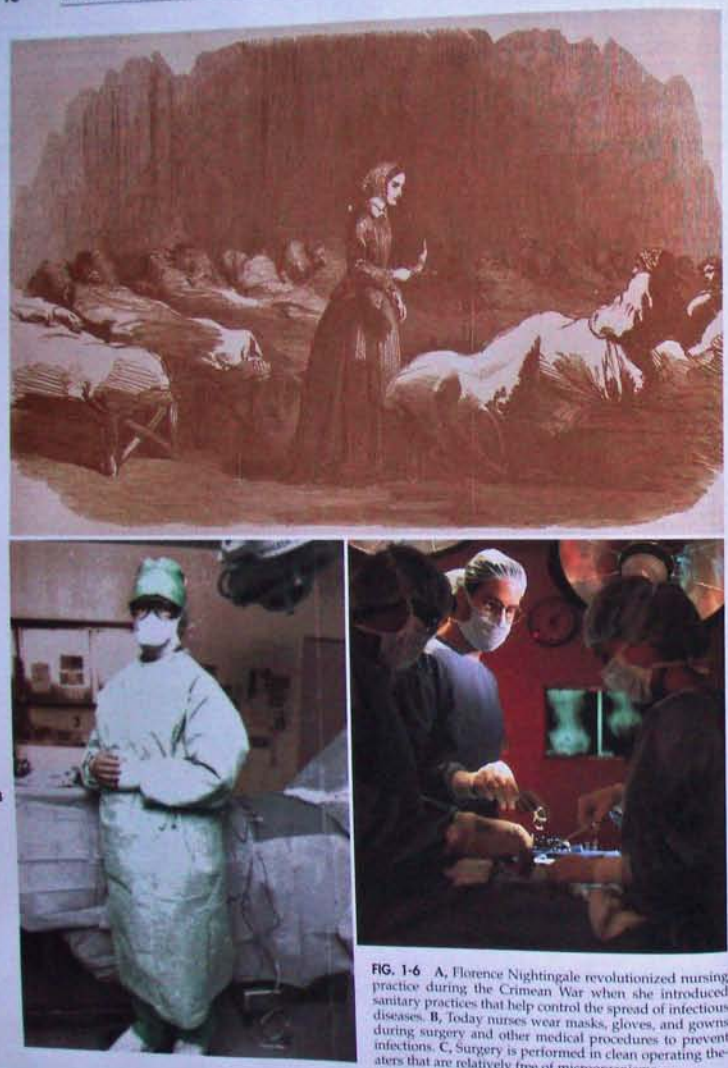


FIG. 1-6 **A,** Florence Nightingale revolutionized nursing practice during the Crimean War when she introduced sanitary practices that help control the spread of infectious diseases. **B,** Today nurses wear masks, gloves, and gowns during surgery and other medical procedures to prevent infections. **C,** Surgery is performed in clean operating theaters that are relatively free of microorganisms.

A mere century ago, 5 of every 10 deaths in the United States were due to microbial infections (FIG. 1.7). Tuberculosis, pneumonia, and gastrointestinal infections were the three leading killers, with diphtheria and bronchitis also listed among the top 10. Today, the only remaining infectious disease caused by microorganisms in this list is pneumonia, a disease that occurs when the defense systems of the body fail—for example, as a consequence of another disease. Although tuberculosis, gastrointestinal infections, bronchitis, and diphtheria still occur, and the number of cases of tuberculosis has recently risen, the percentage of deaths due to these diseases has dropped from over 20% in 1900 to less than 1% of total deaths today.

The reduced mortality from infectious diseases is largely due to the discovery and widespread medical uses of antibiotics and vaccines that have enabled contemporary medicine to reduce the impact of infectious diseases. Antibiotics such as penicillin were not available in medical practice until after World War II. They are now routinely used to control microbial in-

NEWSBREAK

ORIGINS OF VACCINATION

Lady Mary Wortley Montagu, wife of the English ambassador to Turkey (1716-1718), described a crude form of inoculation against smallpox practiced there. In September, old women insert some of "the matter of the best sort of smallpox" into a vein. After 8 days, those so inoculated develop a fever, remain ill for 2 or 3 days, recover within 8 days, and get no pox marks. Lady Montagu tried to persuade the English to adopt this practice, but without success. Edward Jenner got the idea for the technique of vaccination against smallpox from a young girl, a milkmaid it is said, who told him that she would not catch smallpox because she had had cowpox. Jenner is credited with the introduction of vaccination in Europe.

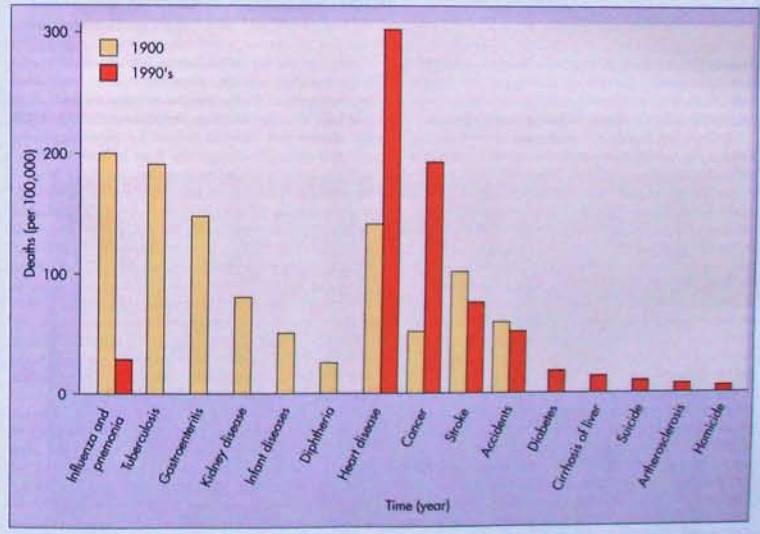


FIG. 1-7 Mortality due to infectious deseases has declined significantly during the twentieth century because of an increased understanding of the roles of microorganisms in human disease and the introduction of practices that reduce the occurrence and effects of microbial infections. Deaths due to many once deadly diseases such as diphtheria, gastroenteritis, and even tuberculosis are very low (near 0 per 100,000) in the 1990s.

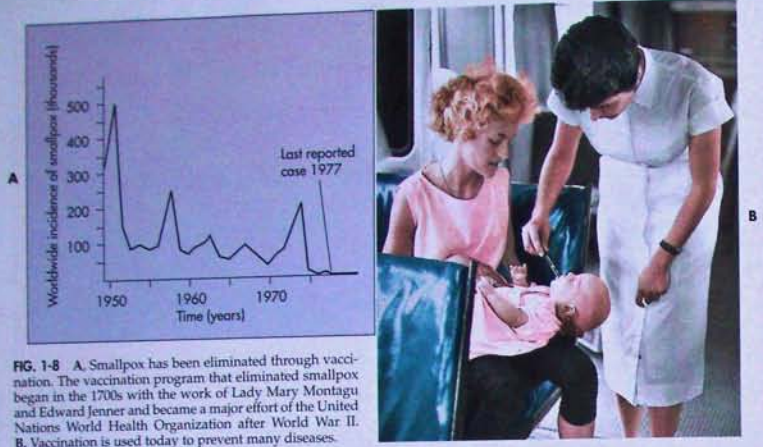


FIG. 1-8 **A,** Smallpox has been eliminated through vaccination. The vaccination program that eliminated smallpox began in the 1700s with the work of Lady Mary Montagu and Edward Jenner and became a major effort of the United Nations World Health Organization after World War II. **B,** Vaccination is used today to prevent many diseases.

fections. Clinical microbiology laboratories assist physicians by rapidly identifying pathogens in infected individuals and determining which antibiotics can be used to control that disease-causing microorganism.

Vaccines are extremely important in preventing disease. An amazing accomplishment of vaccination is the elimination of smallpox. Once 80% of all people on earth could expect to contract smallpox. Now the world is free of this disease. Three centuries of vaccination against smallpox and a massive 20-year public health effort by the World Health Organization finally were victorious over the smallpox virus (FIG. 1-8). The last natural case of smallpox occurred in Somalia, Africa, in 1977. For the first and only time, an infectious disease was eliminated from earth. Effective vaccination programs have also reduced the occurrences of other diseases, such as polio, measles, diphtheria, and whooping cough.

Microbiologists continue to search for new vaccines (substances that stimulate the body's defenses, rendering an individual immune to disease) and antibiotics and to use the techniques of molecular biology to engineer genetically new microorganisms that can produce useful products such as antibiotics and vaccines. Such genetically engineered microorganisms will help meet our future technological needs and improve human health and well-being.

The reduced impact on humankind of diseases caused by microorganisms is due in part to advances in our scientific knowledge of microorganisms and their interactions with humans.

Against the tide of enormous advancement in our understanding of the microorganisms has come the emergence of new infectious diseases, including acquired immunodeficiency syndrome (AIDS). AIDS has shown that diseases caused by microorganisms can still radically change the lives of infected individuals and also of entire societies. Daily, we read in the newspapers, hear on the radio, and see on television reports about the AIDS virus and other diseases caused by microorganisms. Illness and death spark public concern, and the medical aspects of microbiology usually are of the most interest to many of us.

So far, scientists have been unable to develop an effective means of preventing AIDS or drugs that will rid patients of the human immunodeficiency virus (HIV) that causes it once an infection occurs (FIG. 1-9). The Centers for Disease Control estimate the incidence of infection with HIV in the United States in 1995 to be 3 in 1,000, with the infection more common in males than females. The World Health Organization estimates that in Africa the incidence in 1995 will be much higher, with 1 in 10 men and women infected with the AIDS causing HIV virus. The cost of treating AIDS patients in New York City alone is running well over $1 billion per year. Only changes in behavioral patterns that reduce the risk of infection with HIV hold any immediate promise for reducing the incidence of this disease. These include the avoidance of unprotected sex with individuals who may carry HIV and avoidance of exposure to contaminated blood through transfusions or shared

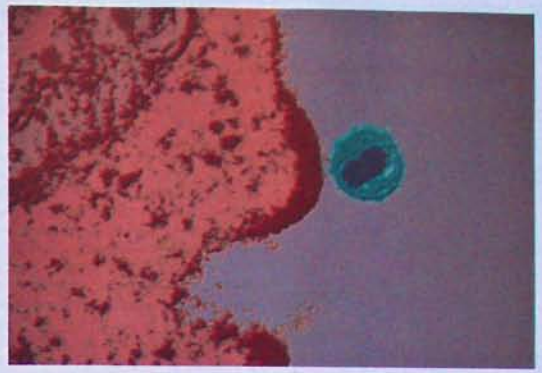


FIG. 1-9 Colorized micrograph of the human immunodeficiency virus (HIV) *(blue)*, that causes AIDS, on the surface of a cell *(red)*.

intravenous needles. If behavioral changes do not occur, and if no cure or preventive is found, the impact of this disease on society will be staggering.

AIDS is just the latest major outbreak of disease to afflict humankind. All societies, both contemporary and ancient, have suffered from the ravages of diseases caused by microorganisms (FIG. 1-10). Plague, tuberculosis, polio, smallpox, and various other diseases caused by microorganisms followed explorers, traders, missionaries, immigrants, or soldiers. Microorganisms have had a significant impact on the course of human civilization, including warfare, reli-

FIG. 1-10 During the Middle Ages there were major epidemics of plague. The disease followed the trade routes, as did the rats carrying the flea vectors infected with *Yersinia pestis*.

gion, migration of populations, art, agriculture, science, and technology. Major disease outbreaks, often plague, altered the course of history many times and contributed to the decline of empires. Widespread disease in ancient Athens led to that city–state's loss to its rival Sparta, during the Peloponnesian War. This prevented Athens from establishing a unified Greek empire. When the central government of the Roman Empire was weakened by the debilitating effects of plague, it could not consolidate its power over its vast empire and the influence of Rome declined.

Population patterns have often been determined by the actions of microorganisms. A fungal infection of potatoes in Ireland in the mid-nineteenth century caused widespread famine. This led to the migration of large numbers of Irish people to the United States. European settlers in the New World thought that the diseases with which they infected the natives were God's way of showing approval for their colonization. Entire tribes of native North Americans were eradicated due to outbreaks of smallpox. These outbreaks were sometimes initiated when Europeans gave them smallpox infected blankets.

Microorganisms have had major impacts on human health and society.

Beneficial Uses of Microorganisms

We have long depended on microorganisms for the production of many of the foods and beverages we enjoy. Even early societies produced beer, wine, and bread using microorganisms. We have built sewage treatment facilities where microorganisms are grown to degrade our wastes. We have also been able to greatly reduce the incidence of many diseases through the use of antibiotics and vaccines produced by microorganisms (FIG. 1-11). Indeed, the vaccines, antibiotics, and other pharmaceuticals that are used to prevent or to treat many human diseases are microbial products.

Many microorganisms benefit humans, producing foods we eat, removing wastes we produce, and making antibiotics and vaccines for use in medicine.

Many agricultural practices encourage the beneficial metabolic activities of microorganisms and discourage their harmful effects. Spacing of crops, the development of disease resistant plant varieties, and the use of chemical pesticides aim to limit infections due to plant pathogens. They also limit crop damage due to insects and other nonmicrobial pests. Crop rotation uses plants such as alfalfa and soybeans that are associated with nitrogen-fixing bacteria. These bacteria can convert atmospheric nitrogen (N_2) into forms of nitrogen that can be used by plants. Plant growth depends on the forms of nitrogen provided by these bacteria or on the addition of nitrogen fertilizers by farmers. Without the microbial activities that transform decaying dead material to substances that can be reused by the living, life on earth would soon cease. We could not survive without microorganisms.

Microorganisms are essential for maintaining the balance of nature and sustaining life on earth.

Clearly the microorganisms are complex and difficult to understand—powerful but small, necessary yet sometimes deadly. Through the science of microbiology we have increased standards of living, improved agricultural crop yields, helped maintain environmental quality, and decreased the incidence of disease. By studying the microorganisms, we hope to develop the necessary understanding to control microorganisms for the benefit of the future of humankind.

NEWSBREAK

Source of Cholera in London

In the mid-1800s a cholera epidemic was devastating London. John Snow, a British physician, sought to find the cause of the epidemic. He believed that the disease was spread by contaminated food and water, not by bad air or casual contact. Snow studied medical records of patients in the Broad Street area of London who had died of cholera. He discovered that most of the victims obtained their drinking water from the Broad Street water pump. He hypothesized that the water from the pump was contaminated with raw sewage containing the cause of cholera and that shutting off that source of water would end the cholera outbreak. The Broad Street pump was shut down and the number of cholera cases dropped dramatically. These important findings led the way to understanding the causes and modes of transmission of infectious diseases.

NEWSBREAK

Biotechnology to Cure Global Warming

Microorganisms may have a role in reversing global warming. They may be used to remove carbon dioxide from the atmosphere. Japanese researchers are investigating the possibility of using genetically engineered algae to remove carbon dioxide from the atmosphere and maintain levels of atmospheric carbon dioxide that will not cause global warming. Already, Japanese researchers have found an alga that has ten times the carbon dioxide fixing capacity of trees. This would be a novel employment of biotechnology for the maintenance of global environmental quality. The idea is to genetically engineer algae with high photosynthetic capacities that produce polymers that are not easily biodegraded. In this way, carbon dioxide would be drawn out of the atmosphere and incorporated into compounds that are not easily degraded back to carbon dioxide.

FIG. 1-11 **A,** Some viral vaccines are produced by culture in eggs or tissure culture. **B,** Vaccines are often produced by growing microorganisms and harvesting them.

FIG. 1-11 cont'd **C,** Some vaccines are produced by growing recombinant vaccine strains in fermentors; the fermentor in this photograph is used to produce hepatitis B vaccine employing a recombinant strain of *Escherichia coli* containing the viral genes.

SUMMARY

Microorganisms (pp. 3-13)

- Microorganisms are very small life forms.
- The observation of microorganisms depends on the use of microscopes.
- Antonie van Leeuwenhoek made the first recorded microscopic observations of bacteria.

The Organizational Structure of Microorganisms (pp. 5-13)

- Even microorganisms, the smallest forms of life, are highly organized living systems.
- The cell is the fundamental unit of all living systems.
- The plasma membrane acts as a barrier that controls the flow of material into and out of the cell.
- Cells contain hereditary information in DNA and carry out metabolism that transforms energy and materials.
- Many microorganisms are unicellular (single celled organisms).
- Some microorganisms are multicellular (composed of many cells).
- Bacteria are cellular organisms, with simple cells that lack organelles.
- Bacterial cells are prokaryotic.
- Fungi, algae, protozoa, plants, and animals (including humans) all have eukaryotic cells.
- Eukaryotic cells have organelles, including a nucleus that houses the genetic information.
- Organelles are membrane-bound compartments within a cell.
- Viruses are not made of cells.
- Viruses only replicate within living cells.

Importance of Microorganisms to Humankind (pp. 14-21)

- Some microorganisms cause disease.
- Diseases caused by microorganisms have a major impact on individuals and human society.
- Today's reduced rates of sickness and death are a direct result of our ability to control the growth of microorganisms and our basic understanding of the involvement of microorganisms in infectious diseases.
- Most microorganisms are nonpathogens that serve useful industrial and essential environmental functions.
- Microorganisms are used to make various foods, beverages, and other useful products.
- Microorganisms are essential for life to continue on earth.

CHAPTER REVIEW

Review Questions

1. What kinds of organisms are studied by microbiologists?
2. What is a microorganism?
3. How can you distinguish living from nonliving microorganisms?
4. How would you define a living organism?
5. What are the arguments for viruses being living organisms? Against?
6. Why is microbiology considered a relatively new field of science?
7. Describe the fundamental organizational structure of living organisms.
8. What are the differentiating characteristics of prokaryotic and eukaryotic cells?
9. What is the difference between unicellular and multicellular organisms? Which microorganisms are unicellular and which are multicellular?
10. How are microorganisms distinguished from higher plants and animals?
11. What are the characteristics that make eubacteria similar to archaebacteria? What are the characteristics that differentiate them?
12. What are the characteristics that make fungi, algae, and protozoa similar? What are the characteristics that differentiate them?

CRITICAL THINKING QUESTIONS

1. How have microorganisms affected your life? Consider the adverse effects microorganisms may have had, such as infectious diseases you, your friends, and your relatives may have had, and the benefits you may have experienced, such as foods and beverages you may have enjoyed.
2. What would life be like without microorganisms? What would be the consequences of eliminating all pathogens from earth? What changes would occur in society if there were no infectious diseases?
3. If you were sending a mission to Mars to explore for life, what would you look for? How would you know whether life existed or not? How would you recognize the difference between an intelligent computer and a living organism?
4. Within the last few years, a new bacterium has been isolated from the intestinal tracts of surgeonfish in the Red Sea. These bacteria are so large that they are visible to the unaided eye. What does this mean with respect to the definition of microorganisms?

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CHAPTER 2

Diversity of the Microbial World

CHAPTER OUTLINE

Classifying Microorganisms 25
- Early Classification Systems
- Modern Classification Systems
- *Highlight:* Evolution of Microorganisms

Survey of Microorganisms 30
- Acellular Nonliving Microorganisms
  - Viruses
  - Viroids
  - Prions
- Prokaryotes
  - Naming Bacteria
  - Characteristics Used in Classifying and Identifying Bacteria
- *Methodology:* DNA Hybridization: A Method for Analyzing Genetic Relatedness
  - Major Groups of Bacteria
- *Highlight:* Microview of Bacterial Diversity
- Eukaryotes
  - Fungi
  - Algae
  - Protozoa

PREVIEW TO CHAPTER 2

In this chapter we will:

- Learn how microorganisms are classified.
- See how microorganisms evolved.
- Gain an overview of the microbial world by examining the fundamental characteristics of the various types of microorganisms.
- Examine characteristics of the acellular, nonliving microorganisms—viruses, viroids, and prions.
- Examine characteristics of the prokaryotic microorganisms—archaebacteria and eubacteria.
- Examine characteristics of the eukaryotic microorganisms—fungi, algae, and protozoa.
- See that the structural differences between acellular, prokaryotic, and eukaryotic microorganisms have practical importance in medical practice.
- Learn the following key terms and names:

algae
Archaea
bacteria
bacteriophage
Bergey's Manual
binomial name
DNA hybridization
endospores
endosymbiotic theory of evolution
eubacteria
eukaryotes
evolution
evolutionary relatedness
filamentous fungi
five-Kingdom classification system
fungi
Gram-negative bacteria
Gram-positive bacteria
Monera
morphology
mycelia
prions
Protista
protozoa
species
taxonomy
viroids
virus
yeasts

CLASSIFYING MICROORGANISMS

The microbial world is extremely diverse. Some organisms have common features that permit grouping into common categories. Scientists use a hierarchical (ranking system) organizational structure in which organisms are classified (grouped) according to their degrees of similarity (FIG. 2-1). Classification systems are used to systematically establish the characteristics of different groups of organisms so that specific types of organisms can be described and identified. The broadest groups in a classification system are called Kingdoms and the smallest are called species. Related species are grouped into genera. For example, *Mycobacterium tuberculosis* and *Mycobacterium leprae* are both in the genus *Mycobacterium*.

The science of identifying and classifying organisms is called **taxonomy**, and scientists who study the classification of organisms are called **taxonomists**. Arguments among taxonomists are often quite heated. Some taxonomists tend to classify many similar organisms into large groups. Others favor small groups that emphasize even minor differences between organisms. Interpreting the evidence rests on personal judgement on the relative importance given certain criteria. Is shape, color, the ability to metabolize a given compound in a particular way, or something else the most important characteristic that should be used to distinguish one microbial species from another? Many modern taxonomists use evolutionary relationships—determined by molecular biological analyses—as the basis for establishing order in classifying organisms.

Classification systems arrange living organisms into groups based on common factors and show how they are related.

It has been difficult to classify microorganisms based on how they are related to each other in terms of their evolutionary relationships. We use fossils that were preserved in the Earth's crust to piece together evolutionary relationships of plants and animals. Microorganisms lack structures such as bones and woody tissues that are preserved as fossils. There is only a limited fossil record that provides a basis for comparing ancient forms with contemporary forms of microorganisms. Direct examination of the ancestors of today's microorganisms is for the most part impossible. As a result, the classification of microorganisms generally has been based on relationships inferred from observed similarities and differences in *morphology* (form and structure) and *physiology* (functions) among actual living microorganisms.

The **species** is the fundamental grouping used in classifying all organisms. In higher organisms the species is defined by the ability to interbreed and produce fertile offspring. Organisms that can interbreed are considered members of the same species.

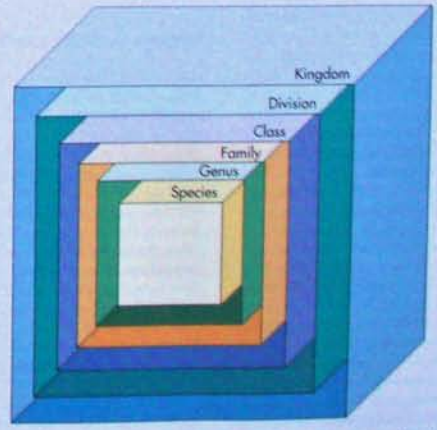


FIG. 2-1 The system used for the classification of living organisms employs hierarchical levels of organization: the highest level of organization (most encompassing) is the Kingdom; the species is the fundamental level of organization.

Those that cannot sexually reproduce fertile offspring are defined as members of different species. Many microorganisms, however, reproduce asexually, with a single cell dividing to form progeny. Therefore, defining microbial species based on the ability to reproduce sexually is not meaningful.

Microbiologists have been hard pressed to find a working definition of a microbial species. Often, they consider a species as a group of microorganisms that have similar characteristics and that are significantly different from those of other groups. Thus, a bacterial species is a group of very closely related bacteria. Many bacterial species that are only genetically distantly related, however, have evolved similar morphological and physiological characteristics, making this definition ambiguous. DNA molecules (hereditary molecules) of all members of the same bacterial species have a very high degree of homology (similarity) to each other, and therefore modern classification systems increasingly rely on molecular analyses of DNA and RNA.

Early Classification Systems

Early classification systems date from the time of Aristotle, before the existence of microorganisms was known. These early systems recognized only two major groups—the plant and the animal Kingdoms—which could be easily distinguished based on motility. All organisms that moved were classified as animals and all those that remained stationary were called plants. In the eighteenth century, just after the microbial world was discovered, the noted Swedish botanist, Carl Linnaeus, established the first comprehensive classification system of all living things. He recognized that some microorganisms were motile and others remained stationary. Linnaeus placed all microorganisms into a single genus, which he appropriately named *Chaos* because he could not establish objective criteria for distinguishing among them.

Gradually, attempts were made to change *Chaos* into an orderly classification system. As is the case for higher organisms, identifying and naming a microorganism requires a system of classification in which the critical characteristics are systematically established that distinguish one microorganism from another. But because many microorganisms look exactly alike, we are usually unable to simply look at a microorganism and identify it. In most cases, to identify a microorganism, it is necessary to consider not only its form (morphology) but also its metabolism (physiology) and its evolutionary history as revealed in its genetics (its molecular sequences).

In 1866, Ernst Heinrich Haeckel proposed a classification system based on inferred evolutionary relationships among species. This was the first attempt to include evolution theory within a classification system. **Evolution** is the process of change that results from the interaction between the genetic information and the environment of organisms of a species. Evolution results in the formation of new organisms that are better adapted to the environment. Modern classification systems attempt to reflect the gradual evolutionary changes that have resulted in the appearance of new organisms. Such **phylogenetic classification systems** (from the Greek *phylo*, meaning kind or type, and *geny*, meaning origin) categorize organisms based on their evolutionary relatedness.

A phylogenetic classification system takes into account evolutionary relatedness.

Haeckel's system contained three Kingdoms: Protista, Animalia, and Plantae. Haeckel proposed that the microorganisms—bacteria, fungi, algae, and protozoa—all belonged to one primary Kingdom, which he called the **Protista.** The Protista were characterized by their simple structural organization, that is, by their lack of specialized tissues. Haeckel believed that the Protista was the first Kingdom to evolve and that both the Plantae and Animalia evolved from the Protista. They both had tissues. The Plantae had specialized tissues and were photosynthetic, using light energy for growth. The Animalia also had specialized tissues but were heterotrophic, meaning that they used organic compounds for energy and growth. Although this system was somewhat naive, it did show a great deal of insight. Its recognition of microorganisms as a single Kingdom distinct from plants and animals is appealing to microbiologists even today.

Modern Classification Systems

Robert H. Whittaker, in 1969, proposed a modification of Haeckel's system that became widely accepted by biologists (FIG. 2-2). Whittaker tried to organize his classification system along evolutionary lines, which he inferred from observed characteristics of living organisms. Whittaker proposed a five-Kingdom classification system: Animalia, Plantae, Fungi, Protista, and Monera. The microorganisms constitute three of the Kingdoms. The Kingdom **Monera** contains the bacteria, which are separated from all other organisms based on their prokaryotic cell structure. The Kingdom **Protista** contains the unicellular algae and protozoa based on the fact they have eukaryotic cells and are unicellular. The fungi, some of which are unicellular and some of which are multicellular, are in the Kingdom **Fungi,** based on their lack of specialized tissues.

According to Whittaker, the Monera (bacteria—prokaryotic organisms) were the first organisms on Earth and the Protista (protists—eukaryotic unicellular organisms) evolved directly from the Monera. Whittaker proposed that fungi, plants, and animals evolved from the protists via three separate directions of evolution. These evolutionary lines were based on differences in how the organisms met their nutritional needs. According to Whittaker's hypotheses, the fungi evolved as the most complex multicellular organisms that obtained their nutrients by absorption, that is, by taking up the chemicals that they needed for growth and reproduction. Animals evolved based on the ability to ingest other organisms to meet their nutritional needs. Plants evolved based on their photosynthetic capacity for self-feeding, that is, their ability to synthesize organic compounds from inorganic compounds using light as an energy source. Among the plants, he included the multicellular algae, such as the structurally complex brown and red algae. Whittaker's system, like the earlier ones, however, lacked direct evidence to support its validity.

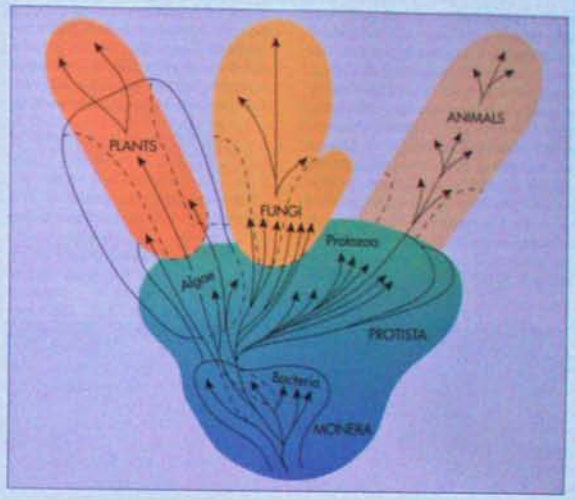


FIG. 2-2 Robert H. Whittaker in 1969 proposed a five-Kingdom classification system that emphasized evolutionary divergence from a presumed common ancestral prokaryotic bacterium. The lines of evolution followed nutritional strategies. Microorganisms occupy three of the five Kingdoms in this system.

The five-Kingdom classification system of Whittaker is based on evolution of species from the prokaryotes (bacteria) along strategies for obtaining nutrition.

In the early 1970s, developments in the field of molecular biology provided the first tools for directly examining the evolutionary relationships among microorganisms. Methods were developed that permitted determination of the degree of relatedness of the DNA from two different organisms. DNA contains the genetic information that is passed to progeny cells generation after generation. It is the primary substance of heredity. DNA provides a molecular record of relatedness that is far more accurate than the fossil record for tracing the path of evolutionary change for any organism. Additionally, RNA can be used to infer evolutionary relatedness among organisms. RNA is made by directly using the information in the DNA. Therefore it can be used to infer hereditary and, hence, evolutionary relatedness.

A precise determination of microbial relatedness can be made based on DNA or RNA nucleotide sequences.

Carl Woese, in the 1970s, analyzed RNA to explore the evolution of microorganisms. Woese reasoned that the RNA of ribosomes was changed only relatively slowly during the evolution of new organisms. Ribosomes are the sites where proteins are made within all cells. So, ribosomal RNA (*r*RNA) should not have changed much as a result of small evolutionary steps. Rather, *r*RNA should change significantly only as a result of major steps in evolution. Before Woese's studies, it was generally accepted that

all bacteria were closely related because they all were prokaryotic cells. Woese's analyses of rRNA molecules revealed that the bacteria actually fell into two distinct and only distantly related groups. One of these groups is as distantly related to organisms with eukaryotic cells as it is to the other group of bacteria.

Woese proposed a new and radically different classification system that defines these three groups as three primary Kingdoms for all living things: *Archaebacteria (Archaea)*, *Eubacteria*, and *Eukaryotes* (FIG. 2-3). According to Woese's theory, three separate paths of evolution from a common progenitor cell produced three different types of cells: the archaebacterial cell, the eubacterial cell, and the eukaryotic cell. Further studies on the physiological characteristics of the organisms designated by Woese as archaebacteria appear to substantiate his proposal. The chemical composition of the plasma membranes of archaebacteria is totally different from all other cells. Additionally, the archaebacteria have metabolic capabilities not found in any other organisms.

There are three distinct lines of cellular evolution that have led to the archaebacteria, eubacteria, and eukaryotes.

With regard to the evolution of the modern eukaryotic cell, with its multiple organelles, Woese accepted an idea put forth a few years earlier by Lynn Margulis—the **endosymbiotic theory of evolution.** Margulis proposed that some bacterial cells had begun to live within the predecessors of modern eukaryotic cells in a mutually beneficial (symbiotic) relationship that probably helped each meet its metabolic energy and nutritional requirements (FIG. 2-4). Living together, the prokaryotic and eukaryotic organisms could help each other live under conditions where either might not be able to live independently. Eventually the endosymbiotic bacteria lost their capacity for independent existence and developed into two of the cellular organelles (mitochondria and chloroplasts) involved in energy transformations that are found in eukaryotic cells today.

Endosymbiosis accounts for the acquisition of organelles by eukaryotes.

Woese was not concerned with the further evolution of the eukaryotic cell. He believed that the origins of the different types of cells, as revealed by the molecular record of evolution retained within DNA and rRNA molecules, should be used as the primary criteria in defining Kingdoms. Most microbiologists who concentrate their studies on organisms composed of one or relatively few cells readily accept this concept. However, many biologists who study higher organisms have difficulty in accepting Woese's system because it places all organisms with eukaryotic cells—including fungi, protozoa, algae, plants, and animals—into a single Kingdom, the eukaryotes. It is not surprising that lumping humans into the same Kingdom as fungi and algae meets some resistance and it is likely that further modifications to Woese's system will be made to subdivide the eukaryotes.

Classification systems continue to develop based on our abilities to examine the characteristics of organisms. Classification of living organisms has changed from systems based on first observational glimpses at the microbial world to systems based on detailed molecular analyses. In the progression of classification systems, we see a quest to change chaos into order and to establish a system of classification that reflects evolutionary relatedness.

Increased technological ability to examine the characteristic properties of organisms has permitted the establishment of more detailed and accurate classification systems for living things.

Interestingly, none of these classification systems considers the viruses. The viruses, perhaps as they should be, are treated as nonliving entities. An examination of the genetic molecules (RNA or DNA) of viruses indicates that they probably evolved from their respective host cells. They probably did not evolve in a hereditary lineage from one virus to the next. Hence, it is appropriate to classify viruses in relation to their host cells, for example, as a tobacco mosaic virus or a human immunodeficiency virus.

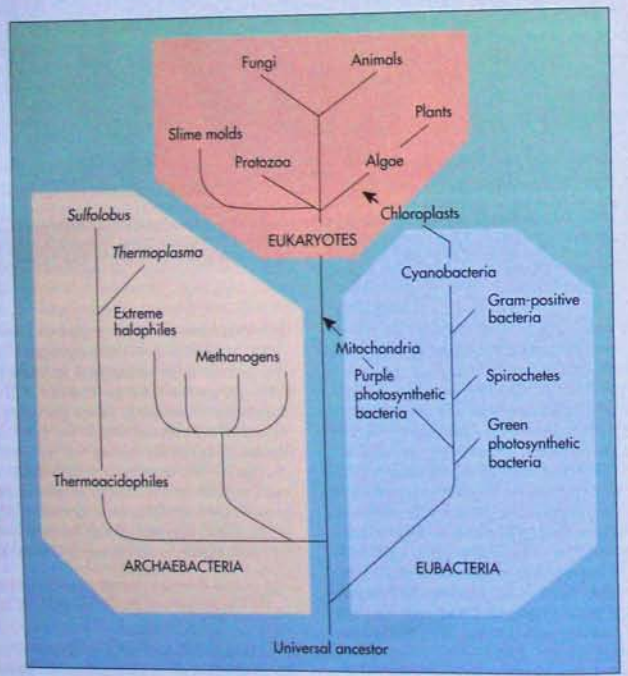


FIG. 2-3 The three-Kingdom classification system proposed by Carl Woese was developed using the modern techniques of molecular biology and, in particular, the examination of the RNA macromolecules of ribosomes. Unlike previous classification systems, the analysis of conserved gene products permits a direct assessment of genetic and, thus, evolutionary relatedness. Based on rRNA analyses, Woese found that there were three primary lines of evolution leading to the archaebacteria (Archaea), eubacteria, and eukaryotes. Modern eukaryotes have cells that incorporated mitochondria (derived from purple photosynthetic bacteria) and chloroplasts (derived from cyanobacteria); mitochondria and chloroplasts became organelles of eukaryotic cells by endosymbiosis.

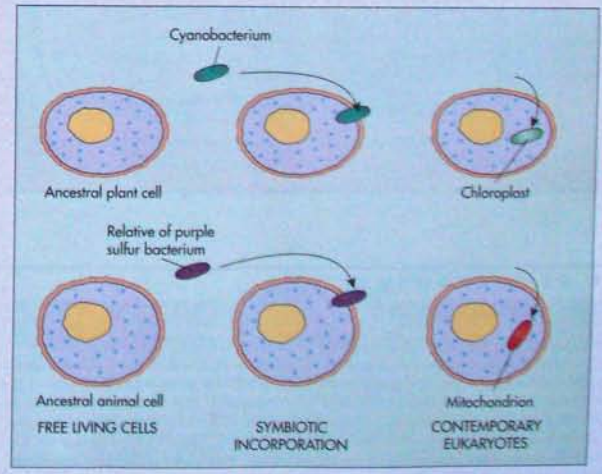
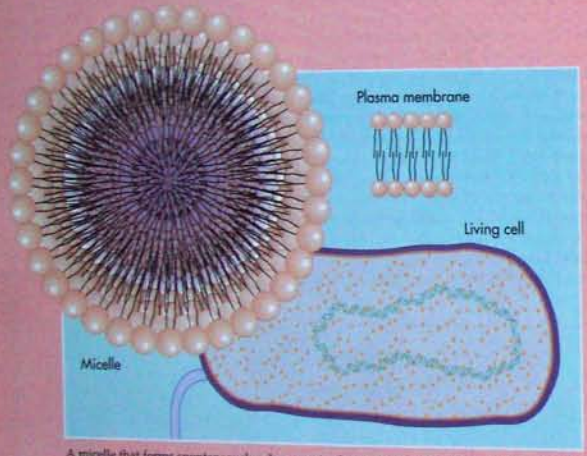


FIG. 2-4 According to endosymbiotic theory the chloroplasts and mitochondria of contemporary eukaryotic cells evolved from prokaryotic cells living within ancestral eukaryotic cells that lacked these organelles, providing photosynthetic and respiratory capabilities, respectively.

HIGHLIGHT

Evolution of Microorganisms

We do not know exactly how the first living organism developed. However, it is possible to show in laboratory experiments that some chemicals that could have accumulated in the primitive atmosphere of Earth spontaneously collect into spheres when wet with water (see Figure). These spheres, called *micelles*, resemble the cells that are the fundamental organizational units of all living systems. A micelle is separated from the surrounding environment by a chemical boundary layer. Its structure allows restricted exchange of materials with the surroundings while permitting the maintenance of a high degree of internal organization. It is likely that about 3.6 billion years ago the chemicals that accumulated within a micelle, including nucleic acid molecules



A micelle that forms spontaneously when certain chemicals interact with water resembles the structure of a living cell.

that can transmit hereditary information, permitted essential life functions to occur. These functions were the ability to process materials and energy and to reproduce. Thus the first living microorganism could have evolved on Earth. Paleobiologists believe they have found fossilized imprints of microbial communities that existed 3.5 to 3.8 billion years ago in western Australia and Greenland.

Of the chemicals that may have accumulated in a micelle, RNA (ribonucleic acid) appears to have had a key role. RNA, like the hereditary molecule of all contemporary living organisms, DNA (deoxyribonucleic acid), can encode genetic information. Also some RNA molecules can act as catalysts for chemical reactions, a role most often played by protein catalysts (enzymes) in living cells. In 1993, scientists at the Scripps Research Institute, made an artificial RNA molecule. When they mixed it with proteins it began to reproduce. As long as they continued to supply proteins, the RNA molecule churned out copies of itself.

In a similar manner the first microorganism probably used RNA as catalyst and guiding template and energy from the organic compounds that accumulated spontaneously in the primordial atmosphere or on the Earth's surface. Generally speaking, organic compounds are substances that contain carbon and hydrogen. With these compounds a living cell could have carried out chemical reactions. (Collectively these reactions are called metabolism.) From these reactions the living cell could obtain energy and transform substances into the materials needed to survive and reproduce. There would have been no molecular oxygen (O_2) in the primitive atmosphere. Therefore this first microorganism would have been an anaerobe, that is, an organism that grows and reproduces without using molecular oxygen.

Reproduction of this first microbial cell produced other living cells. Each of the new cells also reproduced, forming new cells with hereditary information that could be passed on to their progeny. In this continuous chain of descendents, errors or changes in the replication of DNA occasionally occurred so that some of the new cells received somewhat differing hereditary characteristics. Thus new microorganisms evolved. Some of the microorganisms that evolved could synthesize complex organic compounds from the carbon dioxide in the atmosphere. Some of these microorganisms were photosynthetic and were able to use light energy to make organic compounds. The metabolism of these microorganisms gradually changed conditions in the environment so that other organisms could evolve.

The first photosynthetic microorganisms were able to grow only in the absence of oxygen. Later—probably 2 billion years ago, based on geologic evidence—microorganisms evolved that could produce molecular oxygen from water. This oxygen-producing photosynthesis made possible other ways for cells to obtain energy, including aerobic respiration (a life-supporting process that uses oxygen). Over the next 0.6 billion years, the pace of evolution apparently quickened. While many microorganisms became extinct, an astonishing number of new organisms developed, including many types of plants, animals, and microorganisms.

SURVEY OF MICROORGANISMS

Acellular Nonliving Microorganisms

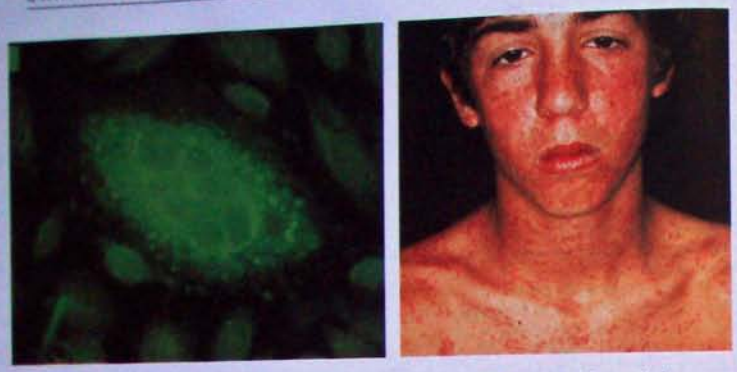
Viruses, viroids, and prions are acellular (noncellular), nonliving microorganisms that can replicate within the confines of a living cell of a compatible host organism (Table 2-1). It is this capacity for multiplying (replicating one's own structure) within a host cell that gives them their "lifelike character" and distinguishes them from other nonliving chemical combinations of molecules. A **host cell** is a compatible cell of a living organism within which a specific virus, viroid, or prion is capable of being replicated.

Viruses, viroids, and prions are obligate intracellular parasites that do not have an independent capacity to carry out life functions. When these acellular microorganisms are replicated within the cells of host organisms, they use the living cells' metabolic functions. Often in doing so they disrupt normal cellular functions, producing diseases in those organisms. Each virus, for example, produces characteristic symptoms when it replicates within a host organism, as typified by the characteristic mosaic pattern on the leaves of a tobacco plant when tobacco mosaic virus

TABLE 2-1

Some Characteristics of Acellular, Nonliving Microorganisms

MICROORGANISM	DESCRIPTION
Virus	Highly structured; contains DNA or RNA as the genetic informational molecule surrounded by a protein coat; may have an additional outer lipid-containing structure called an envelope; replicates within specific hosts (bacteriophage replicate in bacterial cells, plant viruses replicate in plant cells, and animal viruses replicate in animal cells), using the synthetic capabilities of the host cells.
Viroid	Infectious RNA molecule that can be replicated within specific plant cells
Prion	Infectious protein molecule that can be replicated within specific animal cells



A B

FIG. 2-5 **A,** Micrograph of cells infected with measles viruses. **B,** A child with a typical measles rash.

(TMV) infects the cells of that plant. We easily recognize the red rash of measles when the measles virus replicates within certain human cells (FIG. 2-5).

Viruses, viroids, and prions are nonliving acellular microorganisms that can only replicate within living cells.

Viruses

A **virus** is made up of two essential parts: a central genetic nucleic acid molecule and a protein coat called a **capsid** (see FIG. 1-5). The capsid surrounds and protects the viral nucleic acid. The capsid gives the virus a characteristic shape. It also helps establish the specificity of the virus for a particular host cell. Some viruses also have an external **viral envelope** made up of plasma membrane that they acquire from host cells within which they replicate. Unlike prokaryotic and eukaryotic cells, viruses contain only one type of nucleic acid—RNA or DNA—as their hereditary molecule. Some viruses are RNA viruses and others are DNA viruses.

Viruses are composed of a central genetic core—DNA or RNA—surrounded by a protein coat called a capsid.

Viruses are not capable of carrying out metabolism independent of a host cell. They replicate only in specific host cells. For example, viruses that replicate within a bacterial cell do not replicate within the cells of other organisms. This specificity, as well as other characteristics of viruses, is the basis for believing that they evolved from host cells rather than beginning as primitive or independent entities.

Interactions between the viral capsid and the outer layer of the host cell determine whether the viral nucleic acid will be able to enter the host cell. Viral replication within host cells causes numerous plant and animal diseases, including, in humans, the common cold, influenza, AIDS, herpes, measles, mumps, chickenpox, yellow fever, hepatitis, and many more.

There are three groups of viruses. **Animal viruses** infect and replicate within animal cells, **plant viruses** within plant cells, and **bacteriophage** or **phage** within bacterial cells. Viruses that infect bacterial cells cannot infect and replicate within cells of other organisms. Often the specificity is even greater than this. A virus that infects a tomato plant generally cannot infect other plants. This results in greater specificity of viral diseases. A phage that infects the bacterium *Escherichia coli* often cannot infect other bacteria or even other subtypes of *E. coli*. It is possible to utilize this specificity to identify bacteria, a procedure called phage typing.

Viroids

Unlike viruses, **viroids** are composed exclusively of RNA that contains their genetic information. They have no structures surrounding their genetic molecules. Inside a suitable host cell the RNA of a viroid is capable of initiating its own replication. This sometimes manifests as disease symptoms in the host organism. It was discovered in the early 1970s that viroids cause potato spindle tuber disease, chrysanthemum stunt, citrus exocortis and a few other plant diseases. Thus far they appear to affect only plants.

Viroids are infectious RNA molecules found in plants.

Prions

The most recently discovered and least understood microorganisms are the **prions** (for proteinaceous infectious particles). What is so unusual about prions is that they seem to be composed only of protein. Like viroids, these "organisms" have no structures. They are only individual protein molecules that contain the information that codes for their replication when they infect a suitable host cell. They are properly called infectious proteins.

Unlike cellular and even other acellular organisms, prions do not store their genetic (hereditary) material in nucleic acid molecules. This presents a problem in understanding how prions replicate. Prions do not fit into our current understanding of how genetic information in nucleic acid molecules is replicated and how it can determine the specific structural and functional characteristics of each organism. Scientists do not know how a protein can direct its replication, and thus they do not understand how prions replicate.

Prions are infectious proteins.

Prions can cause diseases of the nervous system. They were discovered during the search for the cause of scrapie, an infectious and usually fatal disease of sheep. This disease was known to be caused by an agent that could pass through a filter that could trap bacteria and larger organisms. Therefore it was believed to be caused by a virus. No virus could be found, and eventually scrapie was shown to be caused by an infectious protein, that is, a prion. Prions have been found to cause some exotic human diseases such as kuru, a disease restricted to certain tribes of New Guinea that practice cannibalism. Some scientists have also hypothesized that prions may cause various degenerative nervous disorders, including Alzheimer's disease, that up until now have had no known cause.

PROKARYOTES

Microorganisms with prokaryotic cells include the archaebacteria and the eubacteria (Table 2-2). The prokaryotic cell is structurally not as complex as the eukaryotic cell. However, don't be misled by this statement. All living systems are extremely complex. Eubacteria and archaebacteria, like all other living organisms, must meet their energy and material needs through their metabolic activities, replicate their hereditary (genetic) information, and reproduce. Failure to accomplish any or all of these tasks results in death.

The distinction between archaebacteria and eubacteria is relatively new. Before 1980 all organisms with prokaryotic cells were called bacteria. However, as we discussed earlier, beginning in the late 1970s, scientists discovered that there were two distinct lines of evolutionary descent among the prokaryotes. The distinctions between these two groups will be considered later. One group was given the name **archaebacteria,** implying that these were early evolutionary forms. The archaebacteria have subsequently been called by some the *Archaea* or *archaeobacteria*. The second group was given the name **eubacteria.** Most often these prokaryotes are simply called bacteria.

Naming Bacteria

Like all other living organisms, each bacterial species is given a formal name that distinguishes it from all other organisms (Table 2-3). The formal names of bacteria and all other organisms are given in Latin. Latin is used because it was the classical language of science at the time when formal names were first given to organisms on a systematic basis. When typed or handwritten, species names are italicized, for example, the name *Streptococcus pneumoniae*. (This bacterial species sometimes causes pneumonia in humans.)

The formal name of a species is a **binomial name,** indicating that it has two parts. The use of binomial

TABLE 2-2

Some Characteristics of Prokaryotic Microorganisms

MICROORGANISM	PLASMA MEMBRANE	CELL WALL	GROWTH
Eubacteria (usually referred to simply as bacteria)	Composed largely of phospholipid molecules that have an ester bond linking the fatty acids to the glycerol	Most surrounded by a rigid cell wall composed of a unique molecule called peptidoglycan that has a carbohydrate (glycan) portion and a proteinlike (peptide) portion that contains amino acids not typically found in other organisms	Diverse, including growth on organic compounds, inorganic compounds, and light energy for different species
Archaebacteria (also called archaeobacteria or Archaea)	Composed of lipid molecules that have an ether bond that links the molecules together	Some surrounded by a rigid cell wall composed of unique molecules that resemble but are not the same as peptidoglycan; others have a protein coat or a complex carbohydrate	Many species grow at extreme conditions, such as in high temperature, high acidity, and saline environments

names was introduced by Carl Linnaeus. The first part of the binomial name, for example, *Streptococcus*, is the genus name. A **genus** is a group of closely related species. The first letter of the genus name is capitalized. The second part of the binomial name, for example, *pneumoniae* is the **species epithet**. The species epithet is written in all lower case letters. The **species name** must always contain the genus name and the species epithet. It is permissible, though, to abbreviate the genus name. In this case the genus is indicated by a single capital letter followed by a period—for example, *S. pneumoniae*.

We sometimes designate a subspecies or type to specify some significant characteristics of a particular microorganism. For example, a particular strain of *S. pneumoniae* of the American Type Culture Collection (ATCC) that is resistant to treatment with penicillin is designated *S. pneumoniae* ATCC 35088. Also the pathovar (PV) or serovar (SV) may be noted by letters or numbers, for example, *Escherichia coli* O157:H7, a strain of this common species that is associated with serious dysentery.

TABLE 2-3

Some Names of Bacteria and Their Characteristics.

NAME	MORPHOLOGY		PHYSIOLOGY	
	SHAPE	STAINING	GROWTH	PATHOGENICITY
Bacillus anthracis	Large rod-shaped cells	Gram positive	Aerobic growth; forms heat-resistant endospores	Causes anthrax
B. cereus	Large rod-shaped cells	Gram positive	Aerobic growth; forms heat-resistant endospores	Nonpathogen or food poisoning
B. subtilis	Large rod-shaped cells	Gram positive	Aerobic growth; forms heat-resistant endospores	Nonpathogen
Bordetella pertussis	Short rod-shaped cells	Gram negative	Fastidious (demanding growth requirements)	Causes pertussis (whooping cough)
Chlamydia trachomatis	Small spherical (coccal) cells	Gram negative	Grows only within mammalian cells	Causes a sexually transmitted disease and trachoma
Clostridium botulinum	Large rod-shaped cells	Gram positive	Anaerobic growth; forms heat-resistant endospores	Causes botulism
C. perfringens	Large rod-shaped cells	Gram positive	Anaerobic growth; forms heat-resistant endospores	Causes gas gangrene and food poisoning
C. tetani	Large rod-shaped cells	Gram positive	Anaerobic growth; forms heat-resistant endospores	Causes tetanus
Corynebacterium diphtheriae	Rod-shaped cells; cells remain attached at angles	Gram positive	Fastidious (demanding growth requirements)	Causes diphtheria
Enterobacter aerogenes	Short rod-shaped cells	Gram negative	Aerobic and anaerobic growth	Nonpathogen or opportunistic pathogen
Escherichia coli	Short rod-shaped cells	Gram negative	Aerobic and anaerobic growth	Nonpathogen that lives in human gut; commonly used in microbial genetics; some strains pathogenic
Haemophilus influenzae	Short rod-shaped cells	Gram negative	Fastidious; requires blood factors	Causes upper respiratory infections and meningitis
Klebsiella pneumoniae	Short rod-shaped cells	Gram negative	Aerobic and anaerobic growth	Causes pneumonia
Lactobacillus acidophilus	Short rod-shaped cells, forms chains of cells	Gram positive	Produces lactic acid	Nonpathogen
Legionella pneumophila	Short rod-shaped cells	Gram negative	Fastidious; requires iron and cysteine	Causes Legionnaire's disease and Pontiac fever
Micrococcus luteus	Spherical (coccal) cells occurring in packets	Gram positive	Produces a yellow pigment	Nonpathogen
Mycobacterium leprae	Rod-shaped cells	Acid fast	Slow grower	Causes leprosy
M. tuberculosis	Rod-shaped cells	Acid fast	Slow grower	Causes tuberculosis
Neisseria gonorrhoeae	Large coccal-shaped cells that occur as pairs	Gram negative	Fastidious	Causes gonorrhea
N. meningitidis	Large coccal-shaped cells that occur as pairs	Gram negative	Fastidious	Causes meningitis
Proteus vulgaris	Short rod-shaped cells	Gram negative	Aerobic and anaerobic growth	Nonpathogen or opportunistic pathogen
Pseudomonas aeruginosa	Rod-shaped cells that occur singly	Gram negative	Aerobic growth	Opportunistic pathogen; important in skin infections
Rickettsia rickettsii	Rod-shaped cells	Gram negative	Obligate intracellular parasite	Causes Rocky Mountain spotted fever, transmitted by ticks
R. typhi	Rod-shaped cells	Gram negative	Obligate intracellular parasite	Causes typhus, transmitted by body lice
Salmonella typhi	Rod-shaped cells	Gram negative	Aerobic and anaerobic growth	Causes typhoid fever
Serratia marcescens	Rod-shaped cells	Gram negative	Produces bright red pigment	Opportunistic pathogen
Shigella dysenteriae	Rod-shaped cells	Gram negative	Aerobic and anaerobic growth	Causes dysentery
Spirillum volutans	Helical-shaped cells	Gram negative	At low oxygen concentration	Nonpathogen
Staphylococcus aureus	Coccal-shaped cells that occur in grape-like clusters	Gram negative	Aerobic growth	Causes various diseases, including food poisoning, boils, and upper respiratory infections
Streptococcus pneumoniae	Coccal-shaped cells that occur in chains	Gram positive	Produces lactic acid	Causes pneumonia
Streptococcus pyogenes	Coccal-shaped cells that occur in chains	Gram positive	Produces lactic acid	Causes upper respiratory infections, rheumatic fever, and scarlet fever
Treponema pallidum	Helical-shaped cells that are wound around flagella that are attached to the ends of the cells	Gram negative	Fastidious	Causes syphilis
Vibrio cholerae	Curved, comma-shaped rods	Gram positive	Aerobic and anaerobic growth	Causes cholera
Yersinia pestis	Short rod-shaped cells	Gram negative	Fastidious	Causes plague, transmitted by fleas

Characteristics Used in Classifying and Identifying Bacteria

Many different characteristics are used in classifying and identifying bacteria (Table 2-4). These include general tests that are applied for virtually all bacteria and very specialized tests that are used to identify specific bacterial strains. Often completing the tests to identify a bacterial species, for example, for clinical laboratory identification, will take 1 or more days. Modern identification techniques are reducing this time to less than a day and even to just a few minutes for the identification of some disease-causing bacteria.

The differing morphologies (shapes and structures) of bacteria are used for classification and identification. Microscopic observations are used to view the shapes of bacterial cells. Bacteria are described as *cocci* if the cells are spherical and as *rods* if the cells are shaped like cylinders (FIG. 2-6.) Other shapes include curved rods, spirals, and others. The arrangement pattern of the cells, derived from the number and planes of division, is also an important characteristic that distinguishes one species from another. The cells of some bacteria, such as *Escherichia coli*, occur singly. Other species have cells that occur as pairs, tetrads, chains, or irregular clusters.

Specific staining reactions are also used to describe and classify bacteria. The **Gram stain** is a specific staining procedure, described in Chapter 3; it is especially important in describing and classifying bacteria. The Gram stain reaction is determined by the specific chemical composition of the cell wall, a structure that in most bacterial cells surrounds and protects the cell. Some bacterial species stain blue-purple by the Gram stain procedure and are called **Gram-positive bacteria;** the other bacterial species stain pink-red and are called **Gram-negative bacteria** (see FIG. 3-13). The bacterium *E. coli*, which occurs in the human intestine and is the most widely studied bacterial species, is a Gram-negative rod-shaped bacterium that occurs as single cells. *Streptococcus pneumoniae*, on the other hand, is a Gram-positive cocci that occurs in pairs and sometimes causes pneumonia.

The arrangement of **flagella,** which are filamentous structures involved in the movement of cells, also is important in classification. Some bacterial cells, such as those of *E. coli*, have flagella, called **peritrichous flagella,** that surround the cell. Other bacteria, such as *Pseudomonas aeruginosa*, have one or more flagella, called **polar flagella,** originating from one end of the bacterial cell.

Other special structures are also used to distinguish specific bacteria. For example, the presence of **endospores,** which are dormant heat-resistant structures, is important in classifying some bacteria. *Clostridium* and *Bacillus* species have endospores that make them particularly difficult to kill by heating. This is a particular problem in food canning where heat is used to kill microorganisms that can spoil food and cause disease.

Although morphological and staining characteristics are useful in describing and identifying bacteria, alone they are insufficient to discriminate among bacteria. Too many bacteria look alike. **Growth characteristics,** such as the optimal temperature for growth, and **metabolic characteristics,** such as the ability to produce acid from glucose, are important for classifying and identifying bacteria. Even when examining only a limited number of bacteria, such as those that cause human infections and that are seen in the clinical microbiology laboratory, at least 20 such metabolic characteristics generally must be determined to identify a particular bacterial species (FIG. 2-7).

TABLE 2-4
Some Characteristics Used for Classifying and Identifying Bacteria

CRITERION	EXAMPLE
Cellular Characteristics	
Morphology	Cell shape, cell size, arrangement of cells, arrangement of flagella, capsule, endospores
Staining reactions	Gram stain, acid-fast stain
Growth and nutritional characteristics	Appearance in liquid culture, colonial morphology, pigmentation, energy sources, carbon sources, nitrogen sources, fermentation products, modes of metabolism (autotrophic, heterotrophic, fermentative, respiratory)
Biochemical characteristics	Cell wall constituents, pigment biochemicals, storage inclusions, antigens, RNA molecules
Physiological and ecological characteristics	Temperature range and optimum, oxygen relationships, pH tolerance range, osmotic tolerance, salt requirement and tolerance, antibiotic sensitivity
Genetic characteristics	DNA mole% G + C, DNA hybridization

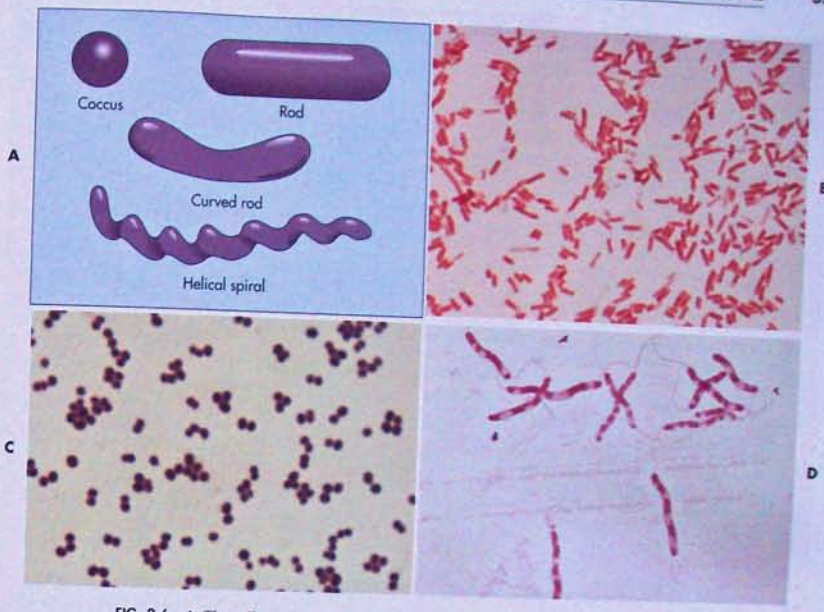


FIG. 2-6 **A,** The cells of each bacterial species have characteristic shapes. The most common shapes of bacterial cells are rods (cylindrical structures), cocci (spheres), and helical spiral (curved cells). **B,** Micrograph showing the rod-shaped cells of *Escherichia coli*, which lives in the human intestines (1,800×). **C,** Micrograph showing the coccal-shaped cells of *Staphylococcus epidermidis*, which lives on the human skin. (1,800×). **D,** Micrograph showing the spiral-shaped cells of the aquatic bacterium *Spirillum volutans*.

FIG. 2-7 Microtiter plate used in clinical microbiology laboratory for determination of metabolic characteristics of isolated bacteria. The color reactions indicate utilization of specific substances.

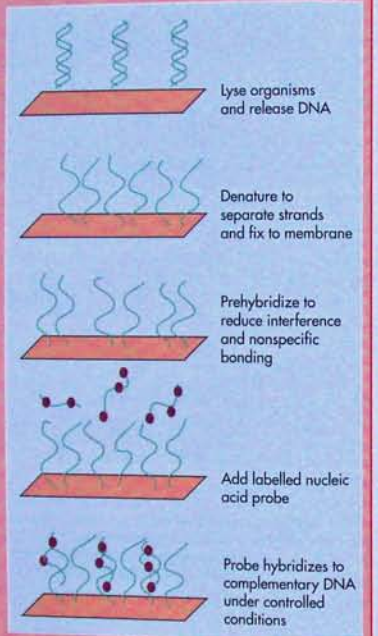
METHODOLOGY

DNA Hybridization: A Method for Analyzing Genetic Relatedness

One method used to assess genetic relatedness among organisms is called DNA hybridization (see Figure). To understand how this DNA hybridization method permits scientists to determine the relatedness of two organisms, we must first recognize that (1) DNA is composed of a series of individual molecules called nucleotides that are linked together in a particular order to establish the informational content of each organism, much in the way that the letters of our alphabet are linked together to form words and sentences, (2) DNA is composed of two complementary strands, and (3) DNA is the universal hereditary substance of all living organisms. The question of relatedness can be explored by determining what sequences of nucleotides the organisms have in common, both in terms of the specific nucleotides and their particular order (sequence) within the DNA molecule.

In the process of DNA hybridization, a radioisotope or a fluorescent dye is used to label or tag the DNA from one of the organisms. Then the DNA from both organisms is converted from double strands to single strands. These labelled and nonlabelled single strands are incubated together, allowing the DNA to reform double strands. Where the labelled DNA matches the nonlabelled DNA, the amount of radioactivity or dye measured will determine the extent to which the DNA from the two organisms combined.

Using this technique, scientists can determine directly the relatedness of organisms from bacteria to mammals. The formation of hybrid double-stranded DNA, that is, a double-stranded DNA molecule in which each strand came from a different organism, is a measure of the similarity, called homology, between the DNA of the two organisms. DNA molecules from organisms that are closely related, such as members of the same species, have a high degree of homology. Because evolution is based on changes in hereditary information and because DNA contains the hereditary information, DNA hybridization provides a tool for directly analyzing evolutionary relatedness for all organisms. No longer is it necessary to infer relationships based solely on appearances or metabolic functions. Using DNA hybridization, microbiologists can determine the relatedness of bacterial species.



In DNA hybridization procedures for gene probe detection, cells are lysed to release double-stranded DNA. The DNA is denatured to convert it to single-stranded target DNA. The single-stranded DNA is affixed to a membrane. A prehybridization solution is used to prevent nonspecific binding to the membrane. A labelled nucleic acid probe (gene probe) is added. (The label may be a dye or a radioactive element.) The labelled probe hybridizes to complementary regions (if any) of the target DNA.

Additionally, **genetic characteristics** are employed in modern classification systems. DNA from different bacteria is compared to reveal the degree of similarity. In some clinical identifications, species are identified using DNA hybridization. Often, short segments of DNA, called *gene probes*, are used to determine the presence or absence of specific diagnostic genes. This is a powerful technique for identifying specific microorganisms

Many morphological, metabolic, and genetic characteristics are used for describing, classifying, and identifying bacteria.

TABLE 2-5

Some of the Major Groups of Bacteria Described in *Bergey's Manual*

GROUP	DESCRIPTION
Spirochetes	Very slender rods that are helically coiled around a central axial filament; includes the bacteria that cause syphilis and Lyme disease
Gram-negative aerobic rods and cocci	Bacteria that have a cell wall structure that results in their staining pink-red by the Gram stain procedure, are cylindrical or spherical in shape, and obtain their energy by respiration
Gram-negative facultatively anaerobic rods	Bacteria that have a cell wall structure that results in their staining pink-red by the Gram stain procedure and obtain their energy either by respiration or anaerobic fermentation
Gram-negative anaerobic bacteria	Bacteria that have a cell wall structure that results in their staining pink-red by the Gram stain procedure and obtain their energy by anaerobic fermentation
Rickettsias and chlamydias	Bacteria that are obligate intracellular parasites, that is, capable of reproducing only within host cells; includes the bacteria that cause Rocky Mountain spotted fever, trachoma, and urinary tract infections
Phototrophic bacteria	Photosynthetic bacteria that derive their energy from light; contain pigmented molecules that are used in photosynthesis; includes the purple and green sulfur bacteria, which do not produce oxygen during photosynthesis, and cyanobacteria, which do produce oxygen during photosynthesis
Gram-positive cocci	Bacteria that have a cell wall structure that results in their staining blue-purple by the Gram stain procedure and that are spherical; include the streptococci and staphylococci
Endospore-forming rods and cocci	Bacteria that form heat-resistant bodies called endospores within their cells; include the bacteria that cause gas gangrene, botulism, tetanus, and anthrax
Gram-positive, nonspore-forming rod-shaped bacteria	Bacteria that have a cell wall structure that results in their staining blue-purple by the Gram stain procedure, are cylindrical in shape, and do not form endospores
Mycobacteria	Bacteria that have a cell wall structure that results in their staining pink-red by the acid-fast staining procedure; include the bacteria that cause tuberculosis and leprosy
Actinomycetes and related organisms	Bacteria that form branching filaments and those that are closely related based on cell wall structure
Mycoplasmas	Bacteria that lack a cell wall; include bacteria that can cause atypical pneumonia that is not treatable with penicillin
Archaebacteria	Physiologically unique bacteria distantly related to other prokaryotes that include methane producers, bacteria that grow at low pH and high temperature, and bacteria that require high salt concentrations

Major Groups of Bacteria

Information on the characteristics of bacterial genera and species is updated periodically and published in a book entitled ***Bergey's Manual of Determinative Bacteriology*** (Table 2-5). *Bergey's Manual* is the standard reference for descriptions of microorganisms—the "bible" of bacterial taxonomy. Examining *Bergey's Manual* quickly reveals that relatively few bacterial species cause disease, even though these may be the ones about which we are most commonly concerned. Most bacteria perform metabolic activities that maintain the ecological balance of the Earth. Only a small portion of the numerous bacterial species described in *Bergey's Manual* are ever seen in the clinical laboratory or ever discussed in introductory microbiology courses. In this book we will be able to examine only a small portion of the diverse bacteria that exist in nature.

Beyond their common prokaryotic cell structure, bacteria exhibit extreme diversity of form and function and in their metabolic characteristics.

Several of the major groups of bacteria described in *Bergey's Manual* are distinguished primarily on morphological characteristics, namely: cell shapes

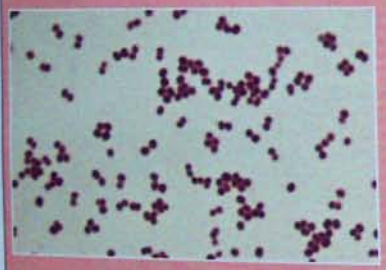
(rods, cocci, curved, or filament forming); spore production (endospores, or other spores); staining reactions (color after staining according to the Gram stain procedure—Gram negative or Gram positive); and motility (nonmotile, motile with peritrichous flagella surrounding the cell, motile with polar flagella projecting from the end of the cell, motile by gliding). Other major bacterial groups are defined based on their metabolism, in particular how they generate energy in the form of ATP (photosynthetic [using light energy], chemolithotrophic [using inorganic compounds], heterotrophic [using organic compounds]), and whether the metabolism is anaerobic (not using molecular oxygen), aerobic (using molecular oxygen), or facultatively anaerobic (capable of aerobic and anaerobic metabolism). Yet others are defined based on combined morphological and physiological characteristics. These characteristics are determined by grouping cultures of bacteria and making observations on those cultures and the cells that grow.

Text continued on p. 51.

HIGHLIGHT

Microview of Bacterial Diversity

Gram-positive cocci include the genus *Staphylococcus*, which typically form grape-like clusters and the genus *Streptococcus*, which occur in pairs or chains. Species of *Staphylococcus* commonly occur on skin surfaces where they live without causing disease. *Staphylococcus aureus*, however, is a potential human pathogen, infecting wounds, membranes, and linings and also causing food poisoning. Some members of *Streptococcus* are also human pathogens. For example, rheumatic fever is caused by *Streptococcus pyogenes*. Several *Streptococcus* species are also responsible for the formation of dental caries, which cause tooth decay.

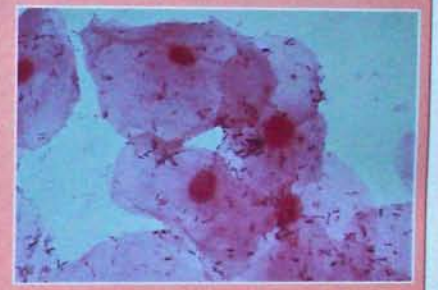


Micrograph of *Staphylococcus aureus*. This bacterium forms grape-like clusters of cells. It causes several human infections, including boils of the skin.

The two most important genera of **endospore-forming bacteria**, *Bacillus* and *Clostridium*, are Gram-positive rods. *Bacillus* species can grow in the presence of air, whereas *Clostridium* species are obligately anaerobic. Food spoilage by *Bacillus* and *Clostridium* species is of great economic importance. Several *Clostridium* species are important human pathogens. For example, *Clostridium botulinum* is the causative agent of botulism, *Clostridium tetani* causes tetanus, and *Clostridium perfringens* causes gas gangrene.

Micrograph of a *Clostridium* species after endospore staining. The spores appear green and the bacterial cells are stained red. The spores of this bacterium are heat resistant, and can survive in boiling water for hours.

The **Gram-positive nonspore-forming, rod-shaped bacteria** include bacteria that produce lactic acid. *Lactobacillus* are Gram-positive nonspore-forming rods that occur in chains. The lactobacilli are extremely important in the dairy industry. Cheese, yogurt, and many other fermented products are made by the metabolic activities of *Lactobacillus* species. They also inhabit regions of the human body, including teeth.



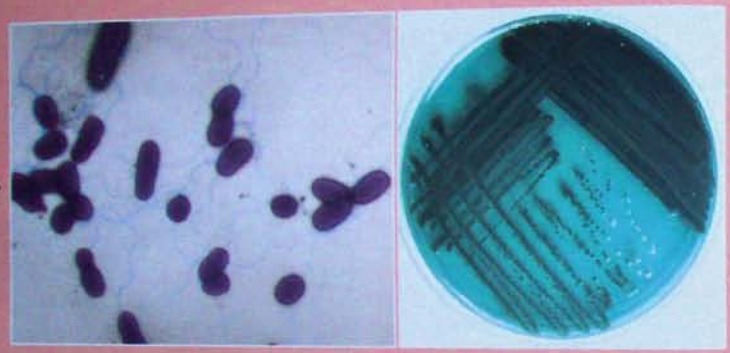
Micrograph of *Lactobacillus* species in a vaginal smear. Lactobacilli colonize many body surfaces. The lactic acid they produce helps protect against infections with disease-causing microorganisms.

The **Gram-negative facultatively anaerobic rods** include intestinal enteric bacteria. They are motile by means of peritrichous flagella and often live in the human intestinal tract. Much of what we know about bacterial metabolism and bacterial genetics has been elucidated in studies using *Escherichia coli*. *E. coli* is employed as an indicator of fecal contamination in environmental microbiology. The genera *Salmonella* and *Shigella* contain many species, many of which are important human pathogens. In particular, typhoid fever and various gastrointestinal upsets are caused by *Salmonella* species and bacterial dysentery is caused by *Shigella*.

Micrograph showing the rod-shaped cells of *Escherichia coli*. This bacterium lives in the human gut and is the most commonly studied.

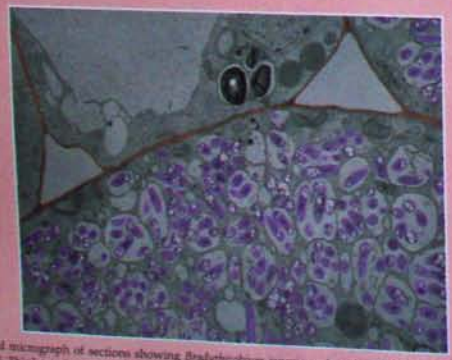
Continued.

The **Gram-negative aerobic rods** encompass a metabolically diverse group of bacteria; all of which can carry out aerobic metabolism. *Pseudomonas* is an aerobic motile bacterial species that is rod-shaped with polar flagella. Many *Pseudomonas* species are nutritionally versatile and capable of degrading many natural and synthetic organic compounds. Some *Pseudomonas* species are plant and animal pathogens. *P. aeruginosa*, can be a human pathogen and is commonly isolated from wound, burn, and urinary tract infections.



Various pseudomonads degrade environmental pollutants. Others cause human and plant diseases. **A**, Colorized micrograph of *Pseudomonas* sp. **B**, *Pseudomonas aeruginosa* growing on an agar plate.

Azotobacter, *Rhizobium*, and *Bradyrhizobium* are Gram-negative aerobic rods that are capable of fixing atmospheric nitrogen. This is a metabolic function unique to a select group of bacteria. *Rhizobium* species infect leguminous plant roots, where they cause the formation of tumorous growths called nodules within which they live in a mutually beneficial relationship. Within nodules, *Rhizobium* cells are irregularly shaped.

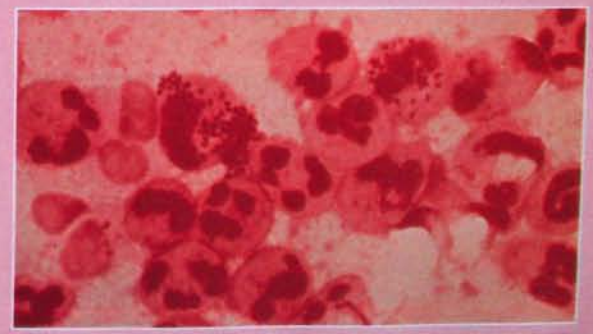


Colorized micrograph of sections showing *Bradyrhizobium japonicum (purple)* within a nodule of a soybean plant cell. This bacterium fixes atmospheric nitrogen and provides nitrogen-containing nutrients to the plant.

The Gram-negative aerobic rod-shaped *Agrobacterium* produces tumorous growths on infected plants. These growths are known as galls. *Agrobacterium tumefaciens* causes galls of many different plants and is an extremely important plant pathogen, causing large economic losses in agriculture.

A tree with crown gall caused by tumorous growth at base of the tree.

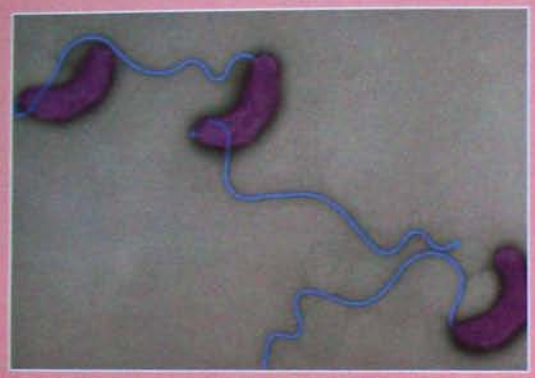
The genus *Neisseria* is a representative example of the **Gram-negative cocci.** *Neisseria gonorrhoeae* causes gonorrhea and *Neisseria meningitidis* causes bacterial meningitis. These bacteria tend to form relatively large cocci that typically look like kidney beans. The cells occur in pairs (diplococci).



Micrograph of intracellular Gram-negative diplococci. The presence of these bacteria in a urethral discharge is diagnostic for gonorrhea and in a vaginal discharge is presumptive for gonorrhea.

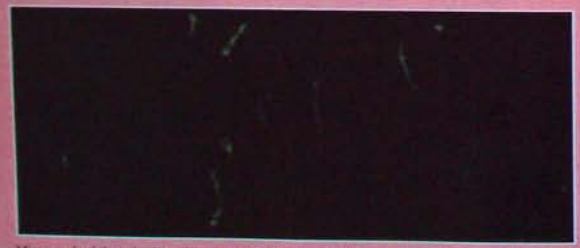
Continued

The **helical** and **curved bacteria** group are helically curved rods that may have less than one complete turn (comma-shaped) to many turns (helical). *Campylobacter fetus* is a curved bacterium that frequently is the cause of gastrointestinal infections in infants. *Bdellovibrio*, which also is curved, has the unique characteristic of being able to penetrate and reproduce within the cells of other bacteria.



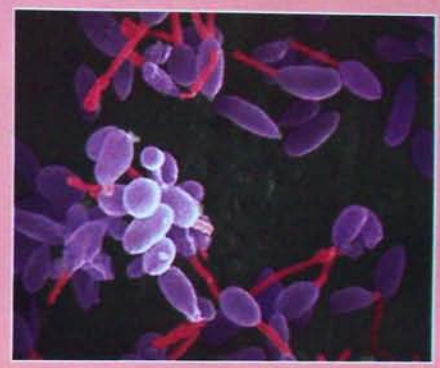
Colorized micrograph of the comma-shaped bacterium *Bdellovibrio bacteriovorus*. This bacterium reproduces within cells of the bacterium *Escherichia coli*.

The **spirochetes** are helically coiled rods, with one or more central axial fibril(s) wound around each cell. Many spirochetes are human pathogens. Several members of the genus *Treponema*, for example, are human pathogens. *Treponema pallidum* causes syphilis, which is a sexually transmitted disease. *Treponema pertenue* causes yaws. *Borrelia burgdorferi*, another spirochete, causes Lyme disease.



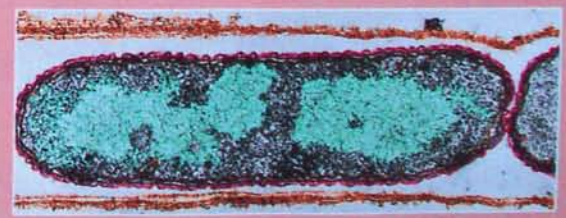
Micrograph of the spirochete *Treponema pallidum* (*green helical-shaped cells*) after fluorescent antibody staining. This bacterium causes syphilis.

The **budding** and/or **appendaged bacteria** are grouped together because they produce cell appendages. Several of these bacteria reproduce by budding. This involves separating a portion of the cell to form a new progeny cell. Many of the appendaged bacteria grow well at low nutrient concentrations. *Caulobacter*, for example, can grow in very dilute concentrations of organic matter in lakes and even is able to grow in distilled water. Some adhere to surfaces of other cells via their appendages.



Colorized micrograph of the stalk-forming bacterium *Hyphomicrobium*. The stalks (*pink*) are part of the cells (*purple*).

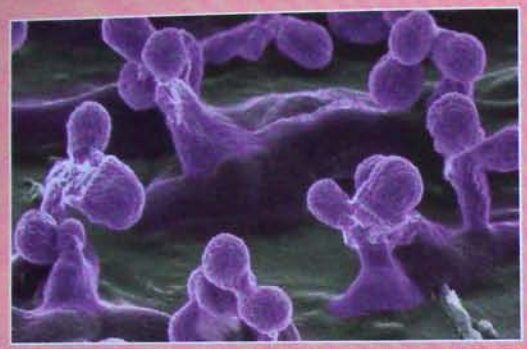
The **sheathed bacteria** comprise bacteria whose cells occur within a filamentous structure known as a sheath. The formation of a sheath enables these bacteria to attach themselves to solid surfaces. It also affords protection against predators and parasites. *Sphaerotilus natans* is a sheathed bacterium that is often referred to as the sewage fungus. This organism normally occurs in polluted flowing waters, such as sewage effluents, where it may be present in high concentrations just below sewage outfalls.



Colorized micrograph of the filamentous bacterium *Sphaerotilus natans*. Cells of this bacterium are enclosed within a sheath (*orange*). It occurs abundantly in rivers below sewage outfalls and is called the "sewage fungus" because of its filamentous (fungal-like) appearance.

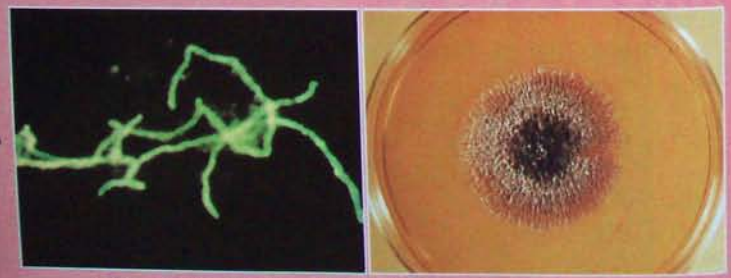
Continued.

Some bacteria are grouped based on their *gliding* motility on solid surfaces. These bacteria lack the specialized structures—flagella—that other bacteria use to propel themselves. The myxobacteria are gliding bacteria that have a unique feature. Under appropriate conditions they aggregate to form *fruiting bodies*. These fruiting bodies represent a stage in the complex reproductive process carried out by these bacteria. The fruiting bodies of myxobacteria occur on decaying plant material, on the bark of living trees, or on animal dung, appearing as highly colored slimy growths that may extend above the surface of the substrate.



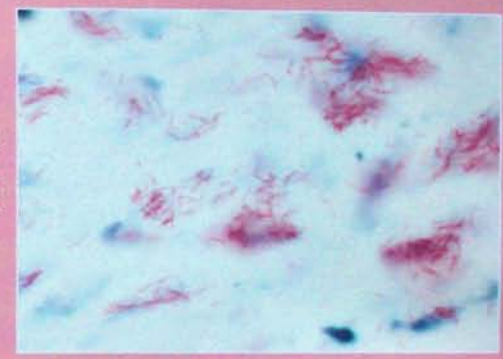
Colorized micrograph of the fruiting myxobacterium *Stigmatella aurantiaca*, formed by the aggregation of many thousands of this bacterium.

Actinomycetes are bacteria that resemble fungi in appearance because they form filamentous growths. The production of antibiotics by actinomycetes, such as *Streptomyces griseus*, is extremely important in the pharmaceutical industry. Many previously fatal diseases are now easily controlled by using antibiotics produced by actinomycetes. Other actinomycetes are human pathogens.



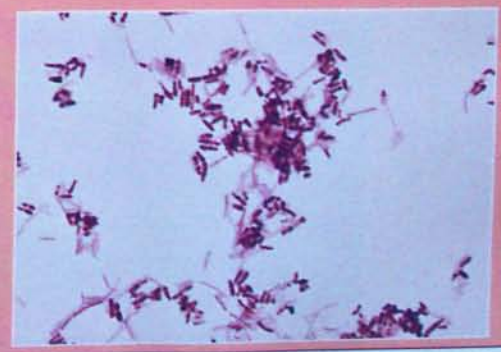
A, Micrograph of the actinomycete *Actinomyces israilii* showing hyphae formation. This bacterium is a human pathogen. **B,** *Streptomyces griseus* growing on an agar plate.

Mycobacteria are acid-fast, meaning that stained cells resist decolorization with acid alcohol, thus remaining red. This genus contains several important human pathogens, including *Mycobacterium tuberculosis* (causative agent of tuberculosis) and *Mycobacterium leprae* (causative agent of Hansen disease [leprosy]).



Micrograph of *Mycobacterium tuberculosis* in a sputum sample of an individual with tuberculosis. The appearance of red rods after acid-fast staining indicates the presence of mycobacteria and is diagnostic of tuberculosis.

The **coryneform bacteria** are defined by a characteristic irregular appearance of the cells. They tend to show incomplete separation following cell reproduction. When coryneforms reproduce, the progeny cells do not completely separate from one another (called snapping division) and form groups resembling "Chinese characters" when viewed under the microscope. Many species of *Corynebacterium* are plant or animal pathogens. For example, *Corynebacterium diphtheriae* is the causative agent of diphtheria.

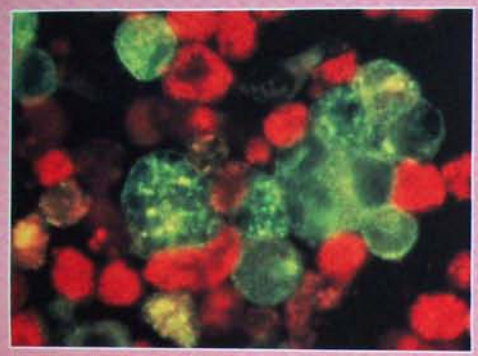


Micrograph of the Gram-positive pleomorphic rod-shaped bacterium *Corynebacterium diphtheriae*. This bacterium causes diphtheria.

Continued.

The **rickettsias** and **chlamydias** are obligate intracellular parasites, that is, they can only reproduce within living host cells. Rickettsias are unable to produce sufficient amounts of metabolic energy to support their reproduction. They obtain energy from the host cells in which they grow. Most rickettsias that cause diseases in humans are transmitted by fleas, ticks, and lice. For example, *Rickettsia rickettsii* is transmitted by ticks and causes Rocky Mountain spotted fever.

Chlamydia reproduction is characterized by a change from a small, rigid-walled infectious form (elementary body) into a larger, thin-walled noninfectious form (initial body). *Chlamydia* cause human respiratory and urogenital tract diseases. They also cause conjunctivitis and trachoma. In birds they cause respiratory diseases and generalized infections. For example, the disease psittacosis, parrot fever, is caused by *Chlamydia psittaci*.

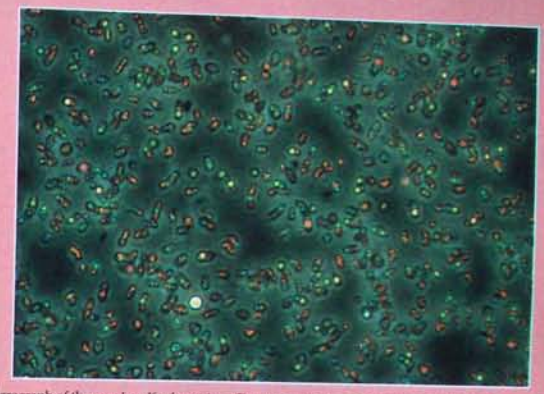


Micrograph showing fluorescent-antibody-stained inclusions formed by clumps of *Chlamydia trachomatis* (*light yellow*) within infected cells (*green*).

The **mycoplasmas** differ from other bacteria in that they lack a cell wall. They are the smallest organisms capable of self-reproduction. Several members of this genus cause diseases in humans. For example, atypical pneumonia is caused by *Mycoplasma pneumoniae*. *Ureaplasma* causes a sexually transmissible disease.

Colonies of *Mycoplasma hominis* with characteristic "fried-egg" appearance.

The **photosynthetic bacteria** are distinguished from other bacterial groups by their ability to use light energy to obtain cellular energy. Some photosynthetic bacteria carry out photosynthesis without the production of oxygen. Such anaerobic (nonoxygen-requiring) photosynthetic bacteria include the purple nonsulfur bacteria, purple sulfur bacteria, green sulfur bacteria, and green flexibacteria.



Micrograph of the purple sulfur bacterium *Chromatium* species. These bacteria deposit sulfur granules within their cells that are iridescent and appear multicolored.

The **cyanobacteria,** or blue-green bacteria (formerly called blue-green algae), carry out a type of photosynthesis that resembles that of higher plants. It results in the production of molecular oxygen.

Micrograph of the cyanobacterium *Anabaena cylindrica* showing vegetative cells and a heterocyst (enlarged cell) in which nitrogen fixation occurs.

Continued.

Chemolithotrophic bacteria use inorganic compounds to generate ATP. The metabolic transformations of inorganic compounds mediated by these organisms cause global-scale cycling of various elements between the air, water, and soil. *Thiobacillus thioxidans* is a chemolithotroph that uses sulfur to generate its energy. It is often found in association with waste coal heaps. The metabolic activities of this organism form sulfuric acid from the sulfur in coal, producing acid mine drainage, a serious ecological problem associated with some coal mining operations. Some chemolithotrophic bacteria, called *nitrifying bacteria*, convert ammonia (NH_4^+) to nitrate (NO_3^-). This conversion is important for the global cycling of nitrogen. This chemical change, however, causes leaching of nitrate into groundwater, which alters soil fertility. Nitrate also can cause a life-threatening disease of human infants. In human infants, nitrate can block the ability of hemoglobin to transport oxygen. Infants that drink water with too much nitrate die of "blue baby syndrome."

Colorized micrograph of membrane systems (*tan*) of the nitrifying bacterium *Nitrosomonas europea*.

The **archaebacteria**, or **Archaea**, represent a distinct evolutionary lineage of prokaryotes. Although they are prokaryotes, they have unique characteristics that distinguish them from eubacteria. Archaea have unusual physiological properties that permit many of them to live in extreme environments such as boiling hot springs, concentrated sulfuric acid, and salt lakes. The thermophilic archaebacteria grow only at temperatures above 85° C. The acidophilic archaebacteria grow at pH values of less than 2. The halophilic archaebacteria grow at high salt concentrations such as in brines that have sodium chloride concentrations of 15%. The methanogenic archaebacteria are very strict anaerobes that grow only in the absence of air and produce methane.

Colorized micrograph of the archaebacterium, *Methanospirillum hungatei* showing cells within a protein sheath (*orange*).

TABLE 2-6

Some Characteristics of Eukaryotic Microorganisms

MICROBIAL GROUP	DESCRIPTION
Fungi	Nonphotosynthetic (heterotrophic) metabolism; cells usually have cell walls, which typically contain chitin; some form spores for reproduction; may reproduce sexually or asexually; generally nonmotile; unicellular (yeasts) or multicellular filamentous forms (molds); no differentiated tissues
Algae	Photosynthetic metabolism; contain chlorophylls and accessory photosynthetic pigments such as carotenoids; no differentiated tissues
Protozoa	Nonphotosynthetic (heterotrophic) metabolism; cells typically lack cell walls; usually unicellular; generally motile; no differentiated tissues

EUKARYOTES

The eukaryotic microorganisms include the fungi, algae, and protozoa (Table 2-6). These microorganisms, like the higher plants and animals, have eukaryotic cells. They evolved along different lines of descent, apparently based on how they obtain nutrition. The algae carry out photosynthesis, obtaining energy from light and carbon from inorganic carbon dioxide for cell growth. The fungi absorb organic nutrients that they use to generate cellular energy and cell constituents. The protozoa tend to engulf nutrients, sometimes growing on other cells.

Fungi

Like the bacteria, the **fungi** are extremely diverse. Unlike bacteria, however, fungi are composed of eukaryotic cells. Most fungi have cell walls, which most often contain chitin, the substance that makes up insect skeletons and crab shells. These cell walls help protect the cells against physical damage and chemical attack. Some fungi—**yeasts**—are primarily unicellular (FIG. 2-8). Others, called **filamentous fungi** or **molds,** form tube-like filaments called **hyphae** (FIG. 2-9). Some hyphae are coenocytic, meaning they lack cross-walls to separate cells; coencytic hyphae are mutinucleate.

Hyphae, which are composed of many cells, can form integrated masses called **mycelia.** Mycelia are the visible structures seen when molds grow on bread and other substrates. In some cases, elongation of hyphae occurs without forming separate cells. Long, multinucleate, fungal hyphae develop. More commonly, separate cells are formed by branches and crosswalls as the hyphae grow. The crosswalls are called **septa.** Even when crosswalls form, cellular materials flow through pores in the septa.

Fungi include the molds, which are filamentous organisms, and single-celled organisms called yeasts.

Fungi obtain their energy from the metabolism of organic compounds. They generally absorb nutrients from their surroundings, often from plant materials. In nature, fungi are very important decomposers. They cause, for example, the decay of dead logs. Un-

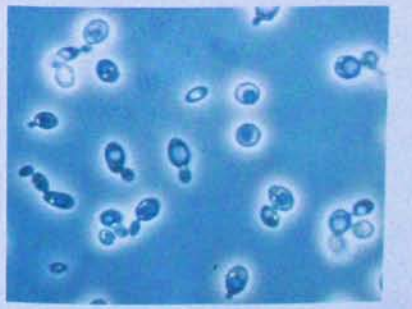


FIG. 2-8 Micrograph of *Saccharomyces cerevisiae* showing budding to produce progeny. (1,075×).

FIG. 2-9 Micrograph of the fungus *Exophiala jeanselmei* that, like other molds, forms long filamentous filaments of intertwined mycelia.

FIG. 2-10 Brown rot of an apricot.

fortunately many are also plant pathogens, causing great losses of agricultural crops. We sometimes use chemical fungicides to protect many cultivated plants (FIG. 2-10). A few also cause human diseases such as athlete's foot, histoplasmosis, coccidioidomycosis, and yeast *(Candida)* infections.

The classification of fungi is based primarily on their sexual reproductive spores (FIG. 2-11). Some sexual spores of fungi are formed within a specialized structure known as the **ascus.** Such spores are called **ascospores** and the fungi that produce them are called **ascomycetes.** Another major group of fungi, the **basidiomycetes,** produce sexual spores on a specialized structure known as the *basidium.* A mushroom is such a basidium. The spores produced by basidomycetes are called **basidiospores.** Other fungi, known as the **deuteromycetes** or **fungi imperfecti,** have no known sexual reproductive phase. As far as we know, they are restricted to asexual means of reproduction. The fungi imperfecti include *Penicillium* and *Aspergillus,* two of the more common fungi that we may observe growing on foods such as bread.

Fungi are composed of eukaryotic cells, may be unicellular or multicellular, often form filaments, usually have cell walls, and typically form reproductive spores.

Fungi are classified primarily based on their sexual means of reproduction.

To distinguish among species of yeasts, we employ a few morphological (structural) observations and numerous metabolic characteristics. The procedure for identifying the unicellular yeasts is very similar to that employed for identifying bacteria. For the filamentous fungi, on the other hand, we rely almost entirely on morphological observations. We use the same basic approach used in identifying plants. Mushroom-producing fungi, for example, are identified based on visual appearance (FIG. 2-12). Great care should be taken in identifying mushrooms. Some mushrooms are edible but others that may look quite similar are deadly poisonous. Even experts have sometimes been fooled and have died after eating mushrooms that they improperly identified.

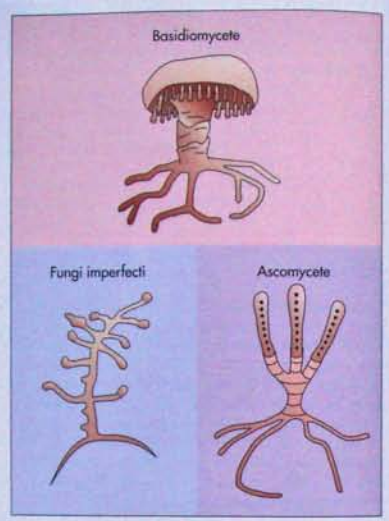


FIG. 2-11 The classification of fungi is based largely on spore formation. Various types of spores and fruiting bodies are formed by different fungi. Basidomycetes form spores on basidia; ascomycetes form spores within asci; fungi imperfecti do not form sexual reproductive spores.

FIG. 2-12 The ink cap mushroom *Coprinus atramentarius.*

Algae

Algae are eukaryotic photosynthetic microorganisms that contain chlorophyll and utilize light energy to generate their chemical energy. They are the only eukaryotic photosynthetic microorganisms. As such, they are the microorganisms most closely related to the plants. They also are able to produce oxygen (O_2) from water. Many of the characteristics used in the classification of algae have been adapted from botanists. These characteristics tend to be those that can be observed. They are generally not metabolic characteristics that must be experimentally determined. The major groups of algae are identified by their characteristic colored pigments and cell morphologies. The major groups of algae include the green algae, euglenoids, golden and yellow-green algae, cryptomonads, and dinoflagellates.

The **diatoms** are unicellular algae that have cell walls containing silicon. They are quite beautiful when seen through the microscope (FIG. 2-13). The euglenoid algae, dinoflagellates, and many green algae are also unicellular. Some green algae are multicellular but do not form structures nearly as complex as the brown and red algae. As examples, the green alga *Volvox* forms a multicellular colonial aggregation of cells and the green alga *Spirogyra* produces a multicellular filament (FIG. 2-14).

Algae are photosynthetic eukaryotic microorganisms.

Most algae are nonpathogens.

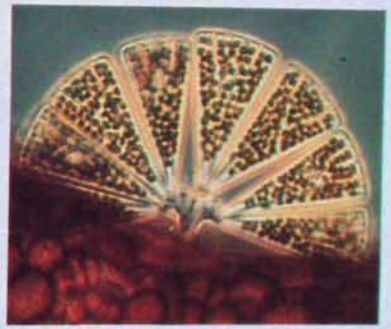


FIG. 2-13 Micrograph of the marine diatom *Licmophora.* (320×).

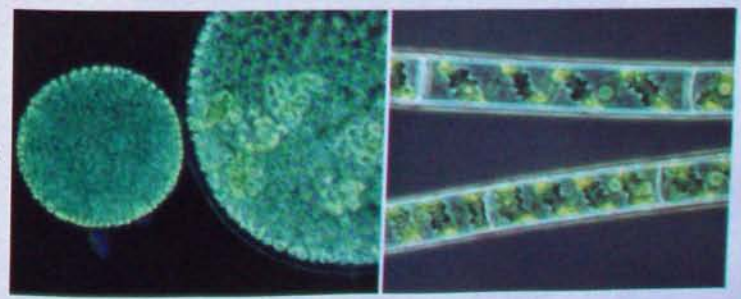


FIG. 2-14 **A,** Micrograph of the green alga *Volvox aureus.* (175×). **B,** Micrograph showing detail of spiral chloroplasts of the green alga *Spirogyra* species. (525×).

Some organisms that have traditionally been classified as algae, namely the red and brown algae, produce complex macroscopic multicellular structures. The kelps, for example, are brown algae that often reach lengths of 50 meters. Kelps have holdfasts, which are rootlike structures that make them plantlike. They are clearly not microorganisms and have been appropriately reclassified as plants instead of algae.

Protozoa

The **protozoa** for the most part are unicellular, nonphotosynthetic eukaryotic organisms. They generally lack a cell wall. Many of the protozoa are motile, meaning they are able to move. Since at one time all organisms that moved were considered animals, protozoa have been studied largely by zoologists. The protozoa have heterotrophic metabolism, meaning that they obtain cellular energy from organic substances such as proteins. Protozoa tend to engulf their food sources, a characteristic that makes them similar to higher animals. Many of the characteristics used in classifying protozoa are analogous to the morphological characteristics used in describing animals.

Protozoa are eukaryotic heterotrophic microorganisms that are classified on the basis of morphological characteristics, especially their means of locomotion.

Motility is a major characteristic traditionally used in classifying protozoa. It is still very important in the latest classification system proposed in 1980. Some protozoa, the **Sarcodina,** form extensions of their cells known as **pseudopodia,** or **false feet** (FIG. 2-15). By forming pseudopodia, these protozoa move from place to place increasing their chances of encountering food. This group includes the genus *Amoeba*. *Amoeba* is an amorphous organism that moves and engulfs food particles by extending its cytoplasm. Unlike the Sarcodina, the cells of the **Ciliophora** are covered by numerous hairlike projections, called **cilia.** Cilia beat continuously, either propelling the protozoan cell or moving food to the protozoan so that it can be engulfed. *Paramecium* is a common example of a ciliate protozoan (FIG. 2-16). The cilia of *Paramecium* propel it from one place to another and drive food toward its "mouthlike" region.

Yet other protozoa, the **Mastigophora,** have flagella that emanate from one end of the protozoan cell. They propel the cell. *Giardia* is a flagellate protozoan that infects the human gastrointestinal tract and causes severe diarrhea. It is becoming an increasing health problem in the United States (FIG. 2-17).

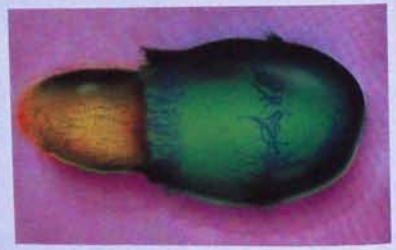


FIG. 2-16 Colorized micrograph of the protozoan *Didinium (green)* consuming the protozoan *Paramecium (yellow).*

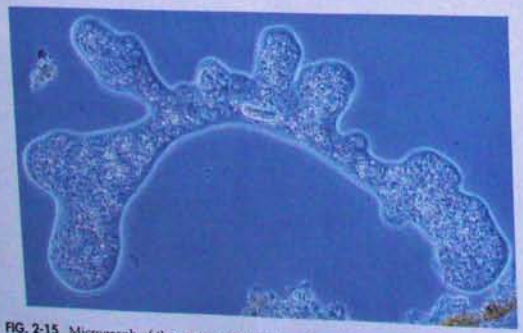


FIG. 2-15 Micrograph of the protozoan *Amoeba proteus*, which moves by extending its cytoplasm to form false feet. (225×).

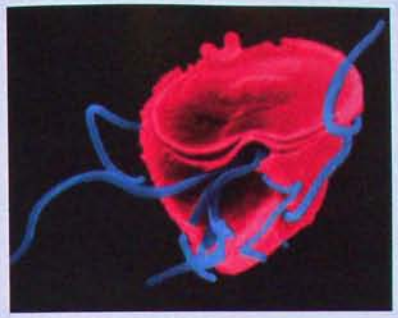


FIG. 2-17 Colorized micrograph of *Giardia lamblia*, a protozoan that is one of the most common causes of gastrointestinal infections.

Another protozoan group that traditionally has been recognized is the **Sporozoa.** Members of the Sporozoa generally are nonmotile, produce spores during their life cycles, and grow only on or in a host organism from which they derive nutrition.

In the protozoan classification system proposed in 1980, the Sporozoa were reclassified into several smaller groups based on morphological characteristics. Many of these characteristics can only be observed with an electron microscope. Various other protozoa were also placed into these smaller groups regardless of their means of locomotion. For example, *Plasmodium*—the protozoan genus that includes the species that cause malaria—was placed into the group Apicomplexa because it produces a small umbrella-like structure at the end of the cell.

The classification of protozoa now includes electron microscopic observations of previously unseen morphological characteristics.

SUMMARY

Evolution of Microorganisms (pp. 25-30)
- Microorganisms evolved from abiotically formed micelles.
- As microorganisms evolved they altered their surroundings, permitting new forms of life to develop.

Classifying Microorganisms (pp. 25-30)

Early Classification Systems (p. 26)
- Early classification systems were based on observable characteristics of microorganisms, such as shape and motility.
- The species is the fundamental unit used in classifying all organisms.

Modern Classification Systems (pp. 26-29)
- Modern classification systems attempt to reflect evolutionary relationships.
- The contemporary classification system of Woese, which is based on genetic analyses at the molecular level, recognizes three primary Kingdoms: archaebacteria (primitive specialized prokaryotes), eubacteria (common prokaryotes or "true bacteria"), and eukaryotes (all other cellular organisms, including plants, animals, protozoa, algae, and fungi).

Survey of Microorganisms (pp. 30-55)

Acellular Nonliving Microorganisms—Viruses, Viroids, and Prions (pp. 30-33)
- The acellular microorganisms include the viruses, viroids, and prions.
- Viruses have two parts: a protein coat (the capsid) and a hereditary molecule, which may be DNA or RNA.
- Viroids are composed of only RNA.
- Prions are infectious proteins.

Prokaryotes—Eubacteria and Archaebacteria (p. 33-50)
- The eubacteria and archaebacteria are prokaryotic cells.
- Like other organisms, bacteria are named using a binomial system of genus and species.
- The major groups of bacteria are distinguished based on morphological, metabolic, and genetic characteristics.
- The bacteria are described in *Bergey's Manual*, which is revised periodically.

Eukaryotes (pp. 51-55)

Fungi (pp. 51-52)
- The fungi are heterotrophic eukaryotic microorganisms.
- Fungal cells commonly have cell walls.
- Some fungi form multicellular filamentous growths called mycelia and others are unicellular (yeasts).
- The fungi are classified into major groups based on their modes of reproduction and, in particular, the reproductive spores they produce.

Algae (pp. 53-54)
- The algae are photosynthetic eukaryotic microorganisms.
- The major groups of algae are defined based on their characteristic pigments and cell morphologies.

Protozoa (pp. 54-55)
- The protozoa are heterotrophic microorganisms.
- The cells of protozoa generally lack cell walls.
- The major groups of protozoa are classified based on their means of motility or on specialized morphological characteristics.

CHAPTER REVIEW

Review Questions

1. Why is it hard to classify microorganisms by their evolutionary relationships?
2. Compare and contrast the classification systems of Linnaeus, Haeckel, Whittaker, and Woese.
3. How do viroids differ from viruses?
4. What are prions? Why are prions different from other living organisms?
5. What are the main characteristics of organisms in each of the Kingdoms in the five-Kingdom system of classification?
6. How are bacteria named?
7. What is *Bergey's Manual*?
8. How do humans differ from bacteria?
9. What are some of the morphological and physiological characteristics that have been used to classify bacteria?
10. What are the main features of the endosymbiont theory of evolution?
11. What are the modern methods used to classify organisms?
12. What were the characteristics of the first microorganisms?
13. How could life have evolved on Earth? Could life have evolved on other planets? If so, how?
14. What are the differences between classification and identification. How can clinical laboratories identify pathogens based on only 20 tests?
15. Why has the proposal by Carl Woese for three primary Kingdoms (eubacteria, archaebacteria, and eukaryotes) upset many traditional plant and animal biologists?

CRITICAL THINKING QUESTIONS

1. You have recently discovered a new microorganism. What criteria would you use to help classify it?
2. You would like to develop a new system of classification that groups all the eukaryotic microorganisms into one category. What characteristics are fundamentally the same for all eukaryotic microorganisms? What characteristics are fundamentally different for the major groups of eukaryotic microorganisms?
3. Compare methods of categorizing organisms. Are any of them better than the others or are they just different ways of looking at the same thing?
4. Protozoologists classified many organisms as protozoa that have traditionally been considered as fungi or as algae. These organisms include the slime molds and the euglenoid algae. How would you resolve the disputes between mycologists and protozoologists over the slime molds and between phycologists and protozoologists over the unicellular euglenoid algae?
5. Should the photosynthetic "blue-greens" be considered cyanobacteria or should they be considered blue-green algae?

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CHAPTER 3

Science of Microbiology: Methods for Studying Microorganisms

CHAPTER OUTLINE

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PREVIEW TO CHAPTER 3

In this chapter we will:

- Examine the scientific method that scientists use in their investigations.
- See how the scientific method was used to disprove the theory of spontaneous generation.
- Learn how the studies of Louis Pasteur began the science of microbiology.
- Examine how the microscope is used to view microorganisms.
- Learn about the principles of microscopy that determine how large an object can be magnified and how small an object can be seen.
- Learn about the different types of microscopes—their applications and limitations—including light and electron microscopes.
- Learn how microbiologists grow pure cultures of microorganisms so that they can study their physiological properties.
- Learn the following key terms and names:

acid-fast staining
agar
aseptic technique
autoclave
bright-field microscope
colony
compound light microscope
controlled experiments
culture
dark-field microscope
differential medium
differential staining procedures
electron microscope
endospore staining procedure
Gram stain procedure
Gram-negative bacteria
Gram-positive bacteria
hypothesis
immunofluorescence microscopy
magnification
microscope
negative staining
objective lens
ocular lens
oil immersion objective
Louis Pasteur
Petri plate
phase contrast microscope
pure culture
resolving power
scanning electron microscope (SEM)
scientific method
selective media
simple staining procedures
spread plate method
staining
sterile
sterilization procedures
streak plate method
theory of spontaneous generation
transmission electron microscope (TEM)

SCIENTIFIC METHOD AND THE DEVELOPMENT OF THE SCIENCE OF MICROBIOLOGY

Suppose you saw a piece of rotting meat covered with wormlike fly larvae. You ask whether these larvae, called maggots, are always found on rotting meat. You search out other pieces of rotting meat and see whether all of them are covered with maggots. If you note that maggots are present every time you observe a piece of rotting meat, you could generalize that when meat rots, maggots appear. Such generalizing from specific observations is called **inductive reasoning.** You could also begin with a generalization. By reasoning from it, you could arrive at a specific conclusion. This process is called **deductive reasoning.** In reaching a conclusion by deductive reasoning, one essentially says that if this happens, then that will happen; if I observe this, then I will observe that. If I see a piece of meat rotting, then I will find maggots on the meat. This type of "if-then" reasoning is essential in science.

However, not all conclusions reached by such deductive reasoning are correct. For example, you could erroneously conclude, based on the above observations, that maggots arise spontaneously from rotting meat. In fact, the repeated observation of maggots on rotting meat led in part to the *theory of spontaneous generation.* This theory held that living organisms could arise spontaneously from nonliving matter. The belief that maggots spring forth by spontaneous generation from decaying meat persisted in various forms from before the time of Aristotle in the fourth century B.C. to the late seventeenth century. Almost all scientists and philosophers until the seventeenth century believed that living animals could be generated from nonliving matter. In this case, the theory of spontaneous generation was logical but wrong. Demonstrating the fallacy of the theory of spontaneous generation required the use of an approach known as the **scientific method** (FIG. 3-1).

FIG. 3-1 In the scientific method, a hypothesis is proposed that can be tested. Experiments typically are run to determine whether predictions based on the hypothesis are accurate. The hypothesis is rejected if the predictions are not validated by experimental observations (data). An alternate new hypothesis may then be proposed and new experiments performed to assess its validity. When predictions based on a hypothesis are experimentally confirmed, the hypothesis is accepted.

The philosophy for this method of inquiry was developed by the English philosopher Francis Bacon in the seventeenth century. The scientific method relies on observations and deductive reasoning. It also demands that conclusions be subjected to thorough testing to develop objective evidence that can be evaluated before their credibility is accepted.

Scientific inquiry depends on systematic observation and tests.

In the scientific method a scientist first poses a question, for example, where do the maggots observed on rotting meat come from? The scientist then proposes a tentative answer to that question, for example, the maggots arise spontaneously. This tentative answer is called a **hypothesis.** Then the scientist tests the validity of the hypothesis by making *systematic observations.* Scientists often run **controlled experiments** to determine whether the tentative answers (hypotheses) are correct. In a controlled experiment, scientists attempt to hold all factors constant except the ones under study. By properly designing their experiments, scientists manipulate conditions so that they can control the factors that may cause a particular phenomenon. This way they can explore the cause-and-effect relationships among phenomena in the universe.

The design of a controlled experiment includes a **control group** and an **experimental group.** The control group serves as the reference. It has a set of conditions that the scientist does not vary. In contrast, in the experimental group, the scientist varies some factor or factors. By comparing the experimental group to the control group, the scientist is able to determine the effect(s) of the factor(s) that is varied on some other parameter(s).

Testing hypotheses through controlled experiments is the basis of scientific inquiry.

Showing that Maggots Do Not Arise Spontaneously from Decaying Meat

At about the same time that the scientific method was developed, Francesco Redi devised a controlled experiment to test the validity of the hypothesis that maggots arise spontaneously from rotting meat (FIG. 3-2). Redi was an Italian poet and physician. He placed a piece of loosely woven cloth over one piece of meat. This prevented insects from reaching the meat. He left a second piece of meat uncovered as a control. The presence or absence of the cloth was the only difference between the two pieces of meat. Redi hypothesized that if the maggots really arose spontaneously from the meat they would be trapped inside the cloth. He reasoned that if the maggots reached the meat from some other source, the cloth would prevent the maggots from getting to the meat. Then maggots would be observed only on the outside of the cloth.

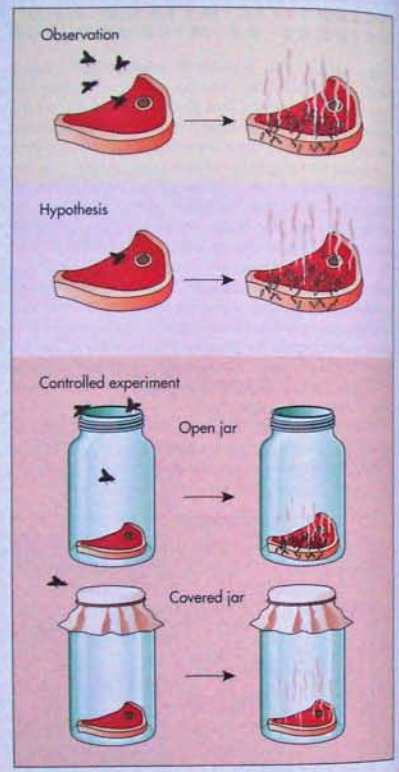


FIG. 3-2 Francisco Redi demonstrated that maggots do not arise spontaneously from rotting meat. When he covered the meat with cheesecloth, no maggots developed in the meat.

Redi observed that as the meat rotted, maggots appeared on uncovered meat. Maggots only appeared on the outside of the cloth of the covered meat. Thus Redi showed that maggots do not arise from within the decaying meat. It was important that the experiment conducted by Redi included a control that showed that the meat would rot and that maggots would appear on the meat in the absence of the cloth covering. The design of the experiment permitted him to determine by direct observation that the maggots came from outside the meat. The outcome of this experiment disproved the theory of spontaneous generation for large multicellular organisms such as insects. Other experiments by other scientists later demonstrated that maggots appear when eggs deposited by adult flies hatch.

It was significant that the experiments performed by Redi to test his hypothesis were repeatable by other scientists. You could easily repeat his controlled experiment today. Your experiment would also show that maggots do not arise spontaneously from rotting meat. Objectivity and the ability to repeat an experiment are essential parts of the scientific method. Reporting of the results of all experiments must be free of subjectivity, or bias, on the part of the scientist. Scientists always question the design and accuracy of experiments performed by their colleagues and the conclusions that are drawn from their experiments. No idea can be put forward with the expectation that its validity will not be challenged. As new information is obtained and experiments are repeated independently, scientists may gain confidence that accepted explanations are correct. Alternatively, they may determine that a once accepted hypothesis is incorrect and that a new hypothesis to explain the available data is needed.

Scientific experiments must be repeatable.

Showing that Microorganisms Do Not Arise Spontaneously During Decay and Fermentation

Scientists can only accept or reject hypotheses based on the evidence at hand. When Redi, for example, demonstrated in 1668 that maggots do not arise spontaneously, scientists did not know that microorganisms existed. It was not until 1670, 2 years after Redi performed his experiment, that Antonie van Leeuwenhoek peered through a microscope and recorded the first observations of bacteria "swimming" in rainwater and other liquids. The observation of microorganisms meant that new questions could be asked, new hypotheses put forth, and new controlled experiments performed to test these hypotheses.

In the eighteenth century, scientists using microscopes began to ask where the large numbers of microorganisms came from that they were now able to observe "swimming" in vats of spoiling wine and pots of rotting beef broth. What had been learned about maggots was not immediately applied to microorganisms. Many scientists, including numerous noted chemists, held that the rotting of meat (decay) and the conversion of sugar to alcohol (fermentation) as occurs when the sugar in grapes is made into the alcohol in wine were strictly chemical processes. They believed that the microorganisms seen in fermenting grape juice or rotting beef broth arose by spontaneous generation. Again, scientists had to demonstrate by using the scientific method that living organisms, in this case, living microorganisms, do not arise by spontaneous generation during decay and fermentation.

Scientists had a difficult time performing controlled experiments with microorganisms they could not see with the naked eye. Woven cloth could not prevent very small microorganisms from entering fermenting grape juice or decaying beef broth. Microorganisms could not be eliminated from these liquids without taking steps that also potentially altered the chemistry of the liquid and the air over it. Hence, the validity of experiments aimed at showing that, like maggots, microorganisms do not arise spontaneously was difficult to establish.

Lazzaro Spallanzani, an eighteenth century Italian priest, was among the first to use the scientific method to investigate whether or not microorganisms arise by spontaneous generation. Spallanzani hypothesized that microorganisms do not arise spontaneously. He hypothesized that, like maggots, microorganisms arise from the reproduction of living organisms. He performed controlled experiments to test his hypothesis.

In his controlled experiments Spallanzani placed various kinds of seeds in a flask. He then sealed the flask by covering the mouth with a stopper. By sealing the flask he prevented any microorganisms that were not already in the flask from reaching the liquid. Spallanzani then boiled the liquid in one flask for 45 minutes to kill any living microorganisms it contained. As a control Spallanzani did not boil the liquid in another otherwise similarly prepared flask. A few days later Spallanzani collected samples from each flask and observed them under a microscope. He saw many microorganisms in the samples from the flask that had not been boiled. He saw no micro-

HIGHLIGHT

Spontaneous Generation of Frogs and Mice

The ancient Greek philosopher Aristotle said that anything that becomes humid and any humid thing that dries produces animals. He taught that insects and worms came from dewdrops and slime, that mice were generated by dank soil, and that eels and fish sprung forth from sand, mud, and putrefying algae. In the seventeenth century, others expanded this belief into the theory of spontaneous generation and created recipes for producing frogs from the mud of ponds, eels from river waters, and mice from straw.

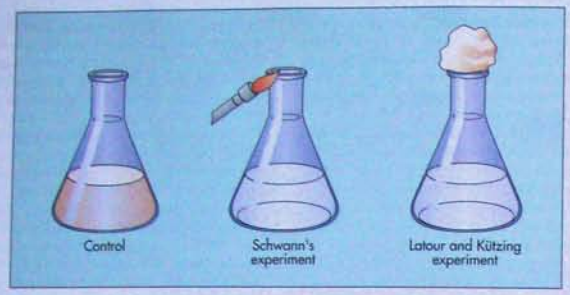


FIG. 3-3 Several scientists, including Latour, Kützing, and Schwann, attempted to disprove the theory of spontaneous generation. They used heat and sealed vessels, which chemists argued destroyed or eliminated oxygen, the "vital force of life." Thus these experiments did not definitively disprove the theory of spontaneous generation.

ganisms in the samples from the flask that he had boiled. The liquid that had been boiled in the sealed flask remained clear, free of microorganisms, and unspoiled indefinitely. Spallanzani concluded that boiling killed the microorganisms and that new microorganisms could not arise spontaneously from the nonliving liquid in a sealed flask.

However, nineteenth century advocates of the theory of spontaneous generation of microorganisms assailed Spallanzani's experiments. They claimed that by sealing the flasks Spallanzani had eliminated molecular oxygen. They argued that the oxygen in air was an essential *"life force"* necessary for the spontaneous formation of microorganisms. Thus, Spallanzani's experimental design was criticized. His opponents claimed that his conclusions were invalid. Chemists held fast to the view that changes in organic chemicals occurred by strictly chemical processes and were not brought about by living organisms.

In the 1830s, several scientists proposed hypotheses and conducted experiments to demonstrate that microorganisms do not arise spontaneously during the course of the decay of organic matter. Like Spallanzani, these scientists used boiling to kill the microorganisms in liquids contained in flasks. However, they did not seal the flasks to prevent microorganisms from entering the liquid broth. Theodor Schwann in Germany placed a flame at the mouth of an open flask to kill microorganisms that might be in the air, thereby preventing microorganisms from entering the flask. Charles Cagniard de Latour in France and Friedrich Kützing in Germany used cotton plugs to prevent microorganisms from entering the flasks and reaching boiled broths (FIG. 3-3).

These experiments showed that when microorganisms were prevented from entering the liquid in the flask, decay and/or fermentation did not occur. Neither were microorganisms observed in the broth when their entry was prevented, whereas microorganisms always were seen in the broths when microorganisms could enter the flasks. However, these experiments were also subject to criticism. Chemists claimed each experiment destroyed or eliminated some essential component called the *"life force"* in the air. They said air was needed for the decay of the organic matter and the spontaneous generation of microorganisms. Remember that such criticism is part of the scientific method. Scientists always ask how do you know that your results are correct, that your interpretation of the experimental results is the best possible one. Only experimental results and interpretations that withstand intense criticism and review become accepted in science.

The results of an experiment are carefully and critically reviewed before their validity is accepted by the scientific community.

Pasteur and the Final Refutation of the Theory of Spontaneous Generation

In 1862, **Louis Pasteur** (FIG. 3-4) designed a controlled experiment that overcame all the criticisms of the chemists, thereby disproving spontaneous generation. Pasteur was a Frenchman who had been trained as a chemist. This provided him with the experience needed to use the scientific method in studies on microorganisms. It also gave him some degree of credibility with other professional scientists. Pasteur believed that the origin of microorganisms had

FIG. 3-4 Louis Pasteur (1822-1895) began as a chemist but soon became a pioneer microbiologist. Pasteur's work encompassed pure research and many areas of applied science that produced several important practical discoveries. Among his many accomplishments, Pasteur discredited the theory of spontaneous generation, introduced vaccination to treat rabies, and solved industrial problems related to the production and spoilage of foods.

to be understood before microbiology could be established as an experimental science. He also held that "one cannot expect the doctrine of spontaneous generation to be abandoned as long as serious argument can be presented in its favor."

When he turned his attention to microorganisms, Pasteur initially asked two questions: Are microorganisms needed for decay and/or fermentation to occur?; and do microorganisms arise spontaneously during decay and/or fermentation? Pasteur hypothesized that microorganisms cause decay and fermentation and that microorganisms do not arise by spontaneous generation. By using controlled experiments, Pasteur left flasks containing boiled beef broth or boiled grape juice open to the air and showed that decay of the beef broth and fermentation of the grape juice did not occur unless microorganisms could reach the liquid from the outside.

For these experiments Pasteur designed a flask that could be left completely open to the air and still prevent microorganisms from reaching the broth contained within the flask (FIG. 3-5). He made glass flasks with several curves in the neck of each flask. Microorganisms would settle with dust particles in the depressions of the neck and never reach the liquid within the flask. The neck of the flask was curved to look like a "swan's neck" and the flask was therefore called a **swan-neck flask.** As a control Pasteur used a flask with a straight neck of the same length. The only difference between the flasks he used in his experiments was the shape of the neck of the flask.

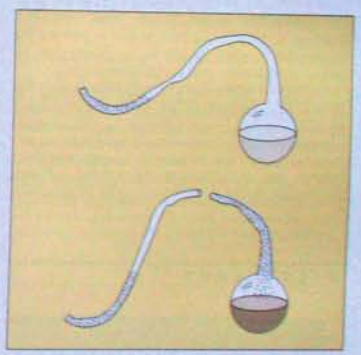


FIG. 3-5 To discredit the theory of spontaneous generation, Pasteur used various shapes in the design of his swan-necked flasks. Pasteur boiled the liquid containing nutrients to kill any microorganisms that were already there. He then left the flasks open to the air. The curved necks of the flasks trapped dust particles, preventing them from carrying microorganisms to the liquid broth growth medium so that the broth remained clear and free of microorganisms (*top*). These experiments demonstrate that spontaneous generation does not occur. When he broke the necks of some of the flasks (*bottom*), dust carried a microorganism into the broth growth medium. The microorganism grew in the broth, making it turbid (cloudy).

Chemists could not claim that this difference affected the *"life force."*

In his experiments, Pasteur found that liquids subjected to boiling remained free of living microorganisms in the flasks with the swan necks. Liquids boiled in the same manner soon swarmed with microorganisms in the flasks with the straight necks. He concluded that, although air could freely enter the flask, the shape of the swan neck of the flask prevented airborne microorganisms from entering the liquid.

As an additional control to demonstrate that the microorganisms that cause decay and fermentation do not arise spontaneously, Pasteur broke off the neck of one of the swan-neck flasks. This allowed microorganisms to enter the medium in the flask that had, until that point, remained free of living microorganisms. Shortly thereafter, Pasteur observed numerous microorganisms in the broth contained in that flask. This demonstrated that the medium in the flask could in fact support microbial life if microorganisms were introduced. The flask with the intact swan neck remained free of microorganisms. He concluded that the microorganisms in the broth came from living microorganisms that were floating unseen in the air. Pasteur had shown that microorganisms are like other forms of life—that living cells come only from the reproduction of pre-existing living cells. Pasteur's swan-neck flasks, now sealed to prevent the evaporation of water, are still free of microorganisms and on exhibit at the Pasteur Institute in Paris.

The results of Pasteur's experiments were challenged. His experiments had to be repeated many times before they were accepted by the scientific community. This is characteristic of science. No scientist, no matter how famous, escapes the critical review of other scientists. A final demonstration of his experiments before a tribunal of other scientists was convened to critically evaluate his disproof of the spontaneous generation of microorganisms. At this time Pasteur declared: "There is no condition known today in which you can affirm that microscopic beings came into the world without germs, without parents like themselves. Those who allege it have been the sport of illusions, of ill-made experiments filled with errors that they have not been able to perceive." Referring to the liquids in his swan-neck flasks he concluded: "I have kept from them the one thing that is above the power of humans to make; I have kept from them the germs which float in the air. I have kept them from life."

The results of Pasteur's experiments discredited the theory of spontaneous generation. They established once and for all that living microorganisms are responsible for the chemical changes that occur during fermentation and decay and that microorganisms do not arise spontaneously. With the success of his experiments, Pasteur proclaimed: "No more shall spontaneous generation rear its ugly head!"

Pasteur's refutation of the theory of spontaneous generation was a critical milestone in the development of microbiology as a scientific discipline. It demonstrated that scientists could successfully use the scientific method for examining microorganisms. It also showed that microorganisms are like other living organisms, that only living organisms give rise to other living organisms. It further revealed the importance of microorganisms in bringing about chemical changes. It was the activities of the microorganisms that transformed grape juice into wine and caused meat to decay. Pasteur went on to study many other aspects of microorganisms and to help pioneer the birth of microbiology as a scientific discipline.

With the studies of Pasteur, microbiology emerged as a scientific discipline.

MICROSCOPY

Making observations is an essential part of the scientific method. It is not surprising, therefore, that microbiology did not develop as a field of science until the necessary instruments and methods were developed for observing microorganisms. Unlike organisms that could be described and studied by the unassisted eye, microorganisms could not be studied until instruments were available with sufficient magnifying power so that they could be seen. The **microscope** is an instrument for producing enlarged images of objects that are too small to be seen unaided. Microscopic observations generally provide information only about the morphology of microorganisms and not their physiological characteristics. The light microscope can be used for viewing objects as small as bacteria. The electron microscope extends the range to even smaller objects, such as viruses and large molecules (FIG. 3-6).

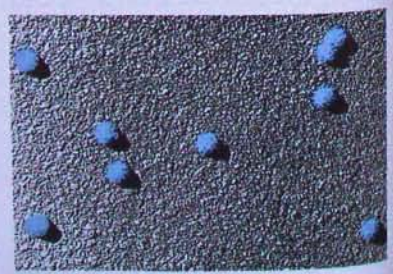


FIG. 3-6 **A,** Colorized transmission electron micrograph of polioviruses observed through an electron microscope.

B

FIG. 3-6, cont'd **B,** Micrograph of a bacterial biofilm viewed by confocal fluorescence scanning microscopy. Color indicates depth within specimen (purple nearest surface and red deepest in biofilm).

By using microscopes to magnify their images, microorganisms can be observed and studied.

Principles of Light Microscopy

Magnification

Because the observation of microorganisms through the microscope is fundamental to the study of microbiology, we should consider some of the principles of microscopy. Let us first discuss how magnification is achieved. The apparent size of an object viewed directly by the eye depends on its distance from the eye. As an object is brought nearer to the eye, the apparent size of the object increases. However, if the object is too close to the eye, the eye no longer can form a clear image. Placing a curved glass lens between the object and the eye can restore the sharpness of the image. This is because the lens changes the direction (angle) of the light rays reaching the eye. The bending of the light rays by a glass lens is called **refraction.** It forms an image of the object that is larger than

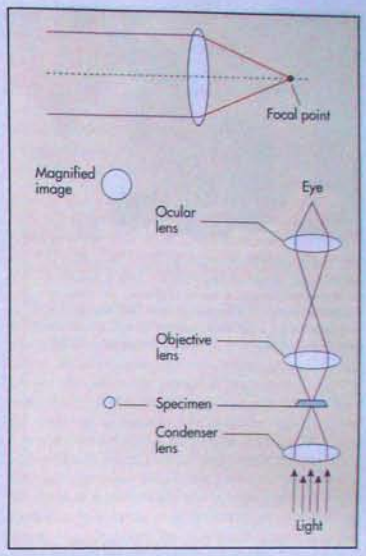
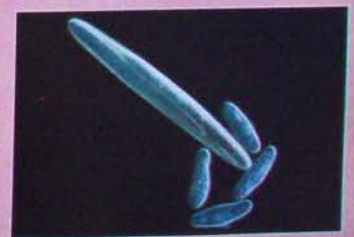


FIG. 3-7 A convex-convex lens theoretically focuses light at a single focal point. However, red light (long wavelength light) focuses more distantly from the lens than blue light (short wavelength light), causing chromatic aberrations. Also, light rays passing through the periphery of the lens (axial rays) focus more distantly from the lens than light rays that pass through the center of the lens (marginal rays), causing spherical aberrations. Various lenses greatly inprove the performance of the microscope lens by correcting for these aberrations. Modern microscopes use several such lenses to produce a high-quality magnified image.

NEWSBREAK

Visible Oxymoron

Although microorganisms are supposed to be invisible to the naked eye, a large bacterial species has been discovered in the guts of surgeonfish. These bacteria are over 0.5 mm long and can be seen with the naked eye. They have been placed in the genus *Epulopiscium*, which means "guest at a banquet of a fish." Because of their size, these bacteria were initially mistaken for protozoa, but they are prokaryotes and, hence, true bacteria. One million typical bacteria, such as the *Escherichia coli* that live in the human intestine, could fit into a cell of *Epulopiscium*. This largest bacterium is a visible oxymoron.



Micrograph of the large bacterium *Epulopiscium* with four *Paramecium* (smaller cells).

FIG. 3-8 A light microscope allows the formation of an enlarged image of a specimen. Light is refracted as it moves through a series of lenses. In a bright-field microscope the condenser lens focuses light on a specimen; the light then passes through the ocular and objective lenses to produce a magnified image.

the object itself (FIG. 3-7). This is because the light reaching the eye from the top to the bottom of the object is at a greater angle than it would be if the lens had not caused the light to bend.

The microscope produces an enlarged (magnified) image of a specimen through the bending (refraction) of light rays by curved glass lenses.

The **compound light microscope** has multiple lenses that refract light and achieve magnification of the image of a specimen (FIG. 3-8). The lenses can be moved to achieve focus. Light, typically from an incandescent bulb source, is focused onto a specimen by a **condenser lens.** The specimen is normally on a clear glass microscope slide that can be moved by a mechanical stage. The light passes through the specimen and enters the **objective lens,** which refracts the light and begins the magnification process. The light then passes through the **ocular lens,** which further refracts the light and completes the magnification process. The light enters the eye and the magnified image is seen.

The degree of **magnification** (enlargement of the image) that can be achieved with a compound microscope is the product of the individual magnifying powers of the ocular and objective lenses. The total magnifying power of a microscope is calculated by multiplying the power of the objective by the power of the ocular. The magnifying power obtained with a 100× objective and a 10× ocular, for example, would be 1,000×. The most commonly used light microscopes have objective lenses with powers of 10×, 40×, and 100× and an ocular lens of 10× so that one can obtain magnifying powers of 100, 400, and 1,000 times with them.

The magnifying power of a compound microscope is calculated by multiplying the magnifying powers of the ocular and objective lenses.

Resolution

One could well ask, why don't we simply continue to increase the magnifying capacity of the lenses to make more and more powerful microscopes? Why not combine a 100× objective lens with a 100× ocular lens so that an image could be magnified 10,000×? The answer is that such an image, while larger, would not show additional details of the specimen. **Resolution** is the ability to distinguish detail in the object that is viewed. In biological specimens, resolution is equated with the ability to see structures of the organism.

Resolving power is a distance measure, sometimes called the resolving distance, that is defined as the closest spacing between two points at which they can still be seen clearly as separate entities. We measure the resolving power of a microscope in units called nanometers (nm), which are billionths of a meter (10^{-9} m). Objects that are closer than the resolving power of the microscope cannot be seen as separate and distinct, and objects that are smaller than the resolving power cannot be seen at all.

The ability to see detail is determined by the resolving power and not the magnifying power of the microscope.

Resolution describes the amount of detail that can be seen in an image.

The resolving power of the light microscope is about 200 nm. This is much better than the 0.1 mm resolving power of the unaided eye. Because all cellular organisms, including bacteria, are larger than 100 nm, the light microscope permits the observation of the microbial world. Most viruses and other acellular microorganisms, which are all smaller than 100 nm, however, cannot be seen with the light microscope.

The resolving power (R) of a microscope is approximately one half the wavelength of the light (λ) that is used to illuminate the specimen divided by the numerical apertures (NAs) of the objective lens and the condenser lens (FIG. 3-9).

$$R = \lambda/(NA_{\text{objective lens}} + NA_{\text{condenser lens}})$$

The numerical aperture represents the amount of light from an object that actually enters a lens. The greater the amount of light that can enter the lens, the higher the numerical aperture. The higher the numerical aperture, the smaller R and hence the better the resolving power. Since the numerical apertures of the objective lens and the condenser can be about the same, the formula for resolving power normally is written as:

$$R = \lambda/(2 \times NA)$$

For visible light, wavelength is seen as color. Blue light has a short wavelength and red light has a long wavelength. The best resolution of a light microscope can be achieved by using a blue light source (short wavelength light) to illuminate the specimen and a lens with a high numerical aperture.

Resolution depends on the wavelength of light (λ) and the numerical aperture (NA) of the lens.

The best resolving power of a light microscope occurs when short wavelength light and a high numerical aperture objective lens are used.

To achieve a high numerical aperture, we insert a drop of clear oil between the specimen and the objective lens. This requires a special type of objective lens called an **oil immersion objective.** Using oil improves the resolving power of the microscope because the oil allows light to pass in a straight line from the specimen to the objective lens. The typical oil immersion objective has a numerical aperture of 1.25 to 1.30, whereas the numerical aperture of a non-oil immersion lens is less than 1. As a rule, the useful magnification of a microscope above which greater detail cannot be seen because of the limitation to resolving power is 1,000 times the numerical aperture of the objective lens being used. It is possible, using an oil immersion lens, to achieve magnifications of just over 1,000×, which is essential for viewing bacteria.

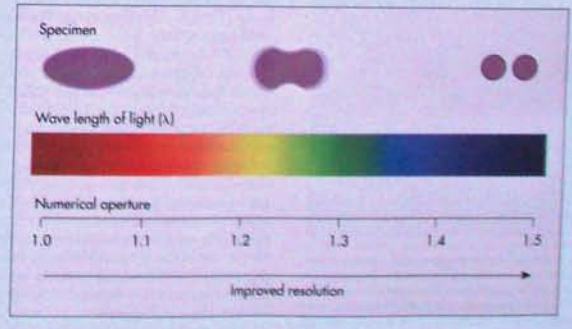


FIG. 3-9 Microscopy depends on the ability to see detail, that is, to resolve distinct points. At low resolution, structures blur; at greater resolution, more detail can be observed. Resolution depends, in part, on the wavelength of light. Blue light, which has a short wavelength (380 nm), gives superior resolution to red light, which has a long wavelength (750 nm).

Contrast and Staining

Staining (dyeing) is used to increase the contrast between the specimen and the background. This usually is necessary to create adequate contrast between the specimen and the background so the specimen can be seen. Most microorganisms are colorless and cannot be seen readily without being stained. Many types of staining procedures, which employ dyes of differing colors, are used for different purposes in microbiology. Most staining procedures for light microscopic observation of microorganisms begin with the transfer of a suspension of microorganisms to a glass microscope slide (FIG. 3-10). The microorganisms are spread as a thin film across the slide that is allowed to dry in the air. Then the slide is quickly passed through a flame to *fix* the cells to the slide. Fixing preserves the shape of the cells and prevents them from being washed off during staining. A stain is added and allowed to penetrate and to react with the cells on the slide for a period of time. Excess stain is then rinsed from the slide and the specimen is ready for viewing under the microscope.

Staining creates contrast between a specimen and its background so it can be seen.

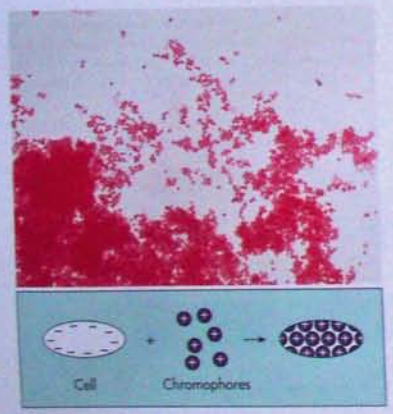


FIG. 3-10 In a simple staining procedure, microorganisms are affixed to a glass slide and stained with an appropriate dye (colored chromophore). This increases the contrast between the cells and the background so they can easily be seen using a light microscope. Because the outer layer of a cell is negatively charged, a positively charged stain chromophore is attracted to the cell; this is the basis of positive staining procedures.

FIG. 3-11 Light micrograph of the bacterium *Bacillus cereus* after simple positive staining with carbolfuchsin. (1,400×). The cells of the rod-shaped bacterium appear red in contrast to the clear background.

Simple staining procedures use a single stain. A stain is a salt comprised of a positively charged ion and a negatively charged ion. If the colored portion of the stain is positively charged, it will be attracted to microbial cells, which have negative charges. Staining with a positively charged dye is called **positive staining**. Stained cells that look dark or colored against a light or clear background will be seen. Methylene blue, for example, will stain microbial cells blue by positive staining. This stain is useful for seeing the shapes of bacterial cells (FIG. 3-11). If the colored portion of the stain is repelled by the negatively charged microorganisms, clear unstained microorganisms will be seen against a dark background. Such staining with a negatively charged dye is called **negative staining**. India ink and nigrosin, which have negatively charged black particles, are examples of dyes that can be used for negative staining (FIG. 3-12). Like positive staining, negative staining is used to reveal the shapes of microorganisms.

Differential staining procedures use multiple stains to distinguish different cell structures and/or cell types. In differential staining procedures, specific types of microorganisms and/or particular structures of a microorganism exhibit different affinities for certain stains. By using multiple stains and washing steps in a differential staining procedure, structural differences between microbial species can be revealed. These differences aid in the classification and identification of microbial species.

The **Gram stain procedure** is the most widely used differential staining procedure in bacteriology

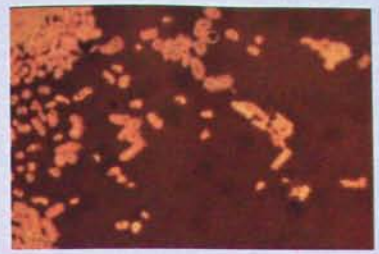


FIG. 3-12 Light micrograph of the bacterium *Klebsiella pneumoniae* after simple negative staining with India ink. The cells appear clear against a dark background because the ink does not penetrate the cells.

today (FIG. 3-13). It was developed in 1884 by the Danish physician Hans Christian Gram. Gram was trying to develop a method for seeing bacteria within mammalian tissues. He failed at that task but instead discovered a method for the diagnostic differentiation of bacterial species based on the way they are stained by his procedure. This Gram staining procedure begins with primary staining with crystal violet, which stains all bacterial cells blue-purple. Then Gram's iodine is applied as a mordant. A mordant is a substance that increases the affinity of the primary stain for the bacterial cells. The cells are rinsed with acetone-alcohol or another decolorizing agent to try to wash out the primary stain. A red counterstain (safranin) is then applied, which stains the bacteria that were decolorized in the previous step so that they can be easily seen.

The decolorization step of the Gram stain procedure is the critical step. It differentiates bacterial species based on their cell wall structure. The cell wall structure of certain bacteria does not permit decolorization. These bacteria remain blue-purple and are called **Gram-positive bacteria.** Gram-positive bacteria appear blue-purple at the end of the Gram stain procedure. *Staphylococcus* and *Streptococcus* are examples of Gram-positive coccal-shaped (round) bacteria. The remaining bacterial species, called **Gram-negative bacteria,** are decolorized and appear red-pink following counterstaining at the completion of the Gram stain procedure. *Escherichia coli*, which occurs in huge numbers in the human intestine, is a Gram-negative bacterial species.

Gram-positive bacteria remain blue-purple after the Gram stain procedure; Gram-negative bacteria appear red-pink.

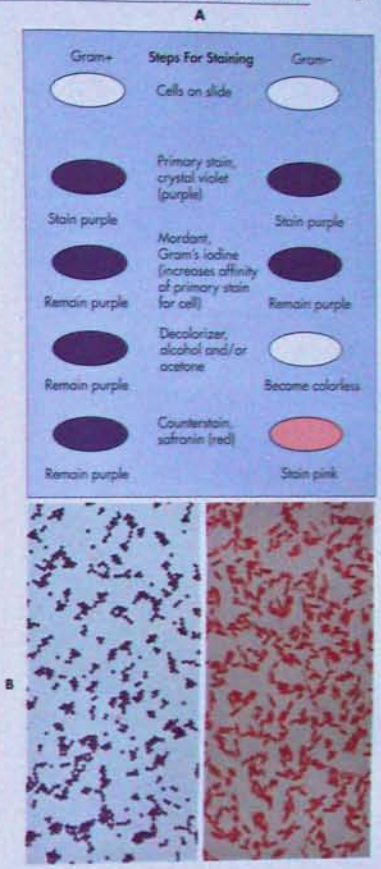
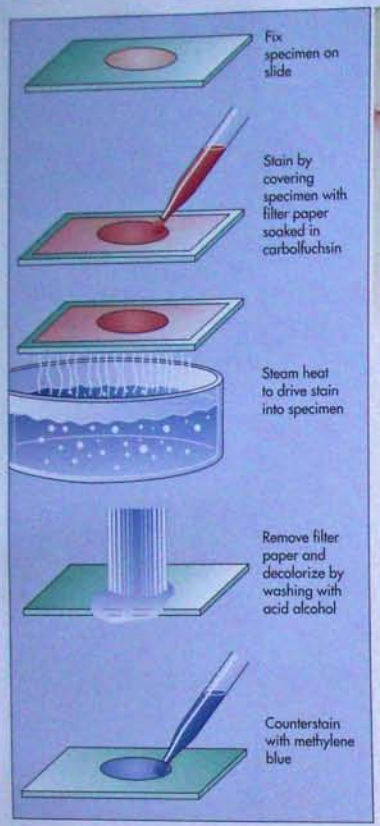


FIG. 3-13 **A,** The Gram stain procedure is widely used to differentiate major groups of bacteria. Gram-positive bacteria stain blue and Gram-negative bacteria stain pink-red by this staining procedure. Gram-positive and Gram-negative bacteria stain purple with the primary stain. The primary stain is removed from Gram-negative cells by the decolorizer and they are then stained pink by the counterstain. Gram-positive cells retain the primary stain and remain purple. **B,** Cells of the Gram-positive bacterium *Staphylococcus aureus* appear as blue-purple cocci. **C,** Cells of the Gram-negative bacterium *Escherichia coli* appear as pink-red cocci.



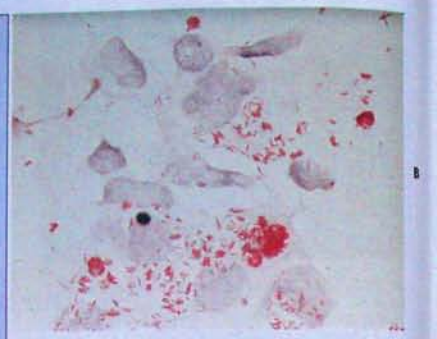


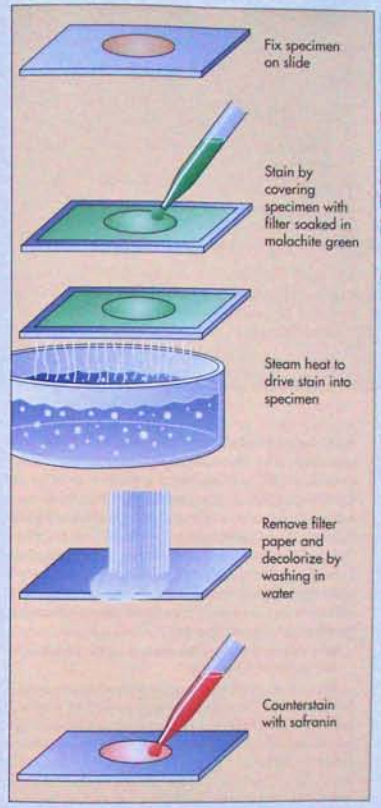
FIG. 3-14 **A,** The acid-fast staining procedure uses the red dye carbolfuchsin to stain cells. Acid-fast bacteria, such as mycobacteria, retain this stain when treated with acid alcohol. Nonacid-fast bacteria and tissue cells are decolorized by acid alcohol; they are counterstained with methylene blue. **B,** Acid-fast mycobacteria, including *Mycobacterium leprae* (the cause of leprosy), appear red after this staining procedure, as shown in this light micrograph.

Acid-fast staining is another differential staining procedure frequently used in bacteriology (FIG. 3-14). The acid-fast stain procedure is especially useful in identifying members of the bacterial genus *Mycobacterium* and is important in identifying the causative organisms of tuberculosis *(M. tuberculosis)* and leprosy *(M. leprae)*. In the acid-fast staining procedure, the red stain carbolfuchsin is used as a primary stain. Next, acid-alcohol is used as a decolorizer. The acid-alcohol will remove the red stain from bacteria, such as *Escherichia coli*, that are not acid-fast. The acid-fast mycobacteria will remain red. The acid-fast bacteria probably retain the carbolfuchsin because of waxy chemicals that occur in their cell walls. These chemicals are not found in nonacid-fast bacterial cells. To complete the acid-fast procedure, cells are counterstained with methylene blue and observed under the microscope. Acid-fast bacteria appear red and nonacid-fast bacteria appear blue. The red mycobacteria can thus be easily recognized even in a sputum sample containing numerous other blue bacterial cells.

Acid-fast staining is used to identify *Mycobacterium*, which are acid-fast and appear red after staining.

Nonacid-fast bacteria appear blue after acid-fast staining.

The **endospore staining procedure** is used to reveal specifically the presence or absence of endospores (FIG. 3-15). Endospores are heat-resistant resting bodies produced by only a few bacteria, such as species of the genera *Clostridium* and *Bacillus*. In the endospore staining procedure, the primary stain malachite green is driven into the endospores by steaming. Water is then used as a decolorizing agent. Water will wash the malachite green from the cells but not from the endospores. Next, safranin, a counterstain, is used to stain the cells. When viewed under the microscope, endospores—if they are present—appear as green spheres. They are either within the red-pink cells that produced them or free if those cells have died.



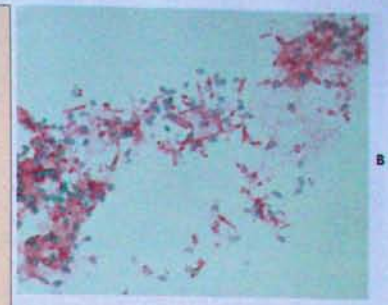


FIG. 3-15 **A,** The endospore staining procedure involves primary staining with malachite green. This stain is retained by endospores but washed out of cells with water. Cells are then counterstained with the red dye safranin. The spores appear green and the cells pink-red after staining by this procedure. **B,** Micrograph of *Clostridium tetani* after endospore staining. The spores appear green and the bacterial cells are stained red.

Types of Light Microscopes

Just as there are different staining procedures that are used for different purposes, there are different types of microscopes (Table 3-1, p. 72). These microscopes differ in magnification and resolution capacities. Some have specialized applications, and others have widespread applications in microbiology.

Bright-field Microscope

Virtually every microbiology laboratory has a **bright-field microscope,** so named because the field of view is brightly illuminated (FIG. 3-16). The typical bright-

FIG. 3-16 The bright-field microscope is used routinely by students of microbiology and practicing microbiologists, such as the technician in the clinical microbiology laboratory seen here.

TABLE 3-1

Comparison of Various Types of Microscopes

TYPE OF MICROSCOPE	MAXIMUM USEFUL MAGNIFICATION	RESOLUTION	DESCRIPTION
Bright-field	1,500×	100-200 nm	Extensively used for the visualization of microorganisms; usually necessary to stain specimens for viewing
Dark-field	1,500×	100-200 nm	Used for viewing live microorganisms, particularly those with characteristic morphology; staining not required; specimen appears bright on a dark background
Fluorescence	1,500×	100-200 nm	Uses fluorescent staining; useful in many diagnostic procedures for identifying microorganisms
Phase contrast	1,500×	100-200 nm	Used to examine structures of living microorganisms; does not require staining
TEM (transmission electron microscope)	500,000-1,000,000×	0.1 nm	Used to view ultrastructure of microorganisms, including viruses; much greater resolving power and useful magnification than can be achieved with light microscopy
SEM (scanning electron microscope)	10,000-100,000×	1-10 nm	Used for showing detailed surface structures of microorganisms; produces a three-dimensional image

field microscope is a compound microscope that has two lenses that contribute to the magnification of the image. The lenses are actually sets of several individual lenses that are designed to minimize distortions of light, called *aberrations*, that would interfere with the clear observation of a specimen. Besides its compound lenses, the modern bright-field microscope also has a built-in light source, a condenser lens that focuses the light on the specimen, and a mechanical stage that holds and permits controlled movement of the specimen.

Fluorescence Microscope

The **fluorescence microscope** is specifically designed for use with fluorescent stains (FIG. 3-17). These stains emit light of a different wavelength (color) than the wavelength of light used to illuminate the specimen. For example, when fluorescein isothiocyanate (FITC) is illuminated with blue or ultraviolet light, it emits an apple-green light. The fluorescence microscope has one filter that sets the wavelength of light used to illuminate the specimen and another filter that determines the wavelength of light that is viewed. As with the bright-field microscope, the specimens usually are stained. Some bacteria, such as *Pseudomonas*, contain fluorescent pigments and can be viewed without staining.

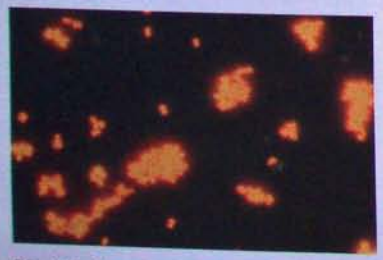


FIG. 3-17 Micrograph of clusters of staphylococci in blood after staining with acridine orange; the cells of this bacterium fluoresce orange.

Microscopy that uses fluorescent dyes is known as fluorescence microscopy.

Fluorescence microscopy is important because fluorescent dyes can be chemically linked to antibodies to produce **fluorescent-conjugated antibodies.** Antibodies are molecules that take part in animal defense systems against invading microorganisms. They have very specific chemical targets with which they react. This means that scientists can design and create fluorescent-conjugated antibodies that will react only with specific microorganisms to the exclusion of all others. By attaching a fluorescent dye to an antibody, the presence of a particular target can be visualized (FIG. 3-18). If the target is a bacterial cell, this forms the basis for **immunofluorescence microscopy** (differential staining using fluorescent-conjugated antibodies). Many pathogens, including the bacterium *Treponema pallidum*, which causes syphilis, can be quickly and positively identified by immunofluorescence microscopy without having to grow cultures in the laboratory. Diagnosis of disease is accomplished rapidly so that appropriate therapy can be initiated as quickly as possible.

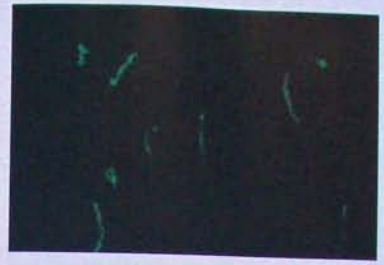


FIG. 3-18 Light micrograph of the spirochete *Treponema pallidum (green)* after fluorescent antibody staining. This bacteria causes syphilis.

Dark-field Microscope

The **dark-field microscope** is designed to enhance the contrast between the specimen and the background without the use of staining. Most staining procedures kill microorganisms. Using the dark-field microscope we can view live as well as dead specimens. The dark-field microscope has a special condenser that does not permit light to be transmitted directly through the specimen and into the objective lens (FIG. 3-19). This special dark-field condenser focuses light on the specimen at an oblique angle, such that the only light entering the objective is reflected off the object under observation. Thus only light that reflects off the specimen will be seen. In the absence of a specimen the entire field will appear black. Bacteria viewed with a dark-field microscope appear very bright on a black background (FIG. 3-20). The contrast between the specimen and the background is sufficient to permit easy visualization of bacteria and other cellular microorganisms.

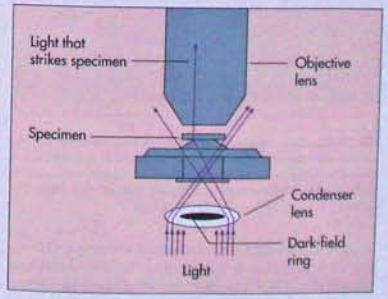


FIG. 3-19 Diagram of a dark-field microscope showing the path of light. The dark-field ring in the condenser blocks the direct passage of light through the specimen and into the objective lens. Only light that is reflected off a specimen will enter the objective lens and be seen.

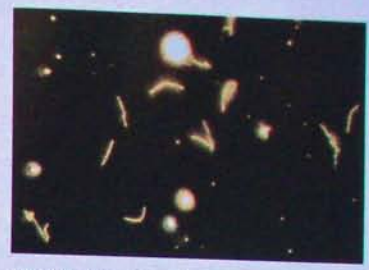


FIG. 3-20 Light micrograph of the bacterium *Treponema pallidum*, which causes syphilis, viewed by dark-field microscopy.

Phase Contrast Microscope

The **phase contrast microscope** also provides a means for achieving adequate contrast for visualizing microorganisms without the necessity of staining. The design of this microscope relies on the fact that light passing through a cell is slowed by the difference in density between it and the surrounding medium. Differences in brightness are created and this contrast is exactly what is needed to visualize specimens (FIG. 3-21). Consequently, with the phase contrast microscope, living organisms can be clearly

FIG. 3-21 Light micrograph of the protozoan *Paramecium caudatum* viewed by phase contrast microscopy. (264×).

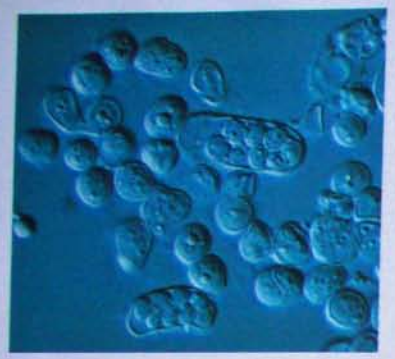


FIG. 3-22 Micrograph of the yeast *Schizosaccharomyces* viewed by Nomarski interference microscopy. (1,200×).

observed in great detail without staining them. This permits the study of their movements and their appearances in their natural surroundings.

Interference Microscope

There are several types of **interference microscopes,** such as the Nomarski interference microscope, that are now used in many microbiology laboratories. Like the phase contrast microscope these interference microscopes work on the principle that light from two light waves can be combined to increase or to decrease brightness. These microscopes are designed so that a beam of light can be split. One part of the light passes through the specimen and another part goes around the specimen. The beams are then recombined in a specialized prism and viewed. The result is a colored image that has a three-dimensional appearance (FIG. 3-22).

Electron Microscopy

An **electron microscope** uses an electron beam instead of visible light. Because an electron beam has a much shorter wavelength than the wavelengths of visible light, the electron microscope can achieve superior resolution. The electron microscope, therefore, permits higher useful magnifications than can be achieved with light microscopy. Consequently, viruses and even the smallest structures of a bacterial cell can be seen by using an electron microscope.

Electron microscopes use electron beams instead of visible light. Electron microscopes achieve better resolution and higher useful magnification than light microscopes.

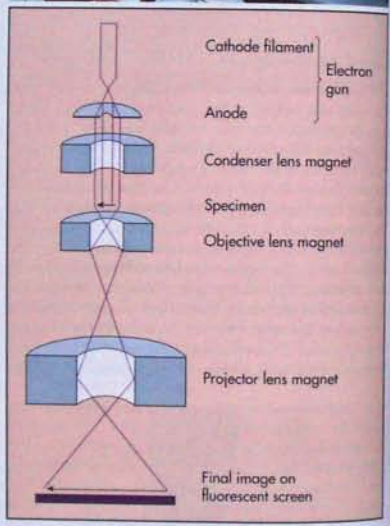


FIG. 3-23 The transmission electron microscope (TEM) allows the visualization of the fine detail of the microbial cell. The TEM uses an electron beam and electromagnets instead of the light source and glass lenses used in light microscopy.

In the **transmission electron microscope** (TEM), the electron beam is transmitted through the specimen (FIG. 3-23). The other type of electron microscope is called the **scanning electron microscope** (SEM). In the SEM an electron beam is scanned across the surface of the specimen (FIG. 3-24). In either type of electron microscope, the electron beam must be transmitted through a vacuum. Otherwise the electrons would collide with the molecules in air and scatter. This produces a blurred image, if any image is produced at all. Much of the apparent complexity and bulk of an electron microscope is the result of the need to produce a high vacuum system for the transmission of the electron beam. Also, because we can not view the electron beam directly, we view the magnified image of an electron microscope on a phosphorescent screen and sometimes make a permanent image on photographic film. The images produced by electron microscopes are called electron micrographs or just micrographs.

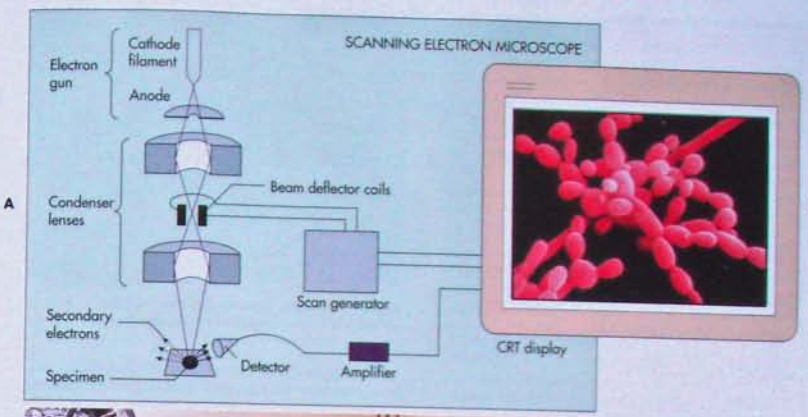


FIG. 3-24 **A,** The scanning electron microscope (SEM) is used for viewing surface structures and their three-dimensional spatial relationships. An electron beam is scanned across a specimen. The electrons emitted from the surface of the specimen determine the intensity of the image. The relative lengths of the scans across the specimen and the cathode ray tube (CRT) display determine the magnification. **B,** Colorized micrograph of the fungus *Candida albicans* viewed using a SEM. (6,300×). **C,** Photograph of a scanning electron microscope.

Transmission Electron Microscope

The principles of operation of the **transmission electron microscope** (TEM) are very similar to those of the compound light microscope. Instead of a light source, the TEM uses an electron beam. In place of glass lenses, the TEM uses a series of electromagnets to focus the electron beam and to magnify the image of the specimen. The electron microscope has a theoretical resolution of approximately 0.1 nm. This is about a thousand times better resolving power than can be achieved by using light microscopy. Consequently, the useful magnification for an electron microscope is higher than 500,000×. Magnifications this high permit the visualization of all microorganisms, including viruses. The internal structures of microorganisms can also be seen in fine detail (FIG. 3-25).

The transmission electron microscope (TEM) permits very high magnification (>500,000×) and superior resolution (0.1 nm).

The TEM permits the visualization of the detailed structures of all microorganisms, including viruses.

There are some special problems in viewing biological specimens with the electron microscope. The specimens must be killed before being placed in a high vacuum chamber and exposed to a high voltage electron beam. Because of the high magnifications achieved with the electron microscope, there is a great potential for creating artifacts that could be mistakenly viewed as real structures in electron micrographs. An artifact is the appearance of something in an image or micrograph caused by the magnification system or procedures used for preparing specimens.

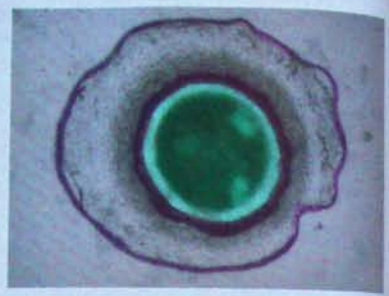


FIG. 3-25 Colorized transmission electron micrograph of a thin section of an endospore of *Bacillus sphaericus*.

An artifact is not a true representation of the features of the specimen on view.

To minimize artifacts, special preparation procedures must be used in electron microscopy (FIG. 3-26). Biological specimens containing water cannot simply be placed under high vacuum because the water would boil. The integrity of the organisms would be destroyed. Therefore, before viewing a microorganism with a transmission electron microscope, it is necessary to preserve (fix) and to dehydrate (remove water from) the specimen. Water is usually removed by transferring the specimen through a series of alcohol baths of increasing alcohol concentrations. Inadequate fixation or insufficient dehydration will result in the formation of artifacts.

Additionally, as surprising as it may seem, whole microorganisms often are too thick to view with a transmission electron microscope. To see the fine detail that is possible with the high resolving power of the TEM, it is normally necessary to slice microorganisms into thin sections or to fracture them into pieces before trying to observe their detailed structures. Microorganisms can be sliced by first embedding them in plastic. A specialized instrument called a *microtome*, equipped with a glass or diamond knife, is used to cut a thin film containing the sliced microorganisms. To break them into pieces, microorganisms can be frozen in liquid nitrogen and then bludgeoned with a dull instrument, such as a hammer. Here again there is a possibility that artifacts will be created in processing the specimen. Therefore great caution must be observed in interpreting electron micrographs.

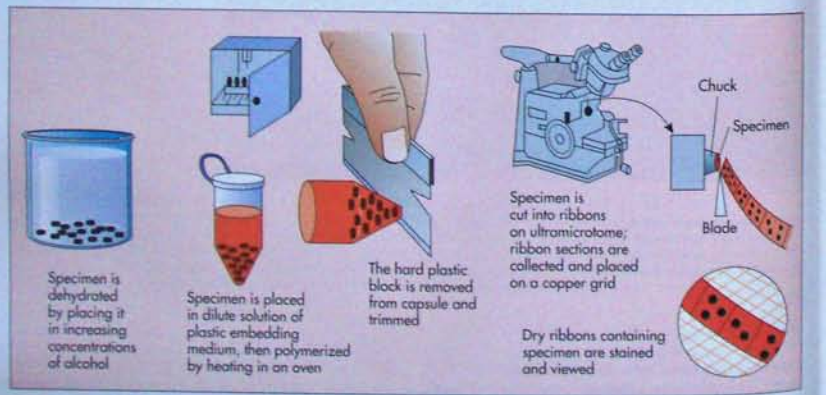


FIG. 3-26 Extensive preparation of a specimen is generally needed for viewing by transmission electron microscopy. Water must be removed; this dehydration of the specimen usually is achieved using alcohol. Many specimens must be cut into thin sections before they can be viewed in the electron microscope. Sectioning is accomplished by placing the specimen in a plastic and then cutting the sections with an ultramicrotome.

HIGHLIGHT

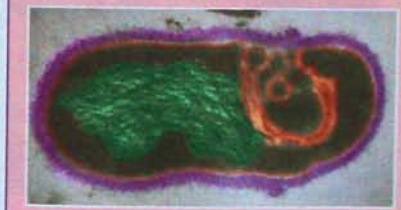
Mesosomes: Real Structures or Artifacts?

Sometimes, when thin sections of bacteria are viewed by transmission electron microscopy, extensive folds of membranes are seen within the cell (see Figure). These membranes are called mesosomes. Careful observations of these mesosomes indicate that they are continuous with the plasma membrane. Having observed mesosomes in many routine electron microscopic preparations, several independent investigators tried to identify their functions. Although many functions were hypothesized for mesosomes, establishing their real function proved elusive. For each proposed function, cells were found without mesosomes that carried out the same functions as cells with mesosomes. For example, because mesosomes were often observed in the region where a bacterial cell divides when it reproduces, one proposed function was that they were involved in cell division. However, mesosomes are not always seen when bacterial cells divide, nor, when they are present, do they always occur near the site of cell division.

After considering the observations made in many studies, it became obvious to the scientists who had first described mesosomes that the evidence for the existence of mesosomes had to be reevaluated. These scientists asked why mesosomes were only sometimes observed and why their absence didn't result in the loss of some function. They hypothesized that mesosomes might be an artifact. Based on numerous observations they found that mesosomes were almost always found to be attached or closely associated with DNA within the cell. They reasoned that the DNA might be shrinking due to dehydration during preparation for electron microscopy. Also, since bacterial DNA is attached to the plasma membrane, such DNA shrinkage might pull part of the plasma membrane inward. If this occurred, they hypothesized, it would create an artifact that appeared as a mesosome.

To see if this were the case, cells were frozen in liquid nitrogen and then exposed to X radiation (X-rays) to break up the DNA before the cells were dehydrated during preparation for electron microscopic viewing. When this procedure was followed, no mesosomes were observed. Mesosomes, however, were observed in control specimens in which the DNA had not been broken up by X radiation prior to dehydration. This suggested that these observed structures were artifacts of preparation for electron microscopic observation, rather than real structures of the bacterial cell.

After years of investigation, the evidence, therefore, supports the hypothesis that mesosomes are artifacts and not real structures. This is a good example of why scientists must always repeat their observations, test their hypotheses, and continue to evaluate their interpretations until they exhaust all possible alternative explanations for their observations.



Colorized transmission electron micrograph of a thin-sectioned bacterium, *Corynebacterium* species, shows the invaginated membrane (tan) that has been called a mesosome.

Caution must be used in interpreting electron micrographs because the techniques used in their preparation/creation have a high potential for producing artifacts.

Scanning Electron Microscope

The operation and design of the **scanning electron microscope** (SEM) are quite different from those of the TEM. In SEM, a narrowly focused beam of electrons is rapidly scanned across the surface of the specimen. The primary scanning electron beam knocks electrons out of the specimen surface. These secondary electrons are collected by a detector, amplified, and used to generate an image on a cathode ray tube (CRT) screen. The image on the CRT screen has a three-dimensional appearance due to the shadowing effects of the sweep beam (FIG. 3-27). The primary beam that scans across the specimen is synchronized with a beam that scans across the CRT display so that the image on the CRT screen is an accurate magnified representation of the specimen. A typical scanning electron microscope is capable of achieving a useful magnification of 10,000× to 100,000× with a resolution of 10 to 20 nm. The SEM is used primarily for viewing surface details.

FIG. 3-27 Colorized scanning electron micrograph of the diatom *Achnanthes exigna*. (875×). The frustule that forms the outer shell of the diatom is composed of silicon dioxide and has two overlapping halves.

The scanning electron microscope (SEM) is used primarily for viewing surface structures.

PURE CULTURE METHODS

Many microorganisms look exactly alike. Examining their physiological characteristics, therefore, is essential for studying and identifying microbial species. In macroorganisms the metabolism of a single organism can be measured. For example, the rate of respiratory production of carbon dioxide by a human athlete can be measured. However, the metabolism of a single microbial cell doesn't transform sufficient material for the scientist to measure. A population of identical microorganisms, on the other hand, will transform measurable amounts of materials. Microbiologists culture (grow) colonies of microorganisms that contain millions of individuals. This way sufficient material to study and determine their metabolic characteristics is provided (FIG. 3-28).

A **pure culture** of bacteria is a population of identical bacteria all derived by asexual reproduction from a single bacterial cell. Bacteria most often reproduce asexually by a process called *binary fission*, in which a single cell divides into two equal-sized identical progeny cells. Scientists, thus, can grow pure bacterial cultures starting with a single bacterial cell (FIG. 3-29). Cultures of bacteria contain millions of identical bacterial cells. These cultures can be used by scientists to study the metabolism and growth characteristics of the individual unseen bacterium. Cultures grown in the laboratory can also be used to study the rates of microbial growth under different environmental conditions.

Microbial growth is essentially synonymous with cell reproduction. The growth of a bacterium, for example, is measured as an increase in cell number due to reproduction. Using cultures, scientists can observe under what conditions a microorganism will reproduce. For example, they can determine the temperature range over which the microorganism can grow and the temperature at which the microorganism grows fastest. The ability to produce new cells is often used to determine whether or not a bacterium is alive. Living or viable bacteria will reproduce, whereas nonviable bacteria will not.

FIG. 3-28 Microbial cultures are grown in pure culture so they can be studied and used for various purposes.

A pure culture of a microorganism, a population of identical bacteria, can be grown to observe the characteristic properties of that microorganism.

FIG. 3-29 Colorized transmission electron micrograph showing the reproduction of *Streptococcus pyogenes* by binary fission. The inward growth of the septum *(purple)* divides the parent cell to produce two equal-sized progeny cells. Other components of the cell have already been synthesized and segregated into the new cells.

Early Development of Pure Culture Methods

Many of the methods for culturing bacteria were developed in the late nineteenth century in Germany by Robert Koch and his assistants. They developed simple methods for the isolation and maintenance of pure cultures of microorganisms. They grew cultures on the surfaces of solid media containing the nutrients needed to support the growth of the microorganisms. Before then, microorganisms were grown only in liquid broths, making it very difficult to separate one microorganism from another. By growing cultures on solid media, individual cells could be separated from other cells. This facilitated the isolation of the progeny of a single cell for the establishment of pure cultures. It also permitted observation of populations of single species of microorganisms.

At first, Koch grew bacteria on solid fruits and vegetables, such as slices of boiled potato, but many bacteria cannot grow on such substances. Koch developed a way of solidifying liquid broths that could support the growth of a greater variety of microorganisms. Initially he used gelatin and later an algal extract called **agar** as the solidifying agent. The suggestion for using agar originated with the wife of one of the investigators at Koch's Institute, Frau Fanny Hesse, who had seen her mother using agar to make jellies. Agar is particularly useful because it melts at 100° C but does not resolidify until it cools to 42° C. Tubes or flasks containing agar can be placed into a boiling water bath to melt the agar. Agar can be poured into other containers while it is still liquid. Bacteria can even be added to the agar while it is liquid at 42° C and survive. Once agar has cooled and solidified it will remain solid at the temperatures used for the culturing of bacteria. Most bacteria that are human pathogens are grown at 37° C. Other bacteria from nature often are grown at lower or higher temperatures.

Agar is a useful medium for the culture of bacteria because it melts at 100° C and resolidifies at 42° C, can be poured, can have bacteria added when liquid, and remains solid at typical bacterial growth temperatures.

One of Koch's students, Richard J. Petri, suggested placing the solidified media into a new type of container. This container was circular in shape and had an overlapping lid that prevented microorganisms in the air from contaminating the cultures placed into the container. The basic design has become known as the **Petri plate.** It is used in virtually all microbiological laboratories in essentially the same design described by Petri (FIG. 3-30). If all microorganisms in the medium in a Petri plate are killed by heating, the plate will remain free of microorganisms, that is sterile, until the lid of the plate is lifted. If the lid is removed for only a very short time in a clean room, specific microorganisms can be placed on the surface

FIG. 3-30 Petri plates are routinely used for culturing microorganisms. **A,** The Petri plate consists of two overlapping halves that permit air to enter but prevent contamination by extraneous microorganisms. **B,** Microorganism grown on a solid medium within the Petri plate, forming visible colonies.

of the solid medium without much risk of contamination by other microorganisms in the air.

It is in this manner that microorganisms can be transferred. They can be transferred from some source, such as a blood or urine specimen collected from a patient, into a Petri plate with the assurance that all microorganisms in the plate came from that source. Once introduced, microorganisms reproduce by using the nutrients in the medium in the Petri plate to support their metabolism. If a single microbial species is introduced into the Petri plate, its reproduction will produce a pure culture. There will be no other microbial species. The Petri plate, with solidified agar, thus permits the establishment of pure cultures of microorganisms. Most microbiological studies, including those used to identify the microorganisms causing a particular disease, require pure cultures for study to establish that a particular effect—such as a disease—is caused by a specific microorganism.

Pure culture methods using agar media and Petri plates are fundamental techniques used in virtually all microbiology laboratories in the world today.

Sterilization

To establish pure cultures of microorganisms, the media, containers, and all the implements used for manipulating microorganisms must initially be free of living microorganisms. An environment that is totally free of all living organisms is **sterile.** Sterilization procedures are used to kill or to remove all living microorganisms from a specified area. Since living organisms, including microorganisms, cannot arise by spontaneous generation, a sterile area will remain sterile unless a living microorganism enters that area naturally from somewhere else or until a scientist intentionally introduces a microorganism.

There are several ways of sterilizing the liquids, containers, and instruments used in pure culture procedures (FIG. 3-31). These methods include: (1) filtration to remove microorganisms; (2) exposure to elevated temperatures (usually over 100° C), (3) exposure to toxic chemicals (such as ethylene oxide), and (4) exposure to ionizing radiation (such as gamma radiation) to kill microorganisms. These methods are discussed in detail in Chapter 11. Sterilization procedures are absolutely essential if pure cultures are later to be obtained for clinical diagnosis, scientific investigation, or industrial use. It also is essential to sterilize instruments and materials for other purposes, such as in surgical procedures where it is critical to avoid the introduction of microorganisms into the body, thereby preventing infection and disease.

Removal of microorganisms from liquids and gases by filtration generally is accomplished by passage of the substance through a filter with 0.2 to 0.45 μm diameter pores. Most bacteria are trapped on the filter, but viruses and some very small bacteria may pass through it. When viruses were first discovered they were referred to as filterable agents because they passed through bacteriological filters (filters with pore sizes less than 0.2 μm). Clean rooms, such as operating theaters, often employ high efficiency particulate air filters (HEPA filters) to remove microorganisms from the air. HEPA filters remove particulate material larger than about 0.3 μm from the air.

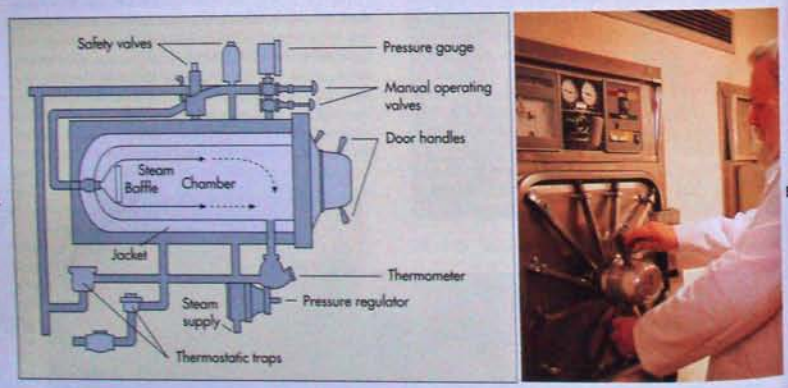


FIG. 3-31 **A,** Diagram of an autoclave. This instrument is routinely used for sterilization of media and other items in the microbiology laboratory. In an autoclave, steam is introduced under pressure into a chamber containing the material to be sterilized. The pressure generally is adjusted to 15 lb/in² so that a temperature of 121° C (250° F) is reached. Valving of the autoclave permits rapid entry of steam from a preheated jacket into the chamber and the subsequent slow exhausting of steam from the chamber; this process permits rapid heating of the material and prevents liquids from boiling out of their containers, as would happen if the pressure was suddenly reduced. **B,** A technician is loading an autoclave with media for sterilization—a routine operation in most microbiology laboratories.

Heat sterilization at a temperature that kills all microorganisms, including their heat-resistant endospores, is often used to eliminate unwanted microorganisms. Incineration can be used to destroy medical or other microbiological wastes. Incineration (burning) is also used to sterilize inoculation loops that are frequently used in the microbiology laboratory to transfer cultures of microorganisms. Dry heat sterilization requires high temperatures and long exposure periods to kill all of the microorganisms in a sample. Exposure in an oven for 2 hours at 170° C (328° F) is generally used for the dry heat sterilization of glassware and other laboratory items.

Much time is spent in the preparation and sterilization of media for the bacteriology laboratory. Culture media preparation usually employs an autoclave that uses steam under pressure for sterilization. An **autoclave** is an instrument that exposes substances to steam at elevated temperatures. Steam has a high penetrating power and a much higher heat capacity than dry heat. Thus it is very effective at killing microorganisms. Generally, exposure for 15 minutes at 121° C, achieved by using a pressure of 15 lb/in² (SI equivalent = 103.4 kPa), is used to sterilize microbiological culture media.

Exposure to ionizing radiation is useful in sterilizing materials that are destroyed by heat, such as plastics. Many of the Petri plates used in microbiology laboratories are radiation sterilized by using gamma radiation. Radiation exposure can be used to increase the shelf life of various foods and to kill foodborne pathogens such as *Clostridium botulinum*, the bacterial species that causes botulism. Exposure to radiation does not leave any residual radioactivity in the food, but the method is still controversial. Labels are placed on foods that have been radiation treated to inform consumers.

Microorganisms can be killed or removed by several methods to achieve sterility. A sterile environment contains no living microorganisms.

Aseptic Technique

Once substances are sterilized, they stay sterile as long as they remain within containers that do not permit living microorganisms to enter. Scientists, however, often must transfer materials from one container to another. To maintain sterility, they use methods that do not permit the accidental entry of living microorganisms during the transfer process. These methods are collectively called **aseptic technique.** For example, when pouring a sterile liquid from one vessel to another sterile vessel, the openings of the vessels often are passed through the flame of a bunsen burner. This is done immediately after they are opened to kill any microorganisms that may be there and to create convection currents away from the mouth of the tube so that microorganisms in the air don't enter the tube. Then the liquid is quickly poured from one vessel to the other. Any contact with surfaces that contain microorganisms that could contaminate the liquid is avoided. After the liquid has been poured, the openings of the vessels are again flamed to reensure that no microorganisms are there that could enter the sterile liquid. Finally the vessels are resealed. Minimizing the time that the vessels are open and working in a clean area are important parts of aseptic technique and limit the possibility that microorganisms will enter the vessel accidentally.

Aseptic technique also must be used to establish a pure culture. The scientist must ensure that only the desired microorganism enters the culture vessel. To transfer microorganisms from one place to another, microbiologists often use a device called a transfer loop (FIG. 3-32). A transfer loop is a piece of wire with a handle on one end and a small circle of wire on the other end. The metal wire usually is made of

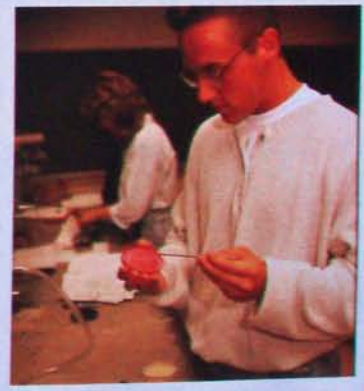


FIG. 3-32 Aseptic transfer procedures are essential for preventing contamination of bacterial cultures and for ensuring that the microorganisms being cultured do not escape into the laboratory.

nichrome or platinum. It can be heated red hot by placing it into an electric heater or the flame of a bunsen burner to kill all the microorganisms on it. The circular wire loop can then be cooled and touched to an already established pure microbial culture. It can also be dipped into a liquid containing microorganisms to pick up living microbial cells.

By avoiding contact with any surfaces containing other microorganisms and by working quickly, the living microorganisms on the loop can be transferred to a new location. For example, a bacterial culture can be moved onto a Petri plate containing sterile medium, without contamination with other living microorganisms. When the transfer loop is touched to the surface of the solid sterile medium some of the living microorganisms fall off. The transfer loop is then heated red hot again to kill any remaining microorganisms. Aseptic transfer technique not only ensures that a pure sample of the microorganism is transferred, it also protects the scientist from contact with the culture. This is especially critical when the culture contains microorganisms that can cause disease. Thus aseptic technique protects microorganism and microbiologist alike from contamination.

Aseptic technique is used to prevent the contamination of a pure culture of a microorganism with extraneous microorganisms and to prevent human contact with potentially dangerous microorganisms.

Isolation of Pure Cultures

Pure cultures of microorganisms can be isolated and maintained using aseptic transfer techniques and sterile media. The isolation of a pure bacterial culture involves separating bacteria into individual cells. These cells are then allowed to reproduce to form a colony on the surface of a plate of a solid medium (FIG. 3-33). A **colony,** which contains millions of cells and can be seen visually, develops from the asexual reproduction of a single cell. Each colony contains a population of a single bacterial species. Separating this colony from all other bacteria results in the formation of a pure culture.

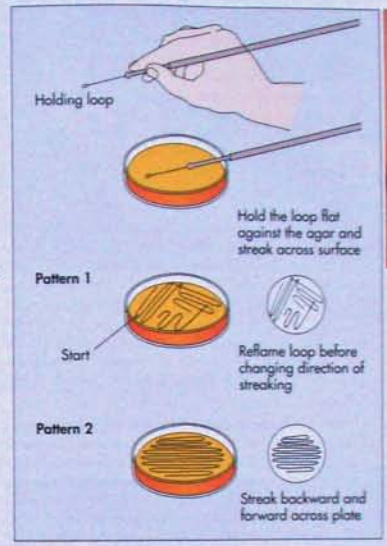
FIG. 3-33 Colonies of *Vibrio parahaemolyticus* growing in a Petri plate on thiosulfate citrate-bile salts-sucrose (TCBS) agar.

Several methods are used to isolate pure cultures of microorganisms. The **streak plate method** is the most commonly used method. In this method a transfer loop is dipped into a substance containing bacterial cells. The source can be a liquid, such as blood, urine, or water, or a solid, such as soil (FIG. 3-34). The bacterial cells that adhere to the loop are picked up. The loop is gently dragged across the solid agar surface of the nutrient medium. The cells are thus deposited. The surface of the medium has been solidified by the addition of agar. In the initial portions of this continuous streak, many cells will be deposited close together. When the plates are incubated, these cells will reproduce to form colonies. Because the cells are deposited close together, the colonies will overlap. The cells in the overlapping colonies do not originate from a single cell. The colony may therefore contain more than one bacterial species. As the transfer loop is streaked further across the plate, there are progressively fewer cells still on the loop that can be deposited on the plate. Often, the microorganisms are dragged across part of the plate, the loop is resterilized, and a new streak is made at an angle to the original streak. Bacterial cells in the latter portions of the streak will be deposited further apart. The reproduction of these cells will give rise to separate, well-isolated colonies. Because each well-isolated colony arises from the reproduction of a single bacterium, it is a pure culture. The isolated colonies can then be transferred, using a sterile inoculating loop and aseptic transfer technique, onto a fresh medium in a separate vessel and grown as pure cultures.

Pure cultures can also be isolated by dripping a small volume of a dilute liquid suspension of microorganisms onto the center of an agar plate. The liquid suspension is then spread over the surface of the agar with a sterile glass rod. This method for isolating pure cultures is called the **spread plate method** (FIG. 3-35). By spreading the suspension over the plate, individual cells are separated from the other cells in the suspension. Well-isolated colonies develop in regions where cells are deposited far apart on the surface of the medium.

In the **pour plate method,** dilute suspensions of microorganisms are added to melted agar that has been cooled to approximately 42° to 45° C (FIG. 3-36). Since agar is a liquid until it cools below 42° C and since most bacteria are not killed by exposure to 45° C for several minutes, living bacteria can be mixed with the liquid agar medium and then poured into sterile Petri plates using aseptic technique. When the agar cools below 42° C and solidifies, individual bacterial cells become trapped at locations throughout the medium. Although the medium holds bacteria in place, it is soft enough to permit reproduction of bacteria and the formation of discrete isolated colonies. As with the other isolation methods, individual colonies are then picked up and transferred to separate vessels where they can be grown as pure cultures.

The streak plate, spread plate, and pour plate methods are used to isolate pure cultures of microorganisms.



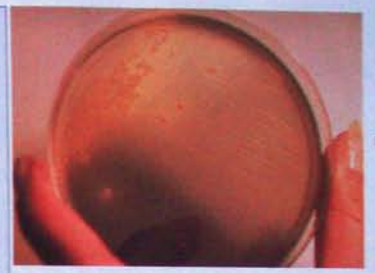


FIG. 3-34 **A,** Streaking for the isolation of pure cultures, showing two different streaking patterns. In this procedure, a culture is diluted by drawing a looptul of the organism across a medium. For a dilute culture, only a single streak may be used (Pattern 2). For more concentrated cultures, a second streak is drawn across the first streaks so that some cells are picked up and further diluted; several additional streaks are made to ensure sufficient dilution so that only single cells are deposited at a given location (Pattern 1). The growth of each isolated individual cell results in the formation of a discrete colony. **B,** Streak plate of *Vibrio cholerae* on thiosulfate citrate-bile salts-sucrose (TCBS) agar.

FIG. 3-35 The spread plate technique for isolating and enumerating microorganisms. 1, A sample is aseptically pipetted onto an agar medium. 2, a spreading rod is sterilized by dipping in alcohol and flaming. 3, the sterile rod is used to spread the suspension over the surface of the medium.

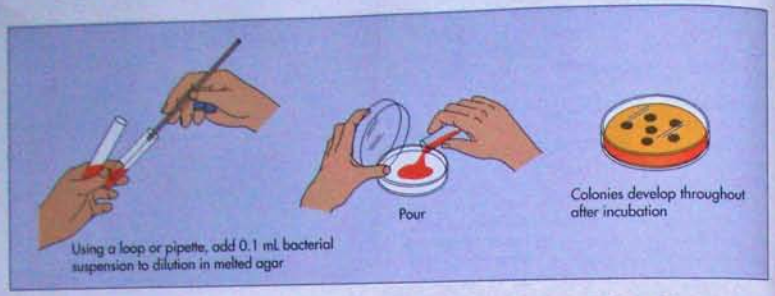


FIG. 3-36 The pour plate technique for isolating and enumerating microorganisms. A sample of a known dilution is mixed with a liquefied agar medium that has been cooled to 45° to 50° C and poured into a Petri plate. After incubation the numbers of colonies that develop are counted and the concentration of microorganisms in the original suspension is calculated.

Conditions and Media for Laboratory Growth of Microorganisms

To culture microorganisms, microbiologists must establish the conditions necessary for microbial growth in the laboratory. They must provide suitable environmental conditions and the necessary nutrients so that the microorganism can carry out its metabolism and reproduce. Under optimal growth conditions, most microorganisms reproduce very rapidly. Visible colonies of many bacteria, for example, develop in less than 24 hours if the culture is incubated under optimal conditions. The ability to obtain pure cultures within a matter of hours is especially important in the clinical microbiology laboratory where speed of identification is essential. Many microbial identifications can be completed in less than a day. This permits the physician to begin appropriate treatment quickly.

Incubators

Temperature is one of the most important environmental factors to control for optimal microbial growth. Microorganisms only survive within certain temperature limits. Rates of microbial metabolism are greatly affected by temperature. Each microbial species has an optimal growth temperature, which is the temperature at which that microbial species reproduces at its fastest rate. Incubators are used to grow bacteria in the laboratory. Within an **incubator** the temperature can be controlled. The temperature is usually set near the optimal growth temperature of the microorganism of interest.

Incubators are temperature control chambers in which microbial cultures are grown.

As with temperature, different microbial species will grow best at different concentrations of molecular oxygen (O_2). **Obligate aerobes** require molecular oxygen for growth and will not grow in its absence. To grow in the laboratory, microbiologists must use procedures that assure the absence of molecular oxygen. Like temperature and oxygen, other environmental factors can also be controlled for culturing microorganisms. For example, some pathogenic microorganisms grow best in an atmosphere with increased concentrations of carbon dioxide. Scientists can use special incubators that control carbon dioxide concentration to cultivate such microorganisms.

Scientists control environmental conditions to grow pure cultures of microorganisms in the laboratory.

Culture Media

To culture microorganisms, microbiologists must ensure that the culture medium contains the variety of organic and inorganic nutrients that are required for microbial metabolism. All organisms require carbon, nitrogen, oxygen, hydrogen, phosphorus, sulfur, and various other substances for growth. These substances must be available in a usable chemical form to meet the nutritional requirements of a particular microorganism and to permit that organism to grow. Not all microorganisms have the same nutritional requirements. In fact, the specific nutritional requirements for different microorganisms vary greatly.

Commonly used culture media contain protein that have been digested with enzymes or acids and/or carbohydrates as growth substances that a microorganism can utilize. These media also generally contain sources of nitrogen—such as ammonium nitrate, phosphate, and sulfate. Additionally, magnesium, sodium, potassium, and chloride ions are needed to meet the inorganic metabolic needs of the microorganism. Because of the difficulty in defining the specific nutritional requirements of individual microbial species, microbiologists often use **complex media,** which are media that contain various substances whose precise chemical compositions are unknown. Such complex culture media will support the growth of many different types of microorganisms that require organic compounds as their source of energy. Many complex media contain beef extract, peptones, and yeast extract. In some cases, scientists must add specific compounds to get a microorganism to grow in the laboratory. Clinical microbiologists often incorporate blood into media that are designed for the culture of disease-causing microorganisms to provide necessary but unspecified nutrients for the growth of these microorganisms. Some disease-causing microorganisms, called **fastidious microorganisms,** are nutritionally demanding. Some require factors in blood or other specific substances to support their growth. They often are difficult to culture in the laboratory.

A growth medium contains the necessary nutrients to support the nutritional requirements of cultured microorganisms.

Different types of media are needed for the cultivation of different microorganisms.

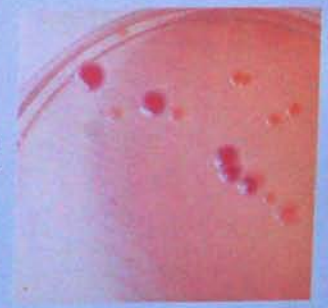
Fastidious microorganisms have specific and demanding growth requirements.

Scientists sometimes design media to prevent the growth of unwanted microorganisms or to permit the differentiation of specific types of microorganisms. Media are called **selective media** if they favor the growth of specific microorganisms and **differential media** if they permit the recognition of specific types of microorganisms; in some cases a medium is both differential and selective (Table 3-2). Selective media usually are designed to allow some microorganisms to grow while preventing growth of others.

TABLE 3-2

Some Differential and Selective Media

MEDIUM	DESCRIPTION
MacConkey Agar	
MacConkey agar is a differential plating medium for the selection and recovery of Enterobacteriaceae and related enteric Gram-negative rods. Bile salts and crystal violet are included to inhibit the growth of Gram-positive bacteria and some fastidious Gram-negative bacteria. Lactose is the sole carbohydrate.	Lactose-fermenting bacteria produce colonies that are varying shades of red because of the conversion of the neutral red indicator dye (red below pH 6.8) from the production of mixed acids. Colonies of non-lactose-fermenting bacteria appear colorless or transparent.



Colonies of *Escherichia coli* growing on MacConkey agar showing characteristic colony morphology and coloration. *E. coli* frequently causes urinary tract infections and also is a common cause of bacterial meningitis in infants.

Continued

TABLE 3-2—cont'd

Some Differential and Selective Media

MEDIUM	DESCRIPTION
Eosin Methylene Blue (EMB) Agar	
EMB agar is a differential plating medium that can be used in place of MacConkey agar in the isolation and detection of the Enterobacteriaceae and related coliform rods from specimens with mixed bacteria. The	aniline dyes (eosin and methylene blue) in this medium inhibit Gram-positive and fastidious Gram-negative bacteria. They also combine to form a precipitate at acid pH, thus also serving as indicators of acid production.

Escherichia coli growing on eosin-methylene blue (EMB) agar showing characteristic colonies with green metallic sheen.

Desoxycholate-citrate (DCA) Agar	
DCA agar is a differential plating medium used for the isolation of members of the Enterobacteriaceae from mixed cultures. The medium contains about three times the concentration of bile salts (sodium desoxycholate) of MacConkey agar, making it most useful in selecting species of *Salmonella* from specimens	overgrown or heavily contaminated with coliform bacteria or Gram-positive organisms. Sodium and ferric citrate salts in the medium retard the growth of *Escherichia coli*. Lactose is the sole carbohydrate, and neutral red is the pH indicator and detector of acid production.

Colonies of *Salmonella arizonae* growing on DCA. This bacterium produces characteristic pink colonies on this medium.

MEDIUM	DESCRIPTION
Hektoen Enteric (HE) Agar	
HE agar is devised as a direct plating medium for fecal specimens to increase the yield of species of *Salmonella* and *Shigella* from the heavy numbers of normal microbiota. The high bile salt concentration of this medium inhibits the growth of all Gram-positive bacteria and retards the growth of many stains of coliforms.	Acids may be produced from three carbohydrates, and acid fuchsin reacting with thymol blue produces a yellow color when the pH is lowered. Sodium thiosulfate is a sulfur source, and H_2S gas is detected by ferric ammonium citrate, producing a black precipitate.

Salmonella enteritidis growing on Hektoen enteric (HE) agar showing characteristic black colonies due to production of hydrogen sulfide.

Xylose Lysine Desoxycholate (XLD) Agar	
XLD agar is less inhibitory to the growth of coliform bacteria. It was designed to detect *Shigella* species in feces after enrichment in Gram-negative broth. Bile salts in relatively low concentration make this medium less selective than the other media included in this table. Three carbohydrates are available for acid pro-	duction, and phenol red is the pH indicator. Lysine-positive organisms, such as most *Salmonella enteritidis* strains, produce initial yellow colonies from xylose utilization and delayed red colonies from lysine decarboxylation. The H_2S detection system is similar to that of HE agar.

Salmonella typhimurium on xylose lysine desoxycholate agar (XLD) showing the black growth due to production of hydrogen sulfide.

For example, the stain methylene blue is more toxic to Gram-positive bacteria than to Gram-negative bacteria. By incorporating 0.5% methylene blue into a culture medium, the growth of Gram-positive bacteria can be inhibited. This will not interfere with the growth of Gram-negative bacteria. Eosin methylene blue (EMB) agar contains methylene blue and, therefore, is a selective medium. It is frequently used for the selective culture of Gram-negative bacteria, such as *Escherichia coli*, from samples, such as stool specimens, that contain both Gram-negative and Gram-positive bacteria. EMB agar is also a differential medium because colonies of different bacterial species growing on it will appear different based on the type of metabolism that they carry out. The bacterium *E. coli*, which metabolizes lactose, produces colonies with a green metallic sheen when growing on EMB agar. These lactose-utilizing bacteria can be easily differentiated from any other bacteria growing on EMB agar.

Selective media contain substances that favor the growth of one type of microorganism and discourage the growth of other types.

Differential media contain substances that permit recognition of different types of microorganisms.

SUMMARY

Scientific Method and Development of the Science of Microbiology (pp. 59-64)

- All fields of science, including microbiology, rely on the scientific method in which a scientist asks a question, proposes a hypothesis, makes systematic observations, and conducts controlled experiments to test the validity of that hypothesis.
- Scientists use deductive reasoning ("if-then" thinking) and test their predictions by conducting experiments.
- Controlled experiments are designed to yield unambiguous answers regarding whether a hypothesis is correct or incorrect.
- The results of experiments must be repeatable, and interpretations of experimental results become accepted only after they have been critically evaluated.
- The struggle to disprove the theory of spontaneous generation represents a good example of the scientific method at work.
- Louis Pasteur's experiments with swan-neck flasks provided the final refutation of the theory of spontaneous generation.

Microscopy (pp. 64-78)

Principles of Light Microscopy (pp. 65-70)

- The usefulness of a particular type of microscopy depends on the ability to produce a magnified image of a microorganism that (a) is large enough to be seen; (b) has not been distorted by the method of preparation or viewing; (c) has sufficient resolution to distinguish structures of interest; and (d) has sufficient contrast so that the organisms and the structures of interest can be distinguished from the surrounding background.
- The light microscope focuses light onto and through a specimen and produces a magnified image; images can be magnified 1,000 times and structures larger than 100 nm can be resolved.
- Magnification is useful only if detail can still be resolved. The resolving power of the microscope (the amount of detail that can be seen) determines the useful magnification.
- The resolving power of the light microscope depends on the wavelength of the illuminating light source and the numerical aperture of the objective lens. The shorter the wavelength of light and the higher the numerical aperture of the objective lens, the better the resolving power.
- Staining increases the contrast between a specimen and its background so it can be seen under the microscope.
- The Gram stain procedure is a widely used differential staining procedure that distinguishes between species that stain red-pink (Gram-negative bacteria) and species that stain blue-purple (Gram-positive bacteria).
- Other differential staining procedures include the acid-fast staining procedure and the endospore staining procedure.

Types of Light Microscopes (pp. 71-74)

- The bright-field microscope is most widely used in microbiology.
- Fluorescent microscopes are used to observe microorganisms that have been stained with a fluorescent dye.
- Certain immunofluorescent procedures, in which fluorescent dyes are coupled with specific antibodies, are widely used for the specific diagnosis of infectious diseases.
- The dark-field microscope relies on the reflection of light striking the specimen at an oblique angle.
- The phase contrast and interference microscopes enhance contrast without the need to stain the specimen.

Electron Microscopy (pp. 74-78)

- The short wavelength of an electron beam permits better resolution and hence higher useful magnifications using electron microscopes than can be achieved with the light microscope.
- There are two types of electron microscopes: the transmission electron microscope (TEM) and the scanning electron microscope (SEM).
- Both TEM and SEM require extensive procedures to prepare the specimen for viewing, while at the same time preventing the production of artifacts.
- The TEM is used to see finely detailed structures. The SEM is most useful for the observation of surface structures.

Pure Culture Methods (pp. 78-88)

- The scientific study of microorganisms requires the growth of large numbers of organisms in pure culture, where the metabolism and other characteristics of living microorganisms can be observed.
- Obtaining and maintaining pure cultures necessitates the elimination of all microorganisms from the growth medium (sterilization), the separation of the microorganism that is being cultured from a mixture of microorganisms (isolation), the movement of the microorganism from one place to another without contamination (aseptic transfer technique), and the growth of the microorganisms in the laboratory.
- Streak plate, spread plate, and pour plate methods are isolation techniques designed so that individual microbial cells are sufficiently separated on the surface of solidified agar so that when they reproduce they develop into well-isolated colonies.
- Colonies are macroscopic clumps of microbial cells.
- A culture medium contains the nutrients that are required by the microorganism for growth. Media used to grow microorganisms in the laboratory generally contain a source of carbon, nitrogen, phosphorus, iron, magnesium, sulfur, sodium, potassium, and chloride. Microorganisms require all of these for metabolism and reproduction.
- In some cases selective and/or differential media are employed to isolate specific microbial species. Such media are designed to suppress the growth of some microorganisms and to permit the recognition of others.

CHAPTER REVIEW

Review Questions

1. Compare and contrast inductive and deductive reasoning.
2. Describe the rise and fall of the theory of spontaneous generation.
3. What is the scientific method? How are hypotheses tested by scientists?
4. Describe the advantages of the bright-field microscope and what kinds of observations are made using it.
5. Define resolution. What is its importance to microscopy? How does wavelength affect resolution?
6. Why is staining usually done?
7. What is the difference between a simple staining procedure and a differential one?
8. Describe the steps in the Gram stain.
9. What advantages does the dark-field microscope provide?
10. What advantages does the phase-contrast microscope provide?
11. What advantages does the fluorescence microscope provide?
12. What is a pure culture? Why was the ability to isolate a pure culture important to the development of microbiology as a science?
13. How do clinical microbiologists culture bacteria? Why does it often take over a day to positively diagnose a disease?
14. What is a selective medium? How is such a medium used to help identify microorganisms in the case of a gastrointestinal tract infection?
15. What is a differential medium? How is such a medium used to help identify microorganisms in the case of a respiratory tract infection?

CRITICAL THINKING QUESTIONS

1. You are working in the clinical microbiology laboratory when a blood sample from a critically ill patient arrives. The physician wants to know as soon as possible whether there are any disease-causing microorganisms in the blood and, if so, the identity of the infecting agent. What would you do?
2. There is an outbreak of a mysterious illness in the southwestern United States. Several individuals who are native American Indians have already died. Someone proposes that the deaths are due to a viral infection. How would you go about proving or disproving that hypothesis? Assuming it was correct, how would you go about finding the source of the disease?
3. There is an outbreak of an illness in Philadelphia. Several individuals who attended a convention suddenly died and several others are ill and have been hospitalized. Someone proposes that the deaths are due to a terrorist attack that introduced a poison into the food that was served at a banquet. Another hypothesis is that a pathogenic bacterium has spread through the air-conditioning cooling system. How would you go about proving or disproving these hypotheses?
4. You are admitted to nursing school. You are able to attend all the scheduled classes but because you are also raising a family you are unable to attend all the laboratory sessions—particularly when they have extended open hours. You therefore decide to purchase a microscope so that you can examine specimens at home and not fall behind in your studies. How would you go about purchasing a microscope that would permit you to examine human tissue specimens and also prepared stained slides of microorganisms? What features would you consider most important in comparing various microscopes?

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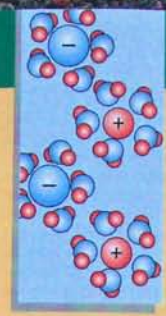

CHAPTER 4

Chemistry for the Microbiologist

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Preview to Chapter 4

In this chapter we will:

- Review some fundamental chemical principles essential for understanding microbial structure and function.
- Discuss the structure of the atom and its relationship to the chemical properties of elements.
- Examine how atoms form chemical compounds by exchanging or sharing electrons to establish chemical bonds that hold molecules together.
- See why water is essential for life and how carbon forms the backbone of the organic chemicals that comprise living systems.
- Examine the types of chemical reactions that occur between molecules that allow cells to obtain energy and materials for growth and reproduction.
- Discuss the essential role of enzymes in the chemical reactions that constitute the metabolism of cells.
- Examine the major types of chemicals that make up living systems and learn about their properties, which we later will relate to microbial structure and function.
- Learn the following key terms and names:

acid
activation energy
amino acids
anion
atom
ATP (adenosine triphosphate)
base
buffer
carbohydrates
cation
coenzyme
covalent bonds
deoxyribonucleic acid (DNA)
electrons
enzymes
functional groups
hydrogen bond
ionic bond
isomers
lipids
macromolecules
molecule
nucleic acids
nucleotides
oxidation
oxidation-reduction reactions
peptide bond
pH
phosphodiester bond
phospholipids
polymers
polypeptide chain
polysaccharides
reduction
ribonucleic acid (RNA)
substrate

ORGANIZATION OF MATTER

Early biological studies centered on the observation of living organisms—what they looked like, where they lived, what they ate. Naturalists cataloged the species of plants and animals in a region, recorded their distributions, and observed their appearances and behaviors. Early microbiologists continued in this tradition, looking at microorganisms and describing their morphologies and movements. Antonie van Leeuwenhoek, for example, recorded the shapes and movements of the "animalcules" he observed. Such microscopic observations revealed the existence of the living microbial world, but gave only limited insight into how microorganisms interact with their environment, or how they obtain the matter and energy needed to sustain life.

In the first half of the nineteenth century chemists developed a fundamental understanding of matter—the physical material of the universe. With the recognition that all matter in the universe has certain unifying chemical and physical properties and that living organisms are manifestations of their underlying chemical composition and the chemical reactions that they carry out, the fields of chemistry, physics, and biology began to be drawn together. Biologists soon recognized that to understand living organisms they had to investigate the chemistry of life. Microbiologists realized that the scientific understanding of the microorganisms, what they are and what they can do, necessitates the understanding of their underlying chemistry. So they incorporated chemistry as an integral part of the field of microbiology. To understand the chemistry of living systems, it is necessary to learn the "language" that chemists use for communicating information about chemicals. It is necessary to become conversant with the chemical terms and principles that are applied to the descriptions of microorganisms and their activities.

Chemical Elements

An **element** is the fundamental unit of a chemical that cannot be broken down further without destroying the properties of that pure chemical substance. There are 92 different naturally occurring elements—such as carbon, hydrogen, nitrogen, and oxygen. Chemists have assigned each element a **chemical symbol** that is a one- or two-letter abbreviation of its English or Latin name. The chemical symbol for the element hydrogen is H, oxygen is O, carbon is C, and so forth. The same chemical symbol is used regardless of the element's name in the language of the country in which it is being used. Thus, even though nitrogen is called *azoto* in Italy and *stickstoff* in Germany, its chemical symbol is always N. These symbols for the chemical elements form the "alphabet" of the language of chemistry. Biologists, generally, are only concerned with the 26 elements that form the major components of living systems. The most abundant elements in living systems are carbon (C), hydrogen (H), oxygen (O), nitrogen (N), phosphorus (P), sulfur (S), sodium (Na), potassium (K), magnesium (Mg), calcium (Ca), iron (Fe), and chlorine (Cl). Of these, carbon is the element that forms the backbone of all molecules that comprise living organisms.

Structure of Atoms

The smallest unit of an element that still retains the chemical properties of that element is called an **atom** (Greek, meaning that which cannot be cut). Atoms were thought to be the smallest particles into which matter could be divided. It was not until the twentieth century that physicists showed that atoms are composed of yet smaller subatomic particles. Chemists subsequently discovered that the number of an atom's constituent subatomic particles determines the characteristic properties of the atoms of different elements, such as their capacities to combine with other atoms.

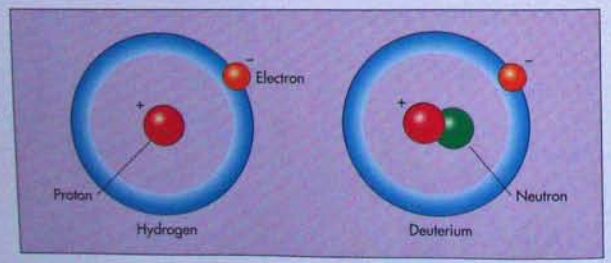


FIG. 4-1 Atoms are the fundamental units of chemical elements. They are composed of subatomic particles (negatively charged electrons, positively charged protons, and, with the exception of hydrogen, neutrally charged neutrons).

These subatomic particles of atoms carry a positive or a negative charge, or no charge (FIG. 4-1). Positively charged particles are called **protons,** uncharged neutral particles are called **neutrons,** and negatively charged particles are called **electrons.** The atom is organized with the protons and neutrons in a central region called the **nucleus.** The electrons move in regions of space around the nucleus.

The nucleus of an atom has a net positive charge because it contains positively charged protons. However, because the number of protons in each nucleus is equal to the number of electrons, the total positive charge of the nucleus's protons equals the total negative charge of the electrons. Therefore each atom has a net charge of zero. An atom is said to be neutral. As discussed later, the chemical properties of atoms, which allow them to participate in chemical reactions, depend on the number and arrangements of their electrons.

Atoms, which are the smallest units of elements, contain positively charged protons, uncharged neutrons, and negatively charged electrons; an atom has a net electronic charge of zero because it contains equal numbers of protons and electrons.

Ions

The number of electrons moving around the nucleus of an atom can increase or decrease. Some atoms have a tendency to lose one or more electrons. Other atoms tend to gain electrons. An atom that has lost or gained an electron is called an **ion.** It is no longer neutral. When a sodium (Na) atom loses an electron, it becomes a positively charged sodium ion (Na^+). Such a positively charged ion is called a **cation.** Other examples of cations are the potassium ion (K^+), magnesium ion (Mg^{2+}), and calcium ion (Ca^{2+}). Atoms of hydrogen can lose an electron and become a stable positive ion (H^+). The formation of hydrogen ions is very important because this is what causes acidity in the watery solutions that are an integral part of biological systems.

When a chlorine (Cl) atom gains an electron, it becomes a negatively charged chloride ion (Cl^-). Such a negatively charged ion is called an **anion.** Other examples of anions are the iodide ion (I^-) and sulfide ion (S^{2-}). Notice that the symbol for an ion is the chemical abbreviation followed by a superscript designating the ion's number of positive (+) or negative (−) charges.

An ion is an atom that has gained or lost one or more electrons.

Atoms that gain electrons form negatively charged ions (anions) and atoms that lose electrons form positively charged ions (cations).

Atomic Number and Weight

Chemists have assigned each element a unique atomic number. The **atomic number** of an element is determined by the number of protons in its nucleus. No two elements have the same number of protons. Therefore each chemical element has a different atomic number.

The atomic number of an atom of an element is the number of protons in the nucleus.

The **atomic weight** of an element is the total number of protons and neutrons in each atom of that element. Each proton and each neutron has one unit of atomic weight. Electrons contribute only negligibly to the weight of an atom. Therefore the atomic weight is calculated by adding only the numbers of protons and neutrons.

The atomic weight of an atom of an element is the sum of the numbers of protons and neutrons in its nucleus.

Isotopes

Isotopes of an element have varying numbers of neutrons. All isotopes of a given element have the same number of protons in their nuclei. Hence, they all have the same atomic number. Their atomic weights differ because they have different numbers of neutrons. The most abundant isotope of carbon (^{12}C), for example, has six protons and six neutrons. Another isotope of carbon (^{14}C) has six protons and eight neutrons.

Many isotopes are stable. They do not change spontaneously into other atomic forms. Some isotopes, though, have unstable combinations of pro-

NEWSBREAK

Beginning of Chemistry and the End of the Chemist Lavoisier

It was the eighteenth century when the science of chemistry emerged from the tradition of alchemy. Antoine Lavoisier introduced quantitative methods in chemistry by demonstrating that substances increase in weight during combustion. He began the use of chemical equations and distinguished between chemical elements and compounds. Lavoisier also explained the process of fermentation and respiration by showing the role of oxygen and carbon dioxide. Despite these brilliant accomplishments, Lavoisier was beheaded by the Revolutionaries during the French Revolution in 1794 because he was a part of the tax-collecting bureau of the monarchy.

tons and neutrons in their nuclei. Such isotopes are called **radioactive isotopes.** A radioactive isotope breaks down or decays by giving off subatomic particles and energy (radiation). For example, carbon-14 (^{14}C) is a radioisotope of carbon because it has too many neutrons in its nucleus to be stable. The instability within the nucleus of ^{14}C results in the breaking apart of a neutron. Energy and a beta particle (an electron formed by the decomposition of a neutron) are emitted from the nucleus. Radioactive isotopes, such as ^{14}C, are useful for labelling biological substances because beta particles can be easily detected. Caution must be used, however, whenever handling radioisotopes because the energy they give off can damage biological systems.

Atoms of the same element that have different numbers of neutrons are called isotopes.

Electron Arrangements and Chemical Reactivity

The protons and neutrons in the nucleus determine the atomic weight of an atom, but electrons of the atom determine its chemical properties. It is the electrons that actually participate in chemical reactions. Each element's atoms differ from those of all other elements in the number and arrangement of their electrons. Electrons move in regions of space around the nucleus.

The regions of space where electrons are likely to be found are called **shells.** Each electron shell represents an energy level. Shells closest to the nucleus have the lowest energy. Shells furthest from the nucleus have the greatest energy. Each shell has a maximal number of electrons that it can hold. The further away from the nucleus a shell is located, the greater the number of electrons that can occupy that shell (FIG. 4-2). The shell closest to the nucleus can hold only two electrons. Electrons first occupy the shells closest to the nucleus. Only after these shells are filled do electrons occupy the shells with higher energy levels.

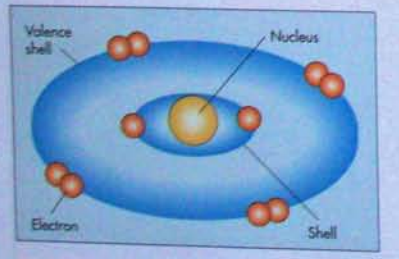


FIG. 4-2 The electrons of an atom are arranged in shells. Each shell represents a different energy level.

The outermost shell is called the **valence shell.** The number of electrons that can occupy the valence electron shell establishes in large part the capacity of that atom to combine with other atoms. The basic principle of atomic reactivity is that when its outermost electron shell is completely full an atom is stable. It will not react with other atoms. By interacting, atoms gain, lose, or share electrons to fill their outer shells. The outer electron shells of the atoms of the major elements in biological systems—hydrogen, carbon, nitrogen, oxygen, phosphorus, and sulfur—are all incomplete. The atoms of these elements, therefore, readily react with other atoms to achieve stable outer electron shells.

The fullness of the outer electron shell (valence shell) of an atom determines the capacity of that atom to combine with other atoms.

Atoms react with each other by losing, gaining, or sharing electrons to fill their outer electron shells.

Molecules and Chemical Bonds

When elements combine with each other they form compounds. A **compound** is a specific combination of elements in which the elements are present in a fixed and unvarying proportion. For example, water (H_2O) is a compound that has a fixed 2:1 proportion of two elements: hydrogen and oxygen. Because the proportion of elements in compounds never varies, they are distinct from **mixtures.** In a mixture two or more elements can be present in different and varying proportions. A mixture can be separated by physical means, such as filtering or sorting. A compound cannot be split into its component parts by such means.

A **molecule** is the simplest form of a compound that still retains the properties of that compound. A molecule is formed when atoms combine with each other. Chemists write **molecular formulas** to describe how many and which specific atoms form a molecule. For example, the molecular formula for water (H_2O) communicates the fact that this molecule is formed when two atoms of hydrogen and one atom of oxygen combine. Likewise the molecular formula for glucose ($C_6H_{12}O_6$) tells us that this sugar is formed by combining six carbon atoms, twelve hydrogen atoms, and six oxygen atoms. If the atoms of elements are the "letters" of the chemical alphabet, then the molecules of compounds are the "words" in the language of the chemist. Like atoms, molecules have specific physical properties, such as density. Molecules also have chemical properties, such as the ability to react with other molecules.

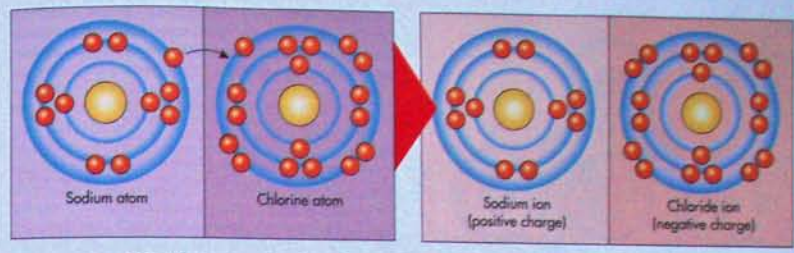


FIG. 4-3 The formation of ionic bonds involves the loss and gain of electrons. In the formation of NaCl, the chlorine atom gains an electron to fill its outer electron shell and the sodium atom loses an electron, so that all the remaining electron shells are filled. After the formation of the ionic bond, the sodium ion has a positive charge and the chloride ion a negative charge.

Chemical bonds are formed between atoms when atoms combine by transferring or sharing electrons. Stable bonds occur when atoms establish complete outer valence shells. The chemical bonds of a molecule hold together the constituent atoms that make up that molecule. The number of bonds that an atom can form depends on the number of electrons required by that atom to fill its outer electron shell. A carbon atom, for example, has four electrons in its outer electron shell that can hold a maximum of eight electrons. A carbon atom, therefore, can establish up to four bonds with other elements.

Stable chemical bonds occur when atoms fill their outer shells with electrons.

Molecules—the fundamental units of compounds—are specific combinations of atoms formed when atoms form chemical bonds. They form these bonds by sharing or transferring electrons.

A molecular formula specifies the numbers and kinds of atoms of elements that are bonded together to form a molecule of a compound.

Three types of chemical bonds can form between atoms. **Ionic bonds** are based on attractions of ions with opposite electronic charges. **Covalent bonds** are based on sharing of electrons. **Hydrogen bonds** are based on interactions of hydrogen atoms with weak opposing electronic charges. Each type of bond is important in establishing and determining the properties of the molecules that make up living systems.

Ionic Bonds

Two ions with different charges can be held together by the mutual attraction of these charges. These are called electrostatic forces. A chemical bond based on such electrostatic forces is called an **ionic bond.** This is the type of bonding that holds sodium and chloride ions together in table salt (NaCl) (FIG. 4-3). Similarly, positively charged hydrogen ions can form ionic bonds with negatively charged chloride ions to form hydrochloric acid. The atoms of certain other elements similarly can lose or gain electrons and thereby establish ionic bonds.

In an ionic bond, two ions with different charges are held together by the mutual attraction of the opposite charges of the two ions.

Ionic bonds readily dissociate (break apart) in water. This is because of the interactions with water molecules. Hydrochloric acid, for example, readily dissociates in water into H^+ and Cl^-. The concentration or relative amount of H^+ formed by such dissociation of acids is what determines the acidity of a solution.

Ionic bonds typically dissociate in water.

Covalent Bonds

Covalent bonds are formed when atoms are held together by sharing electrons. Many of the molecules in living systems are based on the ability of carbon atoms to form covalent bonds. The covalent bonds between carbon atoms is what holds together the molecules that make up the structures of all living systems. The outer shell of carbon contains four electrons and is completed by the addition of four more electrons. Carbon atoms can form four covalent bonds. A carbon atom, for example, can combine with four hydrogen atoms to form methane (CH_4) (FIG. 4-4). Methane is a simple **organic compound.**

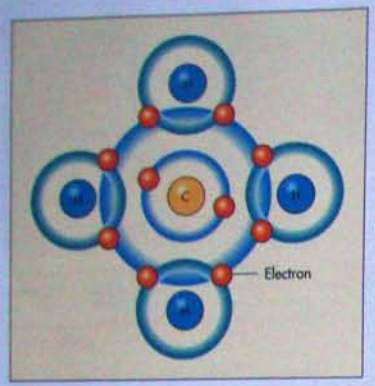


FIG. 4-4 The formation of the covalent bond involves the sharing of electrons. In methane, each hydrogen atom shares an electron with a carbon atom, completing the orbitals of the carbon and hydrogen atoms.

that is, a molecule that contains carbon and hydrogen. In the methane molecule, the carbon atom shares four electrons to complete its outer shell. It shares one electron with each of the four hydrogen atoms. A hydrogen atom has one electron and can hold two in its only shell. Each hydrogen atom completes its shell by sharing one electron from the carbon atom. A carbon atom can also form a covalent bond with another carbon atom, as well as with hydrogen atoms to form a chain of carbon atoms linked to each other. A chain of carbon atoms with hydrogen atoms attached to the carbon atoms is called a hydrocarbon. Similarly, carbon forms covalent bonds with other atoms to establish the large and complex molecules of living systems.

Stable covalent bonds are formed when atoms completely fill their outer electron shells as a result of sharing electrons with other atoms.

The number of covalent bonds that a particular atom can form depends on the number of electrons in its outer electron shell and the number of electrons needed to complete that shell.

Water is an essential molecule for life. When water (H_2O) forms from the elements hydrogen and oxygen, the outer electron shells in both elements reach a stable configuration (FIG. 4-5). The oxygen atom initially has six electrons in the outer electron shell that can hold eight electrons. It completes its outer shell by sharing two electrons—one with each of the two hydrogen atoms. The hydrogen atoms each share an electron with oxygen so that they completely fill their outer electron shells.

In most cases only one pair of electrons is shared to form a **single bond.** A covalent single bond is represented as a line (—). In some cases, atoms share two pairs of electrons. This gives rise to a **double bond,** which is expressed as two lines (═). Double bonds occur most frequently when carbon is double bonded to carbon (C═C) or when carbon is double bonded to oxygen (C═O). They are found in many biologically important molecules. Carbon dioxide (CO_2), for example, is the molecule from which plants, algae, and most photosynthetic bacteria obtain the carbon to build cellular structures. It contains two double bonds between carbon and oxygen (O═C═O).

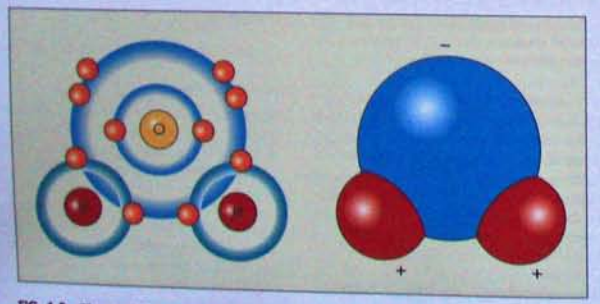


FIG. 4-5 The spatial arrangement of atoms in a water molecule results in a dipole moment because of unequal distribution of electrons between hydrogen and water. As a result, water is a good polar solvent because water can surround both positively and negatively charged ions.

Three pairs of electrons can form a **triple bond.** To form a triple bond, three electron pairs are shared between two atoms. A triple covalent bond is expressed as three single lines (═). Molecular nitrogen (N_2) is an example of a biologically important molecule with a triple bond (N═N) This triple bond structure is very stable and difficult to break. Although it constitutes 78% of the atmosphere, molecular nitrogen cannot be used by most organisms in their metabolism. A few bacterial species, however, are able to use molecular nitrogen. Such species are called nitrogen-fixing bacteria. They incorporate the nitrogen atoms from N_2 into proteins and other chemicals that make up their cellular structures. These nitrogen-fixing bacteria are extremely important because they form nitrogen-containing nutrients that can be used by other bacteria and higher organisms.

By sharing one, two, or three pairs of electrons, atoms can form single, double, and triple covalent bonds.

Covalent bonds are strong. A relatively large amount of energy is required to break them. Atoms held together by covalent bonds generally do not dissociate in water as do ionic bonds. The covalent bonds between carbon atoms are strong enough to form the backbones of the major molecules of living systems. The fact that carbon atoms can form four single covalent bonds is important. This allows carbon to form backbone chains of covalently bonded carbon molecules. It also allows carbon to bond with other atoms, such as hydrogen, oxygen, or nitrogen. Covalent carbon-carbon bonds provide much of the stability needed to establish the very large molecules essential to the operation and reproduction of microorganisms and other living organisms. Such large molecules are called **macromolecules** and include DNA and proteins.

Biochemists have concluded that of all the naturally occurring elements only carbon atoms can form the bonds that will hold together the large molecules of living systems.

Hydrogen Bonds

When hydrogen forms a covalent bond with atoms of oxygen or nitrogen, the relatively large positive nucleus of these larger atoms attracts the hydrogen electron more strongly than the single hydrogen proton. This establishes **polarity** within the molecule. This means that one end of the molecule has a positive charge and the other a negative charge. The positively charged end of the molecule is the end with the hydrogen atoms. The positively charged end can be attracted to the negatively charged end of another molecule. In this way a **hydrogen bond** is formed. When molecules of water (H_2O), for example, come close to each other, a hydrogen atom of one of the water molecules is attracted to the negatively charged oxygen atoms of another (FIG. 4-6). The result is a lattice of water molecules that are held together by these hydrogen bonds established by charge interactions. Such hydrogen bonds do not link atoms together as strongly as do covalent bonds.

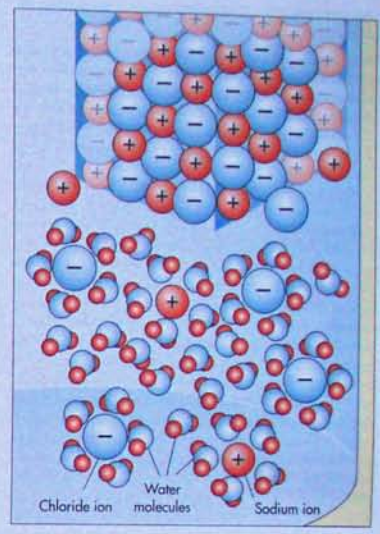


FIG. 4-6 Water forms hydrogen bonds with polar compounds. This allows water to act as a solvent. Most polar substances readily dissolve in water.

A hydrogen bond is formed when a hydrogen atom that is covalently bonded to an oxygen or a nitrogen atom is attracted to a polar atom in another molecule.

A hydrogen bond has only about 5% of the strength of a covalent bond. Although such hydrogen bonds are weak, they are important because they hold different molecules together. They help establish the three-dimensional structures of large molecules by forming weak bonds between atoms with long chains of covalently bonded atoms. They also establish important chemical properties of various molecules. For example, the capacity of water to dissolve many substances is due to water's polarity and its capacity to form hydrogen bonds with ions and polar molecules. Hydrogen bonds are also important in the formation of helical molecules.

Hydrogen bonds are relatively weak bonds that help establish important properties of molecules.

Isomers and the Three-dimensional Structures of Molecules

Molecules that contain identical types and numbers of atoms, but that have different arrangements of those atoms, are called **isomers.** The isomers of a molecule can have very different properties because the ability of each isomer to interact with other chemicals depends in part on the precise position of its constituent atoms in three-dimensional space. For example, glucose and fructose—both of which have the molecular formula $C_6H_{12}O_6$—are isomers that have different chemical properties (FIG. 4-7). Some bacteria can use glucose but not fructose as a source of energy for their metabolism. Other isomers can be distinguished based on how they rotate light: those rotating light to the left are called L-isomers and those rotating light to the right are called D-isomers (L stands for levorotary [left turning] and D for dextrorotary [right turning]). The amino acids that make up proteins are all L-amino acids; D-amino acids occur only in rare and special molecules of living organisms, such as the cell walls of bacteria.

Molecules with different arrangements of the same atoms (isomers) often have different chemical and physical properties.

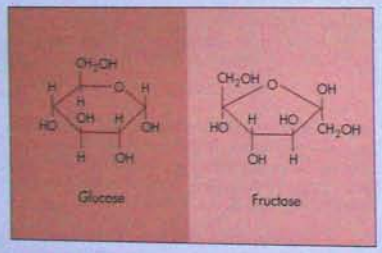


FIG. 4-7 The sugars glucose and fructose are isomers. They have the same elemental composition but their atoms are arranged differently.

Functional Groups

When certain atoms are bound together and behave chemically as a unit that is part of a larger molecule, they are called a functional group. **Functional groups** act in the same way regardless of the molecules in which they occur (FIG. 4-8). Functional groups determine some of the characteristics, such as solubility, and chemical reactivity of the molecules to which they are attached.

Bonding of specific atoms that behave as a unit form a functional group within a molecule.

Functional groups have specific chemical properties, even when they are attached to very different molecules.

The bonding of an oxygen atom to a hydrogen atom forms a polar **hydroxyl (-OH) functional group.** The hydroxyl group has polarity because shared electrons of a covalent bond are drawn closer to the oxygen atom than to the hydrogen atom. This gives the hydroxyl group a slightly negative oxygen atom and a slightly positive hydrogen atom. Alcohols such as ethanol and glycerol are organic molecules that have hydroxyl functional groups. They tend to be relatively soluble in water. Alcohols dissolve in water because of the hydrogen bonding between water molecules and the hydroxyl group of the alcohol.

The bonding of a nitrogen atom with two hydrogen atoms forms a polar functional group, called an **amino ($-NH_2$) functional group.** The bonding of carbon with oxygen and a hydroxyl group forms a polar **carboxyl (-COOH) functional group.** Amino acids, which are the building blocks of proteins, have both amino and carboxyl functional groups.

Functional groups are important because they permit molecules to react with each other in predictable ways. In some cases, reactions between functional groups form bonds that link molecules together. This is how small molecules can be joined to form large molecules. Proteins, for example, are large molecules that are assembled by linking smaller amino acid molecules. Other functional groups have characteristic properties and engage in chemical reactions that are essential for sustaining microorganisms and other living systems.

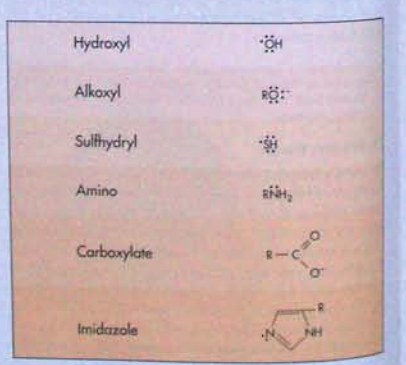


FIG. 4-8 The chemical characteristics of a substance are determined in large part by its functional groups.

CHEMICAL REACTIONS

So far we have considered molecules as if they were fixed structures that remain stationary and do not change. In reality, molecules are in constant motion. They possess **kinetic energy,** the energy of motion. The faster molecules move, the more kinetic energy they have. When moving molecules collide, there may be sufficient kinetic energy to break bonds apart. When this occurs, the atoms can form new bonds. This permits new arrangements of molecules to form. During each chemical reaction, existing chemical bonds are broken and new chemical bonds form to yield different molecules. When molecules react and form new molecules, the combinations of atoms get rearranged. The total kinds and number of atoms always remain unchanged. Atoms can be neither formed nor destroyed in any chemical reaction. This **conservation of matter** is a fundamental law governing the universe.

Kinetic energy, the energy of motion, is used to break chemical bonds.

The law of conservation of matter states that atoms cannot be created or destroyed in chemical reactions.

Chemical Equations

The conservation of matter always applies to all chemical reactions, including the chemical reactions occurring in living systems. There must be a balance between what goes into a chemical reaction, the **reactants,** and what is produced by that reaction, the **products.** The **chemical equation** describes the relationship between the reactants and products. The reactants are shown on the left side and the products are shown on the right side of the equation. If elements are the "letters" of chemistry and molecules are the "words," then chemical equations are the "sentences" in the language of chemistry. A chemical equation identifies the reactants and products by name or chemical formula. It permits chemists to describe the changes that occur during chemical reactions.

In a **balanced chemical equation,** the number of atoms of each element in the reactant molecules must equal the number of product molecules of that element. For example, the equation for the reaction of sodium chloride (NaCl) in water to form sodium (Na^+) and chloride (Cl^-) ions is written:

$$NaCl \longrightarrow Na^+ + Cl^-$$

The numbers of sodium and chlorine atoms on both sides of the equation are the same. The equation is properly balanced. Water is required for this reaction to occur. It is not shown in the equation because it is not changed or transformed in the process of the reaction. When a substance acts as a solvent and does not participate directly in the reaction, it is not shown within the equation. Sometimes, however, the solvent is indicated above the arrow to show that its presence is necessary for the reaction to occur.

$$NaCl \xrightarrow{water} Na^+ + Cl^-$$

Chemical equations show the changes that occur during a chemical reaction.

The chemical equation shows the balance between reactants and products.

Equilibrium

Virtually all chemical reactions are reversible. In a reversible reaction, the reactants can become the products and the products the reactants. The direction of the reaction depends in part on the concentrations of reactants and products. The likelihood of molecules colliding with sufficient kinetic energy to break bonds depends on the relative abundances of molecules with sufficient kinetic energies to react. Concentrations of reactants and products are expressed in units called moles. A **mole** is a measure of the number of molecules (6×10^{23} molecules). A mole is equal to the weight in grams of the molecular weight of a molecule. Thus, 1 mole of water weighs 18 grams because the molecular weight of water is 18, the sum of the molecular weights of two ^1H atoms and one ^{16}O atom.

The greater the concentration of reactants, the greater the opportunity for collisions to occur and, hence, the faster the forward reaction. The greater the concentration of products, the faster the reverse reaction. As more and more product molecules form, fewer and fewer reactant molecules remain. This lowers the rate of the forward reaction. As the concentration of product molecules increases, they will collide more frequently with each other than before. In some cases the reaction is reversed so that the original reactants are reformed. Eventually, a balance—called **equilibrium**—is achieved between the reactants and products of the forward and reverse reactions. At equilibrium the rates of the forward and reverse reactions are equal. This does not mean that the amounts of the products and reactants are equal. In this state there is no net change in the concentrations of reactants and products even though the molecular reactions are still continuing.

Chemical reactions are reversible and chemical reactions tend toward a state of equilibrium.

At equilibrium, the rates of the forward and the reverse reactions are equal and there is no further net change in the concentrations of reactants and products.

Energy and Chemical Reactions

There is only a finite amount of energy in the universe. This energy cannot be created or destroyed. Energy, however, can be converted from one form to another. The various forms of energy include the chemical energy stored in molecular bonds (*potential* or *stored energy*), kinetic energy (energy of motion), electrical energy (energy produced by movement of electrons), and radiant energy (heat or light energy) from the sun. During chemical reactions chemical bonds are broken and new bonds are formed. Energy is transformed during these reactions. In a chemical reaction there always is a net balance between the energy required to break chemical bonds, the energy released by the new bonds that are formed, and the energy—such as heat energy—that is exchanged with the surroundings.

Energy is neither created nor destroyed in chemical reactions; however, energy can be converted from one form to another.

The products of chemical reactions can end up with either less or more energy than the reactants had. Some chemical reactions release energy. Others require the input of energy. Energy-requiring reactions can occur only when extra energy enters into the reaction. The extra energy must come from some other system.

Chemical reactions involve energy changes.

Most often the energy needed to drive energy-requiring chemical reactions in living systems is supplied by **ATP (adenosine triphosphate).** ATP is called an energy-rich or high-energy compound. It contains chemical bonds that can release a relatively large amount of energy (FIG. 4-9). The release of this energy from the ATP molecule can be coupled to energy-requiring reactions. In this fashion, energy-releasing reactions drive the energy-requiring reactions of cell growth, movement, and transport. ATP serves almost universally in biological systems as the energy source for energy-requiring reactions. Cellular processes requiring energy most likely depend on the use of ATP. As such, ATP can be termed the *universal currency of energy* in biological systems.

ATP has a central role in the flow of energy through living systems.

Types of Chemical Reactions

Enzymatic Reactions

For a chemical reaction to occur, the reactant molecules must collide with sufficient kinetic energy to bring about the reaction. The amount of energy needed is called the **activation energy.** The activation energy is the amount of energy needed to start the reaction. It is not the amount consumed or released by the breaking and forming of chemical bonds. A chemical reaction can occur only when the energy of activation is provided to start the reaction. Chemists often heat chemicals with a Bunsen burner to provide the energy needed for chemical reactions to occur. But cells operate at nearly constant temperatures. Cells employ other methods to overcome the energy barrier to starting a chemical reaction presented by the activation energy.

Biological systems depend on enzymes to lower the activation energy of a chemical reaction (FIG. 4-10). **Enzymes** are proteins that act as biological catalysts; some RNA molecules, called ribozymes, can similarly act as biological catalysts. A catalyst speeds

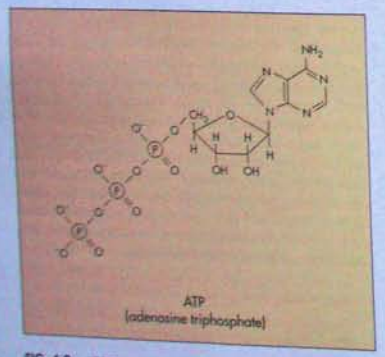


FIG. 4-9 ATP is a compound with high-energy phosphate bonds.

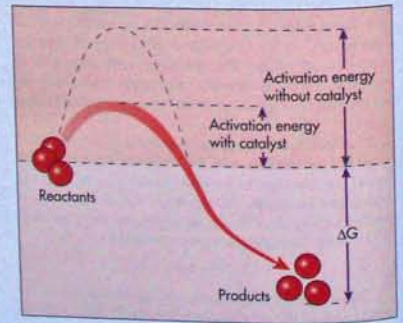


FIG. 4-10 An input of energy called the activation energy is needed to start a chemical reaction. A catalyst lowers the activation energy. In biological systems, enzymes serve as the catalysts to lower the activation energy.

NEWSBREAK

Catalysis by Cell-Free Enzymes

Jons Berzelius discovered the principle of catalysis, based on the recognition that certain substances have a catalytic force that enables them to decompose other compounds and rearrange the decomposed products without being chemically altered in the process. The German chemist Eduard Buchner received a Nobel Prize in 1907 for his demonstration of an alcoholic fermention without yeast cells. He showed that sugar could be changed into alcohol by soluble proteins from yeasts. He called the active proteins "zymase," from which we derive the term enzyme. Today many cell free enzymes are used, for example, in detergents to help remove proteins and in the production of high-fructose corn syrup sweetener for soft drinks.

up a reaction without being consumed in that reaction. At a given temperature, a catalyzed reaction proceeds more rapidly than an uncatalyzed reaction. Because catalysts are not consumed in the reaction, enzymes theoretically may continue to function indefinitely. Since they can be reused, only small amounts of enzymes are often required.

Without enzymes, chemical reactions would not occur fast enough within a cell to support life functions. The rapid rates at which chemical reactions occur in living systems are possible because of the role played by enzymes in lowering the activation energy. Each microbial cell must possess many enzymes, thousands in fact, to carry out the essential metabolic activities involved in its growth and reproduction.

An enzyme works by binding with a molecule called the **substrate.** An enzyme can bond only to a specific substrate. The degree of substrate specificity exhibited by enzymes reflects the fact that the enzyme and the substrate must fit together in a specific way (FIG. 4-11). The precision of fit between enzyme

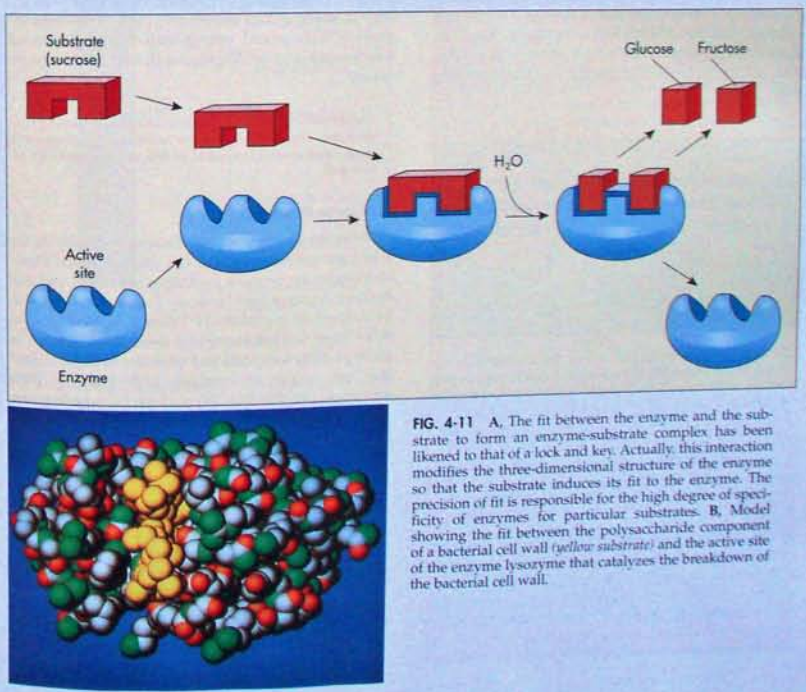


FIG. 4-11 **A,** The fit between the enzyme and the substrate to form an enzyme-substrate complex has been likened to that of a lock and key. Actually, this interaction modifies the three-dimensional structure of the enzyme so that the substrate induces its fit to the enzyme. The precision of fit is responsible for the high degree of specificity of enzymes for particular substrates. **B,** Model showing the fit between the polysaccharide component of a bacterial cell wall (*yellow substrate*) and the active site of the enzyme lysozyme that catalyzes the breakdown of the bacterial cell wall.

and substrate molecules permits the establishment of exactly the right spatial orientation so that the numerous chemical reactions of an organism can occur with greater speed.

Enzymes are proteins that act as biological catalysts.

Enzymes are highly specific, both in terms of their substrates and the reactions they catalyze.

Oxidation-reduction Reactions

Oxidation-reduction reactions are based on the transfers of electrons between molecules (FIG. 4-12). **Oxidation** is the process of removing one or more electrons from an atom or molecule. **Reduction** is the process of adding one or more electrons to an atom or molecule. Oxidation and reduction are coupled because they involve the simultaneous removal of an electron from one substance and the addition of that electron to another. Often in biological systems a proton or hydrogen ion (H^+) is transferred with the electron during oxidation-reduction reactions. Oxidation reactions that involve the removal of an electron and hydrogen ion are called *dehydrogenation reactions*. Reduction reactions that involve the addition of an electron and hydrogen ion are called *hydrogenation reactions*.

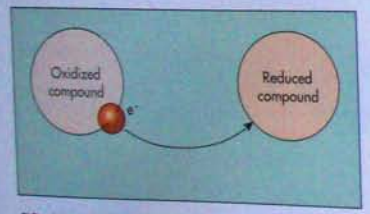


FIG. 4-12 Oxidation-reduction reactions involve an exchange of electrons. The substance that accepts an electron becomes reduced and the substance that donates an electron becomes oxidized.

An example of biological oxidation-reduction reactions is shown in the following equation:

$$X_{reduced} + NAD^+ \longrightarrow X_{oxidized} + NADH + H^+$$

In this example, molecule X serves as the source of two electrons and two protons that are transferred to a molecule of nicotinamide adenine dinucleotide (NAD^+). Molecule X becomes oxidized (two electrons are removed) and NAD^+ becomes reduced (two electrons are added). At the same time, two protons are removed from molecule X. One of these is added to NAD^+ (so that the reduced form of NAD is written as NADH) and the other proton (H^+) is released into the medium.

Oxidation is the removal of electrons and reduction is the addition of electrons.

The oxidation of one substance must always be coupled with the reduction of another.

Oxidation-reduction reactions are important in the metabolism of cells for energy changes and the formation of new cellular material. Oxidation-reduction reactions can release energy that is used to form ATP. For example, during cellular respiration the oxidation of glucose to form carbon dioxide is coupled with the reduction of oxygen to form water. This provides the energy to drive ATP production. Living organisms use the energy released from such oxidation-reduction reactions for growth, reproduction, and other life processes—such as movement.

Oxidation-reduction reactions also are used by living systems to store energy. Thus, when carbon dioxide is reduced to glucose during photosynthesis, energy is stored within the organic molecules of the organism. This stored energy can later be released when organisms oxidize sugars during cellular respiration.

Oxidation-reduction reactions can release energy and are used to fuel cellular reactions, to store energy, and for biosynthesis of the macromolecules of the cell.

Acid-base Reactions

Another important type of chemical reaction is the acid-base reaction. An **acid** is a substance that dissociates into one or more hydrogen ions (H^+) and one or more negative ions (anions). Thus an acid can also be defined as a proton (H^+) donor. A **base,** on the other hand, is a substance that dissociates into one or more positive ions (cations), plus one or more anions that can accept or combine with protons. Thus sodium hydroxide (NaOH) is a base because in water it dissociates to release hydroxyl ions (OH^-), which have a strong attraction for protons. Bases that produce hydroxyl ions are among the most important proton acceptors.

Acids increase the concentration of hydrogen ions in solution.

Bases decrease the concentration of hydrogen ions in solution.

The amount of H^+ in a solution is expressed by a logarithmic pH scale that ranges from 0 to 14 (FIG. 4-13). The **pH** of a solution is the negative logarithm to the base 10 of the hydrogen ion concentration.

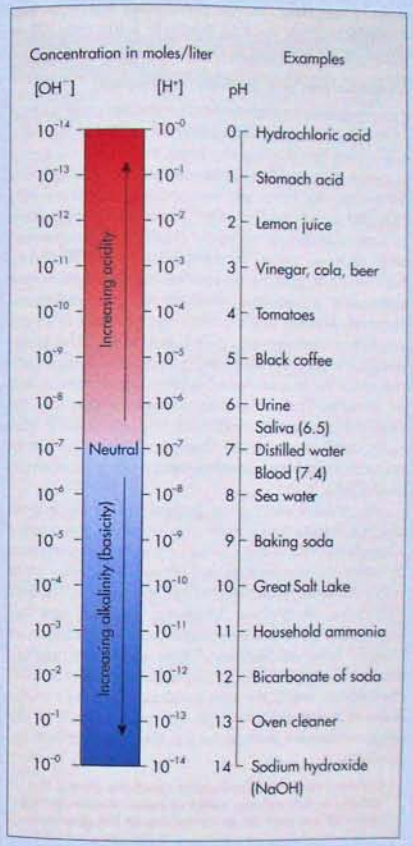


FIG. 4-13 The pH scale showing pH values of some common substances.

$$pH = -\log[H^+]$$

The greater the hydrogen ion concentration the lower the pH. Because the pH scale is logarithmic, a change of one whole pH unit represents a tenfold change from the previous concentration of hydrogen ions. Thus a solution with a pH 1 has 10 times more H^+ ions than one with a pH 2 and 100 times more H^+ ions than a solution with a pH 3. Acidic solutions contain more H^+ ions than OH^- ions and

METHODOLOGY

Measuring pH

It often is critical to determine the pH of a solution. Microorganisms grow only within certain pH ranges. Adjusting the pH of a growth medium is essential to provide a favorable condition for microbial growth. Adjusting or maintaining the pH of many foods so as to ensure unfavorable conditions for microbial growth is used as a food preservation method. *Clostridium botulinum*, the bacterium that causes a fatal form of food poisoning called botulism, for example, will not grow at low pH (acidic conditions). Acidic foods such as red tomatoes are unsuitable for the growth of this deadly bacterium. Other foods such as low-acid yellow tomatoes provide favorable conditions for growth of *C. botulinum* when they are preserved by canning. Therefore higher temperatures are used when canning yellow tomatoes to protect against botulism.

Several methods are used to determine pH. The pH can be determined electronically using a pH meter. The probe of a pH meter is placed into a solution and the instrument responds to the hydrogen ion concentration by registering the pH. pH meters are simple to use and rapidly provide information on the pH of a solution. Various chemical pH indicators are also used to determine pH (see Table). These indicators change color at different pH values. A drop of a solution can be added to the pH indicator and the color reveals the approximate pH.

Color Reactions of Some pH Indicators

INDICATOR	pH RANGE	COLOR	
		Acid	Base
Cresol Red	1.0-2.2	Red	Yellow
Thymol Blue	1.2-2.8	Red	Yellow
Bromphenol Blue	3.0-4.8	Yellow	Blue
Methyl Orange	3.1-4.4	Red	Yellow
Bromcresol Green	4.0-5.6	Yellow	Blue
Methyl Red	4.4-6.2	Red	Yellow
Bromcresol Purple	5.2-6.8	Yellow	Purple
Bromthymol Blue	6.0-7.6	Yellow	Blue
Phenol Red	6.4-8.0	Yellow	Red
Phenol-phthalein	8.0-10.0	Colorless	Red
Thymol-phthalein	9.4-10.6	Colorless	Blue
Alizarin Yellow	10.0-12.1	Yellow	Red

have a pH lower than 7. Basic or alkaline solutions have more OH^- ions than H^+ ions and a pH higher than 7. In pure water the concentrations of H^+ and OH^- are equal and the pH is 7. This pH level is called **neutral.**

pH describes the concentration of hydrogen ions.

Acidic solutions have pH values less than 7; basic solutions have pH values greater than 7; water is neutral (pH = 7).

When an acid reacts with a base, there is a reaction between the hydrogen ions produced by the acid and the hydroxyl ions produced by the base. Acid-base reactions result in the formation of water and a salt. For example, when hydrochloric acid reacts with sodium hydroxide, the products are sodium chloride and water.

$$HCl + NaOH \longrightarrow NaCl + H_2O$$

$$H^+ + Cl^- + Na^+ + OH^- \longrightarrow Na^+ + Cl^- + H_2O$$

If the amounts of acid and base are balanced, all the free hydrogen ions react with all the free hydroxyl ions. This is known as a **neutralization reaction** because it results in a neutral solution of the salt. The hydrogen ion and hydroxyl ion concentrations are balanced and thus achieve a neutral pH of 7.

As living organisms take up nutrients, carry out chemical reactions, and excrete wastes, they may change the balance of acids and bases. This change may occur both within their cells and in the surrounding solution. When bacteria are grown in laboratory medium, for example, some of the chemicals produced by their metabolism are acids that can alter the pH of the medium. Unchecked, the pH of the medium would become too acidic for the bacteria to live. To prevent this, microbiologists add pH buffers to the culture medium. A **buffer** limits the change of pH by reacting with acids or bases to form neutral salts. Phosphate buffer containing K_2HPO_4 and KH_2PO_4 is often used to maintain a pH near 7.0 in culture media.

Condensation and Hydrolysis Reactions

Condensation reactions involve the bonding of two molecules into one. Condensation reactions are very important in forming the large molecules of living systems. In a **condensation reaction,** a hydrogen ion (H^+) removed from one functional group of a molecule and a hydroxyl ion (OH^-) from another combine to form a molecule of water (H_2O). The component molecules are joined by a covalent bond (FIG. 4-14). For example, glucose molecules combine into larger molecules containing multiple glucose subunits. Large molecules formed from the bonding of many subunit molecules are called **polymers.** The polymers produced by condensation reactions may contain millions of individual subunit molecules, called **monomers.** These monomers may or may not be identical. As a rule polymers are less soluble and more stable (long-lived) than monomers. Polymers are important components of many biological structures (Table 4-1).

The reverse reaction is **hydrolysis.** A hydrolytic reaction breaks down polymers into their component monomers (FIG. 4-15). Covalent bonds between parts of molecules are broken and H^+ and OH^- ions from water become attached to the component subunit molecules. Hydrolysis reactions, such as the hydrolysis of ATP, are important for the extraction of energy from molecules. They yield the energy needed to support energy-requiring reactions in cells. Hydrolysis reactions also produce the small molecules that are used by cells for the synthesis of the large molecules that make up the structures of organisms.

Condensation and hydrolysis reactions permit the linking and breaking apart of molecules, including some of the very large molecules of living systems.

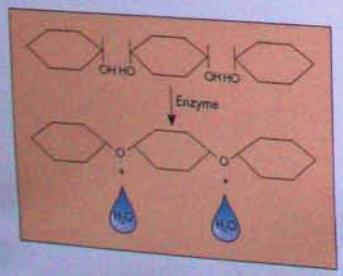


FIG. 4-14 Polymers are formed when smaller chemical units join together in a condensation reaction.

TABLE 4-1
Chemical Compositions of Some Biologically Important Polymers

POLYMER	MONOMER SUBUNITS	BIOLOGICAL FUNCTION
Polysaccharides	Monosaccharides	Structural components that support and protect cells; nutrient storage within cells
Cellulose	Glucose	Structural component of cell walls of algal and plant cells; protection of cell
Starch	Glucose	Storage of carbon as nutrient source within some algal and plant cells
Glycogen	Glucose	Storage of carbon as nutrient source within some protozoan and animal cells
Chitin	*N*-acetylglucosamine	Structural component of cell walls of many fungal cells; protection of cell; structural component of skeletons or shells of some animals
Peptidoglycan	*N*-acetylglucosamine and *N*-acetylmuramic acid	Structural component of cell walls of most bacterial cells; protection of cell
Nucleic Acids	Nucleotides	Storage, transmission, and utilization of cellular hereditary information
DNA	Deoxyribonucleotides	Storage and transmission of hereditary information from one generation to the next in living cells and some viruses
RNA	Ribonucleotides	Structural component of ribosomes; transfer of genetic information from DNA for use in directing protein synthesis in living cells; hereditary informational molecule in some viruses
Proteins	L-Amino acids	Structural component of almost all cell structures; enzymes that catalyze cellular chemical reactions; transport of most chemicals into and out of cells; protective coat of viruses

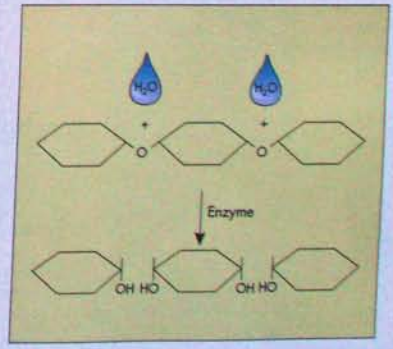


FIG. 4-15 Large molecules are broken down into smaller molecules in hydrolysis reactions.

MOLECULES OF LIVING SYSTEMS

A common feature of all living systems is that they are based on carbon atoms. Organic molecules that contain carbon form the essential components of all living organisms. Carbon, hydrogen, oxygen, and nitrogen atoms comprise 99% of the mass of living organisms. Carbon atoms are able to establish strong bonds with these atoms, as well as with other carbon atoms. Therefore carbon is well suited for uniting the atoms of living systems into stable macromolecules. Microorganisms are composed of various organic macromolecules representing four major classes of chemicals: carbohydrates, lipids, proteins, and nucleic acids. In addition, microorganisms are also composed largely of water. All living systems depend on the availability of water and various other inorganic molecules, such as carbon dioxide and phosphate.

WATER

Of the inorganic compounds of living systems, water is without doubt the most abundant and important. Life cannot exist in the absence of water. Water usually comprises over 75% of the weight of a living cell. Water serves as a solvent that permits the dissociation of chemicals, allowing chemical reactions of many molecules to occur within living cells that produce numerous new combinations of molecules.

Water's structural and chemical properties make it particularly suitable for living cells (FIG. 4-16). The hydrogen (H^+) and hydroxyl (OH^-) portions of water (HOH) can split apart and later rejoin. This enables water to participate as a reactant or a product in many chemical reactions. Water molecules, for example, are involved in many chemical reactions as an important source of the hydrogen and oxygen atoms that are incorporated into the numerous organic compounds that make up living cells.

Because the oxygen region of the water molecule has a slightly negative charge and the hydrogen region has a slightly positive charge, water has a **polar** nature. The polarity of water means that many charged or polar substances dissolve in water by dissociating into individual molecules. Molecules dissolved in water are called **solutes.** The negatively charged part of the water molecule is attracted to the positively charged part of the solute molecule. At the same time the positively charged part of the water molecule is attracted to the negatively charged part of the solute molecule. Solid NaCl, for example, dissolves in water by dissociating into the positively charged sodium ions (Na^+) and chloride ions (Cl^-). The positive sodium ions are attracted to the negatively charged oxygen atom of water. The negative chloride ions are attracted to the positively charged hydrogen atoms of water. Thus the Na^+ and Cl^- ions of solid NaCl are separated by the water molecule and table salt dissolves in water.

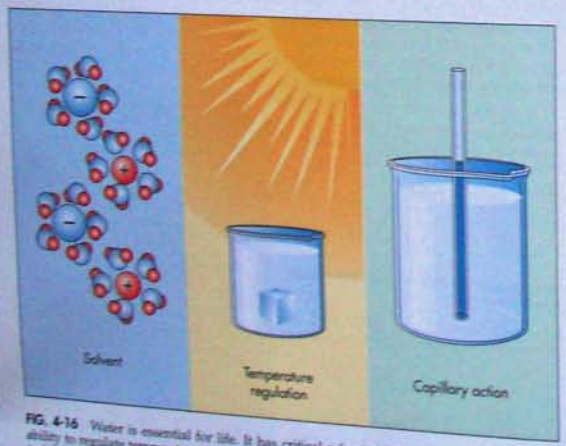


FIG. 4-16 Water is essential for life. It has critical roles in life's functions, including its ability to regulate temperature and to act as a solvent, that are based largely on its ability to form hydrogen bonds.

This polarity of the water molecule also means that hydrogen bonds are formed between nearby water molecules. The hydrogen bonds between water molecules make water an excellent temperature regulator. Cells are mostly water and live surrounded by water. Water readily maintains a constant temperature and tends to protect cells from sudden environmental temperature changes. Also, a great deal of heat energy is required to separate water molecules—held together by hydrogen bonds—from each other to form water vapor, that is, to convert liquid water into gaseous steam. Water exists in the liquid state at temperatures of 0° to 100° C. Liquid water is available on most of the Earth's surface and readily available for use as a solvent.

CARBOHYDRATES

Carbohydrates are a large and diverse group of organic compounds. This group includes sugars and compounds such as starch that are derived from sugars. Each sugar molecule has a fixed ratio of carbon:hydrogen:oxygen of 1:2:1. Therefore, all **carbohydrates** have the same basic chemical formula—$C_n(H_2O)_n$, where n is a whole number equal to or greater than 3.

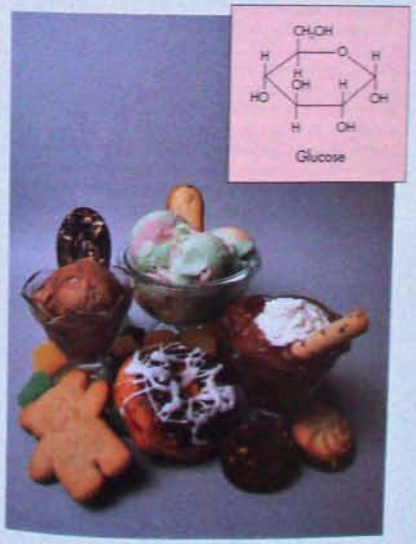


FIG. 4-17 A monosaccharide is the fundamental chemical unit of carbohydrates.

Carbohydrates include the **monosaccharides** (saccharide is the Greek word for sugar). Monosaccharides are simple sugars with three to seven carbon atoms (FIG. 4-17). Monosaccharides may be linked to form larger carbohydrate molecules. A **disaccharide** contains two monosaccharide units, an **oligosaccharide** contains three to ten monosaccharide units, and a **polysaccharide** contains more than ten monosaccharide units. Monosaccharides with five or more carbon atoms tend to form ring structures when dissolved in water. Thus, within cells, **pentoses**, which have five carbon atoms, and **hexoses**, which have six carbon atoms, form molecules with ring structures. Pentoses and hexoses are biologically significant. They both serve as energy sources and as the structural backbones of large informational molecules. Deoxyribose is a pentose found in deoxyribonucleic acid (DNA), the genetic material of the cell. Ribose, another pentose, is found in ribonucleic acid (RNA). RNA is the molecule used to transfer genetic information within cells for the expression of genetic information. Glucose is a common hexose and the main energy-supplying molecule of living cells.

Disaccharides are formed when two monosaccharides join in a condensation reaction (FIG. 4-18). For example, molecules of two monosaccharides, glucose and fructose, combine to form a molecule of the disaccharide sucrose (table sugar). Sucrose is the form in which carbohydrates are transported through plants. The disaccharide lactose is formed by the bonding of one glucose and one galactose subunit. Lactose occurs in the milk of mammals. The bond linking the monosaccharides in these disaccharides is a type of covalent bond known as a glycosidic bond. In a **glycosidic bond** an oxygen atom forms a bridge between two carbon atoms.

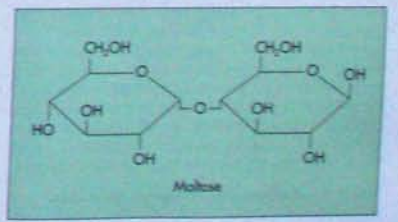


FIG. 4-18 A disaccharide is composed of two monosaccharides.

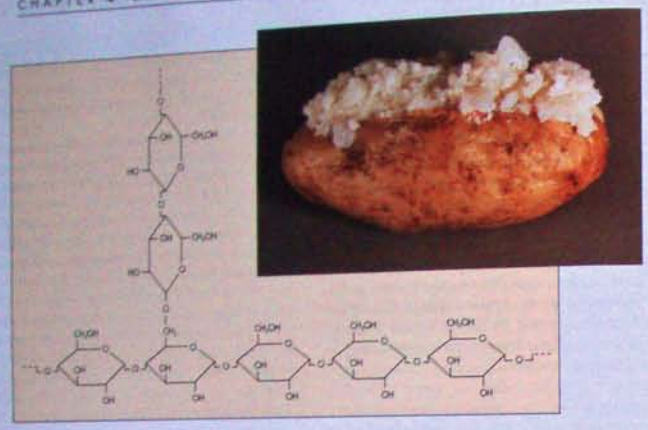


FIG. 4-19 A polysaccharide is composed of numerous monosaccharides.

Many monosaccharide units can likewise be linked to form **polysaccharides** (FIG. 4-19). Polysaccharides, like starch and glycogen, are composed of many units of glucose that are linked together. They function as important carbon and energy reserves in bacteria, plants, and animals. Other polysaccharides, such as cellulose, function as structural supports as in the cell walls of algal and plant cells.

Carbohydrates serve as sources of energy for cells and make up key structures, such as the walls that surround some cells.

Lipids

Like carbohydrates, **lipids** are organic compounds composed of atoms of carbon, hydrogen, and oxygen. Lipids, however, are mostly made up of carbon and hydrogen. They have very little oxygen compared to carbohydrates. Therefore lipids are nonpolar and **hydrophobic.** Being hydrophobic means that they do not readily dissolve in water. Although most lipids are insoluble in water, they dissolve readily in nonpolar solvents such as ether, chloroform, and alcohol. Some lipids function in the storage and transport of energy. Others are key components of membranes, protective coats, and other structures of cells.

Lipids are Hydrophobic Nonpolar Molecules

Many lipids have fatty acid components (FIG. 4-20). A **fatty acid molecule** consists of a carboxyl (–COOH) functional group attached to the end of a long hydrocarbon chain composed only of carbon and hydrogen atoms. Thus fatty acids contain a highly hydrophobic hydrocarbon chain, usually 16 to 18 carbon atoms long, and a carboxyl functional group that is highly hydrophilic. Being hydrophilic means that it is attracted to water molecules. This gives fatty acids interesting chemical properties, such as the ability of part of the fatty acid molecule to associate with water molecules while the other part is pushed away. The carboxyl functional group can donate hydrogen ions in a chemical reaction with the alcohol group of another molecule. In this way fatty acids can combine with alcohols such as glycerol to form fats.

Fats consist of fatty acids bonded to the 3-carbon alcohol glycerol (FIG. 4-21). In the fat molecule the fatty acid is usually stretched out like a flexible tail. A fat molecule is formed when a molecule of glycerol combines with one, two, or three fatty acid molecules to form a **monoglyceride, diglyceride,** or **triglyceride,** respectively. The chemical bond formed between a fatty acid and an alcohol group of glycerol is called an **ester linkage.** Plants and animals store lipids as triglycerides. Glycerides are the most abundant lipids and the richest source of energy in the human body. They are insoluble in water and tend to clump into fat globules.

Complex lipids have additional components such as phosphate, nitrogen, or sulfur, or small hydrophilic carbon compounds such as sugars. For example, the cell wall of *Mycobacterium tuberculosis,* the bacterium that causes tuberculosis, is distinguished

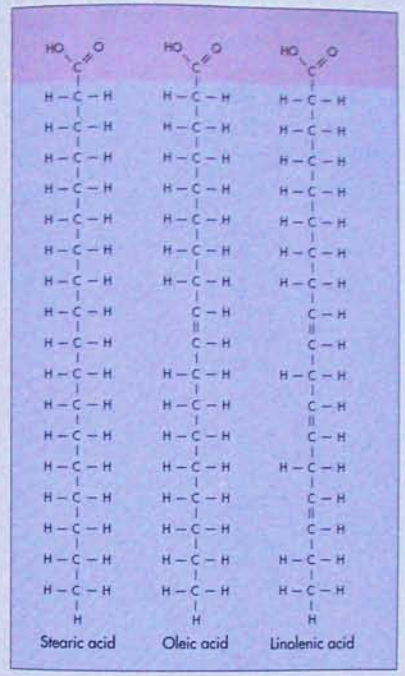


FIG. 4-20 A fatty acid is an organic acid. The portion of the fatty acid with the functional carboxylic acid group is polar and hydrophilic (attracted to water), whereas the remaining hydrocarbon portion is hydrophobic (repelled by water).

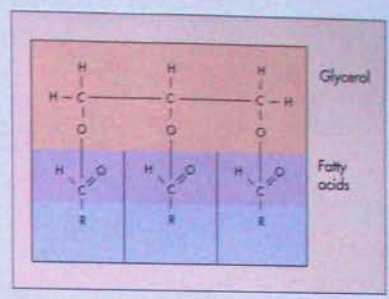


FIG. 4-21 A triglyceride is composed of glycerol and three fatty acids.

by the presence of abundant glycolipids (carbohydrates that are joined to lipids). These glycolipids give the bacterium a wax-like covering that contributes to its distinctive acid-fast staining characteristic.

Phospholipids are complex lipids made up of glycerol, two fatty acids, and a phosphate functional group (FIG. 4-22). Phospholipids are the major chemical component of biological membranes, including the plasma membrane. Their molecules contain both hydrophobic and hydrophilic portions. This enables phospholipids to aggregate into bilayers in which the hydrophobic components of each layer interact with each other and the hydrophilic components are exposed to the aqueous interior or exterior of the cell. The chemical properties of phospholipids make them effective structural components of a cell's plasma membrane. Water soluble (polar) substances are un-

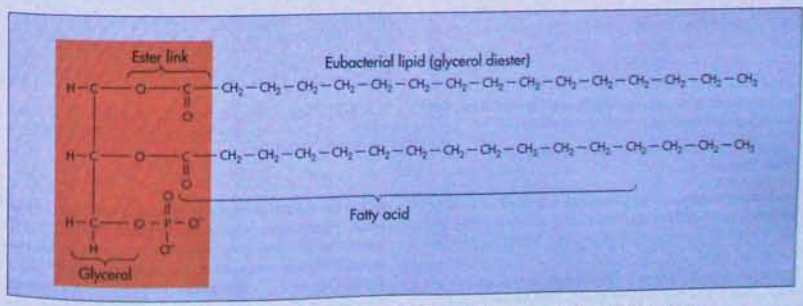


FIG. 4-22 Phospholipids are composed of glycerol linked to two fatty acids and a phosphate group.

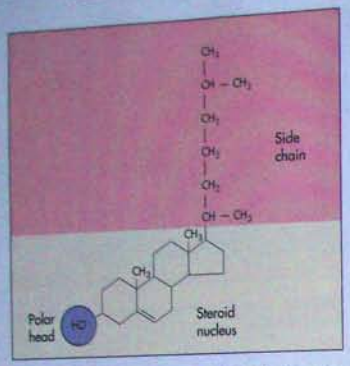


FIG. 4-23 A steroid is a nonpolar lipid with four rings. Cholesterol is an example of a steroid.

able to flow through the hydrophobic fatty acid portion of the bilayer. Phospholipids, thus, enable the plasma membrane to restrict the flow of materials into and out of the cell.

Phospholipids, which have hydrophilic and hydrophobic portions, form an integral part of the plasma membrane.

Steroids are also lipids but they are structurally very different from the lipids described previously. Cholesterol is a steroid compound that contains a —OH group, making it a **sterol** (FIG. 4-23). Cholesterol is an important component of the plasma membrane of eukaryotic animal cells. Other eukaryotic cells such as fungal cells contain different sterols in their plasma membranes. Cholesterol and other sterols wedge between phospholipids in the plasma membrane, maintaining membrane fluidity. Cholesterol and other sterols generally are absent from the plasma membrane of a prokaryotic cell.

PROTEINS

Proteins are large molecules made up of hundreds or thousands of amino acid subunits. **Amino acids** are the building blocks of proteins. An amino acid contains at least one carboxyl (—COOH) functional group and one amino ($-NH_2$) functional group attached to the same carbon atom. This carbon atom is called the alpha- carbon (α-carbon). There are only 20 amino acids naturally found in proteins (FIG. 4-24). Amino acids exist in mirror images called **stereoisomers.** They are designated as either L or D forms (FIG. 4-25). The amino acids found in proteins are always **L-amino acids.** Also attached to the alpha-carbon is a side or **R group.** These R groups are the amino acid's distinguishing factors. The R group can be a hydrogen atom, an unbranched or branched carbon chain, or cyclic ring structure. It can also contain functional groups—such as the sulfhydryl (—SH), hydroxyl (—OH), or additional carboxyl or amino groups.

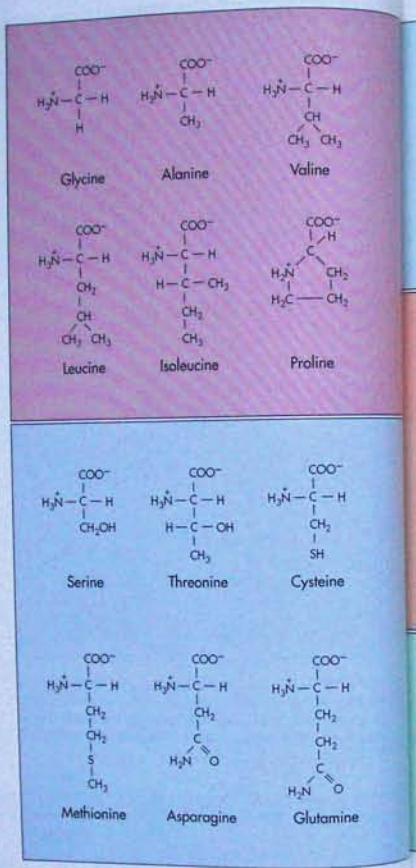
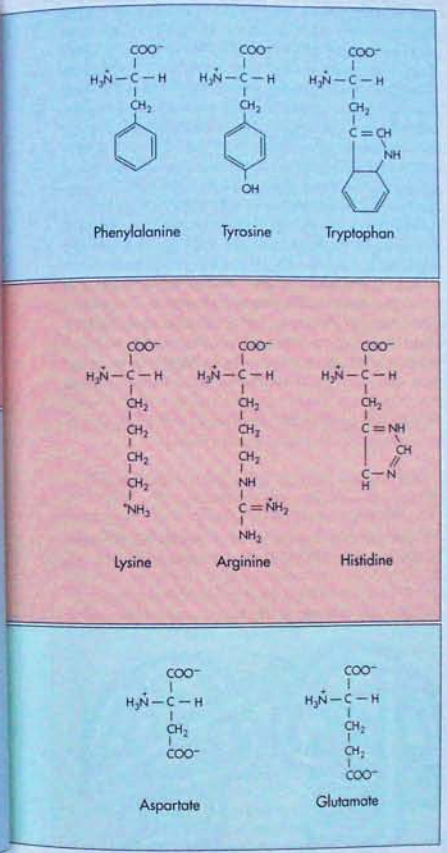


FIG. 4-24 The structural formulas of 20 common amino acids. Each is an L-α-amino acid. The structures differ in the other constituents.



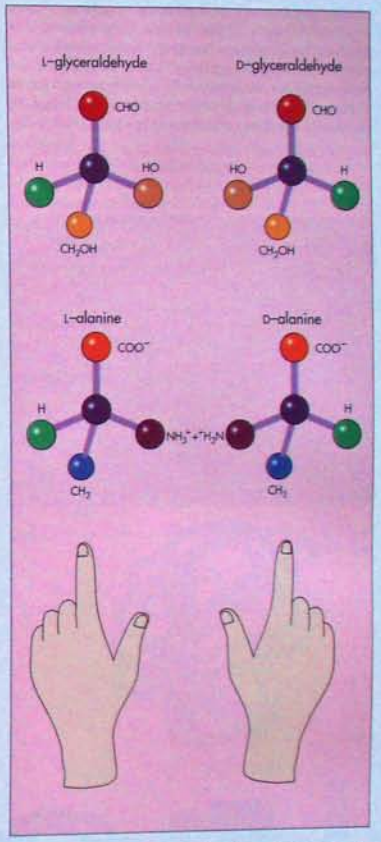


FIG. 4-25 An asymmetric carbon allows molecules to exist in two different forms (isomers) that are mirror images. The D and L forms of gylceraldehyde molecules and their relation to L-alanine. In determining the absolute configuration of a carbohydrate, the D designation is used to indicate that the groups H, CHO, and OH, in that order, are situated in a clockwise fashion about the asymmetric carbon atom, when the CH_2OH group is directed away from the viewer. The designation L is used if the order is counterclockwise. In determining the configurations of amino acids, we still use glyceraldehyde as the reference, with the NH_2 group substituting for OH and the COOH group substituting for CHO.

The L-amino acids of a protein molecule are linked by covalent bonds. These are called peptide bonds. A **peptide bond** forms between the amino group of one amino acid and the carboxyl group of another. The bonding of two amino acids by a peptide bond forms a dipeptide. Three or more amino acids linked by peptide bonds form a **polypeptide chain** (FIG. 4-26).

A protein is composed of a chain of L-amino acids held together by peptide linkages.

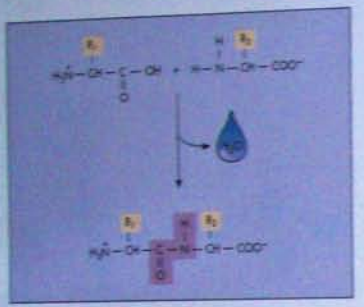


FIG. 4-26 A polypeptide has a free amino end and a free carboxyl end.

Protein Structure

Proteins have very highly organized three-dimensional structures (FIG. 4-27). Both the number and order of the specific amino acids within the polypeptide chain are important. They establish the structure and functional properties of protein molecules. Proteins have different lengths, different quantities of the various amino acid subunits, and different specific sequences in which the amino acids are bonded. Hence, the number of proteins is practically endless. Every living cell produces many different proteins.

There are only 20 different L-amino acids found in proteins, and virtually every protein contains the same amino acids. Yet each different protein has a unique sequence of amino acids. This sequence of amino acids forms the **primary structure** of a protein. The primary structure influences the three-dimensional shape of a protein, its function, and how it will interact with other substances. Alterations in amino acid sequences can have profound metabolic effects. For example, a single incorrect amino acid in a blood protein can produce the deformed hemoglobin molecule characteristic of sickle cell anemia.

The primary structure of a protein is the sequence of amino acids in its polypeptide chain.

The primary structure of a polypeptide determines how the molecule can fold and twist. The positioning of the R groups of the amino acids is dictated by the primary structure of the peptide chain. The R group position forces the polypeptide to twist and fold in a specific way. The term ***secondary structure*** refers to the helical or extended protein structures that result when different amino acids are positioned close enough to allow hydrogen bonding to occur. Most often, hydrogen bonds form between every fourth amino acid. They hold the chain in a specific structure, called the α-helix, in which a helical coil is wound about its own axis. In other cases the chain is almost fully extended and hydrogen bonds form between different chains. These bonds hold many chains side by side in a sheetlike structure. In the β-sheet or pleated sheet, the chain of amino acids in the polypeptide folds back and forth on itself. R groups are thus exposed that can undergo extensive hydrogen bonding. The R groups of some amino acids tend to favor helical patterns; others tend to favor sheetlike patterns.

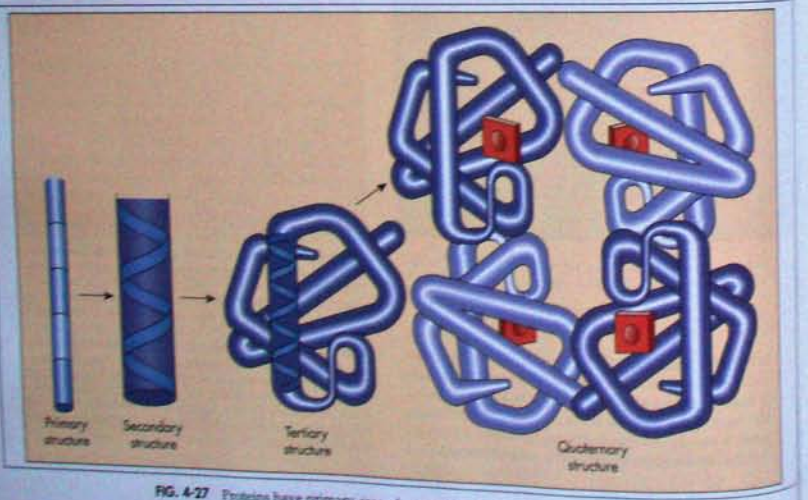


FIG. 4-27 Proteins have primary, secondary, tertiary, and quaternary structures.

The secondary structure of a protein is stabilized by hydrogen bonding between the amino acids of the polypeptides.

Most helically coiled chains become further folded into some characteristic shape. The folding of polypeptide chains is called **tertiary structure.** Folding of a helical polypeptide accomplishes two things. The polypeptide becomes a unique shape that is compatible with a specific biological function, and the folding process converts the molecule into its most chemically stable form. The tertiary structure is based on interactions between various R groups of specific amino acids. Hydrophobic R groups associate with each other at the interior of folded chains. Hydrophilic R groups assume exterior positions where they can form weak bonds with other polar R groups or with water. The highly nonpolar regions of the polypeptide are brought close together by tertiary folding. They contribute stability to the folded structure by preventing the penetration of water into these regions. In addition, sulfhydryl groups (—SH) on two amino acid subunits can form a covalent, disulfide bond (—S—S—). This bond further stabilizes the folding of the protein molecule, contributing to the tertiary structure of the protein.

The actual shape of a protein is the result of the interactions of the polar covalent bonds of the peptide linkage and the combined interactions of the polar and nonpolar side chains of the individual amino acids.

Some proteins consist of more than one polypeptide chain. Their structures are even more complex. In some cases, the polypeptide chains are linked by disulfide bridges. For example, the antibodies that help protect the human body against disease are composed of four peptide chains that are linked by disulfide bonds. Such proteins have a quaternary structure. The **quaternary structure** describes the arrangement in space of multiple peptide chains when they make up the structure of a protein.

The three-dimensional shape formed by the secondary, tertiary, and quaternary structure of a protein is essential for the function of all proteins, including those that act as enzymes. The sequence of the amino acids and the three-dimensional shape they assume determines where a substrate can bind and the catalytic properties of the active site. Enzymes with different three-dimensional shapes at their active sites catalyze different metabolic reactions.

Primary, secondary, tertiary, and quaternary structures contribute to the three-dimensional shape of a protein that is essential for its proper functioning.

The ability of a protein to function as an enzyme and catalyze chemical reactions depends on its three-dimensional shape.

Denaturation of Proteins

If the three-dimensional structure of a protein is disrupted, the protein is **denatured** (FIG. 4-28). Denatu-

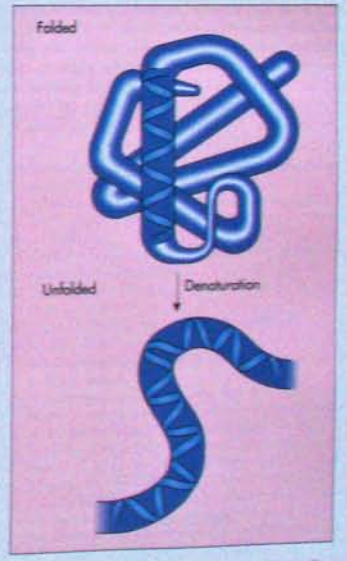


FIG. 4-28 Denaturation occurs when the three-dimensional shape of a protein is altered so that it no longer functions properly. Heat, salt, and various other factors can disrupt the tertiary structure of a protein, denaturing it.

ration occurs when there is a change in the three-dimensional structure of the protein that results in the loss of proper function of the protein. If the protein is an enzyme, denaturation results in the loss of catalytic capacity. The critical three-dimensional configuration of a protein can be disrupted without breaking the covalent peptide bonds of the polypeptide chain. Exposure to high temperatures, typically above 60° C, or certain chemical agents can disrupt the hydrogen bonds, sulfhydryl bonds, and hydrophobic interactions on which secondary, tertiary, and quaternary structures are based. This is one reason that high temperatures can be used to kill microorganisms. High salt or high H^+ concentrations can also denature proteins. They alter the weak bond interactions that maintain the structure of the protein molecule.

Proteins lose their functional capabilities when they are denatured because denaturation disrupts critical three-dimensional structures.

Nucleic Acids

Nucleic acids are polymers composed of monomeric **nucleotides.** Each nucleotide has three different parts: a nitrogen-containing base, a pentose, and a phosphate group (FIG. 4-29). These three parts of the nucleotide are joined by covalent bonds. The nitrogen-containing base is either adenine, guanine, cytosine, thymine, or uracil. Adenine and guanine are double-ring structures. Collectively they are referred to as **purines.** Thymine, uracil, and cytosine are smaller, single-ring structures. They are called **pyrimidines.**

A single strand of a nucleic acid consists of nucleotide units strung into long chains. A phosphate bridge connects their sugars. Bases stick out to the side. The type of bond that links the nucleotides in a nucleic acid is called a **phosphodiester bond** (FIG. 4-30). The backbone of the nucleic acid molecule is always the same. It consists of alternating sugar and phosphate units. The nitrogenous bases attached to the sugar portion of the nucleotides vary in the chain. Hence, when chemists refer to a specific sequence of nucleotides in a nucleic acid, they really are describing the sequence of nitrogenous bases. The sequence of bases in a DNA or RNA molecule carries the genetic information necessary to produce the proteins required by the organism.

Nucleic acids are polymers composed of nucleotide monomers held together by phosphodiester linkages.

DNA

All cells contain hereditary material called genes. Each gene is a segment of a **deoxyribonucleic acid (DNA)** molecule. Genes determine all hereditary traits. They control all the potential activities that take place within living cells. When a cell divides, its hereditary information is passed on to the next generation. This transfer of information is possible because of DNA's unique structure. DNA contains four

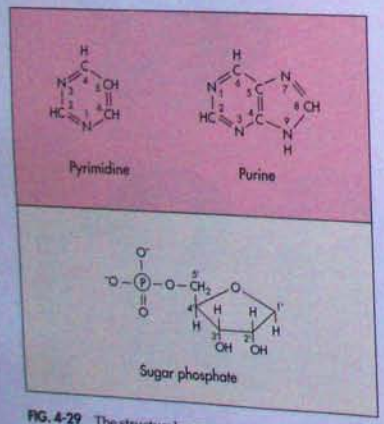


FIG. 4-29 The structural components of a nucleotide—the basic unit of the informational macromolecules of living systems. The carbon atoms of the sugar are assigned sequential number primes.

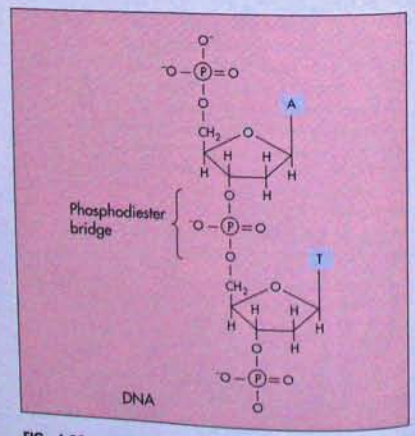


FIG. 4-30 Nucleotides are linked by phosphate diester bonds to form dimers and polymeric units (A, adenine; T, thymine). The linked nucleotides form long chains in DNA and RNA macromolecules.

nucleotides that each contain one of four nucleic acid bases: adenine (A), guanine (G), cytosine (C), and thymine (T). The ordering of the nucleotides determines the hereditary information contained in DNA. Nucleotides are held together by phosphodiester bonds between deoxyribose, the sugar found in the backbone of DNA. There are two strands of DNA held together by hydrogen bonds to form a coiled molecule called a **double helix.** Hydrogen bonds can form between adenine (A) and thymine (T) or between guanine (G) and cytosine (C).

The four nucleic acid bases in DNA are adenine, guanine, cytosine, and thymine.

RNA

Ribonucleic acid (RNA) differs from DNA in several respects. The five-carbon sugar in the RNA nucleotide is ribose. Ribose has one more oxygen atom than does the deoxyribose in DNA. One of RNA's bases is uracil (U) instead of thymine. Whereas DNA is normally double stranded, RNA is usually single stranded.

The four nucleic acid bases in RNA are adenine, guanine, cytosine and uracil.

Other Nucleotides

The nucleotide **adenosine triphosphate (ATP)** is the principal energy-carrying molecule of all cells. It stores the chemical energy released by some chemical reactions. It also provides the energy for energy-requiring reactions when needed. ATP consists of adenine, ribose, and three phosphate groups. ATP is called a high-energy molecule because it releases a large amount of usable energy upon hydrolysis of a phosphate group. The product is **adenosine diphosphate (ADP).** The production and utilization of ATP are essential to the bioenergetics of the cell. All of the metabolic pathways of microorganisms are involved in producing or consuming ATP.

Nucleotides also serve as coenzymes. A **coenzyme** is a temporary carrier of substances such as electrons. During metabolism, coenzymes transport hydrogen atoms and electrons. Nicotinamide adenine dinucleotide (NAD^+) and flavin adenine dinucleotide (FAD) are two of these coenzymes. These coenzymes gain electrons and hydrogen during some chemical reactions and donate them during other chemical reactions. Much of the metabolism of microorganisms involves chemical reactions requiring ATP and/or coenzymes. This accounts for the importance of these nucleotide molecules in the chemical reactions of living systems.

SUMMARY

Organization of Matter (pp. 92-98)

Chemical Elements (p. 92)
- An element is the fundamental unit of a chemical.
- Chemists use letter abbreviations (symbols) for the chemical elements (H, C, O, N, and so forth).

Structure of Atoms (pp. 92-94)
- All matter is made of atoms that are composed of a central nucleus, containing protons and neutrons, and electrons moving around the nucleus.
- The atoms of each element have a unique number of protons.
- The number of protons is the atomic number of an element.
- The sum of protons and neutrons is the atomic weight of an element.
- Electrons are arranged in electron shells of differing energy levels.
- Each shell can contain a fixed maximum number of electrons.
- An atom is most stable, and therefore least reactive, when its outermost shell is either completely full or completely empty.

Molecules and Chemical Bonds (pp. 94-98)
- Atoms combine to form molecules, which are fixed combinations of elements in which atoms are held together by chemical bonds formed when atoms transfer or share electrons.
- There are three principal types of chemical bonds: ionic, covalent, and hydrogen bonds.
- When atoms form ions by gaining or losing electrons, they can interact to form ionic bonds based on charge interactions.
- Covalent bonds are strong bonds formed when two atoms share electrons.
- If atoms share electrons equally they form a nonpolar covalent bond.
- If atoms share electrons unequally they form a polar covalent bond in which one of the atoms has a relative negative charge and the other atom has a relative positive charge.
- A hydrogen bond is the attraction between charged atoms of different molecules or distant parts of a large molecule that result from polar covalent bonds.
- Isomers are molecules that contain different arrangements of the same types and numbers of atoms, which gives the molecules different properties.
- Functional groups are specific combinations of atoms that act the same no matter to which molecules they are attached. They determine the characteristics, solubilities, and reactivity of the molecules.

Chemical Reactions (pp.99-105)
- In chemical reactions, molecules combine, break up, or transfer atoms or electrons.

- As a result of chemical reactions, new molecules are formed and energy can be released from the bonds of molecules to support the energy-requiring activities of living systems.

Chemical Equations (p. 99)
- Chemical equations are symbolic representations of what changes take place in a chemical reaction.

Equilibrium (p. 99)
- Chemical reactions are reversible and tend to move toward a state of equilibrium.

Energy and Chemical Reactions (p. 100)
- Chemical reactions involve energy changes.

Types of Chemical Reactions (pp. 100-105)
- Enzymes are proteins that act as catalysts.
- An enzyme lowers the activation energy, that is, the energy needed to start a reaction. By lowering the activation energies of chemical reactions, enzymes permit chemical reactions to occur rapidly at temperatures where living systems can maintain their structural integrity and organization.
- The activity of an enzyme depends on its three-dimensional shape, which allows it to bind with a substrate and catalyze a chemical reaction.
- Oxidation is the loss of electrons; reduction is the gain of electrons.
- Oxidation-reduction reactions are always coupled, so that as one atom or molecule is oxidized another atom or molecule is reduced.
- Some of the chemical reactions in cells are acid-base reactions.
- Acids donate hydrogen ions; bases accept hydrogen ions.
- The concentration of hydrogen ions in a solution is measured as pH.
- The pH of a solution = $-\log[H^+]$.
- Condensation reactions in which small molecules combine to form larger molecules are very important for the biosynthesis of the molecules that make up the structures of cells.
- Most large biological molecules are synthesized by linking many smaller subunit molecules; chains of subunits are connected by covalent bonds through condensation reactions.
- Hydrolysis reactions that break down molecules into smaller molecules are important for releasing energy and for forming the subunit molecules that are used for biosynthesis.

Molecules of Living Systems (pp. 106-115)

Water (pp. 106-107)
- The properties of water allow it to act as a solvent for polar molecules and to modulate temperature; these properties are critical for supporting life processes.

Carbohydrates (pp. 107-108)
- Carbohydrates include sugars, and large polysaccharides, such as starch and cellulose.
- Sugars (monosaccharides and disaccharides) are used for storage of energy and for the construction of other molecules.
- Starch and glycogen are polysaccharides that serve for long-term energy storage in eukaryotic cells.
- Cellulose and related polysaccharides form the cell walls of bacteria, fungi, and other organisms.

Lipids (pp. 108-110)
- Lipids are water-insoluble molecules of diverse chemical structure, and include oils, fats, phospholipids, and steroids.
- Lipids are used for energy storage and as the principal component of cell membranes (phospholipids).

Proteins (pp. 110-114)
- Proteins are chains of amino acids linked together by peptide bonds.
- The function of each protein is determined by the sequence of amino acids in the chain.
- The three-dimensional shapes of proteins, determined by their primary structure (covalent bonds) and by higher order interactions (weak hydrogen bonds and hydrophobic interactions), are especially important in establishing the specificity of roles played by enzymes.
- When the three-dimensional structure of a protein is disrupted, it cannot function.

Nucleic Acids (pp. 114-115)
- Nucleic acids are chains of nucleotides.
- Each nucleotide is composed of a phosphate group, a sugar group, and a nitrogen-containing base.
- The two types of nucleic acids are deoxyribonucleic acid (DNA) and ribonucleic acid (RNA).
- Other nucleotides include energy carrier molecules (ATP) and coenzymes.

CHAPTER REVIEW

REVIEW QUESTIONS

1. Describe the four levels of protein structure.
2. Describe the chemical composition of carbohydrates, lipids, proteins, and nucleic acids.
3. Why are most enzymes active only over a particular range of temperatures? Why are most enzymes not active at temperatures above 100° C?
4. What are the most common elements in living systems? What are their chemical symbols?
5. Compare and contrast covalent, ionic, and hydrogen bonding.
6. What are the differences between atoms, ions, and isotopes?
7. Describe how the chemical nature of water affects its properties relative to living systems. Why is water essential for life? What properties of water contribute to functions in living systems?
8. What is the difference between a monosaccharide and a polysaccharide? A peptide and a polypeptide? A nucleotide and nucleic acid?
9. What are the main properties of enzymes?
10. What are the differences between enzymes and coenzymes? Why are both needed for living systems?
11. What role does ATP have in living systems?
12. What role does DNA have in living systems?

CRITICAL THINKING QUESTIONS

1. Describe the different roles that are played by covalent and noncovalent bonds in biological systems. Why do living cells require molecules with both covalent and noncovalent bonds?
2. All living cells are based on organic molecules that contain carbon atoms. Why is this so? Why couldn't hydrogen, which can establish only a single covalent bond with another atom, form the basis for organic molecules? Could life have evolved based on silicon, which like carbon can form four covalent bonds with other atoms?
3. In oxidation-reduction reactions some substance becomes oxidized and another becomes reduced. If one substance causes another one to become reduced, the first substance is called a reducing agent. Will a reducing agent be oxidized or reduced in the process? What do you think an oxidizing agent does? Will an oxidizing agent be oxidized or reduced?

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UNIT
TWO

Cellular and Molecular Microbiology

CHAPTER 5

Cell Structure

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PREVIEW TO CHAPTER 5

In this chapter we will:

- Examine the structures of the cell and see how the chemical composition of each structure relates to the life processes it carries out.
- Discuss the relationships between form and function and see that even minor, seemingly trivial, differences in chemical structure can have a profound influence on the ability of a cell to survive.
- Compare prokaryotic and eukaryotic cells and examine how each meets the essential requirements for sustaining life.
- Explore the practical implications of the structural differences between eukaryotic and prokaryotic cells, such as why certain antibiotics can be used to kill the bacteria causing infections without also killing human cells.
- Learn the following key terms and names:

bacterial chromosome
capsule
cell wall
chemiosmosis
chemosensors
chloroplasts
chromosomes
cyst
cytosis
diffusion
endoplasmic reticulum
endospores
endotoxin
exocytosis
facilitated diffusion
flagella
glycocalyx
Golgi apparatus
Gram-negative bacterial cell wall
Gram-positive bacterial cell wall
group translocation
lipopolysaccharide (LPS)
lysosomes
magnetotaxis
nucleus
osmosis
outer membrane
passive diffusion
peptidoglycan
periplasmic space
peritrichous flagella
permeases
phagocytosis
phagolysosome
phagosome
plasma membrane
plasmids
polar flagella
porins
protonmotive force
protoplast
ribosomes
slime layer
spheroplast
spore
thylakoids
trophozoite
vegetative cells

CELLS

All living organisms are composed of cells, which are the fundamental units of all living systems. A cell is a self-contained system capable of independently carrying out metabolism. Cells also are the units of reproduction for living organisms. They house the hereditary information that is passed from one generation to the next. Cells come only from pre-existing cells.

All cells have certain common functional and structural properties:

1. Each cell has a plasma membrane that surrounds it. The plasma membrane forms a boundary layer between the living cell and its surroundings. The plasma membrane regulates the passage of materials into and out of the cell.
2. Each cell contains a fluid substance, called the cytoplasm. Chemical reactions take place in the cytoplasm. These reactions transform the energy and material needed for cell growth and reproduction. The cytoplasm consists of a solution, called the cytosol, and various particulate structures.
3. Each cell contains a copy of the hereditary information stored in molecules of DNA. The genetic molecules of DNA direct the activities of the cell and pass hereditary information to new cells formed as a result of cellular reproduction.
4. Each cell has thousands of small particles, called ribosomes, where proteins are made. The actual transfer of genetic information from DNA involves the formation of another informational molecule, RNA. Several different types of RNA are involved in the transfer process: messenger RNA (mRNA), transfer RNA (tRNA), and ribosomal RNA (rRNA). Messenger RNA carries the genetic information to the ribosomes where that information is used to direct the synthesis of proteins. There are structural proteins, regulatory proteins, and enzymatic proteins. Enzymes are proteins that are the action molecules catalyzing the metabolic functions of the cell.
5. Each cell utilizes energy from ATP, the "universal energy currency" of living cells. Cells carry out metabolism through which they generate ATP and cell constituents for growth and reproduction.

Every living cell is surrounded by a plasma membrane, contains a fluid called cytoplasm that has a solution portion called cytosol, contains hereditary information in molecules of DNA, processes genetic information, employs RNA intermediates to form proteins at ribosomes, and uses ATP as the cellular form of energy.

PLASMA MEMBRANE: MOVEMENT OF MATERIALS INTO AND OUT OF CELLS

The **plasma membrane** that surrounds the cell acts as a semipermeable barrier, regulating the flow of materials into and out of the cell. Its ability selectively to control which materials enter and which leave the cell is essential, since exchanges with the external surroundings must be selective and restricted to maintain life functions. Cells grow and reproduce by acquiring energy and materials from their surroundings and they also must discharge wastes into those same surroundings. The plasma membrane regulates the flow of materials into and out of the cell. It also allows the maintenance of the highly organized dynamic state that is characteristic of living systems.

The plasma membrane regulates the flow of materials into and out of the cell.

The plasma membrane, which is almost always a lipid bilayer with various proteins distributed within it, has a limited capacity to handle the essential exchanges of materials with the cell's surroundings. This is because of the plasma membrane's limited surface area and, hence, limited passageways through which materials may pass. Most cells are very small—less than 100 μm in diameter. The size of a cell is limited by the relationship between the cell's surface area and its volume. If a cell grows too large in volume, the surface area of the plasma membrane becomes small compared to the volume. This is because volume of a sphere increases much more rapidly than surface area as the diameter increases. If the diameter increases by a factor of 10, the volume increases by a factor of 1,000, but the surface area increases only by a factor of 100.

This does not provide sufficient surface area for substances to move across. Then rates of exchange between a cell and its surroundings are not fast enough to meet the needs of the cell. On the other hand, cells must be large enough to house their genetic information and proteins that are required for metabolism and reproduction. The smallest cells are about 0.1 μm in diameter. Recently, a new bacterium has been discovered that is 0.5 mm (50 μm) long. However, most bacterial cells have diameters of 0.2 to 2 μm.

Enclosed within the plasma membrane is the cytoplasm. Cytoplasm is a semifluid substance contain-

ing various embedded cell structures and solutions of chemicals. The fluid portion of the cytoplasm is called the **cytosol.** It contains various dissolved substances such as amino acids and sugars. The concentrations of chemicals within the cytosol are very different from those of the outside environment. The cytosol receives raw materials from the external environment that pass through the plasma membrane. Enzymatic reactions within the cytosol then degrade them to yield usable energy and new substances that are used for the synthesis of new cellular materials. If the plasma membrane breaks, the cytosol leaks out and the cell dies.

Life depends on the integrity of the plasma membrane and its ability to act as a semipermeable barrier, regulating the flow of materials into and out of the cell.

Structure of the Plasma Membrane

The plasma membranes of most prokaryotic and eukaryotic cells are composed of phospholipid and protein. The chemical nature of a phospholipid explains how it contributes to the ability of the plasma membrane to regulate the flow of materials into and out of the cell. A phospholipid molecule has two parts. It has a phosphate portion, which is hydrophilic and attracted to water. It also has a fatty acid (lipid) portion, which is hydrophobic and repelled by water. When phospholipids are surrounded by water, hydrophobic interactions cause their fatty acid tails to move away from water and to cluster. When a thin cross section of a cell is viewed with a transmission electron microscope, the plasma membrane appears like a railroad track completely encircling the cell (FIG. 5-1). The dark rail-like portions of the membrane correspond to the electron-dense hydrophilic phosphate portions. The clear space between the "rails" corresponds to the lipid portions of the phospholipid molecules.

The basic structure of the plasma membranes of eubacterial and eukaryotic cells is a lipid bilayer. In this structure, the hydrophobic fatty acids of the phospholipid are sandwiched in the middle of the bilayer. The hydrophilic phosphate portions of one layer of phospholipid molecules interact with the water outside the cell and those of the other layer interact with the cytosol within the cell. The individual phospholipid molecules within the bilipid layer move about. They slide sideways, spin in place, and flex their fatty acid tails. These movements prevent the lipids from packing tightly together. They also impart fluidity to the membrane. This is important because it allows some molecules to pass through the plasma membrane without destroying its integrity.

The plasma membranes of most cells contain phospholipids that contribute to their ability to act as a semipermeable barrier.

Whether or not molecules pass through the plasma membrane is determined by their size and polarity. Nonpolar molecules, which dissolve easily in lipids, pass through the phospholipid portion of the plasma membrane more readily than do polar substances, which do not dissolve in lipids. Relatively small uncharged molecules, such as oxygen (O_2), nitrogen (N_2), and hydrogen (H_2), usually pass through the plasma membrane easily. Ions and other charged molecules pass through the membrane only

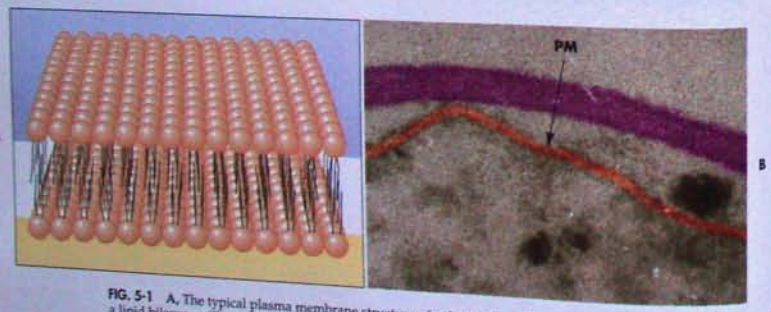


FIG. 5-1 **A,** The typical plasma membrane structure of eubacterial and eukaryotic cells is a lipid bilayer, as illustrated here, showing the orientations of the hydrophilic (*tan spheres*) and hydrophobic (*black*) ends of phospholipids that make up this structure. The hydrophilic portions (phosphate groups) occur near the water inside and outside the cell. The hydrophobic portions (formed from fatty acids) are sequestered in the interior of the membrane. **B,** Colorized electron micrograph of the plasma membrane (*PM*) of the bacterium *Bacillus subtilis* reveals the characteristic railroad track appearance of this lipid bilayer.

very slowly. Their electrostatic charges prevent them from interacting with the lipids in the middle of the bilayer. Large molecules with high molecular weights, such as proteins, cannot pass through the phospholipid portion of the plasma membrane.

However, proteins that compose part of the plasma membrane provide passageways through which polar and relatively large molecules can pass. Proteins are distributed in a patchlike or mosaic pattern in the plasma membrane. Some of the proteins span the bilayer and others are partially embedded in it. The partially embedded proteins are exposed only to the internal cytosol or the external surrounding environment. Since some of these proteins move in the plane of the membrane, scientists have proposed the fluid mosaic model for the plasma membrane. This model explains most of the functional and structural aspects of the plasma membrane (FIG. 5-2). The proteins that span the membrane create channels that connect the outside of the cell with the inside. The passage of some, but not all, materials across the plasma membrane is thus allowed.

Proteins form channels and serve as carriers to move materials across the plasma membrane.

Several antimicrobial agents work because they disrupt the structure of the plasma membrane, causing the death of the cell. These include the polymyxin antibiotics that destroy the phospholipid bilayer of the plasma membrane. Leakage of intracellular contents then occurs, followed by cell death. Various alcohols also destroy plasma membranes and are commonly used to kill microorganisms, such as when alcohol is applied before puncturing the skin with a hypodermic syringe.

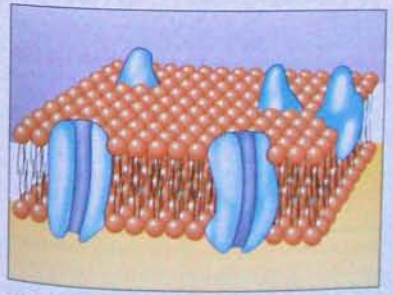


FIG. 5-2 The fluid mosaic model of membrane structure accounts for the facts that proteins (*blue*), as well as lipids (*beige* and *black*), comprise an integral part of membranes and that the structure is dynamic as opposed to static. Some proteins extend through the membrane (integral proteins) and others are associated with one side or the other (peripheral proteins) and do not completely transverse the membrane.

Comparison of Archaebacterial and Eubacterial Plasma Membranes

The plasma membranes of archaebacterial cells have a lipid composition that is fundamentally different from all other organisms (FIG. 5-3). The chemical composition of the archaebacterial plasma membrane indicates that they evolved as a separate Kingdom. It also shows that archaebacterial cells are only distantly related to eubacterial and eukaryotic cells.

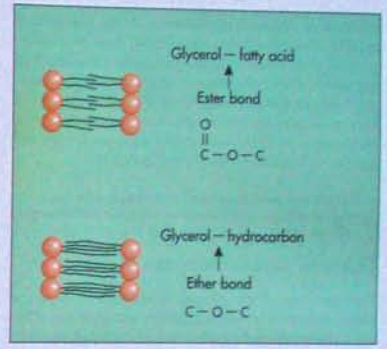


FIG. 5-3 Unlike the lipids that make up the plasma membranes of eubacterial cells, which are glycerol diesters (they have ester bonds formed by the reaction of glycerol and a fatty acid), major lipids of archaebacteria are glycerol diethers, diglycerol tetraethers, and tetrapentacyclic diglycerol tetraethers (they have ether bonds formed by the reaction of a hydrocarbon with glycerol). The glycerol diethers, like the phospholipids (glycerol diesters) of eubacterial and eukaryotic cells, form a lipid bilayer. Diglycerol tetraethers form a single layer with the two glycerols on the outside.

In particular, the presence of ether linkages in the lipids of the archaebacterial plasma membranes is unique in the biological world. Despite their chemical uniqueness, archaebacterial plasma membranes are functionally similar to those of eubacterial and eukaryotic cells. They also serve as selectively permeable barriers. The unique chemical structure permits this membrane to function in extreme environments, such as the very hot, very salty, and very acidic environments where some archaebacteria live. Under these conditions, phospholipids in the plasma membranes of other organisms would not be able to maintain the integrity of the permeability barrier.

Comparison of Eubacterial and Eukaryotic Plasma Membranes

Eukaryotic cells have sterols in their membranes. Prokaryotic cells generally do not. Sterols make the eukaryotic membrane stronger. They permit eukaryotes to survive without cell walls in environments where bacteria cannot survive unless they have other protective structures, such as a bacterial cell wall. Only a few bacteria, such as *Mycoplasma*, have sterols in their plasma membranes. These bacteria are restricted to certain environments. Sterols are an integral part of the eukaryotic plasma membranes of disease-causing eukaryotic microorganisms such as pathogenic fungi. They are often the targets of antimicrobial drugs. Amphotericin B, for example, is used to treat some fungal infections. It reacts with the specific sterols in fungal plasma membranes, such as ergosterol, disrupting membrane function and causing death of the infecting fungi. Amphotericin B has a lesser affinity for the sterols in human cells, such as cholesterol, which is why it can be used therapeutically in individuals with fungal infections.

The plasma membranes of eukaryotic cells contain sterols, whereas those of most prokaryotic cells do not.

Transport Across the Plasma Membrane

The structure of the plasma membrane permits the selective movement of substances into and out of living cells by several different mechanisms (Table 5-1). Different cells have different mechanisms for transporting specific substances into and out of the cell. The specific transport capabilities of a cell determine what substances it can use for growth and reproduction.

Diffusion

The concentrations of many substances differ inside and outside a cell so that there is a *concentration gradient*, that is, a region of high relative concentration on one side of the membrane and a region of low relative concentration on the other side. Unless prevented from doing so, substances will move from regions where they are in relatively high concentration to regions where they are in relatively low concentration. This process of moving from high to low concentration is called **diffusion.** To understand how diffusion works, let us consider a simple example. If a drop of red dye is added to a pool of water, it initially can be seen as a red spot where the dye is in high concentration. Soon, however, the dye begins to disperse, spreading out by diffusion from the initial region of high concentration, forming a concentration gradient from the region of highest to the region of lowest concentration of the dye. The diffusion of the dye continues until the dye is equally distributed throughout the water. If unrestricted, all chemicals will naturally move toward a state of equal concentration.

Diffusion is the movement of molecules down a concentration gradient, moving from a region of relatively high concentration to a region of relatively low concentration.

TABLE 5-1

Comparison of Transport Mechanisms Across the Plasma Membrane

TRANSPORT PROCESS	DESCRIPTION
Passive diffusion	Movement of substances across the plasma membrane due to a concentration gradient; movement is from the region of high to low relative concentration at a rate proportional to the concentration difference
Facilitated diffusion	Movement of substances across the plasma membrane due to a concentration gradient; movement is from the region of high to low relative concentration at a rate that is higher than expected based on the concentration difference; movement of substance is aided by permeases in the membrane, which are protein carriers that help move substances across the membrane; the action of permeases accounts for the relatively high rate of movement
Active transport	An energy-requiring transport mechanism mediated by permeases that allows substances to move against a concentration gradient, that is, from a region of relatively low to a region of relatively high concentration; ATP often supplies the energy for active transport
Group translocation	Occurs only in prokaryotic cells; substances are chemically modified as they move across the membrane; energy-requiring process with energy supplied by phosphoenolpyruvate
Cytosis	Occurs only in eukaryotic cells; the plasma membrane wraps around a substance, enclosing it and releasing it when the plasma membrane subsequently unwraps it

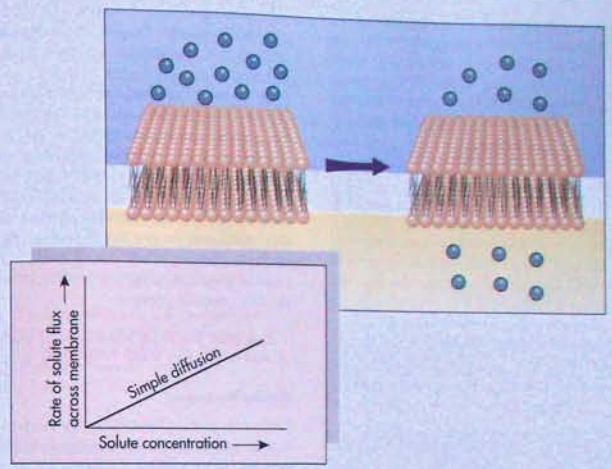


FIG. 5-4 Diffusion across a membrane occurs when substances pass through the membrane and when there is a favorable concentration gradient; this type of transport represents the downhill flow of a substance along a concentration gradient from high to low.

Some substances move across the plasma membrane and enter or exit the cell by a process called **passive diffusion,** so named because it does not require an input of energy or the use of special carriers in the membrane (FIG. 5-4). Substances will tend to move by passive diffusion from a region of higher concentration to one of lower concentration at a rate that is determined by the concentration gradient. The greater the concentration difference across the plasma membrane, the more rapid the rate of diffusion. Many small molecules (including water) can enter and leave cells by passive diffusion. These substances are able to diffuse into and out of cells because there is a concentration gradient across the membrane. The plasma membrane does not prevent their movement. The structure of the plasma membrane, however, restricts movement of large and charged molecules, including ions such as H^+ and Na^+.

The rate of passive diffusion into and out of a cell depends on the relative concentrations of a substance on opposing sides of the membrane.

In some cases, substances move more rapidly across the plasma membrane than can be explained by the concentration gradient alone because of a process called **facilitated diffusion** that increases the rate of passage across the membrane. Like passive diffusion, facilitated diffusion involves the movement of chemicals down a concentration gradient. Faster movement occurs because of proteins that span the plasma membrane and selectively increase the permeability of the membrane for specific substances. Such proteins are called **permeases.** Permeases act as carriers, making it easier for a substance to move through the membrane. Permeases are highly specific and will carry only selected molecules across the plasma membrane. A substance is not chemically modified by a permease as it moves across the membrane. The permease simply picks up the substance at one side of the membrane and pulls it through the membrane. The substance is released unchanged at the other side.

Facilitated diffusion involves permeases that carry substances across the plasma membrane and thereby increase the rate of diffusion when concentration gradients are small.

Osmosis

While water can move across the plasma membrane through channels without restriction, the movement of many substances dissolved in water is restricted. Substances dissolved in water are called *solutes*. When a solute cannot move across the plasma membrane in response to a concentration gradient, water will move across the membrane. This occurs because the presence of solute changes the concentration of

water. If there is a high concentration of solute on one side of the membrane the water concentration is correspondingly lower. In this case, water will move across the membrane from the region of high water concentration to the region of low water concentration. Such movement of water across a semipermeable membrane is called **osmosis.**

Osmosis is the movement of water across a semipermeable membrane due to diffusion.

In a medium where the solute concentration is higher outside the cell than inside the cell, water will flow out of the cell. The cell will shrink. This process is called **plasmolysis** (FIG. 5-5). The reverse will occur if the cell is in a medium where the solute concentration is lower outside the membrane than inside the cell. In this latter case, water will flow into the cell. The cell will expand. This is often characteristic of microorganisms because the concentrations of chemicals within the cell normally are in considerably higher concentrations than in the surrounding solution.

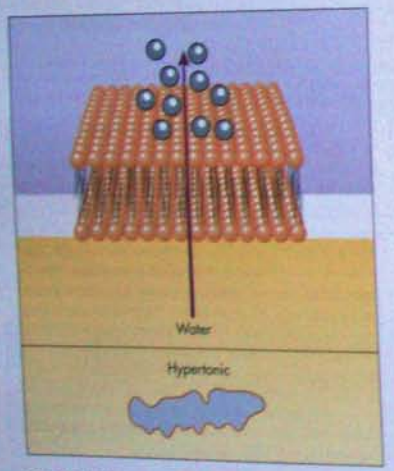


FIG. 5-5 Cells respond to osmotic pressure because water can move across the plasma membrane by osmosis and cause the cell to expand or shrink. Under isotonic conditions (equal concentrations of solute on both sides of the plasma membrane), cell shape is maintained. Under hypertonic conditions (higher solute concentration outside cell), the cell loses water and shrivels. Under hypotonic conditions (lower solute concentration outside cell), water moves into the cell, pressing the cell to expand; because the cell has very limited ability to increase volume without new synthesis of cell wall and plasma membrane components, osmotic pressure increases and the cell lyses due to osmotic shock.

If the flow of water into the cell is unrestricted, the plasma membrane will burst. Pressure is exerted on the plasma membrane by the water entering the cell. This pressure is called **osmotic pressure.** Rupture of the cell is called *cell lysis.* When this rupture is the result of excessive osmotic pressure, the cell is said to have died due to **osmotic shock.** To survive, microorganisms have developed various strategies, such as having a rigid cell wall surrounding the plasma membrane, for preventing cell lysis due to excessive osmotic pressure.

Cell lysis will occur if the flow of water into a cell due to osmosis is not controlled.

Active Transport

Diffusion only allows the movement of chemicals down a concentration gradient. Substances can be moved across the plasma membrane against a concentration gradient by a process called **active transport.** To reverse the direction of movement of diffusion, active transport requires an energy input. In active transport, specific membrane proteins (permeases) act as carriers. Energy from ATP or another source is used to move substances across the plasma membrane against a concentration gradient. Many substances that do not freely diffuse through the plasma membrane, such as most sugars, amino acids, ions, and the like, can pass through the membrane by active transport. They can be concentrated to over 1,000 times that of the solution surrounding the cell.

The active transport process can be likened to a pump that uses energy to move water uphill, that is, against an energy gradient. As with any pump, active transport requires that energy be expended and work be performed. In a eukaryotic cell, ATP generated by the metabolism of the cell is used to drive the uptake of substances by active transport. In bacteria, the energy for driving the pump usually comes from the potential energy of a concentration gradient. This gradient is formed by the expulsion of hydrogen ions (protons) from the cell across the membrane. This energy is called the *protonmotive force.* The potential energy of the concentration gradient of protons is used to drive the uptake of nutrients by active transport into bacterial cells.

In active transport, cells use metabolic energy and protein carriers in the plasma membrane to move substances across the plasma membrane against a concentration gradient.

Group Translocation

Group translocation is a form of energy-requiring transport that uses groups of enzymes and alters the chemical as it moves across the plasma membrane. This process occurs only in bacteria. The fact that the transported substance is chemically changed distinguishes group translocation from active transport. Additionally, ATP is not the source of energy for group transport. Phosphoenolpyruvate (PEP) is used by *Escherichia coli* to move glucose and other substances into the cell. The glucose is changed to glucose phosphate in this group transport process.

In group translocation, bacteria use multiple enzymes and phosphoenolpyruvate as the source of energy to pump substances across the plasma membrane and simultaneously alter them.

Cytosis

Cytosis allows substances to move into or out of cells without passing through the hydrophobic internal portion of the plasma membrane. Only eukaryotic cells are capable of cytosis. In cytosis the plasma membrane wraps around a substance. The plasma membrane engulfs that substance to form a membrane-bound sphere called a *vesicle.* A vesicle now can open on the other side of the membrane and release that substance. Alternatively, it can separate from the plasma membrane and remain as an intact vesicle.

The prefixes *endo-* (into) and *exo-* (out of) indicate whether the substance is entering or leaving a cell (FIG. 5-6). **Endocytosis** refers to the movement of materials into the cell. **Exocytosis** denotes movement out of the cell. Both endo- and exo-cytosis are important in moving some substances in bulk into and out of cells. These substances include large and polar molecules that cannot move across the plasma membrane by other processes. In some instances one cell can even engulf and ingest another cell by cytosis. The process of engulfing and ingesting cells or other solid materials is called **phagocytosis** (FIG. 5-7). Many protozoa obtain their nutrients by ingesting bacteria by phagocytosis. The ability of certain white blood cells to engulf microbial cells by phagocytosis, and then later to digest and kill them, is a very important part of our body's defense against infections.

Eukaryotic cells can move substances into and out of the cell by cytosis.

Phagocytosis is the engulfment by one cell of another cell.

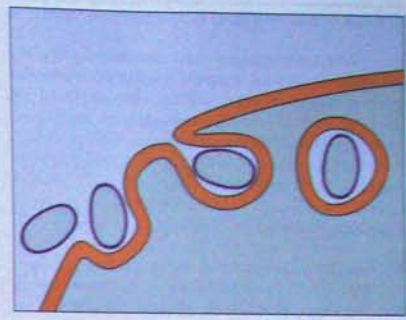


FIG. 5-6 In cytosis, which only occurs in eukaryotic cells, a substance is transported into or out of the cell without actually passing through the membrane. This is important for the movement of large substances and large quantities of substances into and out of the cell.

FIG. 5-7 Colorized electron micrograph showing the phagocytic capture of a rod-shaped bacterium (*purple*) by the soil protozoan *Vahlkampfia.* Many protozoa graze on bacteria as their food sources.

The plasma membrane defines the outer limit of the cell. Many cells have external cellular structures that are critical to their survival. Some of these structures, such as the bacterial cell wall, protect the plasma membrane from physical disruption. Others, such as flagella, move the cell in search of food or to escape hostile environments.

Cell Wall

Because the plasma membrane is a selective barrier that maintains cell viability, it is necessary to protect this structure. Prokaryotic plasma membranes are relatively fluid. Bacterial cells tend to expand under the force of osmotic pressure until they burst. The plasma membrane must be protected by an external structure against excessive expansion. The cell wall provides such protection (FIG. 5-8). Only bacterial cells growing in regions of high external solute concentration, such as in the fluids that occur in the human lungs or the high sugar concentrations of some laboratory solutions, can survive with a defective or missing cell wall.

Protection of the bacterial cell against osmotic shock is the main function of the bacterial cell wall.

The cell wall also establishes the shape of a bacterial cell. Bacteria occur as spheres called *cocci*, cylinders called *rods* or *bacilli*, and helical shapes called *spirilla*, as well as other diverse forms (see FIG. 1-4). The *morphology* (shape) of a bacterial cell is an important characteristic used in its identification. Each bacterial genus has a characteristic shape. For example, *Staphylococcus*, *Streptococcus*, and *Neisseria* are all cocci, whereas *Escherichia*, *Enterobacter*, *Bacillus*, and *Clostridium* are all rods, and *Rhodospirillum* and *Spirillum* are all helical cells (spirilla).

The shape of a bacterial cell affects its ability to survive and grow in different environments. Cocci are round and can usually survive more severe drying than can rod- or spiral-shaped bacterial cells. Hence, many of the bacteria living on the surface of human skin are cocci. For example, staphylococci can withstand the dry conditions that often exist on the skin. Rod-shaped bacteria, on the other hand, have more surface exposed per unit volume than cocci. Therefore they can take up nutrients from dilute solutions more readily than cocci. Most bacteria that live in lakes, rivers, and oceans are rod shaped.

The bacterial cell wall protects the cell against osmotic shock and gives the cell its shape.

The cell walls of most eubacteria consist in large part of peptidoglycan. Peptidoglycan is a polymer that is part peptide and part carbohydrate. Its chemical structure gives it the physical strength to withstand osmotic pressure and prevent lysis. Peptidoglycan is a biochemically unique substance that is found only in eubacterial cell walls. Many archaebacterial and eukaryotic cells also have cell walls. However, the chemical composition of their walls is different. They do not contain peptidoglycan. Archaebacterial cells have a substance called pseudopeptidoglycan, plant cells have cell walls made of cellulose, and many fungal cell walls contain chitin.

Chemical Composition of the Bacterial Cell Wall

The peptidoglycan polymer of the eubacterial cell wall has a peptide portion and a glycan, or sugar, portion (FIG. 5-9). The peptide portion is composed of amino acids connected by peptide linkages. Some of these amino acids are found only in the peptidoglycan of the bacterial cell wall. For example, D- and L-amino acids occur in peptidoglycan whereas only L isomers of amino acids occur in protein. The glycan portion that forms the backbone of the molecule is a complex polysaccharide composed of two amino sugar molecule subunits, *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM). The sugar subunits are linked so that they form an alternating and repeating unit—NAG-NAM-NAG-NAM and so forth.

Short peptide chains are covalently linked to some of the *N*-acetylmuramic acid groups. These peptides hang like tails from the glycan backbone of this structure. They are linked together by additional short peptides. This reduces the flexibility of the entire peptidoglycan molecule and provides the desired rigidity. They also form bridges between adjacent peptidoglycan polymers. The cell wall is a multilayered sheet, linked together into one functional unit.

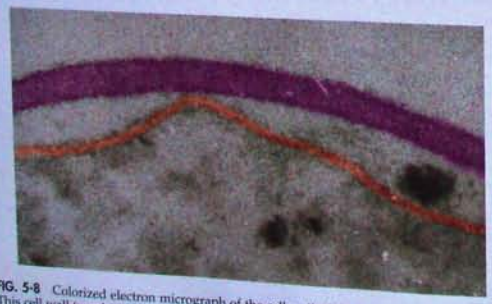


FIG. 5-8 Colorized electron micrograph of the cell wall of the bacterium *Bacillus subtilis*. This cell wall *(purple)* completely surrounds and protects the plasma membrane *(tan)*.

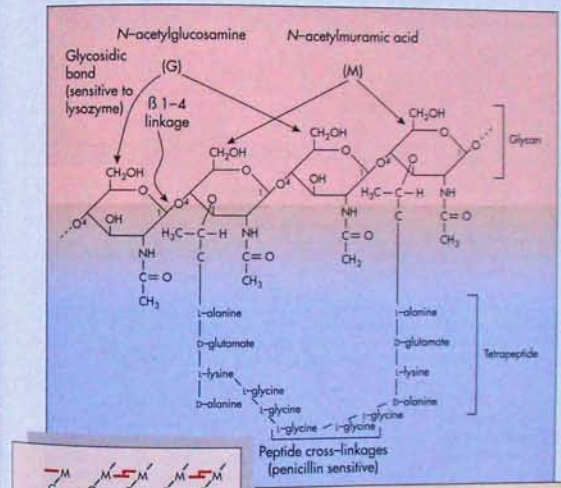
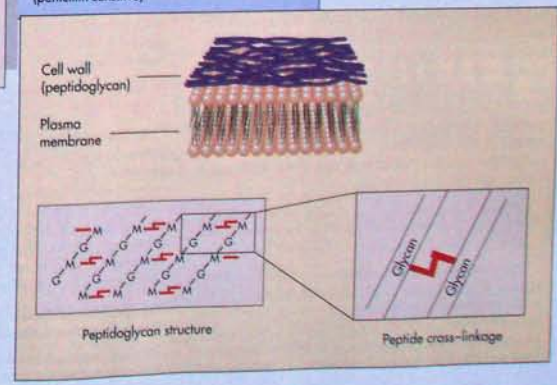


FIG. 5-9 Peptidoglycan is the backbone chemical of the bacterial cell wall; it is composed of repeating alternating units of *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM) and has cross-linked, short peptide chains, some of which have unusual amino acids such as D-alanine. The cross-linkages provide the needed structural support of the wall and are characteristic for specific bacterial genera.



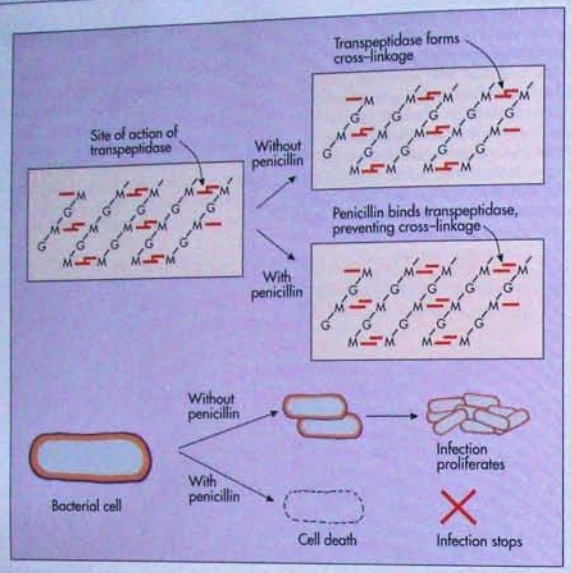


FIG. 5-10 The mode of action of penicillin involves inhibition of the formation of the normal cross-linkages in the peptidoglycan layer of the bacterial cell wall. Penicillin forms an inactive complex with transpeptidase, a key enzyme in cell-wall sythesis, so that the peptide cross-linkages do not form.

Enzymes are involved in cross-linkage of peptides during cell wall formation. Bacterial cell walls are weakened by substances that inhibit these enzymes. When this happens, the cell wall is defective. It cannot adequately protect the bacterial cell against osmotic shock. Such inhibition of peptide cross-linkage formation is the basis for the action of penicillin and cephalosporin. It is the reason that these antibiotics are effective in controlling many bacterial infections (FIG. 5-10). Since human cells lack peptidoglycan, penicillins and cephalosporins will selectively inhibit bacterial growth without adversely affecting human cells. This selective toxicity is why these antibiotics are of high therapeutic value.

Penicillins and cephalosporins work by blocking formation of peptide cross-linkages in the bacterial cell wall.

The enzyme lysozyme breaks apart the backbone glycan portion of the peptidoglycan molecule (FIG. 5-11). Lysozyme occurs as part of various normal human secretions, such as tears and saliva and within phagocytic white blood cells. It provides protection against would-be bacterial invaders. Lysozyme and cell wall–inhibiting antibiotics prevent the growth and/or kill many species of bacteria. However, these agents will not attack the few species that do not have peptidoglycan-containing cell walls. For example, penicillin and cephalosporin are ineffective against *Mycoplasma*, a bacterial genus that lacks a cell wall entirely.

The enzyme lysozyme will destroy the peptidoglycan of the bacterial cell wall and kill bacteria.

In the laboratory, scientists sometimes use lysozyme to remove bacterial cell walls, so they can lyse bacterial cells and recover their internal contents. When bacterial cells are treated with lysozyme, all or only a part of the cell wall may be destroyed. If a portion of the bacterial cell wall remains after lysozyme treatment, the remaining cell is called a **spheroplast.** If the cell wall is removed completely, the remaining cell is called a **protoplast** (see FIG. 5-11).

Comparison of Gram-positive and Gram-negative Bacterial Cell Walls

The cell walls of Gram-negative and Gram-positive bacteria are structurally different (FIG. 5-12). This

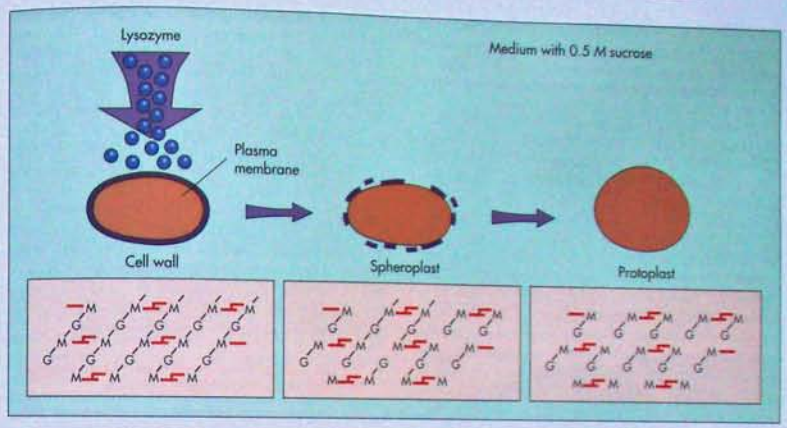


FIG. 5-11 Lysozyme cleaves the glycan portion of the peptidoglycan. The wall is degraded but as long as there is an osmotic support (such as a 0.5 *M* sucrose solution), the cells do not lyse (rupture). Spheroplasts have some intact wall, whereas protoplasts have none. If the osmotic support is removed, both spheroplasts and protoplasts lyse.

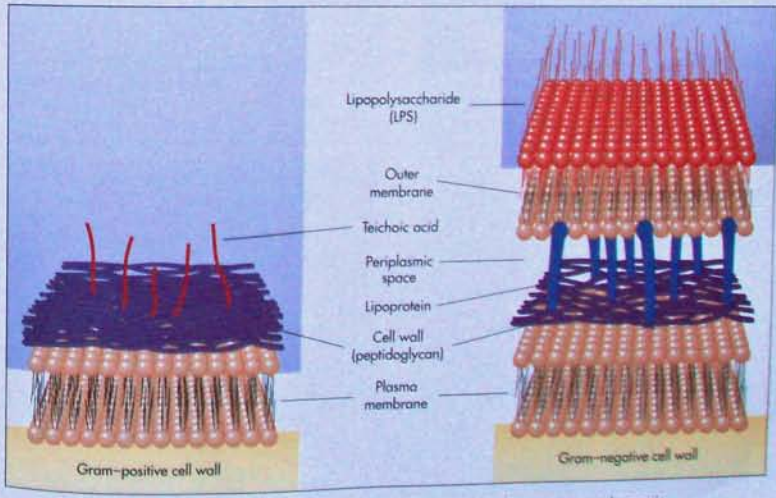


FIG. 5-12 The Gram-negative cell wall has a thin layer attached to an outer membrane via lipoproteins. The outer membrane contains phospholipid on its inner surface and lipopolysaccharide (LPS) on its outer surface. The space between the outer membrane and the plasma membrane is called the periplasmic space.

difference in cell wall structure is critical. It affects the survival of bacteria in nature and the effectiveness of antibiotics against bacterial pathogens. It is also of further practical importance because of the use of the Gram stain in identifying bacterial species.

Over 90% of the Gram-positive cell wall is made of peptidoglycan (see FIG. 5-8). This provides a strong, relatively thick protective layer that protects the plasma membrane from lysis by osmotic shock. Besides peptidoglycan, Gram-positive cell walls contain teichoic acids, which are acidic polysaccharides. Teichoic acids make the Gram-positive cell wall acidic. This is important because cells produce enzymes that break down peptidoglycan. These enzymes are called *autolysins*. At relatively low pH, however, autolysins do not degrade the cell wall. Autolysins are normally involved in cell wall growth and restructuring. Teichoic acids regulate autolytic activity so that autolysins can perform their necessary function without causing self-destruction (autolysis) of the cell.

Gram-negative cell walls are far more complex. Two distinct structures make up the Gram-negative wall: a peptidoglycan layer and an outer membrane (FIG. 5-13). The peptidoglycan layer of the Gram-negative cell is very thin, often comprising only 10% or less of the cell wall. It does not contain teichoic acids as in Gram-positive bacterial cell walls. The peptidoglycan layer and outer membrane are held together by lipoproteins (lipid linked to protein molecules).

The region between the plasma membrane and the outer membrane is called the **periplasmic space,** or **periplasmic gel.** Some substances get trapped within the periplasmic gel. Various chemical reactions occur there. The periplasmic gel occurs only in Gram-negative bacterial cells. Additionally, there are regions where the plasma membrane and the outer membrane are attached to one another.

The **outer membrane** is located at the outer extremity of the Gram-negative cell wall. The outer membrane, like the plasma membrane, is a semipermeable structure. The outer membrane restricts the passage of materials based largely on pore size. Many harmful chemicals are excluded from the cell by this structure. The region is unique to Gram-negative bacteria. The outer membrane is a phospholipid bilayer that contains a unique lipopolysaccharide (LPS). LPS is called an *endotoxin* because it is part of the bacterial cell and can cause a toxic reaction in mammals. The symptoms of some infections caused by Gram-negative bacteria are the direct result of the action of endotoxin. Endotoxin causes fever, lysis of red blood cells, and coagulation of blood in capillaries when it is released from dead Gram-negative bacterial cells and enters the circulatory system.

The cell walls of Gram-positive and Gram-negative bacterial cells are chemically different.

The Gram-negative bacterial cell wall is a complex structure that has an outer membrane containing the endotoxin lipopolysaccharide (LPS).

The outer membrane can render a Gram-negative bacterial cell resistant to antibiotics. It has channels made of proteins called **porins.** Small polar molecules freely pass through porins. The outer membrane blocks the passage of very large molecules, such as lysozyme, or molecules that have both polar and nonpolar ends, such as penicillin. Some antibiotics cannot cross the outer membrane. They cannot reach their targets at the cell wall's peptidoglycan layer or gain entry into the cell itself. Consequently, for treating infections caused by Gram-negative bacteria, physicians often must select different antibiotics that can cross the outer membrane of the Gram-negative bacterial cell wall and can work against other targets within the cell.

FIG. 5-13 Colorized electron micrograph of the cell wall of the Gram-negative bacterium *Escherichia coli*. (154,000×). The outer membrane *(red)* encloses the peptidoglycan *(purple)*. The entire cell wall surrounds the plasma membrane *(tan)*.

Bacterial Capsules and Glycocalyces

In addition to a cell wall and an outer membrane, some bacteria form another protective structure called a **capsule** (FIG. 5-14). A capsule is a surface layer that surrounds the cell outside of the cell wall. It is almost always composed of polysaccharides. Negative staining with India ink and phase-contrast microscopy readily reveal the presence of a capsule in bacteria having this structure. Capsules occur only in some Gram-positive and Gram-negative bacterial species, such as the pneumonia-causing pathogens *Streptococcus pneumoniae, Klebsiella pneumoniae,* and *Haemophilus influenzae.*

The capsule is especially important in protecting bacterial cells against phagocytosis by some eukaryotic cells. When most bacterial cells lacking a capsule infect human tissues, they are engulfed by phagocytic white blood cells and digested. Phagocytic white blood cells are one of the major defenses against infections. Bacterial cells with capsules are much more difficult for phagocytic white blood cells to engulf. Bacteria lacking a capsule are easily destroyed by such cells in the lungs. The presence of a capsule, therefore, can be a major factor in determining the virulence of a bacterium, that is, the ability of that bacterium to cause disease. Capsule-producing bacteria such as *Streptococcus pneumoniae* and *Klebsiella pneumoniae* are relatively resistant to the phagocytic white blood cells that protect the lungs against infections, which is why many of the bacteria that cause pneumonia have capsules. Capsules also protect the bacteria they surround from desiccation (drying) and virus attack.

A capsule is a polysaccharide layer that surrounds some bacterial cells external to their cell walls and protects the bacterial cell against phagocytosis.

In some bacteria, the polysaccharide surface layer is less firmly attached to the bacterial cell and is called a **slime layer.** *Pseudomonas aeruginosa,* a bacterium that produces a significant slime layer, is a particular problem for patients with cystic fibrosis (FIG. 5-15). These bacteria become entrapped in the mucus of the respiratory tract of individuals with cystic fibrosis and their slime layer contributes to difficulty in breathing.

The **glycocalyx** is a less organized structure than the capsule (FIG. 5-16). The glycocalyx, like the cap-

FIG. 5-14 Colorized electron micrograph showing capsules *(red)* of the bacterium *Alcaligenes faecalis*. The capsule surrounds and protects the cell.

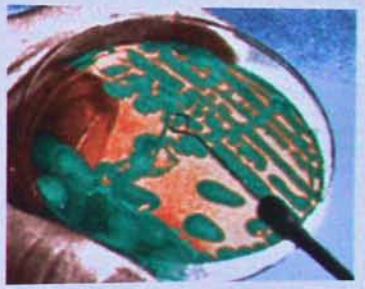


FIG. 5-15 Abundant mucoid material produced by a strain of *Pseudomonas aeruginosa* isolated from a patient with cystic fibrosis. Note adherence of slime with bacterial cells to the inoculating loop.

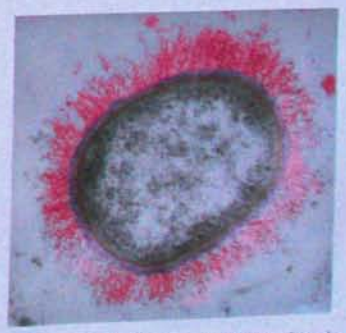


FIG. 5-16 Colorized electron micrograph of the glycocalyx *(red)* of a Gram-negative bacterium. (58,500×).

sule, is often made of polysaccharide and surrounds some bacterial cells. The glycocalyx was not observed until very recently. This structure has a very high water content. This makes it difficult to view by light microscopy. It also makes it easy to destroy during the dehydration steps used in preparing specimens for viewing with the electron microscope.

The glycocalyx allows the bacterial cell to attach to solid surfaces. By attaching to surfaces, bacterial cells maintain themselves in a location that has favorable conditions for growth and survival. Some bacteria in aquatic habitats seem to attach to rocks through their glycocalyces. These bacteria obtain nutrients from water as it flows by. They do not have to expend energy in search of food. Other pathogenic bacteria adhere to the animal tissues they invade via a glycocalyx. This enables them to obtain nutrients from the animal.

Some bacteria adhere to the surfaces of teeth via their glycocalyces. These bacteria produce a glycocalyx that consists of sticky polysaccharides made from sucrose. This extensive polysaccharide material attaches the bacteria to the tooth surface. It also entraps other oral bacterial cells. This layer of polysaccharide and entrapped bacterial cells is called **dental plaque** (FIG. 5-17). When dental plaque secures bacteria to the surfaces of teeth, it also traps lactic acid produced by the adhering bacteria. The trapped lactic acid eats away at the enamel surface of teeth and may result in dental caries, that is, tooth decay. Treatment of teeth with fluorides helps make the enamel more resistant to the action of lactic acid, reducing the frequency of tooth decay.

FIG. 5-17 Glucan *(red)* production allows bacteria to adhere to teeth, forming dental plaque. This colorized electron micrograph shows bacterial cells and the dextran matrix of plaque. (10,200×).

The glycocalyx allows bacteria to attach to surfaces.

Pili

Some bacterial cells produce short (less than 1 μm) hairlike projections made of protein (FIG. 5-18). Such projections are called **pili** or **fimbriae.** Pili are involved in specific attachment (adhesion) processes. For example, pili permit some bacteria to adhere to rocks in flowing rivers. Pili also permit some bacteria to attach to cells and to grow on the surfaces of the cells. Sometimes there is sufficient growth to cause disease in the host organism. For example, *Vibrio cholerae* attaches to the surfaces of cells lining the human gastrointestinal tract via its pili, where it reproduces, causing the disease cholera (FIG. 5-19).

A special type of pilus called the *F pilus* or *sex pilus* is involved in mating between bacteria. During mating, DNA is passed from a donor to a recipient bacterial cell. In the absence of an F pilus, mating between bacterial cells cannot occur. F pili are found exclusively on the donor cells that donate DNA during this process. Recipient cells lack F pili.

Pili are hairlike surface projections that attach bacterial cells to other cells and surfaces.

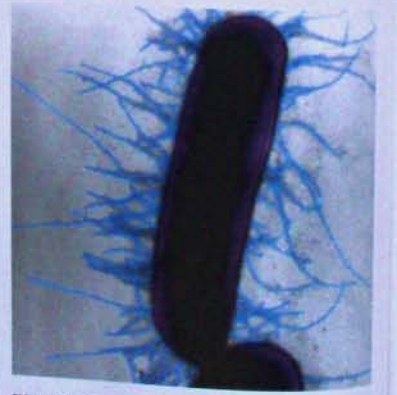


FIG. 5-18 Colorized electron micrograph of pili *(blue)* emanating from the surface of a cell of *Escherichia coli.* (16,400×).

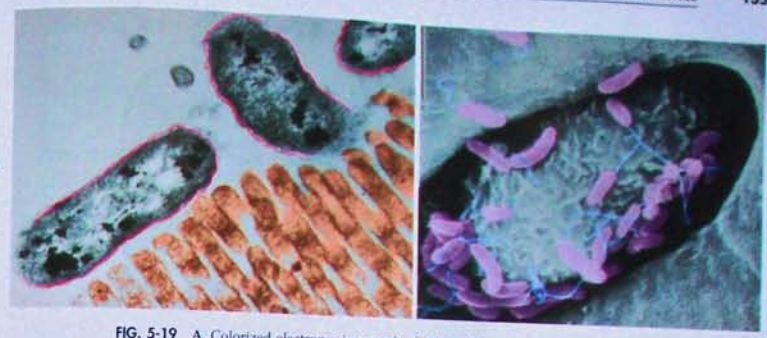


FIG. 5-19 **A,** Colorized electron micrograph of *Vibrio cholerae* adhering to the cell lining *(tan)* of the gastrointestinal tract via its pili. **B,** Colorized scanning electron micrograph of *Vibrio cholerae* attachment to cells in human ileal mucosa via pili *(blue).*

Bacterial Flagella

Flagella are structures that project from the cell surface and propel the cell. Bacteria with flagella are able to move. Some bacteria have only one attached flagellum, whereas others have numerous flagella. The arrangement of the flagella on the bacterial surface is characteristic of a particular genus and is an important diagnostic characteristic used in classifying bacteria (FIG. 5-20). In some bacteria such as *Pseudomonas,* one or more flagella emanate from one end, or pole, of the cell. In other bacteria such as *Spirillum,* flagella may project from both ends of the cell. Whether they emerge from one or both ends of the cell, such flagella are called **polar flagella.** In other bacteria, such as those of the bacterial genus *Proteus,* flagella surround the entire bacterial cell. Flagella that occur around a bacterial cell are called **peritrichous flagella.**

Flagella are structures that propel bacterial cells.

Polar flagella emanate from the ends of cells.

Peritrichous flagella emanate from points all around the cell.

Bacterial flagella extend from the plasma membrane as relatively long, rigid helixes, usually several

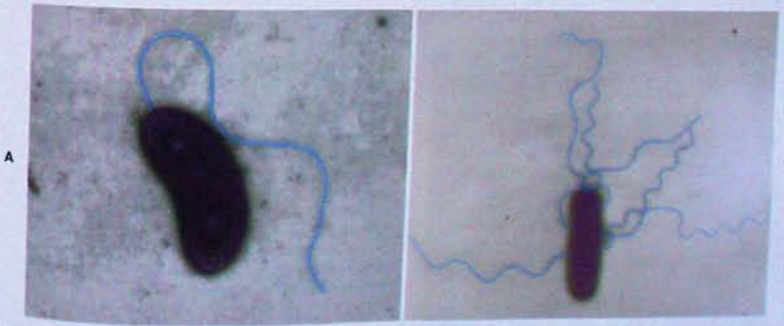


FIG. 5-20 **A,** Colorized electron micrograph of a *Vibrio* sp. shows that a single polar flagellum *(blue)* emanates from the end of the cell. (25,500×). **B,** Colorized electron micrograph of a *Salmonella* sp. shows that peritrichous flagella *(blue)* arise anywhere on the cell. (20,000×).

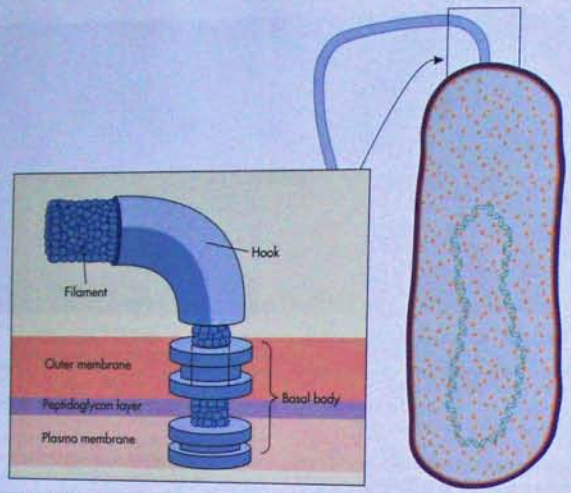


FIG. 5-21 The flagellum is anchored to the cell via a hook and basal body structure. There are rings that join the flagellum to the outer and plasma membranes of a Gram-negative cell. This structure permits the flagellum to rotate. The energy for rotation comes from the protonmotive force.

times longer than the length of the cell. Each bacterial flagellum consists of a single tubelike filament composed of a protein called flagellin. An individual flagellum does not grow from the base, like an animal hair; rather, it grows from its tip. Flagellin molecules formed within the cell pass up through the hollow core of the flagellum and add on at the tip.

Each flagellum is attached to the bacterial cell by a hook and basal body (FIG. 5-21). The basal body is a complex structure consisting of a small central rod that passes through a system of rings. In Gram-negative bacteria, there are two pairs of rings: an outer pair of rings in the cell wall and an inner pair of rings in the plasma membrane. Gram-positive bacteria, which lack the outer lipopolysaccharide layer, only have an inner pair of rings attached to the plasma membrane. The hook is a tubular structure that passes between and extends just beyond the rings of the basal body. The hook has a wider diameter than the filament of the flagellum so that the filament fits into the hook and is thereby attached to the bacterial cell.

The attached flagellum can spin within the rings. It spins much like the shaft of an electric motor spinning within surrounding magnetic coils. The structure of the bacterial flagellum allows it to spin. The propeller-like rotation of the bacterial flagellum propels the bacterial cell from place to place. This adaptive feature increases the bacterium's ability to obtain nutrition or to escape from hostile microenvironments. Bacterial flagella propel the cell by rotating.

Bacterial flagella rotate to propel the cell.

The bacterial flagellum provides some bacteria with a mechanism for swimming toward or away from particular chemicals. This behavior is known as **chemotaxis** (FIG. 5-22). Some bacteria move toward certain chemicals *(attractants)* and away from others *(repellents)*. Sensory structures associated with the cell wall or plasma membrane of the cell, called *chemosensors*, detect certain chemicals and signal the flagella to respond. The chemosensors permit the detection of concentration gradients as the bacterium moves so that the bacterium can detect whether it is moving toward a region of higher or lower concentration of a specific substance.

Some bacteria exhibit chemotaxis in which they move in response to chemical concentration gradients; chemotaxis indicates that some bacteria can sense and respond to their chemical environment.

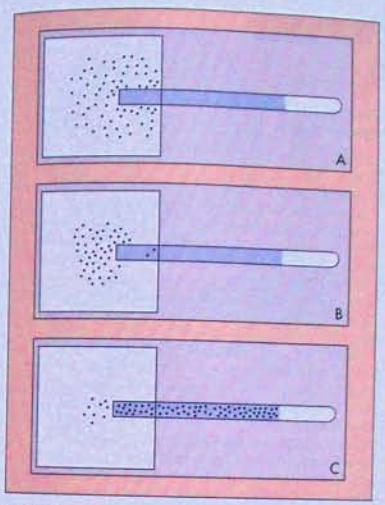


FIG. 5-22 Chemotactic behavior is readily demonstrated and measured by placing the tip of a thin capillary tube containing an attractant solution in a suspension of motile *Escherichia coli* bacteria. The suspension is placed on a slide, under a cover slip. **A,** At first, the bacteria are distributed at random throughout the suspension. **B,** After 20 minutes, they have congregated at the mouth of the capillary tube. **C,** After about an hour, many cells have moved up into the capillary tube. If the capillary tube had contained a repellant, few bacteria, if any, would have entered. Using this technique, it is possible to show which chemicals attract bacteria and which do not.

When bacteria move, they periodically change direction. They do not reach their destination by swimming in a single straight line. Their movement consists of a series of *runs* (straight line movements) and *tumbles* (turning motions). Counterclockwise rotation of the flagella produces runs. Tumbling occurs when the flagella rotate in a clockwise direction. The direction of flagella rotation, and hence the length of a run, is determined by the interactions of the chemosensors with attractants or repellents in the cell's plasma membrane, or in the periplasmic gel. An increasing concentration of attractant, for example, interacts with the chemosensors to decrease the frequency of tumbling. A decreasing concentration of attractant causes increasing tumbling, and hence shorter runs. The reverse is true for the interactions with repellents, where increasing concentration of the repellent causes increased tumbling. The net effect of this process is an overall movement toward an attractant or away from a repellent.

Eukaryotic Flagella and Cilia

In contrast to the rather simple structure of the bacterial flagellum, the flagella and cilia of eukaryotic microorganisms are larger, far more complex, and operate by entirely different mechanisms. **Eukaryotic flagella** also propel cells. They originate from the polar region of the eukaryotic cell (FIG. 5-23). **Cilia** generally are also involved in cell movement. They surround the cell and are more numerous and shorter than flagella (FIG. 5-24). Unlike the rigid bacterial flagella that rotate, cilia and flagella of eukaryotic cells undulate in a flexible wave-like motion to propel the cell.

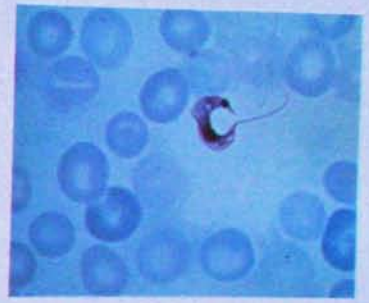


FIG. 5-23 Photomicrograph of a flagellate protozoan *Trypanosoma cruzi*, which is a human pathogen.

FIG. 5-24 Colorized scanning electron micrograph of the ciliate protozoan *Tetrahymena*.

HIGHLIGHT

Magnetotaxis: Specialized Movement of Bacteria in Response to Magnetic Fields

A few bacteria can sense and respond to magnetic fields. The movement of bacteria in response to a magnetic field is known as *magnetotaxis*. These bacteria orient their movements in relation to the Earth's magnetic field. The magnetotactic bacteria were discovered by Richard Blakemore while viewing the movement of some Gram-negative bacteria obtained from sulfide-rich lake mud. He observed that the bacteria were all moving in the same direction across the microscope's field of view. Even when he turned the microscope around or moved it to another location in the room these bacteria continued to swim in the same geographic direction.

The bacteria did not appear to be swimming in response to any particular chemical stimulus. They continued to swim in one direction even in a darkened room. Blakemore hypothesized that the bacteria might be orienting their movements to the Earth's magnetic field. To test his hypothesis, Blakemore placed a magnet adjacent to the microscope slide. He observed that the bacteria instantly began to swim toward the end of the magnet that attracts the north-seeking end of a compass needle. When he turned the magnet around, the bacteria also turned around. They began to swim back toward the same end of the magnet that they had been previously moving toward. These observations clearly showed that these bacteria could orient the direction of their movements in response to a magnetic field.

Blakemore then hypothesized that if he collected bacteria from the Southern Hemisphere they would show the opposite responses to magnetic fields. When he examined magnetotactic bacteria from New Zealand, in the Southern Hemisphere, they swam toward the south pole and away from the north pole of a magnet. Magnetotactic bacteria from the Northern and Southern Hemispheres swim in opposite directions in response to a magnetic field.

Blakemore continued his studies by observing the cells of these bacteria with an electron microscope. He observed dense particles within the bacterial cells of these bacteria. He did not observe such particles in bacteria that did not exhibit magnetotactic behavior (see Figure). Analysis of these particles showed that they were composed of iron oxide (Fe_3O_4), a magnetic metal compound. The iron oxide is stored in structures, called *magnetosomes*, that act like magnets and allow bacterial cells containing them to respond to magnetic fields and, thus in their natural environments, to orient themselves along the Earth's magnetic field. This enables magnetotactic aquatic bacteria to point their cells downward toward sediments where nutrients are more abundant and where oxygen concentrations are favorable for their growth.

Colorized electron micrograph of a section of *Aquaspirillum magnetotacticum* shows characteristic magnetosomes (*red granules*) that allow this bacterium to respond to a magnetic field. (42,900×).

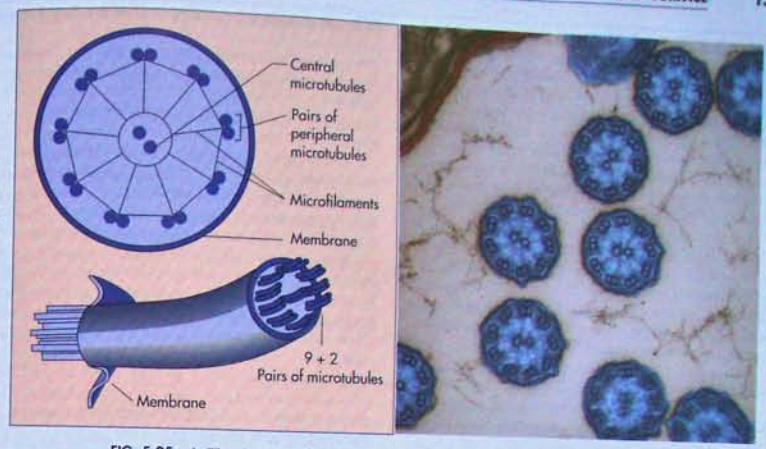


FIG. 5-25 **A,** The structure of the eukaryotic flagellum and cilia has nine pairs of peripheral microtubules surrounding a central pair of microtubules. The microtubules are connected by microfilaments. The peripheral microtubules slide past the central microtubules, causing the flagellum or cilia to bend. **B,** Electron micrograph of a cross section of the cilia of the ciliate protozoan *Mesodinium*. (98,800×).

Both eukaryotic flagella and cilia consist of a series of microtubules (FIG. 5-25). These microtubules are hollow cylinders composed of proteins surrounded by a membrane. The protein that makes up the microtubule is called tubulin. The arrangement of microtubules in eukaryotic flagella and cilia is known as the "9 + 2" system. It consists of nine pairs of peripheral microtubules that form a circle around two single central microtubules. Attached to each of the peripheral pairs of microtubules are molecules of a protein called dynein. Dynein is involved in the conversion of chemical energy from ATP into the mechanical energy of flagellar movement. The movement of flagella or cilia is based on a sliding microtubule mechanism. The peripheral pairs of microtubules slide past each other, resulting in bending of the flagella or cilia. This causes the flagella or cilia to bend and propel cells with a whiplike motion.

Eukaryotic flagella flex to propel cells based on the sliding movement of microtubules.

Cilia may also be involved in moving materials such as food particles past the cell surface while the eukaryotic cell remains stationary. Paramecia use such movement of cilia to obtain food. Some human tissues have cells with cilia. For example, the cells lining the human respiratory tract have cilia that beat with an upward wave-like motion. They sweep mucus outward and prevent particles such as bacteria and viruses trapped in the mucus from reaching the lungs. Cilia thus help to defend the human respiratory tract against microbial infections (FIG. 5-26).

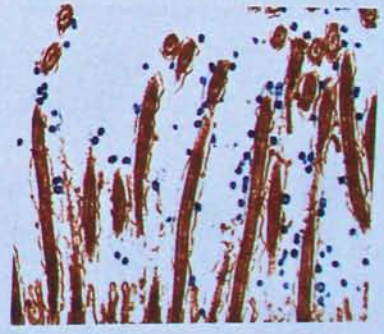


FIG. 5-26 Colorized micrograph of the cilia (*tan*) lining the human respiratory tract. These cilia help prevent viruses, such as those shown in blue, from entering the body via the respiratory tract.

DNA is sometimes called the "master molecule" of all living cells. It contains the hereditary information that is passed from generation to generation. DNA determines the potential metabolic and morphological characteristics of the cell. It also regulates the expression of genetic information. The total genetic material of an organism is called the **genome.** The genome is divided into functional segments, or genes. Each **gene** specifies the amino acid sequences of specific proteins or regulates when particular proteins or RNA molecules are to be made. The DNA in a bacterial cell typically contains about 6,000 genes. Passage of replica copies of the DNA to progeny cells during reproduction transmits the hereditary information from one generation to the next.

The genetic information **(genotype),** which is stored in the DNA, is expressed through the production of functional proteins. It controls the **phenotype** (appearance) of the cell, that is, the cell's morphology and metabolism. In this way, the genetic information determines the properties and structural characteristics of the cell. The actual steps in the expression and transmission of genetic information, which are two essential life functions, will be considered in detail when we examine microbial genetics in Chapter 7. Here, we will only discuss the way in which genetic information is stored in prokaryotic and eukaryotic cells and the structures involved in the expression of this information.

Bacterial Chromosome

In the bacterial cell, almost all the genetic information is contained within a single DNA macromolecule called the **bacterial chromosome.** The cytoplasmic region occupied by the bacterial chromosome is referred to as the *nucleoid region*. This region is not actually surrounded by a covering or membrane. It can move within the cell (FIG. 5-27).

The bacterial chromosome is composed virtually exclusively of DNA. The DNA forms a double helix—two strands wound around each other, giving the appearance of a spiral staircase with two handrails. The bacterial chromosome is a closed loop of DNA. If this circular bacterial chromosome were to be cut open, the linear DNA molecule formed would be approximately 1 mm (1,000 μm) long. Since most bacterial cells are less than 5 μm in length, bacterial DNA must be extensively coiled into a twisted molecule. This twisted molecule is called supercoiled DNA. Supercoiled DNA is very compact and fits within the bacterial cell. This molecule houses all the essential information that determines the structural and functional characteristics of the bacterial cell.

The bacterial chromosome is circular and houses the essential genetic information of the cell.

Plasmids

Some bacterial cells also contain one or more small circular macromolecules of DNA that store additional specialized information. These are known as plasmids. **Plasmids** contain a limited amount of specific genetic information. This information supplements the essential genetic information contained in the bacterial chromosome. Plasmids contain only 1% to 5% as much DNA as is in the bacterial chromosome, that is, about 20 genes. Nevertheless, the supplemental genetic information contained in plasmids can be quite important. They can establish such characteristics as resistance to antibiotics and tolerance to heavy metals. The gene products of plasmids may permit the survival of the bacterium under conditions that are normally unfavorable for growth and survival. Having a plasmid that codes for resistance to an antibiotic, for example, can mean 100% survival for a bacterium. Plasmids can be transferred from one bacterial cell to another, sometimes even from one bacterial species to another. Thus plasmids conferring antibiotic resistance can become prevalent among bacteria surviving in areas where antibiotics are widely used, such as within hospitals. For this reason physicians limit the use of antibiotics to situations where they will be especially useful in treating a specific disease.

Plasmids are small circular DNA molecules that contain supplemental genetic information that can enhance survival potential, including resistance to antibiotics.

FIG. 5-27 Colorized electron micrograph of a section of the bacterium *Mycobacterium phlei* showing the nucleoid region *(green)* within the cytoplasm where the bacterial chromosome occurs.

Nucleus of the Eukaryotic Cell

The DNA of eukaryotic cells is contained in a specialized organelle called the **nucleus.** The nucleus is segregated from the rest of the cell because it is surrounded by two membranes, the **nuclear envelope** (FIG. 5-28). The nuclear envelope is rather porous so that exchange of molecules such as RNA and proteins can easily occur between the cytoplasm and the fluid within the nucleus. This fluid is called the **nucleoplasm.** DNA and associated proteins are suspended in the nucleoplasm. The nucleus also contains a region called the **nucleolus** where ribosomes are assembled.

DNA in eukaryotic cells is contained within the nucleus, an organelle surrounded by a nuclear envelope that separates the nucleoplasm from the cytoplasm.

The critical functions that occur within the nucleus are the processing and transmission of hereditary information. When a eukaryotic cell is about to divide, the DNA and its associated proteins fold and twist into condensed structures called **chromosomes.** Chromosomes can be observed through a light microscope (see FIG. 5-28). Chromosomes pass the hereditary information from one generation to the next. Eukaryotic chromosomes are linear and generally occur in pairs. Each individual chromosome in the pair has virtually the same genes. Thus there generally is a duplication of genetic information in eukaryotic cells that is not found in bacterial cells. Most eukaryotic cells are *diploid* (having two copies of each gene), whereas prokaryotic cells are *haploid* (having one copy of each gene).

When a nondividing eukaryotic cell is stained for observation through a light microscope, all that can

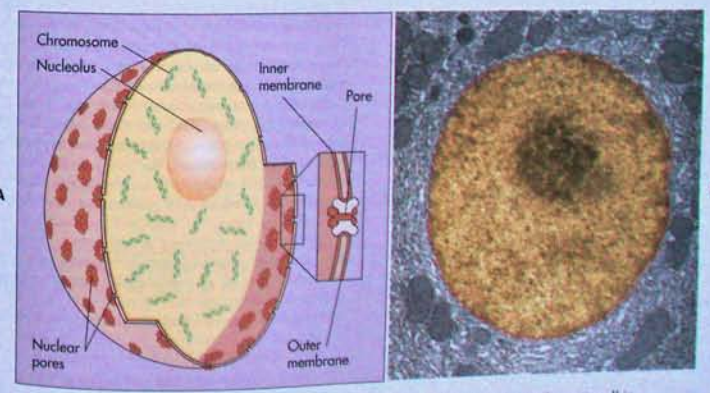


FIG. 5-28 **A,** The nucleus that contains the hereditary information in a eukaryotic cell is surrounded by two membranes: an inner and an outer membrane. The nucleolus within the nucleus is the site where ribosomal subunits are made. There are pores in the membranes through which materials can move, including messenger RNA that carries information from the DNA within the nucleus to the ribosomes in the cytoplasm. **B,** Colorized micrograph of the nucleus of a eukaryotic cell shows the double membrane structure and the pores of this organelle that contains the chromosomes of eukaryotic cells. (8,400×).

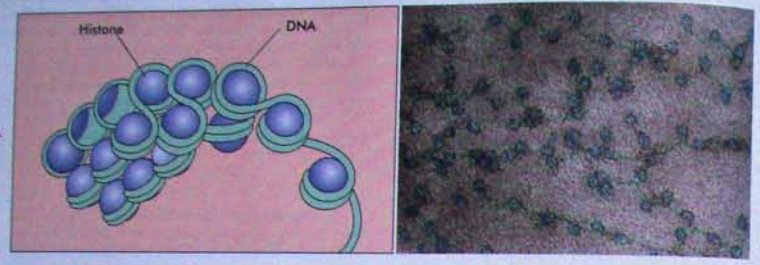


FIG. 5-29 **A,** A nucleosome showing how DNA is wrapped around histones (basic proteins), establishing coiling of DNA within the nucleus of eukaryotic cells. **B,** Colorized micrograph of a region of a chromosome showing the beadlike appearance of nucleosomes.

be seen within the nucleus is a grainy material, called *chromatin*. Chromatin is composed of strung-out DNA and its associated proteins. The DNA of the chromatin directs the metabolic activities of the eukaryotic cell. Some proteins of the chromatin, called **histones**, maintain the highly coiled shape of the DNA macromolecule. The DNA wound around the histones forms subunits of chromatin known as *nucleosomes* (FIG. 5-29). Each nucleosome is composed of about 200 nucleotides coiled around several different histones. When viewed by electron microscopy, nucleosomes appear as spherical particles, like beads on a string.

Chromatin consists of DNA and associated proteins, including histones that help maintain the shape of the DNA molecule.

DNA in chromatin is coiled around the histones to form nucleosomes.

RIBOSOMES

The expression of genetic information requires the formation of proteins—particularly enzymes that are the action molecules that determine what a cell is and what it does. All cells use the genetic information stored in their DNA to direct the production of specific proteins. First, the information in the DNA is transferred to RNA in a process called *transcription*. Following transcription, some RNA molecules, called *messenger RNA (mRNA)*, carry the genetic information to the ribosomes in the cytoplasm of the cell.

Ribosomes are the structures within a cell where proteins are made. It is at the ribosomes that protein synthesis, *translation*, occurs. During translation the information in RNA is used to direct the synthesis of a specific protein. Thousands of different proteins are continuously needed to support the metabolism of a cell. A typical prokaryotic cell may have 10,000 or more ribosomes. Eukaryotic cells contain considerably more.

Ribosomes are the structures within cells where protein synthesis occurs.

Functional ribosomes are made up of two subunits, a larger and a smaller one. Each subunit is made up of specific RNA molecules and a group of different proteins. Protein synthesis can occur only when the subunits are joined. In eukaryotic cells the ribosomal subunits are produced within the nucleus. They then are exported through the pores of the nuclear envelope to the cytoplasm where they are assembled into functional ribosomes.

Ribosomes of bacteria are different from those of eukaryotic cells. Bacterial cells have **70S ribosomes.** The ribosomes in the cytoplasm of eukaryotic cells are **80S ribosomes** (FIG. 5-30). (Eukaryotic cells also have 70S ribosomes within specialized organelles involved in ATP generation: mitochondria and chloroplasts.) "S" stands for Svedburg units. Svedburg units represent a combined measure of molecular weight and shape derived from the rate of sedimentation in an ultracentrifuge. An ultracentrifuge is a centrifuge that spins at very high speeds, in excess of 25,000 revolutions per minute.

Bacterial cells have 70S ribosomes.

Eukaryotic cells have 80S ribosomes in the cytoplasm.

The difference between eukaryotic and prokaryotic ribosomes is significant. Various antibiotics discriminate between 70S and 80S ribosomes. Antibiotics such as erythromycin, tetracyclines, and many others are effective for treating human bacterial infections because they are able to block protein synthesis by specifically binding with the 70S ribosomes of bacterial cells. These antibiotics do not bind to 80S eukaryotic ribosomes in the cytoplasm. They do not disrupt protein synthesis at the 80S ribosomes of human cells. Therefore they can be used to selectively inhibit protein synthesis of infecting bacteria and can be used therapeutically in the treatment of human infections caused by pathogenic bacteria. (A few adversely affect a patient because human cells have 70S ribosomes within mitochondria and these have more limited therapeutic uses.) Once again, a fundamental difference between prokaryotic and eukaryotic cell structure provides the opportunity for selective drug action against infecting bacteria while leaving eukaryotic human cells untouched.

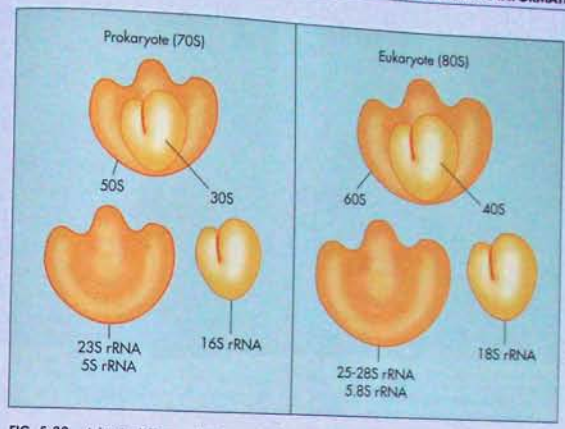


FIG. 5-30 A basic difference between prokaryotic and eukaryotic cells is the nature of the ribosomes in the cytoplasm. The prokaryotic cell has 70S ribosomes composed of 30S and 50S subunits. The 30S subunit contains about 21 proteins and a 16S rRNA molecule, having approximately 1,540 nucleotides; the 50S subunit is composed of approximately 34 proteins, a 23S rRNA, having approximately 2,900 nucleotides, and a small 5S rRNA species having only about 120 nucleotides. A eukaryotic cell has 80S ribosomes in its cytoplasm composed of 60S and 40S subunits. The 40S subunit contains proteins and an 18S rRNA, and the larger 60S subunit has proteins, 25S to 28S rRNA, and 5.8S rRNA. These differences form the basis for the specificity of action of some antibiotics that inhibit protein synthesis.

CELLULAR STRUCTURE AND ENERGY TRANSFORMATIONS

Besides having mechanisms for storing and expressing genetic information, cells must have a means of obtaining energy to carry out life functions. Cells do not create energy. Cells transform the energy they obtain from light or chemicals to produce usable cellular energy stored as molecules of ATP. Many of the chemical reactions that occur during metabolism are involved in generating ATP. Cells must generate sufficient ATP for their maintenance, growth, and reproduction, or they will die. The metabolic reactions that generate ATP in prokaryotic or eukaryotic cells can occur in either of two locations. They can occur within the cytosol or in association with specialized membranes, or at both sites.

Living systems depend on their ability to generate ATP, the universal currency of energy transfer within the cell.

The mechanism by which ATP is generated at membranes was proposed in 1965 by Peter Mitchell. Initially the scientific community rejected Mitchell's hypothesis for ATP generation but later accepted his

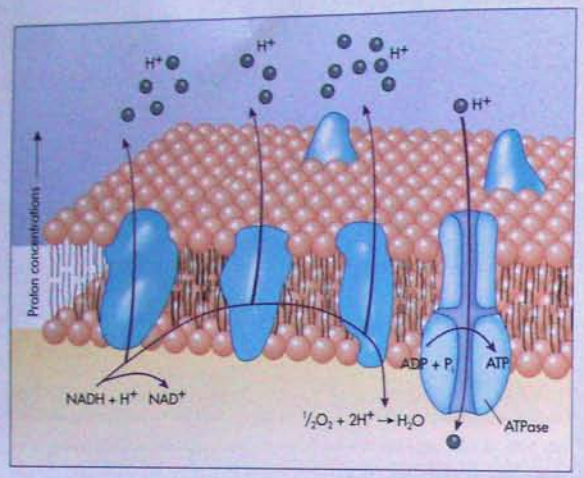


FIG. 5-31 The protonmotive force drives the formation of ATP by chemiosmosis. Protons are extruded across the membrane to establish a proton gradient. The membrane is impermeable to protons, which can only move back across the membrane through protein channels associated with ATPase. As the protons move by diffusion through these channels, energy is transferred to form ATP.

idea and awarded him a Nobel Prize in 1978. Mitchell reasoned that transport of substances across the membrane and the semipermeable properties of biological membranes have a special role in the generation of ATP by many organisms. He proposed that protons (hydrogen ions) are extruded across membranes as a result of oxidation-reduction reactions that occur when electrons are transferred between compounds embedded within the membrane. The metabolism of the cell is thus connected to the establishment of a proton gradient across the membrane (FIG. 5-31). The concentration difference between hydrogen ions on the opposing sides of the membrane represents a potential energy called the **protonmotive force.** The protonmotive force can be used to drive the formation of ATP. This process for the generation of ATP is called **chemiosmosis.** In chemiosmosis, energy from metabolic oxidations is used to move protons (H^+) across a membrane, establishing a concentration gradient. Diffusion of H^+ back across the membrane is coupled to the synthesis of ATP.

Chemiosmosis is the process in which the protonmotive force (potential energy across a membrane based on a concentration gradient of protons) is used to generate ATP.

Sites of Chemiosmotic Generation of ATP in Prokaryotic Cells

In bacterial respiration the plasma membrane is an important site of chemiosmotic generation of ATP. Protons are moved across the plasma membrane out from the cell. The concentration gradient of protons establishes the protonmotive force that is used to generate ATP. When protons diffuse back across the plasma membrane, ATP is generated by chemiosmosis. In some specialized groups of bacteria, internal membranes are similarly involved with chemiosmotic generation of ATP (FIG. 5-32). Such specialized bacteria include the nitrifying bacteria and the photosynthetic bacteria. Nitrifying bacteria are bacteria that oxidize inorganic nitrogen-containing compounds to generate ATP. Photosynthetic bacteria use light energy to generate ATP. In photosynthetic bacteria the internal membranes are the sites where light energy is converted to chemical energy in the form of ATP during photosynthesis. These specialized photosynthetic membranes can be simple extensions of the plasma membrane (as in the purple sulfur bacteria), cylindrically shaped vesicles (as in the green photosynthetic bacteria), or an extensive multilayered system of membranes (as in the cyanobacteria) (FIG. 5-33).

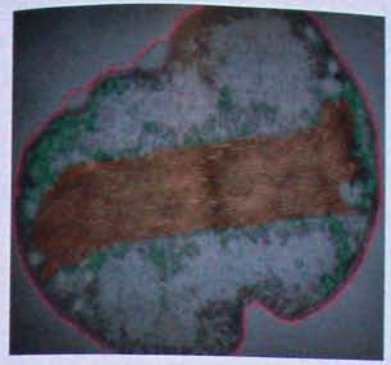


FIG. 5-32 Colorized electron micrograph of a section of the nitrifying bacterium *Nitrosococcus oceanus* showing internal membrane *(tan).*

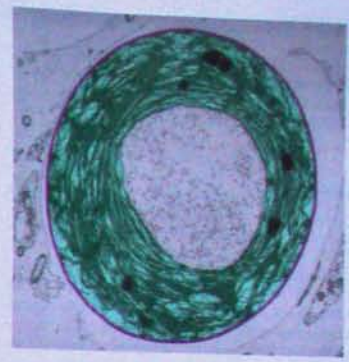


FIG. 5-33 Colorized electron micrograph of the photosynthetic bacterium *Prochloron didemni* reveals that it has extensive internal membranes that have photosynthetic pigments *(green).* (6,400×). These membranes are the sites of chemiosmotic generation of ATP by this bacterium, which derives the energy for ATP formation from light energy.

Sites of Chemiosmotic Generation of ATP in Eukaryotic Cells

Mitochondria

Mitochondria are important sites of chemiosmotic ATP generation in respiring eukaryotic cells (FIG. 5-34). These organelles appear to have arisen as a result of endosymbiotic evolution and, like prokaryotic cells, contain 70S ribosomes and a circular DNA macromolecule. Some yeast cells have as few as two mitochondria per cell. Other eukaryotic cells have many more. Many animal cells, for example, contain 1,000 mitochondria. A mitochondrion is a double membrane–bounded compartment. It has an interior membrane that forms a separate compartment and an outer membrane that acts as the boundary between the mitochondrion and the cell cytosol. The establishment of a proton gradient across the inner membrane is used for the chemiosmotic generation of ATP.

The inner membrane of the mitochondrion has many folds or convolutions. These folds are called *cristae.* They increase the surface area. Particles of ATPase, the enzyme that catalyzes the chemiosmotic generation of ATP, are bound to the cristae. Protein

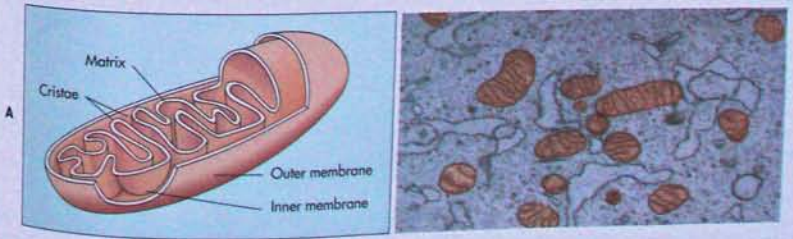


FIG. 5-34 **A,** A mitochondrion is the site of ATP generation by chemiosmosis in eukaryotic cells. There are two distinct membranes and extensive folding of the internal membrane. Protons are pumped across the inner membrane into the space between the inner and outer membranes. This establishes the protonmotive force that drives the formation of ATP. **B,** Colorized electron micrograph of mitochondria *(tan)* of a human cell. (11,900×).

channels in the outer membrane allow passage of any molecule of molecular weight less than approximately 10,000. This permeability permits the ATP produced within the mitochondria to move into the cytosol, where it is used in energy-requiring reactions.

Mitochondria are the sites of chemiosmotic generation of ATP in eukaryotic cells.

Chloroplasts

Chloroplasts are the structures where photosynthetic generation of ATP occurs in algal and plant cells (FIG. 5-35). They are quite similar in many ways to mitochondria in structure and function. They appear to have arisen as a result of endosymbiotic evolution. Chloroplasts are composed of extensively invaginated membranes, contain 70S ribosomes, and have a circular DNA macromolecule—as do the mitochondria. Like the mitochondrion, the chloroplast contains an outer membrane that separates the organelle from the cytosol and an inner membrane.

Chloroplasts also have an additional complex internal membranous system, known as the **thylakoids.** Thylakoids are sac-like membranous vesicles that contain various photosynthetic pigments. These pigments include the chlorophylls that are the primary photosynthetic pigments involved in the conversion of light energy to cellular chemical energy. Groups of thylakoids may be organized to form densely stacked piles called *grana.*

The fluid within the chloroplast, called the *stroma,* is where the fixation of carbon dioxide occurs during photosynthesis. The thylakoid membranes of the chloroplasts are highly impermeable to protons, so that protons moved across the membrane cannot freely diffuse back across the membrane. The proton gradient and associated protonmotive force established across thylakoid membranes is used to drive the synthesis of ATP by chemiosmosis when protons move through ATPase channels in the membranes. This chemiosmotic generation of ATP at the thylakoid membranes of chloroplasts is analogous to the synthesis of ATP in the mitochondria.

Chloroplasts are the structures where photosynthetic chemiosmotic generation of ATP and carbon fixation occur in eukaryotic algal and plant cells.

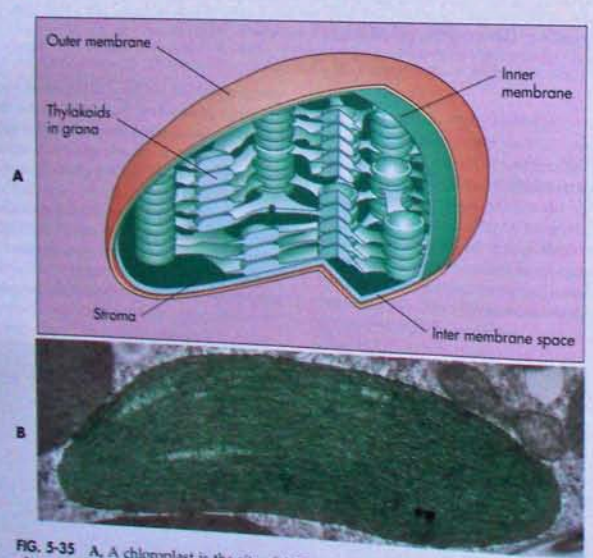


FIG. 5-35 **A,** A chloroplast is the site of ATP generation by chemiosmosis in eukaryotic photosynthetic cells. Light energy is trapped by the chlorophyll in the chloroplast. This initiates a process in which water is converted into oxygen (O_2) and protons (H^+) There are two distinct membranes. Protons are pumped inward across the inner membrane. This establishes the protonmotive force that drives the formation of ATP. The return flow of protons into the space between the inner and outer membranes passes through a protein channel associated with ATPase. **B,** Colorized electron micrograph of a chloroplast (*green*) of the alga *Euglena proxima.* (28,900×).

SEGREGATION AND MOVEMENT OF MATERIALS WITHIN CELLS

In addition to generating ATP as a usable form of cellular energy, cells must move substances from one place to another within the cell. In prokaryotes this is relatively simple. The lack of internal membrane-bound organelles means that substances can freely mix within the cell's cytoplasm. To compensate for the lack of internal compartments, some functions occur extracellularly or within the cytoplasmic membranes of the prokaryotic cells that occur within the organelles of eukaryotic cells. Some bacterial cells form relatively insoluble reserve materials, such as the fatlike molecule poly-β-hydroxybutyric acid (PHB), the phosphate-rich polyphosphate (also called volutin or metachromatic granules), and elemental sulfur granules. Because they are nonpolar molecules and hence do not readily dissolve in cytosol, these reserve materials naturally segregate from the other components within the cell and clump, forming granules (FIG. 5-36).

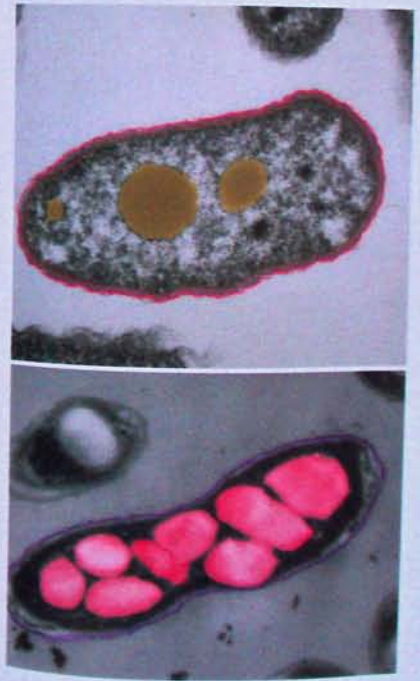


FIG. 5-36 **A,** Polyphosphate (*gold*) accumulates in some bacterial cells such as *Pseudomonas aeruginosa* (colorized electron micrograph; 44,100×). **B,** The polyhydroxybutyrate inclusions (*pink*) of a *Vibrio* species nearly fill the cells of this bacterium (colorized electron micrograph; 32,800×).

Cytomembrane System of Eukaryotic Cells

In marked contrast to the prokaryotic cell, the eukaryotic cell is filled with membranous organelles involved with the processing and storage of materials within the cell (Table 5-2, p. 148). These membrane-bound organelles physically separate different chemicals so that they do not mix haphazardly within the cytoplasm. The extensive internal membrane system of eukaryotic cells also permits the segregation of chemical reactions that might interfere with one another. Organelles furthermore separate different chemical reactions in space and time. Molecules being used and produced are passed from one organelle to another in specific reaction sequences. However, this segregation increases the need to coordinate and manage the functions of the cell's subunit organelles.

Many of the organelles of the eukaryotic cell are linked together. They function in a coordinated manner through what is collectively called the *cytomembrane system.* The cytomembrane system includes the nuclear membrane, the endoplasmic reticulum, the Golgi apparatus, lysosomes, vacuoles, and the cytoskeleton. The endoplasmic reticulum isolates, packages, and transports proteins and other substances. The Golgi apparatus is used for the modification and distribution of materials and to produce various membrane-bound vesicles and vacuoles. These membrane-bound vesicles and vacuoles are involved in the storage, processing, and transport of materials. The movement of materials through this cytomembrane system is highly organized. Functioning together, the outer nuclear membrane, the endoplasmic reticulum, the Golgi bodies, and the secretory vesicles perform a sequence of coordinated activities that move and process materials (often proteins) through the cytoplasm to the exterior of the cell.

Eukaryotic cells contain a network of membranous organelles that are involved in the storage and processing of materials.

Endoplasmic Reticulum

As part of their cytomembrane system, eukaryotic cells contain an extensive membranous network known as the **endoplasmic reticulum** (ER) (FIG. 5-37). The endoplasmic reticulum is composed of a se-

TABLE 5-2

Comparison of Structures in Prokaryotic and Eukaryotic Cells

		PROKARYOTIC CELLS		EUKARYOTIC CELLS				
STRUCTURE	FUNCTION	ARCHAEBACTERIA	EUBACTERIA	FUNGI	ALGAE	PROTOZOA	PLANTS	ANIMALS
Plasma membrane	Semipermeable barrier; regulation of substances moving into and out of cell	+	+	+	+	+	+	+
Cell wall (with peptidoglycan)	Protects cell against osmotic shock or physical damage	–	+	–	–	–	–	–
Cell wall (without peptidoglycan)	Protects cell against osmotic shock or physical damage	+	–	+	+	–	+	–
Flagella* that lack microtubules and rotate	Cell movement by rotation	+	+	–	–	–	–	–
Flagella† with microtubules that undulate	Cell movement by flexion	–	–	+	+	+	+/–	+
Cilia†	Cell movement; movement of materials	–	–	–	–	+	+/–	+
Nucleoid	Region of DNA concentration; heredity control	+	+	–	–	–	–	–
Nucleus	Membrane-bound organelle containing DNA; region of heredity control	–	–	+	+	+	+	+
Nucleolus	Formation of ribosomal subunits	–	–	+	+	+	+	+
Bacterial chromosome	Circular molecule that contains genome (hereditary information)	+	+	–	–	–	–	–
Chromosomes	Linear molecules that contain genomes; DNA stores the hereditary information; protein establishes structure of the chromosome essential for gene expression	–	–	+	+	+	+	+
Ribosome	Translation of genetic information carried by mRNA into proteins; protein synthesis	+	+	+	+	+	+	+
Endoplasmic reticulum	Processing and transport of proteins and other substances through cell; communication of chemicals and coordination of functions within cell	–	–	+	+	+	+	+
Golgi body	Processing of substances and packaging of materials into vesicles for export from cell	–	–	+	+	+	+	+
Lysosome	Containment of digestive enzymes; controlled degradation of substances	–	–	+	+	+	+	+
Cytoskeleton	Organization and support of organelles within cell	–	–	+	+	+	+	+
Mitochondrion	Respiratory chemiosmotic generation of ATP	–	–	+	+	+	+	+
Chloroplast	Photosynthetic chemiosmotic generation of ATP	–	–	–	+	–	+	–
Endospore	Survival; heat resistance	–	+	–	–	–	–	–

*Simple protein structure. †Complex structure.

ries of interconnected membranous tubes and sacs. These tubes and sacs form compartments within the cytoplasm. This membranous network appears to provide a communication system that helps coordinate the metabolic activities of the cell. The ER shows two distinct morphologies when examined by electron microscopy. One type of ER (rough ER) looks "rough" because ribosomes are attached to it. The other type, smooth ER, appears smooth because it is not associated with ribosomes. The attachment of ribosomes to the endoplasmic reticulum permits the coordinated synthesis and sequestering of certain proteins. Proteins made at these ribosomes can be sent through the channels of the endoplasmic reticulum to other organelles within the cell for storage, use, modification, or export. Prokaryotic cells have no analogous membrane structure to which ribosomes can attach. They have no system comparable to the endoplasmic reticulum to coordinate movement of materials within the cell.

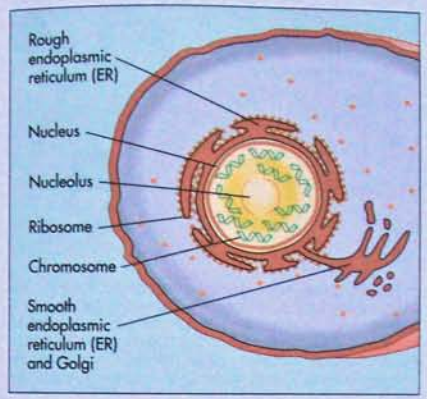


FIG. 5-37 The endoplasmic reticulum (ER) is an extensive membrane network that runs throughout the eukaryotic cell. Regions of the ER that have attached ribosomes are called rough ER; those lacking ribosomes are called smooth ER. These names are derived from the appearances of the ER when viewed by electron microscopy.

The endoplasmic reticulum is an extensive membrane network that coordinates the synthesis, segregation, and movement of proteins in the eukaryotic cytoplasm.

Golgi Apparatus

The **Golgi apparatus** is a series of flattened membranous sacs. It forms a continuous network with the rough endoplasmic reticulum (FIG. 5-38). Substances are packaged at the Golgi and transported to the plasma membrane or organelles within the cell. Vesicles, derived from the endoplasmic reticulum, carry protein and lipids to the Golgi apparatus where these materials can be chemically incorporated into membrane-bound vesicles.

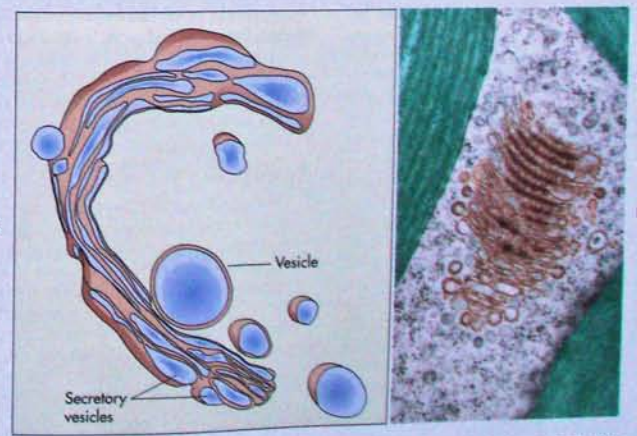


FIG. 5-38 **A**, The Golgi apparatus is involved in the packaging of substances for export from the cell. Secretory vesicles are formed at the Golgi apparatus that carry substances out of the cell. **B**, Colorized electron micrograph of the Golgi apparatus *(tan)* of the alga *Euglena*. (30,400×).

Repackaging of materials into special **secretory vesicles** occurs in the Golgi apparatus. The secretory vesicles move to the plasma membrane where they release their contents through exocytosis. Such a process is important for the construction of structures external to the plasma membrane, such as the cell walls of eukaryotic cells. Digestive enzymes that are used extracellularly (outside the cell) are also packaged at the Golgi apparatus. They are subsequently secreted from the eukaryotic cell. Thus materials are packaged and routed by the Golgi apparatus for distribution to specific sites.

The Golgi apparatus is involved in packaging and distribution of materials.

Lysosomes

Lysosomes are specialized membrane-bound organelles produced at the Golgi apparatus. The lysosomes contain various digestive enzymes that are used within the cell. It is essential that these enzymes do not mix freely with the contents of the cytoplasm. They could digest the substances found there. The lysosome membrane is impermeable to the outward movement of digestive enzymes. It also is resistant to their action. This segregation of digestive enzymes within the lysosome prevents these enzymes from digesting many of the cell's structural components. A lysosome can fuse with a membranous sac, bringing together the contents of each, thus digesting selected materials.

Lysosomes segregate and store digestive enzymes.

When a white blood cell captures a bacterium by phagocytosis, it encloses the bacterial cell in a membranous sac, called a **phagocytic vesicle** or **phagosome** (FIG. 5-39). This membranous vesicle, containing the bacterial cell, fuses with a lysosome. A single structure called a *phagolysosome* is formed. Within the phagolysosome, the lysosomal enzymes can digest the bacterial cell without simultaneously digesting the eukaryotic cell. The remains of the bacterial cell after such digestion are small enough to be transported across the membrane surrounding the phagolysosome into the cytoplasm. Once there, they can be used in the metabolism of the cell. Some bacteria, such as *Salmonella typhi*, *Legionella pneumophila*, and *Mycobacterium tuberculosis*, are resistant to lysosomal digestive enzymes and survive within the phagolysosome. The ability to resist digestion within phagocytic cells is important to the abilities of these pathogens to resist body defenses and to cause human disease.

Vacuoles

Various types of membrane-bound sacs serve different purposes within the cells of eukaryotic microorganisms. These membrane-bound sacs are called **vacuoles.** One type of vacuole segregates reserve materials from other cytoplasmic constituents of eukaryotic cells. This is the **storage vacuole.** Other vacuoles fuse with the plasma membrane to move materials out of the cell by exocytosis. In other cases, endocytosis of a food particle results in the formation of a vacuole that fuses with lysosomes. This establishes a digestive vacuole within which the ingested food is digested to small molecules. These small molecules can diffuse across the membrane of the vacuole into the cytoplasm.

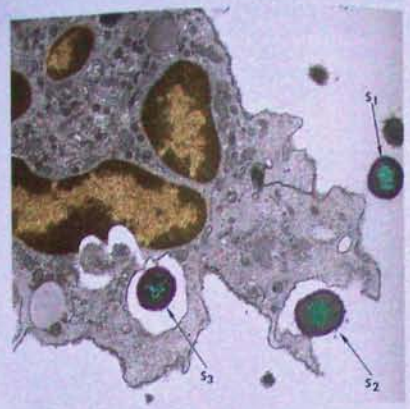


FIG. 5-39 Colorized electron micrograph showing the phagocytosis of the bacterium *Streptococcus pyogenes* by a human polymorphonuclear leukocyte, which is a white blood cell that helps defend the body against infection. (15,000×). One bacterial cell is free (S_1); one is in the process of being phagocytized (S_2); and one is within the phagosome (S_3).

Cytoskeleton

The eukaryotic cell also has a cytoskeleton (FIG. 5-40). The **cytoskeleton** is composed of flexible strands called microfilaments (which are about 5 nm thick) and microtubules (which are about 25 nm thick). The microfilaments and microtubules are organized into a three-dimensional network through the cytoplasm. The cytoskeleton is involved in the support and movement of membrane-bound structures, including the plasma membrane and the various organelles of the eukaryotic cell. It helps the cell maintain or change its shape.

Microfilaments contain a protein that allows them to contract. They are involved in the movement of materials within cells. For example, microfilaments move chloroplasts in response to changes in the position of the sun. During division of a eukaryotic cell, microtubules pull chromosomes apart. Microtubules thus aid in the distribution of chromosomes to progeny cells. Microtubules are also involved in cellular movement. For example, microtubules located just beneath the surface of the plasma membrane apparently provide the basis for vesicle movement during endo- and exo-cytosis. The movement of microtubules permits the extension of the cytoplasmic membrane to form the "false feet" (pseudopodia) used by some protozoa, like *Amoeba*. These protozoa move by extending their cytoplasm in a particular direction as they continuously change shape (FIG. 5-41).

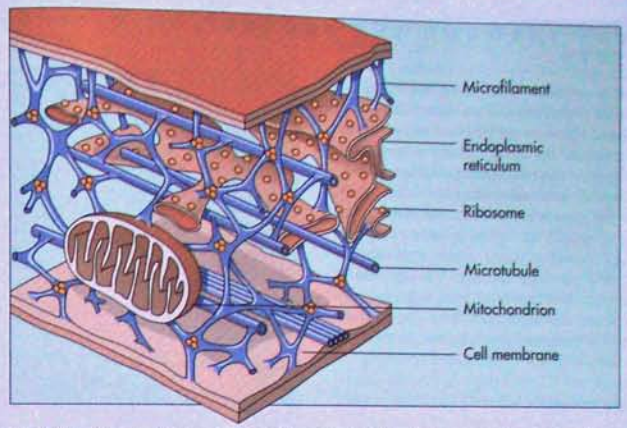


FIG. 5-40 The cytoskeleton is a complex network that links the organelles of the eukaryotic cell. Organelles are attached to microfilaments of the cytoskeleton.

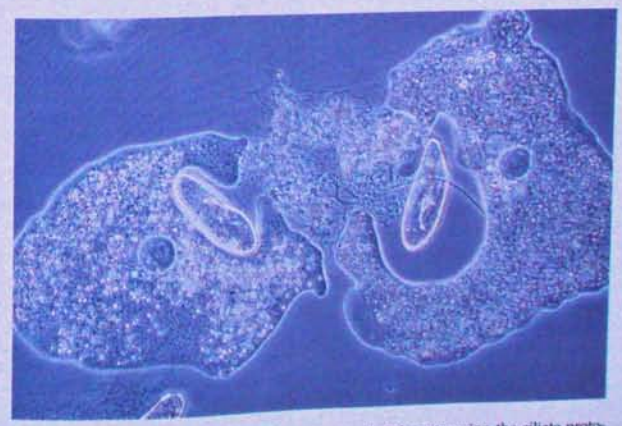


FIG. 5-41 Colorized micrograph of the protozoan *Amoeba* consuming the ciliate protozoan *Paramecium*.

SURVIVAL THROUGH THE PRODUCTION OF SPORES AND CYSTS

Some organisms produce specialized cells that enhance their survival potential. These specialized cells are called **spores.** Spores may be involved in reproduction, dispersal, or the ability of the organism to withstand adverse environmental conditions. They enhance the overall survival potential of the organism. For example, spores involved in the dispersal of microorganisms usually are quite resistant to desiccation (drying), enabling some microorganisms to survive extended passage through the air. Many spores are *dormant* (nongrowing) with minimal metabolism. They are distinguished from the normal *vegetative cells* of an organism, which are the cells involved in growth.

Bacterial Endospores

The **bacterial endospore** is a heat-resistant spore formed within the cells of a few bacterial genera (FIG. 5-42). The bacterial endospore is highly resistant to elevated temperatures, desiccation (drying), and radiation. It can survive at temperatures as high as 100° C for extended periods, whereas bacterial cells are killed by brief exposures to such high temperatures. Placing an endospore in boiling water, even for several hours, often doesn't kill it.

Several structural and chemical factors contribute to the relative resistance of endospores to conditions that kill vegetative cells. The endospore is a complex multilayered structure that contains a spore coat and a cortex. The cortex contains calcium dipicolinate, but little water, which contributes to its ability to survive under adverse conditions.

Bacterial endospores are highly heat-resistant structures that permit survival under adverse conditions.

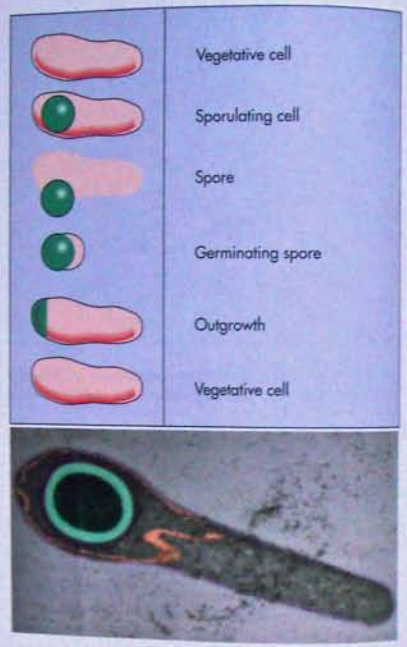


FIG. 5-42 **A,** The cycle of endospore formation, release, and germination. The endospore is a multilayered structure that is heat resistant. It is formed within a vegetative cell and released when the cell lyses. Subsequently, an endospore can germinate under favorable conditions to form new vegetative cells. **B,** Colorized transmission electron micrograph of an endospore of *Clostridium tetani* shows the complex multilayers of this heat-resistant body within a cell.

Endospores are formed when conditions are unfavorable for the continued growth of bacteria, such as when nutrients are not available. Dormant endospores are formed under starvation conditions. In the first observable stage of spore formation (*sporogenesis*), a newly replicated bacterial chromosome and a small portion of cytoplasm are isolated by an inward growth of the plasma membrane. This forms a structure called the *spore septum* (FIG. 5-43). The spore septum becomes a double-layered membrane that surrounds the chromosome and cytoplasm. A structure, called a *forespore*, that is entirely enclosed as a separate compartment within the original cell is thus produced. Thick layers of peptidoglycan and calcium dipicolinate are laid down between the two membrane layers. The presence of calcium dipicolinate contributes to the heat resistance of the spore.

Then a thick *spore coat* of protein forms around the outside membrane for additional protection. When the endospore matures, the vegetative cell wall lyses and the endospore is released.

Only a few bacterial genera form endospores. The most important endospore producers are members of two genera, *Bacillus* and *Clostridium*. *Bacillus* and *Clostridium* are defined as Gram-positive rods that form endospores. *Bacillus* is aerobic, growing in the presence of oxygen. *Clostridium* is obligately anaerobic, growing only in the absence of oxygen. Some members of these genera are human pathogens, such as *B. anthracis* (cause of anthrax), *C. perfringens* (cause of gas gangrene), and *C. tetani* (cause of tetanus). The endospores of these bacteria can survive in soil for extended periods, even years. Contamination of a wound with endospores (for example, those of *C. tetani*) can lead to tetanus—often fatal in unvaccinated individuals.

The ability of *Clostridium* and *Bacillus* species to form endospores presents special problems for the food industry. The food industry must employ special processes that rely on heat to prevent spoilage of products. Some endospores can withstand boiling for more than 1 hour. To ensure that such endospores are killed, it is necessary to heat liquids to a temperature greater than 120° C and hold that temperature for at least 15 minutes. It is especially important to use sufficient heating to kill endospores when canning foods. The endospore-producer *Clostridium botulinum*, the bacterium that causes botulism, sometimes contaminates foods. If the endospores are not killed during canning, this bacterium can grow under the anaerobic conditions in a canned food and produce sufficient toxin to kill anyone who eats that food (antitoxin is available but must be quickly administered).

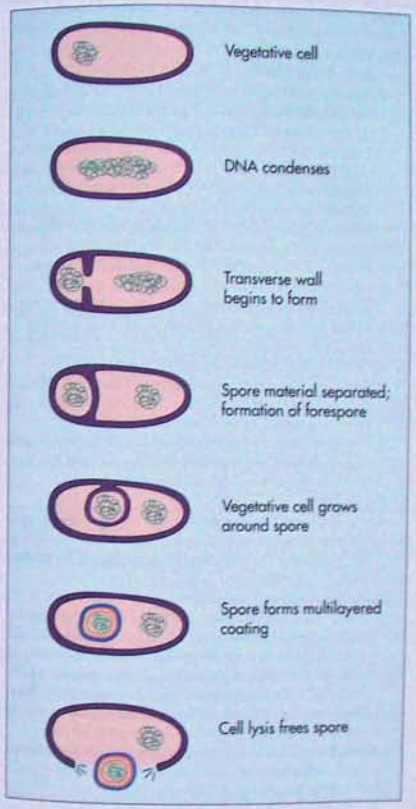


FIG. 5-43 The formation of an endospore is a complex process that occurs in stages. The spore contains a copy of the bacterial chromosome, which is separated from the rest of the cell by several layers that contain peptidoglycan. There is also a layer containing calcium dipicolinate that contributes to the heat resistance of the spore.

Fungal Spores

Some fungi produce spores that enhance their survival under conditions of low moisture, high temperature, or lack of nutrients. These spores are not as heat resistant as bacterial endospores. Various fungal spores perform different functions, including reproduction. (Bacterial endospores are not a means of reproduction.) Fungal spores involved in the dispersal of microorganisms usually are quite resistant to desiccation, and the production of such spores is an important adaptive feature that permits the survival of fungi for long periods of time during transport in the air. The spread of many fungi depends on the successful transport of fungal spores from one place to another. Unfortunately, many of these fungi are plant pathogens and cause great agricultural damage as a result of their ability to move effectively from field to field.

Protozoan Cysts

Some protozoa can form survival structures. The active feeding stage of a protozoan is called a **trophozoite.** Trophozoites are the vegetative stage of several parasitic and free-living protozoa. Trophozoites often cannot withstand harsh environmental conditions. To cope with such conditions many protozoa secrete a thick, resistant covering. They develop into a resting stage called a **cyst.**

A protozoan can produce many cysts, which are readily dispersed in the environment. Since these cysts represent the infectious form of pathogenic protozoa, they represent a high potential for disease transmission. This was clearly evident in Milwaukee, Wisconsin, in the summer of 1993 when there was a major outbreak of the waterborne disease caused by the ingestion of cysts of the protozoa *Cryptosporidium*. Similarly, there have been major outbreaks of infections in cities like St. Petersberg, Russia, due to ingestion of cysts of the protozoan *Giardia*.

The process of encystment represents a type of cell differentiation. Cysts often aid in the spread of disease pathogens.

SUMMARY

Cells p. 121

- Every living organism is made up of one or more cells. The cell is the fundamental unit of living systems.
- Each new cell arises only from cells that already exist.

Plasma Membrane: Movement of Materials into and out of Cells (pp. 121-127)

- All cells are surrounded by plasma membranes, contain cytoplasm, have DNA and ribosomes, and utilize energy from ATP. The plasma membrane is a semipermeable barrier that separates the organism (living system) from the surrounding environment, serving as the boundary layer between the fluid around the cell and the cytoplasm contained within the cell. The plasma membrane controls the flow of materials into and out of the cell, allowing some substances to pass and preventing others from entering or exiting the cell.

Structure of the Plasma Membrane (pp. 122-123)

- The plasma membrane of eubacterial and eukaryotic cells is composed of a phospholipid bilayer and proteins.
- The fluid mosaic model explains the structure of the plasma membrane.

Comparison of Archaebacterial and Eubacterial Plasma Membranes (p. 123)

- Archaebacterial plasma membranes have ether linkages.
- Eubacterial plasma membranes have phospholipids with ester linkages.
- Eubacterial and archaebacterial plasma membranes serve identical functions.

Comparison of Eubacterial and Eukaryotic Plasma Membranes p. 124)

- Plasma membranes of eukaryotic cells contain sterols.
- Plasma membranes of eubacterial cells generally lack sterols.

Transport Across the Plasma Membrane (pp. 124-127)

- Some substances enter and leave the cell by diffusion, moving from regions of high concentration on one side of the membrane to regions of low concentration on the other side of the membrane. When water moves by diffusion, the process is called osmosis. The rate of movement by simple diffusion is dictated by the concentration gradient across the membrane.
- Some substances move across the plasma membrane at accelerated rates—called facilitated diffusion—due to the action of permeases. Active transport uses energy to transport substances through the plasma membrane against a concentration gradient. In eukaryotic cells, substances can enter or leave the cell by cytosis.
- Group translocation modifies substrates as they move across the plasma membrane.
- Phagocytosis occurs when a eukaryotic cell engulfs another cell.

Cell Surface (pp. 128-139)

Cell Wall (pp. 128-132)

- Most bacterial cells have a rigid cell wall surrounding the plasma membrane that protects the cell against lysis due to osmotic shock. Cell walls give bacteria their characteristic shapes. Peptidoglycan makes the cell wall rigid, but if the peptidoglycan is not properly formed or if it is digested, the cell wall is defective and cannot protect the cell from lysis. The Gram-positive cell wall is composed almost entirely of peptidoglycan, a unique biochemical that does not occur in other organisms. The Gram-negative cell wall has a relatively thin layer of peptidoglycan and also additional material, including lipopolysaccharide (LPS).
- LPS is known as endotoxin and can cause adverse reactions in humans.

Bacterial Capsules and Glycocalyces (pp. 133-134)

- Some bacteria have a specialized structure, called a capsule, that surrounds the cell and makes such cells relatively resistant to phagocytosis.
- The glycocalyx, which surrounds some bacterial cells, is responsible for the ability of some bacteria to attach to living tissues and inanimate surfaces.

Pili (p. 134)

- Pili are short hairlike projections that are involved in various attachment processes, such as the mating of bacteria and the attachment of bacteria to human cells.

Bacterial Flagella (pp. 135-137)

- Many bacterial cells are motile by means of flagella. Peritrichous flagella occur all around a bacterial cell. Polar flagella emanate from the ends of the bacterial cell. Bacterial flagella rotate to propel the cell. Motile bacterial cells are able to respond to chemicals, moving toward some and away from others, by a process known as chemotaxis.
- Some very specialized bacteria navigate along magnetic fields by magnetotaxis.

Eukaryotic Flagella and Cilia (pp. 137-139)

- The flagella and cilia of eukaryotic cells undulate in a wave-like motion.

Storage and Expression of Genetic Information (pp. 140-143)

Bacterial Chromosome (p. 140)

- In bacterial cells the DNA is not segregated from the rest of the cell. All of the essential genetic information of a bacterial cell is contained within a single bacterial chromosome.
- The bacterial chromosome is a circular loop of double helical DNA.

Plasmids (pp. 140-141)

- Some bacterial cells have plasmids, which are genetic elements that contain ancillary information.

Nucleus of the Eukaryote Cell (pp. 141-142)

- In eukaryotic cells the DNA is contained within a nucleus.

- Chromosomes of eukaryotic cells are linear.
- Eukaryotic cells often contain multiple pairs of chromosomes.
- The DNA of a eukaryotic chromosome is wound around specialized proteins called histones, which establish the tightly coiled structure of the chromosome.

Ribosomes (pp. 142-143)

- Ribosomes are the sites of protein synthesis.
- The ribosomes of prokaryotic cells are 70S. The ribosomes in the cytoplasm of eukaryotic cells are 80S. Various antibiotics, such as tetracyclines and erythromycin, bind specifically to 70S ribosomes, thereby selectively inhibiting bacterial protein synthesis.

Cellular Structure and Energy Transformations (pp. 143-146)

- Pumping of hydrogen ions across a membrane to establish a hydrogen ion gradient forms the basis of chemiosmotic ATP generation. Mitochondria and chloroplasts are organelles of eukaryotic cells where ATP is generated.

Segregation and Movement of Materials within Cells (pp. 147-151)

- Eukaryotic cells contain various membrane-bound organelles that establish a compartmentalization of function and permit many different and sophisticated specialized activities to occur within an integrated framework. Eukaryotic cells have an extensive internal cytomembrane system that includes the endoplasmic reticulum, the Golgi apparatus, lysosomes, vacuoles and the cytoskeleton.

Survival Through the Production of Spores and cysts (pp. 152-153)

- Members of a few bacterial genera, most notably species of *Bacillus* and *Clostridium*, produce a specialized structure, the endospore, that has great survival value.
- Some endospores can withstand boiling for more than 1 hour.
- Most fungi produce spores that facilitate their dissemination.
- Some protozoa produce resistant resting stages called cysts.

CHAPTER REVIEW

REVIEW QUESTIONS

1. Eubacteria and archaebacteria are prokaryotes. Describe their similarities and differences.
2. Compare and contrast simple diffusion, facilitated diffusion, and active transport.
3. Draw and label the parts of a typical eubacterial cell. Describe the function of each part.
4. Draw and label the parts of a typical fungal cell. Describe the function of each part.
5. Draw and label the parts of a typical algal cell. Describe the function of each part.
6. Describe the cell walls of Gram-positive and Gram-negative bacteria.
7. How do bacteria move? How do they respond to their chemical environment?
8. Describe the structure and function of cilia, flagella, and pseudopodia.
9. Why are endospores known as resting cells?
10. What advantages does having endospores bring to bacteria?
11. What are functions of the bacterial plasma membrane?

CRITICAL THINKING QUESTIONS

1. Why have organelles been hypothesized to be necessary for eukaryotic cells and not for prokaryotic cells? How has the finding of a bacterium with cells of 0.5 mm altered this view?
2. How do bacteria adapt to their environment? How do they find places where they can grow?
3. Eukaryotic and prokaryotic cells have several different structures. How do these differences relate to medical practice and, in particular, the use of drugs to treat diseases caused by microorganisms? Why is it generally easier to cure a patient of a bacterial disease than a protozoan disease?
4. Since eukaryotic human cells have 70S ribosomes in their mitochondria, how can drugs that target the 70S ribosomes—such as erythromycin—be used to treat bacterial infections?
5. How long can endospore-forming bacteria survive? Could there be a million-year-old bacterium that still could be grown in the laboratory?
6. Why are archaebacteria considered to be descendants of prokaryotes that evolved early in the history of life on earth? How do the physiological properties of archaebacteria allow them to survive in extreme environments?

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New techniques in biochemistry and microscopy are used to probe the structure and function of the cytoskeleton.

CHAPTER 6

Cellular Metabolism

CHAPTER OUTLINE

Process of Metabolism Within a Cell 158
- Role of Enzymes
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  - Heterotrophic Metabolism

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  - Aerobic Respiration
  - Anaerobic Respiration
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- Lipid and Protein Catabolism
- Fermentation
  - Ethanolic Fermentation
- *Newsbreak:* Intestinal Yeast Infections Cause Intoxication
  - Lactic Acid Fermentation
- *Highlight:* Production of Cheese
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  - Mixed-acid Fermentation
  - Butanediol Fermentation
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  - Sulfur Oxidation
  - Nitrification
- Nitrogen Fixation
- Methanogenesis
- *Newsbreak:* Explosions That Destroy Houses Traced to Methane from Landfill

PREVIEW TO CHAPTER 6

In this chapter we will:

- Study the chemical reactions mediated by enzymes that constitute the metabolism of a cell.
- Learn about the diverse strategies of metabolism that enable cells to sustain life functions.
- Examine how a cell meets its energy needs by generating ATP.
- Compare various forms of autotrophic and heterotrophic metabolism.
- See the chemical reactions that occur in various metabolic pathways, including fermentation, respiration, photosynthesis, and chemolithotrophy.
- Learn the following key terms and names:

activation energy
active site
adenosine triphosphatase (ATPase)
aerobic respiration
alcoholic fermentation
anabolic pathway
anaerobic respiration
autotrophic metabolism
butanediol fermentation
butanol fermentation
butyric acid fermentation
Calvin cycle
capnophiles
catabolic pathway
cellular metabolism
chemiosmosis
chemoautotrophic metabolism
chemolithotrophic metabolism
coenzyme
electron transport chain
Embden-Meyerhof pathway
enzymes
heterolactic fermentation
heterotrophic metabolism
homolactic fermentation
Krebs cycle
lactic acid fermentation
Methyl Red (MR) test
microaerophiles
mixed-acid fermentation
nitrogen fixation
nitrogenase
photoautotrophic metabolism
propionic acid fermentation
protonmotive force
respiration
substrate-level phosphorylation
substrates
Voges-Proskauer test

The evolution of living cells began with the use of the energy released from a chemical bond. Early organisms evolved that were able to convert this energy into ATP. Proteins evolved diverse catalytic functions, making possible the retention of a larger portion of the chemical bond energy available in abiotic (nonliving) organic molecules. Breaking a series of chemical bonds in successive steps enabled this to occur and allowed cells to carry out metabolism.

Metabolic processes are believed to have evolved in the essentially oxygen-free atmosphere that characterized the atmosphere of the Earth during the time life began on Earth. Primitive life forms are thought to have obtained chemical energy by breaking down organic molecules formed by non-metabolic reactions.

The totality of all of the chemical reactions that a cell carries out is called **cellular metabolism.** Through the process of cellular metabolism, cells bring about chemical changes through which they obtain energy and materials for growth and reproduction. Energy is required for living things to sustain life processes. Cells can store energy or use it for the synthesis of new molecules by controlling the status of chemical bonds. Adenosine triphosphate (ATP) is the central chemical in the energy transformations of cellular metabolism. ATP cannot be stored for long periods of time and therefore must be continually made. Within a living cell, the flow of energy involves the formation and consumption of ATP. Cellular metabolism transforms the energy stored in light or chemicals into ATP. Cellular metabolism also transforms starting materials into the numerous carbon-containing chemicals that make up the structural and functional components of the cell.

Energy and materials are transformed within living cells through a complex integrated network of chemical reactions that collectively constitute the metabolism of the cell, or cellular metabolism.

Role of Enzymes

Cellular metabolism is based on chemical reactions catalyzed by enzymes. **Enzymes** are biological catalysts, which are substances produced by cells that accelerate the rates of chemical reactions. Almost all biological catalysts are proteins but a very few are RNA. Virtually every step in cell metabolism involves an enzyme. Enzymes increase the rates of a cell's chemical reactions by more than a million times.

Energy is required for a chemical reaction to occur. Enzymes bind to molecules and bonds in such a way that the energy required to initiate a chemical reaction, called the **activation energy**, is lowered (FIG. 6-1). This lowering of the activation energy is critical because it permits reactions to occur at life-supporting temperatures. The result is that the reaction occurs more rapidly than it otherwise would at those temperatures.

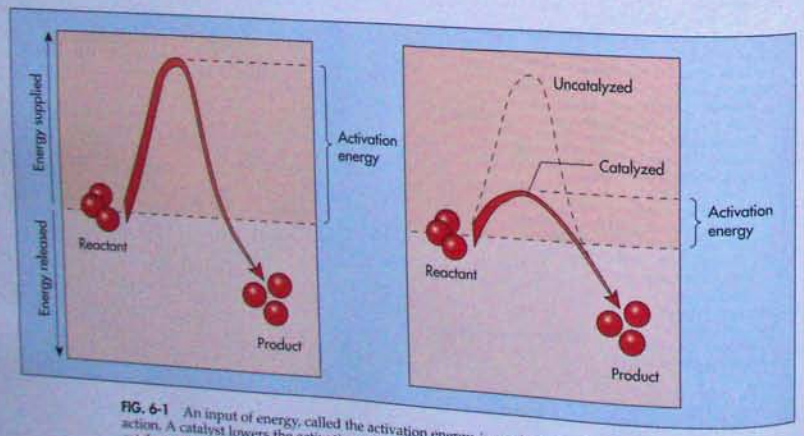


FIG. 6-1 An input of energy, called the activation energy, is needed to start a chemical reaction. A catalyst lowers the activation energy. In biological systems, enzymes serve as the catalysts to lower the activation energy.

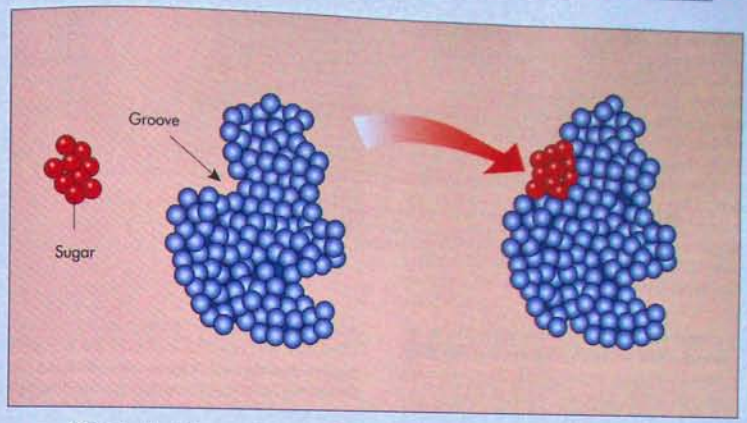


FIG. 6-2 The fit between the enzyme and the substrate to form an enzyme-substrate complex has been likened to that of a lock and key. Actually, this interaction modifies the three-dimensional structure of the enzyme so that the substrate induces its fit to the enzyme. The precision of fit is responsible for the high degree of specificity of enzymes for particular substrates.

An input of energy—the activation energy—is needed to initiate a chemical reaction.

Enzymes are biological catalysts that lower the activation energy so that chemical reactions can occur within living cells.

Some molecules can bind to a particular enzyme so that the enzyme can catalyze a chemical reaction. Such molecules are called the **substrates** of that enzyme (FIG. 6-2). Enzymes can bind to substrate molecules because the three-dimensional shape of the enzyme fits the substrate molecule, sort of like a lock fits a key. Enzymes exhibit great specificity in the reactions that they catalyze. When an enzyme binds to a substrate it forms an enzyme-substrate complex. The enzyme-substrate complex then breaks down, releasing the enzyme and the product(s) of the reaction. The enzyme is not consumed in the overall reaction and can continue to act as a catalyst.

$$\text{Substrate} + \text{Enzyme} \rightleftharpoons \text{Enzyme-substrate complex} \rightleftharpoons \text{Enzyme} + \text{Product}$$

Different enzymes are needed to bring about reactions that transform even very similar chemical compounds. Thousands of enzymes involved in the metabolic reactions of each cell are necessary for cellular growth and reproduction. Enzymes catalyzing key reactions in metabolic pathways govern whether molecules are degraded as an ATP-generating energy source or converted for use in biosynthesis.

The actual site of the enzyme that is responsible for its catalytic action is called the **active site.** Because protein shapes are not rigid, when some enzymes bind to their substrate, there may be a slight alteration in the shape of the enzyme molecule so that there is a good fit between substrate and enzyme. The fit is essential for the enzymatic reaction to occur. If the shape of the enzyme is altered so that it can no longer function as a catalyst, the enzyme is said to be denatured. Heating and certain chemicals can denature enzymes, destroying their catalytic activities.

Enzymes exhibit a high degree of substrate specificity.

The enzymes a particular cell synthesizes will determine which chemical reactions occur in cellular metabolism of that cell.

Coenzymes and Oxidation-Reduction Reactions

Many of the chemical reactions in cellular metabolism are oxidation-reduction reactions in which electrons and protons are exchanged between molecules. Many of these reactions are used to extract energy from organic compounds. In these reactions, electrons and protons often are transferred to a molecule

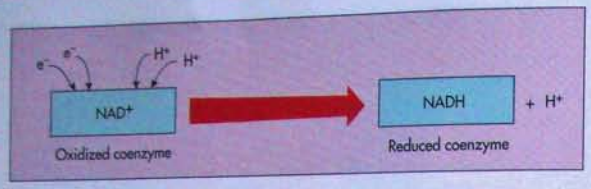


FIG. 6-3 The reduction of the oxidized coenzyme NAD^+ to the reduced coenzyme NADH + H^+ is a critical reaction that often is coupled with the oxidation of substrates within a cell. This reaction can be written as $NAD^+ \rightarrow NADH$.

called a coenzyme (FIG. 6-3). A **coenzyme** is an organic molecule that serves as a carrier of electrons and/or protons during metabolism. The coenzyme NAD^+ (nicotinamide adenine dinucleotide) is the common temporary holder of electrons and protons in many metabolic pathways. An example of such a reaction is:

$$\text{malate} + NAD^+ \longrightarrow \text{oxaloacetate} + NADH + H^+$$

The reduced coenzyme NADH formed in this reaction can then donate an electron and a proton to another molecule so that the coenzyme is reoxidized. Other important coenzymes used in cellular metabolism are NADP (nicotinamide adenine dinucleotide phosphate) and FAD (flavin adenine dinucleotide).

ATP and Cellular Energy

A central concern of cellular metabolism is the flow of energy. All of the activities of living organisms use energy. Living systems can neither create nor destroy energy. Rather, living systems transform energy, capturing energy from one source and using that energy to drive the essential chemical reactions that enable cells to carry out the life functions of growth and reproduction. Some cells capture energy directly from sunlight; others obtain energy from the oxidation ("burning") of organic or inorganic chemicals. Regardless of the source of energy, all cells employ the same strategy of carrying out metabolic reactions that transfer energy to molecules of ATP. Then ATP serves as the molecule for transferring energy within the cell. A growing cell of the bacterium *Escherichia coli* must synthesize approximately 2.5 million molecules of ATP per second to support its energy needs.

During metabolism, energy is transferred to and stored within molecules of ATP.

ATP is the universal energy carrier of all living cells.

In particular, the energy from ATP is used to drive the energy-requiring reactions of biosynthesis that are needed for cellular growth and reproduction. ATP contains a phosphate functional group joined to the rest of the molecule by a high-energy bond (FIG. 6-4). Breaking this bond yields an inorganic phosphate group (P_i) and a molecule of adenosine diphosphate (ADP) and releases a large amount of energy—approximately 7300 calories for every 6.023×10^{23} molecules (mole) of ATP converted to ADP + P_i. This energy can be used to drive the cell's energy-requiring chemical reactions.

In cellular metabolism, energy-requiring reactions occur because they are coupled with reactions that cleave ATP.

The formation of ATP from ADP + P_i is an energy-requiring reaction. This reaction cannot occur spontaneously. When coupled to a spontaneously occurring, energy-releasing reaction, however, the synthesis of ATP from ADP + P_i does take place, because energy is now available to drive the synthesis of ATP. The generation of ATP by coupling energetically favorable reactions to the synthesis of ATP from ADP plus either an organic or inorganic phosphate source is called **substrate-level phosphorylation** (FIG. 6-5).

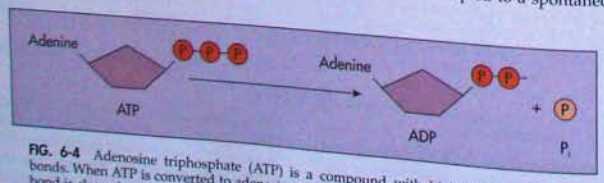


FIG. 6-4 Adenosine triphosphate (ATP) is a compound with high-energy phosphate bonds. When ATP is converted to adenosine diphosphate (ADP) a high-energy phosphate bond is cleaved, releasing about 7.5 kcal/mole that can be used to drive other chemical reactions.

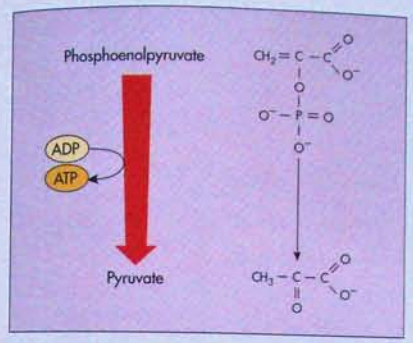


FIG. 6-5 In substrate-level phosphorylation an energy yield reaction directly provides the energy for the generation of ATP; this occurs via a coupled reaction.

In other cellular reactions the chemical formation of ATP is driven by a diffusion force in a process known as **chemiosmosis** (FIG. 6-6). Protons are pumped out of prokaryotic cells across the plasma membrane or the membrane-bound organelles (mitochondria and chloroplasts) of eukaryotic cells as a result of oxidation-reduction reactions that transport electrons and protons through membrane-embedded carriers. As the proton concentration across the membrane becomes higher on one side than the other, the protons on that side of the membrane are driven back across the membrane. The force exerted by these protons to drive them back across the membrane is called the **protonmotive force.** The passage of these protons through specific channels in the membrane provides the energy for the formation of ATP from ADP + P_i.

Metabolic Pathways and Carbon Flow

The chemical reactions of metabolism occur in sequences. In each sequence the product of one chemical reaction becomes the substrate for the enzyme that catalyzes the next reaction. The overall ordered sequences of enzyme-catalyzed chemical reactions are called **biochemical pathways** or **metabolic pathways.** The sequential steps between the starting substrate molecule(s) and the end product(s) constitute the **intermediary metabolism** of the cell.

The enzymatically mediated metabolic reactions of a cell proceed via a series of small discrete steps that establish a metabolic pathway.

Metabolic pathways that involve the breakdown (degradation) of organic molecules are said to be

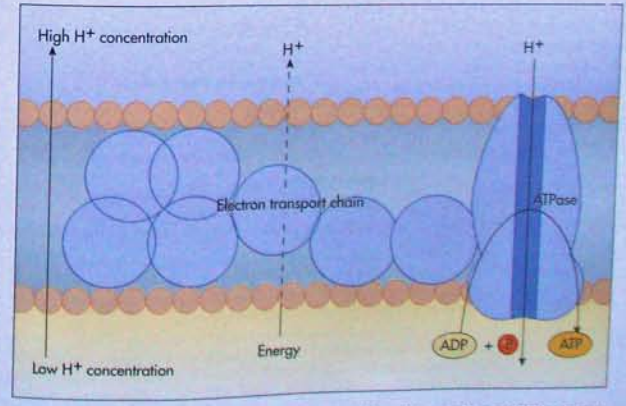


FIG. 6-6 In chemiosmosis the metabolic reactions of the cell are used to establish a proton gradient across a membrane. This proton gradient exerts a force called the protonmotive force that is used to generate ATP.

catabolic (meaning to break down). A catabolic pathway is one in which larger molecules are split into smaller ones. Such pathways can be used to obtain energy from organic chemicals, for example. The processes that expend energy to synthesize organic molecules are said to be **anabolic** (meaning to build up). The catabolic and anabolic pathways of a cell are interconnected so that the substrates used to feed the cell can be changed into the molecules that make up a living cell.

Catabolism means degradative process and anabolism means biosynthetic process.

Cells must be able to process matter from available starting material into their own structural and functional components. As with energy, the chemical reactions of living systems can neither create nor destroy matter. Cells obtain matter from their surroundings and the chemical reactions the cells perform can change the combinations of atoms within that matter to form new molecules. Thus the various available starting substrate molecules are transformed by cellular metabolism into the many different macromolecules of the cell. These macromolecules include, among others, proteins for enzymes, lipids for membranes, carbohydrates for various structures such as cell walls, and nucleic acids for the storage and expression of genetic information.

Carbon is the backbone atom of all organic chemicals. In terms of carbon flow, the basic strategy of the cell is to form relatively small molecules that can act as the basis for the carbon skeletons of larger macromolecules (FIG. 6-7). When a microorganism uses an organic substrate, like glucose, to generate ATP, it follows a catabolic pathway to break that molecule down into smaller compounds; these smaller compounds then act as building blocks—called **precursors**—for the biosynthesis of macromolecules. Then the microorganism uses an anabolic pathway to transform small molecules into larger molecules.

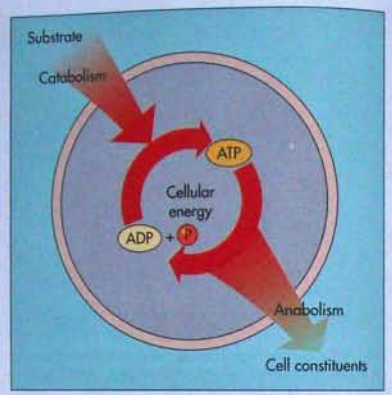


FIG. 6-7 The metabolic strategy of a cell is to break down substances into smaller compounds via catabolic pathways. The small compounds that are formed are used as the substrates for biosynthetic reactions in anabolic pathways.

Autotrophic and Heterotrophic Metabolism

Microorganisms exhibit differing strategies of metabolism for meeting their common needs. These needs include synthesizing ATP and transforming carbon-containing molecules into the macromolecules that constitute the cells of the microorganism. Two distinct modes of microbial metabolism have evolved for accomplishing these tasks: **autotrophy** (meaning self-feeding) and **heterotrophy** (meaning other-feeding).

Autotrophic Metabolism

Microorganisms with **autotrophic metabolism** are called **autotrophs** (Table 6-1). The cellular metabolism of autotrophic organisms uses inorganic carbon dioxide as a source of carbon for the biosynthesis of the molecules of the cell. Also, autotrophic metabolism almost always generates ATP from the oxidation of inorganic compounds or through the conversion of light energy to chemical energy—not from organic compounds. Photoautotrophic (photosynthetic) microorganisms use light energy and chemoautotrophic (chemolithotrophic) microorganisms use the energy derived from oxidation of inorganic compounds to supply energy needed for synthesis of ATP.

Carbon for the macromolecules of autotrophic microorganisms originates from inorganic carbon dioxide.

Autotrophic metabolism does not use organic compounds for the generation of ATP but rather captures light energy or energy from the oxidation of inorganic chemicals; the cellular carbon of autotrophs comes from carbon dioxide.

TABLE 6-1
Types of Autotrophic Microbial Metabolism Used to Generate ATP

TYPE OF METABOLISM	DESCRIPTION
Oxygenic photosynthesis	Uses two connected photosystems and results in evolution of oxygen, as well as generation of ATP; carried out by algae and cyanobacteria, which gain reducing power (H^+) from photolysis of water
Anoxygenic photosynthesis	Uses one photosystem and does not result in evolution of oxygen; carried out by anaerobic photosynthetic bacteria, e.g., green and purple sulfur bacteria, and under some conditions by cyanobacteria, which gain reducing power (H^+) from H_2S or organic compounds
Chemoautotrophic (chemolithotrophic)	Uses oxidation of inorganic compounds such as sulfur, nitrite, nitrate, and hydrogen to establish a protonmotive force across a membrane that results in generation of ATP by chemiosmosis

Heterotrophic Metabolism

In **heterotrophic metabolism** the generation of ATP is based on the use of an organic substrate molecule (Table 6-2). The conversion of the organic substrate to end products occurs via a metabolic pathway that releases sufficient energy to be coupled with the synthesis of ATP. The catabolic pathway involves reactions that break down an organic molecule into smaller molecules. Besides using organic compounds to provide energy for ATP generation, heterotrophs obtain their cellular carbon from organic substrates. In heterotrophic metabolism, organic compounds are broken down into smaller molecules, called **intermediary metabolites,** that subsequently are used for biosynthesis.

Heterotrophic metabolism uses organic chemicals to supply the energy for ATP generation; the cellular carbon of heterotrophs also comes from organic compounds.

Heterotrophic metabolism occurs by either of two processes: respiration or fermentation. Respiration links the metabolism of an organic substrate with the utilization of an inorganic compound. **Respiration** is defined as a type of metabolism involving oxidation-reduction reactions where the final electron acceptor that completes the metabolism is an inorganic molecule. Often the inorganic compound is molecular oxygen (O_2) so that the process is dependent on air (aerobic respiration) but, in some cases, respiration occurs in the absence of air (anaerobic respiration). ATP is formed during respiration both by substrate-level phosphorylation and by chemiosmosis.

Fermentation is an anaerobic catabolic process that releases energy from sugars or other organic compounds. During fermentation the final electron acceptor is an organic molecule. Only substrate-level phosphorylation is used to generate ATP in a fermentation pathway. During fermentation, hydrogen ions and electrons are transferred from NADH to pyruvic acid, which is turned into various end products. Various microorganisms are able to ferment different substrates; the end products depend on the particular microorganism, the substrate, and the activity of the enzymes that are present (Table 6-3, p. 164).

Respiration requires an inorganic substance, often molecular oxygen, to complete the metabolism of an organic substrate; ATP is generated by both substrate level phosphorylation and chemiosmosis during respiration.

TABLE 6-2
Types of Heterotrophic Microbial Metabolism Used to Generate ATP

TYPE OF METABOLISM	DESCRIPTION
Respiration	Uses complete oxidation of organic compounds, requiring an external electron acceptor to balance oxidation-reduction reactions used to generate ATP; much of the ATP is formed as a result of chemiosmosis based on the establishment of a proton gradient across a membrane
Aerobic respiration	Uses oxygen as the terminal electron acceptor in the membrane-bound pathway that establishes the proton gradient for chemiosmotic ATP generation
Anaerobic respiration	Uses compounds other than oxygen, e.g., nitrate or sulfate, as the terminal electron acceptor in the membrane-bound pathway that establishes the proton gradient for chemiosmotic ATP generation
Fermentation	Does not require an external electron acceptor, achieving a balance of oxidation-reduction reactions using the organic substrate molecule; various fermentation pathways produce different end products

TABLE 6-3
Types of Fermentative Metabolism

FERMENTATION PATHWAY	END PRODUCTS
Homolactic acid	Lactic acid
Heterolactic acid	Lactic acid + ethanol + CO_2
Ethanolic	Ethanol + CO_2
Propionic acid	Propionic acid + CO_2
Mixed acid	Ethanol + acetic acid + lactic acid + succinic acid + formic acid + H_2 + CO_2
Butanediol	Butanediol + CO_2
Butyric acid	Butyric acid + butanol + acetone + CO_2

Fermentation does not require an inorganic substance to utilize an organic substrate for generating ATP as a form of usable cellular energy and materials for growth; fermentation does not require air; ATP is generated exclusively by substrate level phosphorylation.

Microorganisms can be grouped into categories based on their requirement for or intolerance of oxygen. **Aerobes** grow in the presence of air that contains molecular oxygen. They generally grow only by respiratory metabolism. Other microorganisms, called **microaerophiles,** grow only at reduced concentrations of molecular oxygen. Such organisms require oxygen for growth but only at concentrations (about 5%) lower than atmospheric levels (20%). Generally, microaerophilic organisms will not grow in air. Some microaerophiles prefer to grow at elevated carbon dioxide concentrations (5% to 10%) and are called **capnophiles.** *Campylobacter jejuni*, a bacterium that causes gastroenteritis and diarrhea, is capnophilic (FIG. 6-8).

Facultative anaerobes can grow in the presence or absence of air. Many facultative anaerobes, such as *Escherichia coli*, switch between respiration and fermentation, depending on the availability of molecular oxygen; they usually carry out fermentative metabolism in the absence of oxygen and respiration in the presence of oxygen. Other facultative anaerobes, such as streptococci, carry out only a fermentative metabolism whether or not oxygen is present.

Other bacteria are **anaerobes** and grow only in the absence of air. **Obligate anaerobes,** such as *Clostridium* species, can only carry out fermentative metabolism. Only a few groups of bacteria and protozoa are obligate anaerobes. Some of these, such as *Clostridium botulinum*, the bacterium that causes botulism, are very sensitive to oxygen and even a brief exposure to oxygen will kill them.

FIG. 6-8 Colonies of *Campylobacter jejuni* after 48 hours of incubation on Campy blood agar in an atmosphere with reduced oxygen concentration.

METABOLIC PATHWAYS

The metabolism of a cell occurs via a specific series of chemical reactions, called **metabolic pathways,** in which energy is transformed to generate ATP. A metabolic pathway has discrete steps between a starting substance (substrate molecule) and the products of the chemical reactions (end products). All of the ATP-generating strategies involve metabolic pathways consisting of multiple discrete enzyme-catalyzed steps. These steps operate in a specific sequence to convert an initial substrate or substrates into the end product or products of the pathway, accompanied by the formation of ATP. Several central metabolic pathways have key roles in the metabolism of microbial cells. The reactions that lead to ATP generation involve various intermediary metabolites linked together in a series of small steps to form unified metabolic pathways. The intermediary metabolites and the cellular energy are used to build the molecules of new cells for growth and reproduction.

RESPIRATION

A respiration pathway has three distinct phases (FIG. 6-9). These three phases for the respiratory metabolism of glucose are: phase 1, glycolysis; phase 2, Krebs cycle; and phase 3, electron transport chain. The overall respiration pathway results in the formation of carbon dioxide from the organic substrate

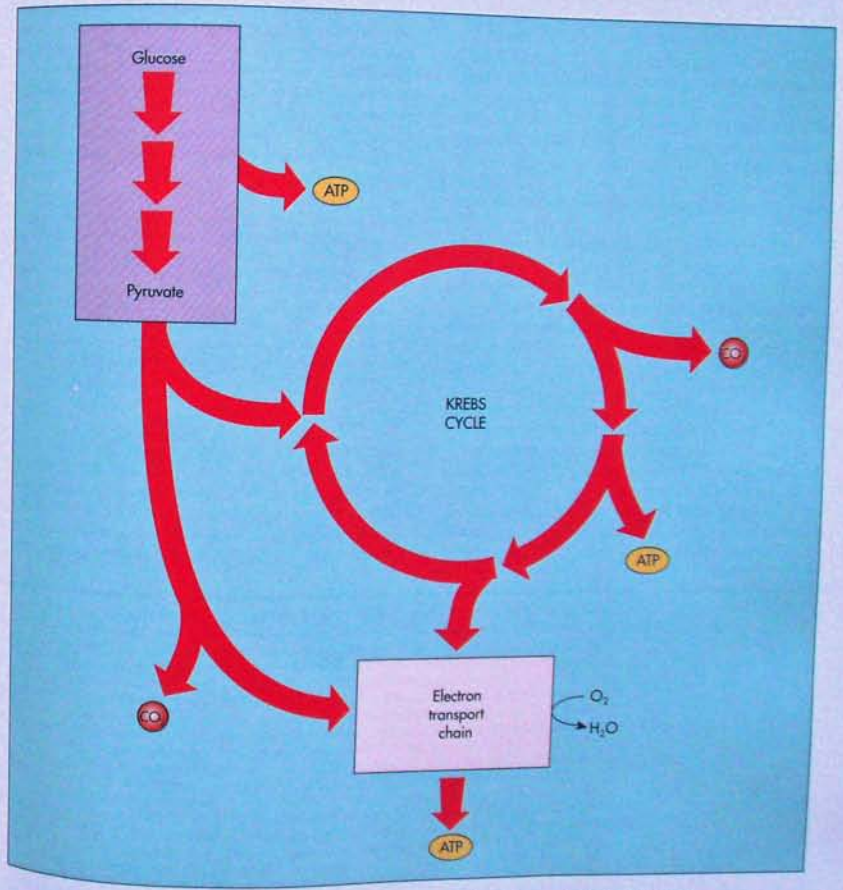


FIG. 6-9 Respiration occurs in three distinct metabolic pathways. In the first pathway, called glycolysis, carbohydrates are broken down to pyruvate. Then in the Krebs cycle the pyruvate is further metabolized to carbon dioxide. Reduced coenzymes formed during glycolysis and the Krebs cycle are used to generate a protonmotive force for chemiosmotic generation of ATP in the third and final phase of respiration.

molecule. In aerobic respiration, water is also produced as a result of the reduction of oxygen. The classic equation that summarizes aerobic respiration using glucose as substrate is:

$$\underset{\text{glucose}}{C_6H_{12}O_6} + \underset{\text{oxygen}}{6O_2} \longrightarrow \underset{\text{carbon dioxide}}{6CO_2} + \underset{\text{water}}{6H_2O}$$

In the first phase of a respiration pathway, the organic substrate is converted into small organic molecules. For example, glucose can be converted to pyruvate in a pathway called **glycolysis.** The word glycolysis comes from the Greek, *glyco,* meaning sweet (sugar), plus *lysis,* meaning to break. For each molecule of glucose that passes through this catabolic pathway, the cell acquires a few ATP molecules by substrate-level phosphorylation. It also produces reduced coenzyme (NADH). This means that glycolysis captures about 2% of the available chemical energy of glucose within the ATP molecules that are formed.

The metabolites produced in this first stage of respiration, for example pyruvate, next enter the **Krebs cycle** (named for its discoverer, Hans Krebs). In the Krebs cycle these small organic compounds are further oxidized, producing inorganic carbon dioxide and generating reduced coenzyme (NADH and $FADH_2$). Whereas glucose and other carbohydrates are initially broken down to pyruvate via glycolysis, other classes of chemicals are converted via different metabolic pathways into small organic molecules that can also enter the second stage of respiration—the Krebs cycle. Proteins are broken down into amino acids, which are further converted to form various small organic acids that can enter the Krebs cycle. Lipids are converted to fatty acids, which are broken down into products that can enter the Krebs cycle.

In the third and final stage of respiration, reduced coenzyme molecules formed during glycolysis and the Krebs cycle are reoxidized and ATP is generated by chemiosmosis. This reoxidation of reduced coenzymes involves the transport of electrons through a series of membrane-bound carriers to the terminal electron acceptor. This transfer of electrons is called **electron transport** and the carriers are referred to as the **electron transport chain.** The process of electron transport through the membrane-embedded carriers also results in the expulsion of hydrogen ions (protons) across the membrane. Because substances move by diffusion from regions of high to low concentration, the difference of proton concentration across the membrane exerts a force. This protonmotive force, caused by the concentration gradient of protons across the membrane, is used to generate ATP by chemiosmosis.

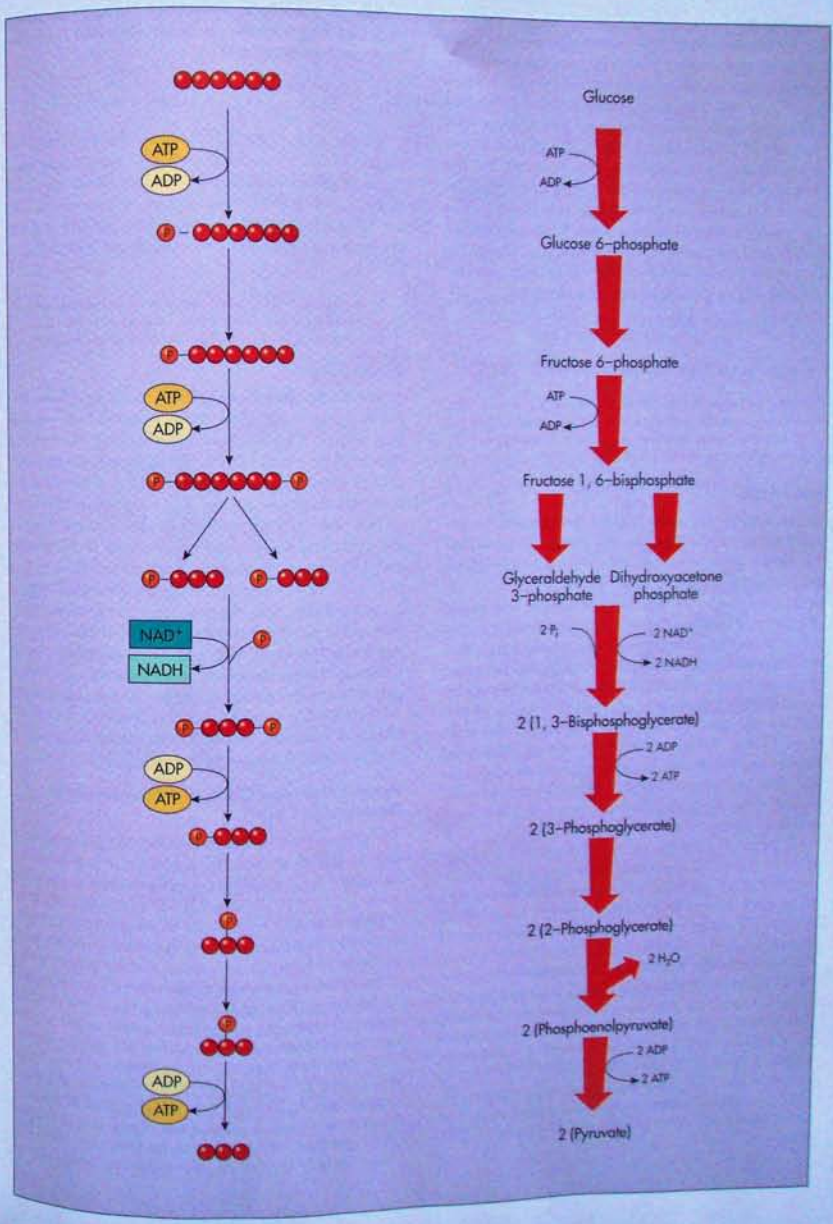
Respiration involves three distinct phases: the catabolic breakdown of a substrate by glycolysis with the generation of some ATP and reduced coenzyme (NADH), the complete oxidation of the intermediary metabolite to produce CO_2 in the Krebs cycle with the generation of additional ATP and reduced coenzymes (NADH + FADH), and the reoxidation of the reduced coenzymes involving an electron transport chain with the resultant generation of ATP by chemiosmosis.

Glycolysis

Glycolysis is the initial stage of all the main pathways of carbohydrate metabolism. It occurs within the cytoplasm in both prokaryotic and eukaryotic cells. The most common pathway of glycolysis is the **Embden-Meyerhof pathway** (FIG. 6-10). In this pathway the 6-carbon molecule glucose is first con-

FIG. 6-10 The Embden-Meyerhof pathway of glycolysis is a central metabolic pathway in various eukaryotic and prokaryotic cells for the conversion of carbohydrates to pyruvate and the formation of ATP. Each red ball represents a carbon atom. In the Embden-Meyerhof pathway a molecule of glucose is converted to two molecules of pyruvate, with the net production of two molecules of ATP and two molecules of reduced coenzyme NADH. In the initial step in the Embden-Meyerhof pathway, glucose is converted to glucose-6-phosphate. ATP is used here rather than produced. The reactive glucose-6-phosphate is then modified and converted to fructose-1,6-bisphosphate. Again, ATP is consumed rather than produced so that now the net ATP balance is minus two. Fructose-1,6-bisphosphate is enzymatically split into two phosphate-containing molecules: glyceraldehyde-3-phosphate (PGA) and dihydroxyacetone phosphate (DHAP). DHAP is converted into PGA so that now there are two molecules of PGA, and beyond this point in the pathway each step occurs twice for each molecule of glucose metabolized. Next, the PGA molecule loses 2 electrons—becoming more oxidized—and the electrons are transferred to NAD^+ to form NADH. Also in this reaction an additional phosphate group is added to form 1,3-bisphosphoglycerate. This is a substrate level phosphorylation reaction in which the generation of ATP does not depend on chemiosmosis. At this point in the glycolytic pathway there is an exact balance between the amount of ATP consumed and the amount of ATP generated.

Now net ATP production begins. The 1,3-bisphosphoglycerate is used to convert ADP to ATP, thus balancing the original energy investment of ATP. The resulting molecule is rearranged to produce phosphoenolpyruvate (PEP). PEP is transformed into pyruvate, the end product of the glycolytic pathway. It is in this last reaction that a phosphate group is added to ADP to form additional ATP, yielding the net gain of two ATP molecules for each glucose molecule degraded via this pathway.



verted to the phosphate-containing compound fructose 1,6 bisphosphate. This conversion requires an input of energy so that two ATP molecules are consumed in the reactions that bring about this conversion. The energy that has been transferred to fructose 1,6 bisphosphate subsequently is used to form ATP. Fructose 1,6 bisphosphate, which like glucose has 6-carbon atoms, is broken down into molecules that have three carbon atoms. This results in the production of two molecules of pyruvate (see FIG. 6-10). It also results in the net production of two molecules of NADH and the net synthesis of two ATP molecules so that the overall equation for the Embden-Meyerhof pathway of glycolysis can be written as:

$$\text{glucose} + 2\,\text{ADP} + 2\,\text{P}_i + 2\,\text{NAD}^+ \longrightarrow 2\,\text{pyruvate} + 2\,\text{NADH} + 2\,\text{ATP}$$

where P_i stands for inorganic phosphate.

In the Embden-Meyerhof pathway of glycolysis, glucose is partially broken down into pyruvate, two NADH molecules are formed, and the energy released leads to a net yield of two ATP.

Krebs Cycle

The second phase of respiratory metabolism occurs when the pyruvate generated by glycolysis feeds into the **Krebs cycle**, which is also known as the **tricarboxylic acid** or **citric acid cycle** (FIG. 6-11). In prokaryotic cells, the Krebs cycle occurs within the cytoplasm. In eukaryotic cells, this metabolic process occurs within mitochondria. In the series of chemical reactions that make up the Krebs cycle, the potential chemical energy stored in intermediate compounds derived from pyruvate is released in a series of oxidation-reduction reactions. As a result of the reactions of the Krebs cycle the pyruvate molecules formed during glycolysis are oxidized to form carbon dioxide. Thus at the end of the Krebs cycle, six carbon dioxide molecules are produced for each 6-carbon glucose molecule metabolized.

To begin the Krebs cycle, pyruvate produced by glycolysis is split and a fragment of it is attached to coenzyme A (CoA). The combined molecule is called *acetyl-CoA*. Acetyl-CoA then enters the Krebs cycle, initiating a series of reactions that release electrons and protons (H^+). NADH is generated during several reactions of the Krebs cycle. Another coenzyme, flavin adenine dinucleotide (FAD), is also reduced to $FADH_2$. One of the reactions of the Krebs cycle is also directly coupled with the substrate-level generation of a high-energy phosphate-containing compound called guanidine triphosphate (GTP). GTP can be converted to ATP, and, for accounting purposes, the GTP generated in this reaction is counted as ATP in determining the net synthesis of ATP during respiration.

The net reaction of metabolism through the Krebs cycle, starting with the pyruvate generated from glucose, can be written as:

$$2\,\text{pyruvate} + 2\,\text{ADP} + 2\,\text{FAD} + 8\,\text{NAD}^+ \longrightarrow 6\,\text{CO}_2 + 2\,\text{ATP} + 2\,\text{FADH}_2 + 8\,\text{NADH}$$

At the end of the Krebs cycle, the cell has converted all of the substrate carbon of the glucose molecule to carbon dioxide. There also has been a net synthesis of four ATP molecules—the production of ten reduced coenzyme molecules as NADH and the generation of two reduced coenzyme molecules as $FADH_2$.

In the Krebs cycle, intermediary metabolites, such as pyruvate, are completely oxidized to CO_2 with the production of reduced coenzymes and some ATP.

The Krebs cycle and glycolytic pathways have important roles within the overall respiratory generation of ATP. They also occupy a central place in the flow of carbon through the cell. As a result of its function of supplying small biochemical molecules for biosynthetic pathways, the Krebs cycle is rarely completed. Some of the intermediates are siphoned out of the cycle, especially into amino acid biosynthesis, and so some of the intermediary metabolites of this pathway must be continuously resynthesized to continue the Krebs cycle. In many microorganisms, only part of the substrate is completely oxidized for driving the synthesis of ATP, and the remainder is used for biosynthesis. Similarly, the reduced coenzymes generated in this pathway can be used for generating ATP or for the synthesis of the reduced coenzyme NADPH (reduced nicotinamide adenine dinucleotide phosphate) for use in biosynthesis.

Electron Transport Chain and Chemiosmotic Generation of ATP

The reduced coenzyme molecules that are generated during glycolysis and the Krebs cycle can be reoxidized and the energy stored in them used to generate additional ATP (FIG. 6-12). The energy-requiring synthesis of ATP from ADP in respiration is driven largely by chemiosmosis. As electrons pass down an electron transport chain, some of their carrier molecules extrude protons from one side of the membrane to the other. Some of the compounds in the electron transport chain can accept and transfer whole hydrogen atoms (that is, a proton and an electron), whereas others can accept only electrons. When only electrons are accepted, the protons (hydrogen ions) must go somewhere. In bacteria they are extruded to the outside of the plasma membrane. Since the phospholipid portions of plasma membranes are normally impermeable to protons, this pumping establishes a

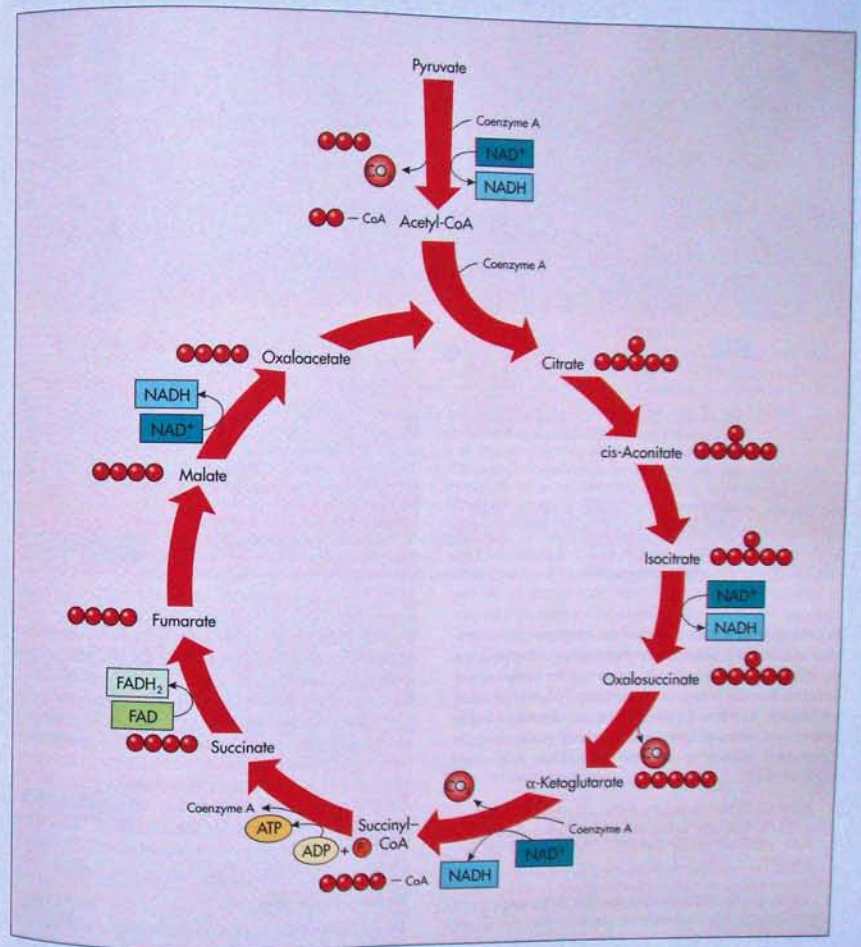


FIG. 6-11 The Krebs cycle is a metabolic pathway central to respiratory metabolism and provides a critical link between the metabolism of the different classes of macromolecules. The metabolism of pyruvate through the tricarboxylic acid Krebs cycle results in the generation of ATP and reduced coenzymes and the formation of CO_2. The oxidation of pyruvate takes place in two stages: the decarboxylation of pyruvate, that is, CO_2 removal, to form acetyl-CoA, and the subsequent oxidation of the acetyl-CoA to form CO_2. When the pathway is completed, the intermediate carboxylic acids are regenerated and continue to cycle throughout the reactions of the Krebs cycle. Each carbon atom in the molecules of the pathway is represented as a red ball.

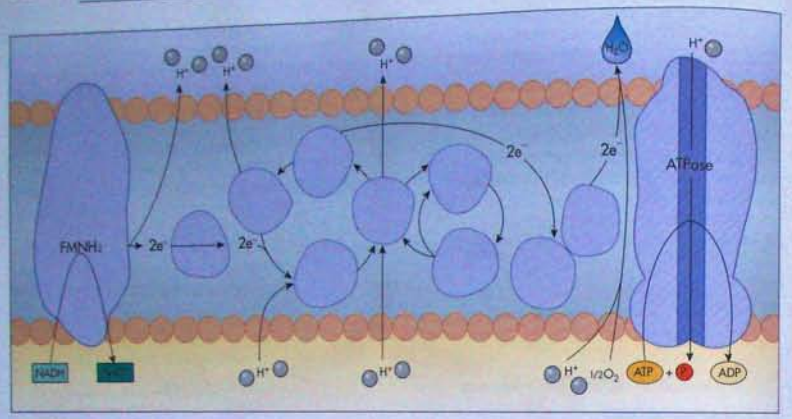


FIG. 6-12 The electron transport chain is a membrane-embedded series of reactions that result in the reoxidation of reduced coenzymes. The transport of electrons through the cytochrome chain of this pathway results in the pumping of protons across the membrane, and the return flow of hydrogen ions resulting from this proton gradient drives the generation of ATP. Electrons that enter the system from NADH are transported through flavin mononucleotide (FMN) to coenzyme Q; those that enter from $FADH_2$ go directly to coenzyme Q. Electrons then flow through a series of cytochromes, designated cyt *b*, *c*, *a*, and a_3, to the terminal electron acceptor. As the electron is transported through each carrier, there is an oxidation-reduction reaction, so that in the case of the cytochromes, for example, iron (Fe) within the cytochrome alternates between the oxidized Fe^{3+} and reduced Fe^{2+} states.

proton gradient, that is, there is a higher concentration outside the membrane than inside. The protons on the outside of the membrane can be transported inward by the enzyme **adenosine triphosphatase (ATPase)**, which is located in the membrane. As this movement occurs, energy is released from the protonmotive force and used by the ATPase to convert ADP to ATP.

Chemiosmosis is based on pumping protons across a membrane and using the energy released by their diffusive return (protonmotive force) to generate ATP.

Through chemiosmosis, energy contained in reduced coenzyme molecules is used to drive ATP synthesis. The hydrogen ion gradient (protonmotive force) across a membrane and chemiosmosis drives the formation of ATP. The reduced coenzyme NADH contains more stored chemical energy than the reduced coenzyme $FADH_2$. For each NADH molecule, three ATP molecules can be synthesized during oxidative phosphorylation, compared to only two ATP molecules for each $FADH_2$. The ten NADH molecules generated during glycolysis and the Krebs cycle, therefore, can be converted to 30 ATP molecules during oxidative phosphorylation. The two $FADH_2$ molecules generated during the Krebs cycle can generate four ATP molecules. This ATP is in addition to that formed during glycolysis and the Krebs cycle, so that a total of 38 ATP molecules may be produced from the respiratory metabolism of each glucose molecule.

Prokaryotic and eukaryotic cells use chemiosmosis for ATP production in both oxidative phosphorylation and photophosphorylation. Electron transport carriers and ATPase are located in membranes, either the plasma membrane of prokaryotes, the inner mitochondrial membrane, or the thylakoid membrane of photosynthetic cells, such as those found in chloroplasts.

Aerobic Respiration

When oxygen (O_2) serves as the terminal electron acceptor, as in the above example, the respiratory metabolism is called **aerobic respiration.** The oxygen is reduced to form water in this process. The overall reaction for the aerobic respiratory metabolism of glucose can be written as:

$$\text{glucose} + 6\,O_2 + 38\,ADP + 38\,P_i \longrightarrow 6\,CO_2 + 6\,H_2O + 38\,ATP$$

Aerobic respiration (respiration in which molecular oxygen serves as the terminal electron acceptor) is remarkably efficient. The initial breakdown of glucose by glycolysis yields only two ATP molecules per molecule of glucose. In aerobic respiration, an additional 36 ATP molecules can be formed. Hence, approximately 40% of the chemical bond energy of the glucose that is broken down in aerobic respiration is recovered as high-energy-containing ATP.

Anaerobic Respiration

Some cells can use substances other than oxygen as the terminal electron acceptor in a respiratory pathway (FIG. 6-13). Bacteria such as *Pseudomonas* and *Bacillus* can use a nitrate ion (NO_3^-) as a terminal electron acceptor. This NO_3^- is then reduced to nitrite ion (NO_2^-), nitrous oxide (N_2O), or nitrogen gas (N_2).

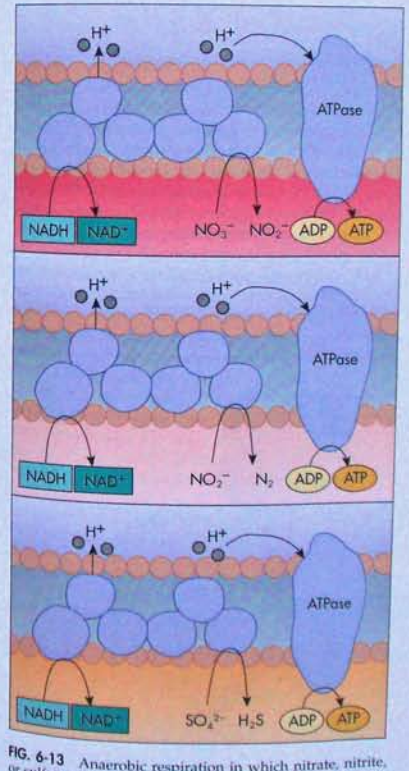


FIG. 6-13 Anaerobic respiration in which nitrate, nitrite, or sulfate serves as the terminal electron acceptor has specific electron carriers. The electron transfer results in the formation of nitrite from nitrate, molecular nitrogen from nitrite, or hydrogen sulfide from sulfate.

HIGHLIGHT

A Microbial Explanation for Ghosts

Under anaerobic conditions, where there is no oxygen, nitrate, or sulfate to serve as terminal electron acceptors for respiration, some microorganisms may still be able to carry out respiration using phosphate as the terminal electron acceptor. The use of phosphate (PO_4^{3-}) as the terminal electron acceptor results in the production of phosphine (PH_3), a reactive green-glowing gas. Conditions where this might occur include areas of extensive organic matter decomposition, such as might be encountered around cemeteries where the graves are not sealed. Mass burial sites were often used in villages where cemeteries were located on hillsides and numerous bodies were placed daily in times of famine and epidemic outbreaks of disease. Imagine the myths that might arise when villagers heard a rumbling noise and saw a green glow rising from the graves on the hillside.

Other bacteria, such as *Desulfovibrio*, use sulfate (SO_4^{2-}) as the terminal electron acceptor to form hydrogen sulfide (H_2S). When a molecule other than oxygen, such as nitrate or sulfate, serves as the terminal electron acceptor, the metabolic pathway is called **anaerobic respiration.** As the name implies, anaerobic respiration does not require the presence of air (oxygen). Anaerobic respiration is often less efficient than aerobic respiration, producing about one third of the ATP made by aerobes. This is because a complete Krebs cycle often does not function in the absence of molecular oxygen and because two rather than three ATP molecules are generated from each NADH by chemiosmosis.

In the absence of molecular oxygen, nitrate can serve as the terminal electron acceptor with the production of molecular nitrogen and water.

In the absence of both oxygen and nitrate, sulfate can serve as the terminal electron acceptor, becoming reduced to hydrogen sulfide and water in the process.

Lipid and Protein Catabolism

Besides glucose and other carbohydrates, microorganisms also utilize lipids and proteins as substrates for metabolism. Because of their high molecular weights these substances can not enter the cell unless they are first broken down outside the cell (extracellularly). Some microorganisms produce extracellular enzymes for the breakdown of proteins and lipids, as well as for attacking polysaccharides such as cellulose, chitin, and starch.

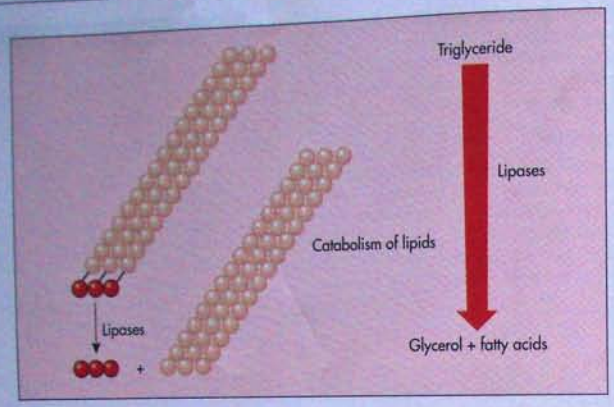


FIG. 6-14 Lipids are broken down by lipases into glycerol and fatty acids. The fatty acids are further metabolized by β-oxidation to smaller fatty acids and acetate.

Various microorganisms produce extracellular enzymes, called *lipases*, that break down lipids (fats) into their fatty acid and glycerol components. These components enter the cell, where they are further metabolized (FIG. 6-14). Many microorganisms convert glycerol into dihydroxyacetone phosphate, one of the intermediates formed during glycolysis. The dihydroxyacetone phosphate enters the glycolytic pathway and is further metabolized to CO_2. Fatty acids are catabolized by *beta-oxidation*. In this process, carbon fragments of long chains of fatty acids are removed two at a time and acetyl-CoA is formed. A fatty acid with sixteen carbons yields eight molecules of acetyl-CoA in seven cleavage steps. As the molecules of acetyl-CoA form, they enter the Krebs cycle, as do the acetyl-CoA molecules formed by the oxidation of pyruvate. In this process, reduced coenzymes are produced and their subsequent reoxidation results in the chemiosmotic generation of ATP.

Microorganisms also produce extracellular enzymes, called *proteases*. Proteases break down proteins into short polypeptides and amino acids (FIG. 6-15). These small subunits enter the cell and are further attacked. Amino acids can be enzymatically

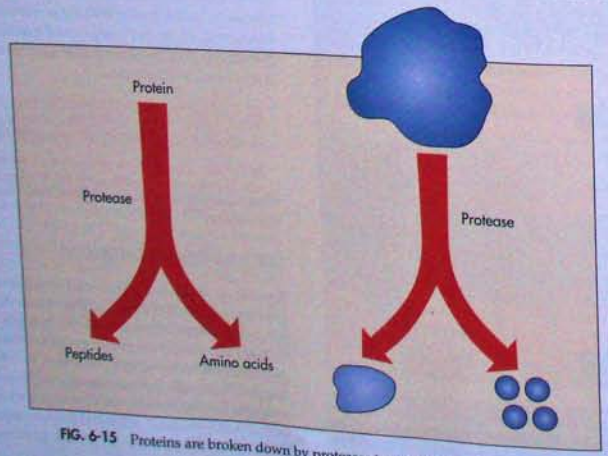


FIG. 6-15 Proteins are broken down by proteases into peptides and amino acids.

deaminated, that is, the amino group can be removed and converted to inorganic ammonia. Removal of an amino group from an amino acid produces a carboxylic acid. The carboxylic acids produced in this manner can enter the glycolytic pathway or the Krebs cycle. Further catabolism results in decarboxylation with the production of CO_2. ATP is generated as a result of the protonmotive force formed by the establishment of a proton gradient across the plasma membrane of a prokaryotic cell or the mitochondrial membranes of eukaryotic cells.

FERMENTATION

In fermentation pathways the organic substrate molecule is converted to another organic molecule that serves as the terminal electron acceptor. There is no net change in the oxidation state of the products relative to the starting substrate molecule. The oxidized products are exactly counterbalanced by reduced products, and thus the required oxidation-reduction balance is always achieved. Such a metabolic pathway can occur in the absence of air because there is no requirement for oxygen or any other inorganic compound to serve as the terminal electron acceptor to balance a change in the oxidation state of the organic molecule. Some microorganisms can carry out fermentation in the presence of air, simply ignoring the presence of oxygen, whereas others can carry out fermentation only in the absence of oxygen.

Fermentation is an anaerobic process that does not require an external electron acceptor to balance the oxidation-reduction reactions in the pathway.

In a fermentation pathway, substrate-level phosphorylation converts ADP to ATP. Unlike respiration, fermentation does not involve an electron transport chain or chemiosmosis for generating ATP. Rather, the synthesis of ATP in fermentation is largely restricted to the amount formed during glycolysis. Fermentation yields far less ATP per substrate molecule than respiration. This is because during a fermentation pathway the organic substrate molecule is not completely oxidized to carbon dioxide. As a result, not as much energy can be released from the substrate molecule to drive the synthesis of ATP.

Fermentation generates ATP by substrate-level phosphorylation.

Fermentation yields far less ATP per substrate molecule utilized than respiration.

The energy obtained from the complete oxidation of glucose to carbon dioxide and water by respiration is more than 10 times greater than that obtained when glucose is metabolized by fermentation. Because more ATP can be generated per molecule of substrate, fewer substrate molecules must be metabolized during respiration than during fermentation to achieve equivalent growth. From both the viewpoints of the energy needs of living organisms and conservation of available organic nutrient resources, respiration is more favorable than fermentation. Organisms that have the metabolic capability to carry out both types of metabolism will generally use the energetically more favorable respiration pathway, when conditions permit, and will rely on fermentation only when there is no available external electron acceptor.

The initial metabolic steps in the fermentation of a carbohydrate are identical to those in respiration. It begins with glycolysis. If the microorganism uses the Embden-Meyerhof glycolytic pathway, it generates two pyruvate molecules, two reduced coenzyme NADH molecules, and two ATP molecules for each molecule of glucose as a result of the glycolytic breakdown of glucose.

In respiration the reoxidation of the coenzymes occurs in the electron transport chain and requires an external electron acceptor. In fermentation the reoxidation of NADH to NAD^+ depends on the reduction of the pyruvate molecules formed during glycolysis to balance the oxidation-reduction reactions. Different microorganisms have developed different pathways for utilizing the pyruvate to reoxidize the reduced coenzyme. These pathways have different ter-

NEWSBREAK

INTESTINAL YEAST INFECTIONS CAUSE INTOXICATION

Some people in Japan have been reported to suffer from a strange form of alcoholism apparently related to a yeast infection in their intestines. These people had not consumed any alcoholic beverages but nevertheless frequently appeared to be inebriated. The evidence indicates that yeast growing in their intestines is carrying out an alcoholic fermentation. This fermentation produced sufficient alcohol to bring about this state of drunkenness. Reduction of the yeast population in their intestines by treatment with antibiotics eliminated this state of intoxication. Thus there is a cure for such infectious intoxication.

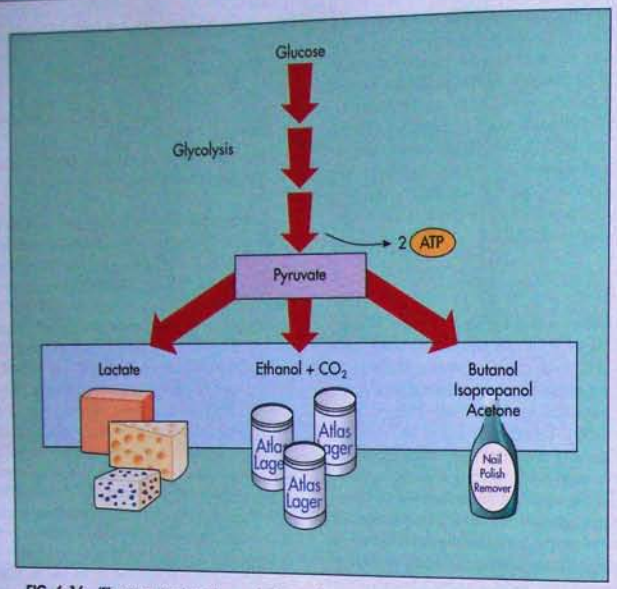


FIG. 6-16 There are various fermentation pathways that produce different end products.

minal sequences and hence the various fermentation pathways result in the formation of varying end products (FIG. 6-16).

A complete fermentation pathway (1) begins with a substrate, (2) includes glycolysis, and (3) terminates with the formation of end products. There is no net change in the oxidative state of the coenzymes during the overall fermentation pathway, and the coenzyme does not appear in the overall fermentation equation. There are several different fermentation pathways that are generally named for the characteristic end products that are formed (see Table 6-3). Many of the products of fermentation pathways have commercial value. As will become apparent from the discussion of the specific fermentation pathways, numerous foods, beverages, and other products we frequently use are the products of microbial fermentations.

Ethanolic Fermentation

In the **ethanolic fermentation,** or **alcoholic fermentation,** pyruvate is converted to ethanol and carbon dioxide. Ethanolic fermentation is carried out by many yeasts, such as *Saccharomyces cerevisiae*, but by relatively few bacteria. The ethanolic fermentation pathway can be written as:

$$\text{glucose} + 2\ ADP + 2\ P_i \longrightarrow 2\ \text{ethanol} + 2\ CO_2 + 2\ ATP$$

This fermentation pathway is very important in food and industrial microbiology. It is used to produce beer, wine, and distilled spirits (FIG. 6-17). For example, the carbohydrates in grains are converted to ethanol by yeasts in the production of beer and spirits. The sugars in grapes are the substrates for ethanol production by the wine-producing yeasts. *Saccharomyces cerevisiae*, also known as baker's yeast, is used in bread making; the carbon dioxide released during ethanolic fermentation causes bread dough to rise in a process called *leavening* (FIG. 6-18). The ethanol is driven from the bread during baking, which, according to the United States Environmental Protection Agency, is an important source of air pollution that must be controlled. The ethanol produced by *S. cerevisiae* is also used as a fuel to augment gasoline in a product known popularly as *gasohol*.

Lactic Acid Fermentation

Lactic acid fermentation is carried out by bacteria that—by virtue of these metabolic reactions—are classified as the lactic acid bacteria. This fermentation pathway produces lactic acid as an end product. When the Embden-Meyerhof scheme of glycolysis is used in the lactic acid fermentation pathway, the overall pathway is a **homolactic fermentation** because the only end product formed is lactic acid. Homolactic fermentation is carried out by *Streptococcus*

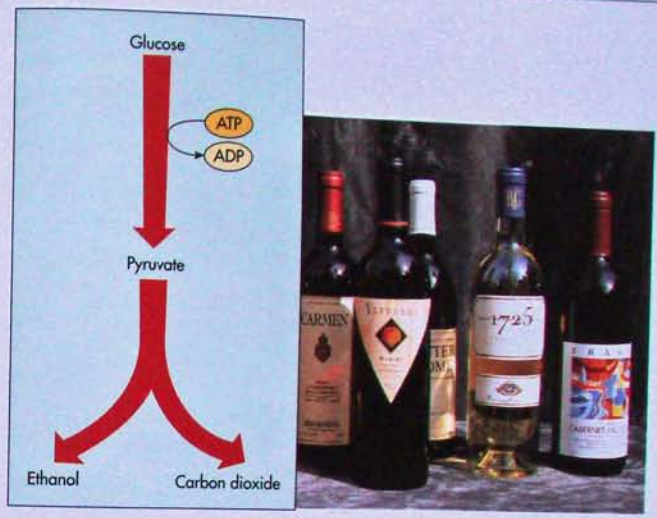


FIG. 6-17 The ethanolic fermentation pathway results in the formation of ethanol and CO_2. The fermentation of carbohydrates to these end products forms the basis of the beer, wine, and spirits industries.

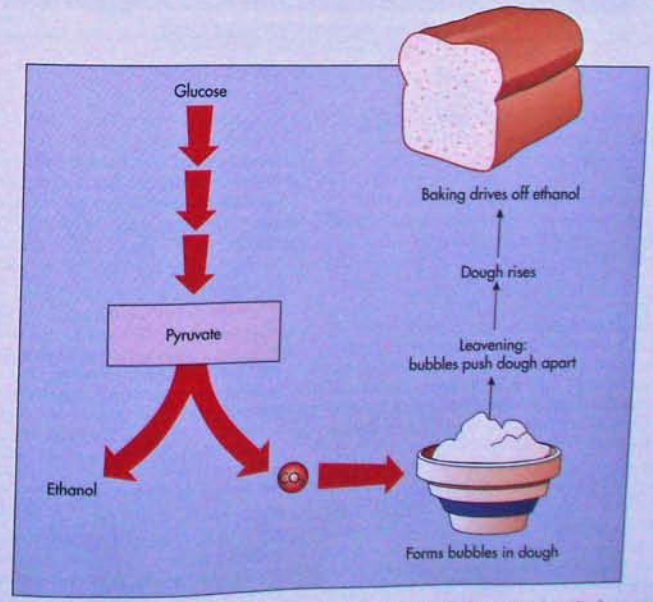


FIG. 6-18 Bread is made by an alcoholic fermentation. The CO_2 from the ethanolic fermentation causes the bread to rise.

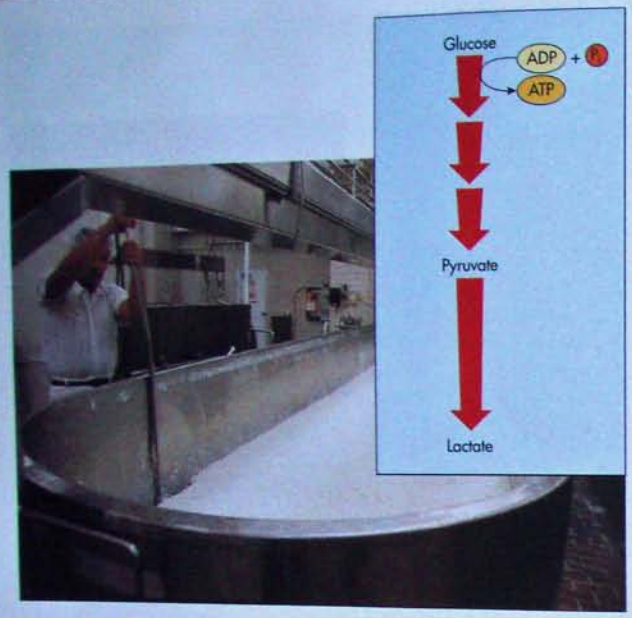


FIG. 6-19 The homolactic acid fermentation pathway results in the production of lactate (lactic acid). This fermentation pathway is the basis for cheese production.

and *Lactobacillus* species (FIG. 6-19). The homolactic acid fermentation pathway can be written as:

$$\text{glucose} + 2\,\text{ADP} + 2\,\text{P}_i \longrightarrow 2\,\text{lactic acid} + 2\,\text{ATP}$$

Streptococci, even though they are metabolically obligate anaerobes, live on human tooth surfaces and produce lactic acid that is held against the tooth by dental plaque. The acid can gradually eat through the enamel of the tooth and create cavities. The lactic acid produced by *Lactobacillus* species in the vaginal tract helps protect against sexually transmitted pathogens. *Lactobacillus* species are the initial colonizers of the human intestinal tract and occur in the human digestive tract, aiding in the digestion of milk and dairy products. Many adults cannot digest the carbohydrates in milk and so *L. acidophilus* is added to various commercial milk products, such as acidophilus milk, to aid those who cannot tolerate milk. The enzymes produced by *L. acidophilus* convert milk sugars to products that do not accumulate and cause gastrointestinal upset. The homolactic acid fermentation pathway is important in the dairy industry because it is responsible for the souring of milk. It is used in the production of many types of cheese, yogurt, and other dairy products. Buttermilk and sour cream are made by using different strains of lactic acid bacteria as starter cultures and different parts of whole milk as the starting substrate.

In some bacteria, however, a different pathway of glycolysis—the Entner-Doudoroff pathway—is used. Such bacteria are termed *heterolactic* because both ethanol and carbon dioxide are produced in addition to lactic acid. The ethanol and carbon dioxide come from the glycolytic portion of the pathway. This fermentative pathway is carried out by *Leuconostoc* and various *Lactobacillus* species and is responsible for the production of sauerkraut (FIG. 6-20). Several types of sausage are produced by allowing meat to undergo heterolactic acid fermentation during curing. The overall reaction for the **heterolactic fermentation** can be written as:

$$\text{glucose} + \text{ADP} + \text{P}_i \longrightarrow \text{lactic acid} + \text{ethanol} + \text{CO}_2 + \text{ATP}$$

Propionic Acid Fermentation

Another fermentation pathway of interest is the **propionic acid fermentation pathway.** This metabolic sequence carried out by the propionic acid bacteria produces propionic acid and carbon dioxide. The bacterial genus *Propionibacterium*, which contributes to acne, is defined as Gram-positive rods that produce propionic acid from the metabolism of carbohy-

FIG. 6-20 The heterolactic acid fermentation pathway results in the production of lactate (lactic acid), ethanol, and CO_2. This fermentation pathway is the basis for sauerkraut production.

HIGHLIGHT

Production of Cheese

Cheeses are produced by microbial fermentations. Cheese consists of milk curds that have been separated from the liquid portion of milk (whey). The curdling of milk is accomplished by using the enzyme rennin and lactic acid bacterial starter cultures. Rennin is obtained from calf stomachs or by microbial production.

Cheeses are classified as: (1) soft, if they have a high water content (50% to 80%); (2) semihard, if the water content is about 45%; and (3) hard, if they have a low water content (less than 40%). Cheeses are also classified as unripened, if they are produced by a single-step fermentation, or as ripened, if additional microbial growth is required during maturation of the cheese to achieve the desired taste, texture, and aroma. Processed cheeses are made by blending various cheeses to achieve a desired product. If the water content is elevated during processing, thereby diluting the nutritive content of the product, the product is called a "processed cheese food" rather than a cheese.

The natural production of cheeses involves a lactic acid fermentation with various mixtures of *Streptococcus* and *Lactobacillus* species used as starter cultures to initiate fermentation. The flavors of different cheeses result from the use of different microbial starter cultures, varying incubation times and conditions, and the inclusion or omission of secondary microbial species late in the fermentation process.

Ripening of cheeses involves additional enzymatic transformations after formation of the cheese curd, using enzymes produced by lactic acid bacteria or enzymes from other sources. Unripened cheeses do not require additional enzymatic transformations. Cottage cheese and cream cheese are produced by using a starter culture similar to the one used for the production of cultured buttermilk and are soft cheeses that do not require ripening. Sometimes a cheese is soaked in brine to encourage development of selected bacterial and fungal populations during ripening. Limburger is a soft cheese produced in this manner. During ripening, the curds are softened by proteolytic and lipolytic enzymes and the cheese acquires its characteristic aroma. The production of Parmesan cheese also involves brine curing.

Various fungi are also used in the ripening of different cheeses. The unripened cheese is normally inoculated with fungal spores and incubated in a warm moist room to favor the growth of filamentous fungi. For example, blue cheeses are produced by using *Penicillium* species. Roquefort cheese is produced by using *P. roqueforti*, and camembert and brie are produced by using *P. camemberti* and *P. candidum*.

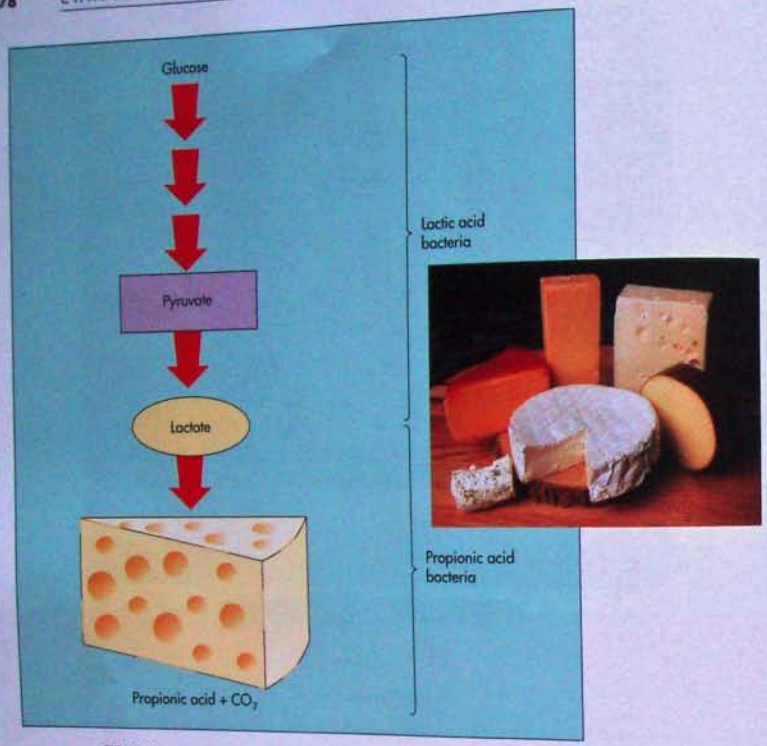


FIG. 6-21 The propionic acid fermentation produces propionic acid and carbon dioxide. The carbon dioxide produced in this fermentation forms the holes in Swiss cheese.

drates (FIG. 6-21). These bacteria have the ability to carry out this fermentation pathway beginning with lactic acid as the substrate. The ability to utilize the end product of another fermentation pathway is quite unusual, but it allows species of *Propionibacterium* to carry out late fermentation during the production of cheese.

Lactic acid bacteria convert the initial substrates in milk to lactic acid. Propionic acid bacteria then convert the lactic acid to propionic acid and carbon dioxide. Propionic acid bacteria begin their fermentation only after the cheese curd has formed through the action of lactic acid bacteria. The release of carbon dioxide during this late fermentation forms gas bubbles in the semisolid cheese curd, forming the holes in Swiss cheese. The propionic acid formed during this fermentation also gives Swiss cheese its characteristic flavor.

Mixed-acid Fermentation

The **mixed-acid fermentation** pathway is carried out by *Escherichia coli*, as well as hundreds of other bacterial species. In the mixed-acid fermentation pathway the pyruvate formed during glycolysis is converted to various products—ethanol, acetate (acetic acid), formate (formic acid), lactate (lactic acid), molecular hydrogen (H_2), and carbon dioxide (CO_2). It is called the mixed-acid fermentation because of these many products. The proportions of the products vary, depending on the bacterial species.

Mixed-acid fermentation can be detected by the **Methyl Red (MR) test,** which is based on the color reaction of the pH indicator Methyl Red (FIG. 6-22). This test is one of several typically used in clinical identification systems, including miniaturized commercial identification systems used for the identification of bacteria, such as *E. coli*, which cause urinary tract and other infections.

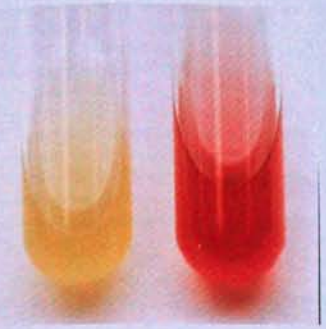


FIG. 6-22 The Methyl Red test, which detects the production of acid, is useful for differentiating various bacterial species, including *Enterobacter aerogenes* and *Escherichia coli*. Negative test result for *Enterobacter aerogenes* is on left. Positive test result for *Escherichia coli* is on right.

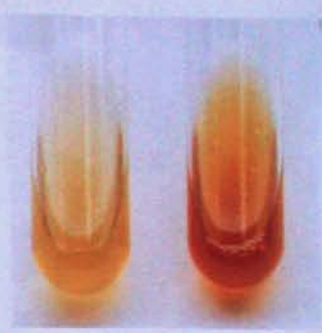


FIG. 6-23 The Voges-Proskauer test, which detects the production of acetoin, is useful for differentiating various bacterial species, including *Enterobacter aerogenes* and *Escherichia coli*. Negative test result for *Escherichia coli* is on left. Positive test result for *Enterobacter aerogenes* is on right.

Butanediol Fermentation

Some bacteria, such as members of the bacterial genus *Enterobacter* and *Klebsiella*, carry out a **butanediol fermentation** pathway. An intermediary product in the butanediol fermentation pathway is acetoin (acetyl methyl carbinol). One of the classic diagnostic tests used for separating *E. coli* from *Enterobacter aerogenes* is the **Voges-Proskauer test,** which detects the presence of acetoin (FIG. 6-23). *E. coli* does not carry out a butanediol pathway, whereas *Enterobacter aerogenes* does. Thus *Enterobacter aerogenes* is Voges-Proskauer positive and *E. coli* is Voges-Proskauer negative.

It is important to distinguish between these organisms because *E. coli* is used as an indicator of human fecal contamination in assessing water quality and safety. The Methyl Red test is also used for separating *Enterobacter aerogenes* from *E. coli*. It detects very low pH resulting from high amounts of acid production. Because *Enterobacter aerogenes* channels part of its substrate into the neutral fermentation end product, butanediol, it does not produce as much acid and thus does not lower the pH as much as *E. coli*, which channels all of its substrate into the mixed-acid fermentation pathway. Thus *E. coli* shows a positive Methyl Red test, whereas *Enterobacter aerogenes* yields the opposite reaction.

Butanol Fermentation

In yet another pathway, members of the genus *Clostridium* carry out a **butanol fermentation.** Different species of *Clostridium* form various end products via this fermentation pathway, with pyruvate being converted either to acetone and carbon dioxide, propanol and carbon dioxide, butyrate, or butanol. Several of these products are good solvents. Chaim Weizmann, a Polish-born microbiologist working in Britain, discovered the butanol fermentation pathway in time to allow the British to produce acetone for the manufacture of munitions for World War I. Their ability to do so was instrumental in the success of the Allied forces. Today acetone for nail polish remover often is produced by microbial fermentation via this pathway.

Photosynthetic Metabolism

Some early organisms evolved a different way of generating ATP by **photoautotrophic metabolism,** or **photosynthesis.** Instead of obtaining energy for ATP synthesis by breaking chemical bonds, these organisms, called **photoautotrophs,** developed the ability to use light as the source of energy that moves protons across membranes of their cells to create a protonmotive force. Photoautotrophs also obtain their carbon from inorganic CO_2.

In photosynthesis, light energy is captured and used to generate ATP.

In photosynthesis, sunlight strikes pigments embedded within the membranes of the cell. In this first stage of photosynthesis, electrons within special re-

FIG. 6-24 Micrograph of the cyanobacterium *Aphanizomenon flos-aquae*.

action centers of certain pigments are excited, meaning that they have absorbed energy from the light. These excited electrons pass from the reaction-center pigments through a series of proteins embedded within the membrane, eventually reaching and activating channels that enable the transport of protons across the membrane. These channels retain the electrons and export the protons across the membrane. The protonmotive force generated in this process is used to produce ATP by chemiosmosis. As a result of electron transport, the reduced coenzyme NADPH also is produced; NADPH is later used to produce sugars from carbon dioxide.

Photoautotrophs

Organisms that use light as a source of energy and carbon dioxide as their chief source of carbon are called **photoautotrophs.** The photoautotrophs include photosynthetic bacteria (green sulfur and purple sulfur bacteria, and cyanobacteria), algae, and green plants (FIG. 6-24). In the photosynthetic reactions of cyanobacteria, algae, and plants, the electrons of water are used to reduce carbon dioxide, and oxygen gas (O_2) is given off (FIG. 6-25). Because this photosynthetic process produces O_2, it is sometimes

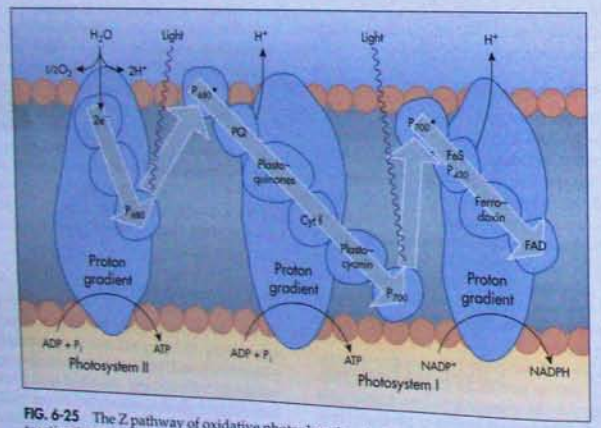


FIG. 6-25 The Z pathway of oxidative photophosphorylation combines two separate photoactivation steps (photosystems) into a unified pathway. These are the excitation of P_{680} to P_{680}^* and the excitation of P_{700} to P_{700}^*. The P_{680} has a sufficiently positive reduction potential to use H_2O as an electron donor. The resulting P_{680}^* is at a considerably more negative reduction potential, such that the resulting electrons can "fall down" a potential gradient in which protons are extruded across the membrane and form a proton gradient (protonmotive force) to generate ATP. The electrons are passed to the P_{700} complex, which when excited is at a potential more negative than that of the $NADP^+$/NADPH redox pair and is thereby capable of reducing $NADP^+$ to NADPH. The pathway is called the *Z pathway* because when these reactions are plotted as a function of reduction potential the resulting figure resembles a Z. The protons originate from the photolysis of water to establish the protonmotive force.

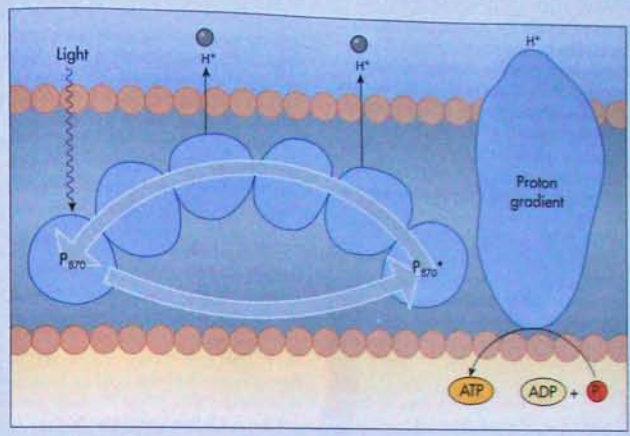


FIG. 6-26 Cyclic photophosphorylation of anaerobic photosynthetic bacteria uses P_{870}. This pathway generates the proton gradient needed to drive the formation of ATP and does not produce reduced coenzyme.

called **oxygenic photosynthesis.** The light-trapping pigment required by green plants, algae, and cyanobacteria is the green compound **chlorophyll *a***. It is located in the thylakoids of chloroplasts in algae and green plants and in the thylakoids that form a part of an elaborate internal membrane structure in cyanobacteria.

Algae and cyanobacteria have chlorophyll *a*, which absorbs light energy.

Oxygen is formed from water during oxygenic photosynthesis.

Besides the cyanobacteria, there are several other families of photosynthetic prokaryotes that are classified according to the way they reduce carbon dioxide. These bacteria cannot use electrons from water to reduce carbon dioxide and cannot carry on photosynthesis when oxygen is present, that is, they must have an anaerobic environment (FIG. 6-26). Their photosynthetic process does not produce oxygen gas, and therefore this type of photosynthesis is called **anoxygenic photosynthesis.**

The green sulfur and purple sulfur bacteria are photoautotrophic microorganisms that carry out anoxygenic photosynthesis. The chlorophyll used by these photosynthetic bacteria is *bacteriochlorophyll*, which absorbs light of longer wavelengths than that absorbed by chlorophyll *a*. The green sulfur bacteria use sulfur, sulfur compounds, or hydrogen gas to reduce carbon dioxide and form organic compounds. Applying the energy from light to bacteriochlorophyll and the appropriate enzymes, these bacteria oxidize sulfur to sulfuric acid, hydrogen sulfide to sulfur, or hydrogen gas to water. The purple sulfur bacteria also use sulfur, sulfur compounds, or hydrogen gas to reduce carbon dioxide. They are distinguished from the green sulfur bacteria on the basis of their biochemistry and morphology.

Oxygen is not produced during anoxygenic photosynthesis.

Anaerobic photosynthetic sulfur bacteria oxidize hydrogen sulfide (H_2S) so that they can reduce carbon dioxide to organic matter.

Photoheterotrophs

Photoheterotrophs use light as a source of energy and can convert CO_2 to sugars, but they also require organic compounds as sources of carbon for growth. Their organic compound sources include alcohols, fatty and other organic acids, and carbohydrates. Among the photoheterotrophs are the green and purple nonsulfur bacteria.

Photosystems and ATP Generation

Chlorophyll and various other colored pigmented compounds are light-trapping pigments that are organized into clusters of 200 to 300 molecules. These compounds harvest or collect light energy in one of their chemical bonds. When this happens, an electron becomes excited and reaches a higher energy level. That extra energy is released and transferred to a

neighboring pigment when the electron returns to a lower energy level. The high energy electron is transferred to an electron acceptor. The capture of light energy and the transfer of electrons and energy occurs via a system called a **photosystem.** These systems consist of pigment molecules that absorb light energy and a series of molecules that alternately accept and donate electrons and protons to form a chain of oxidation-reduction reactions through which electrons and protons are passed. The transfers of protons establishes a protonmotive force that is used for the chemiosmotic generation of ATP.

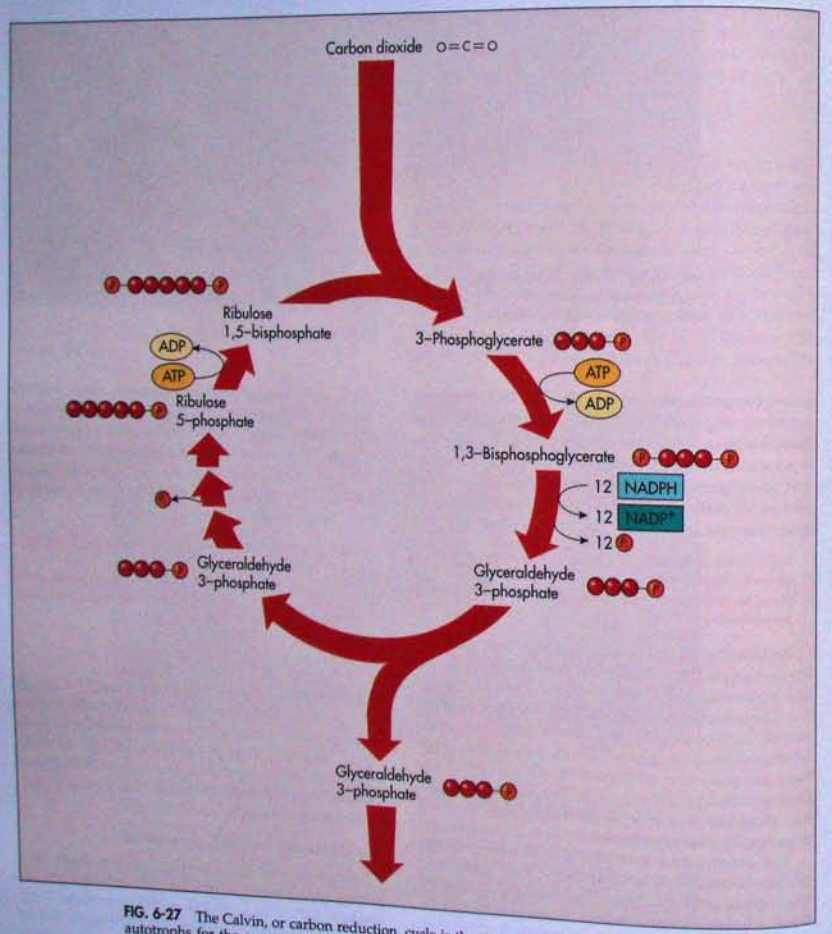


FIG. 6-27 The Calvin, or carbon reduction, cycle is the main metabolic pathway used by autotrophs for the conversion of carbon dioxide to organic carbohydrates. The pathway, which is active in photoautotrophs and chemolithotrophs, requires the input of carbon dioxide, ATP (energy), and NADPH (reducing power).

Calvin Cycle and CO_2 Fixation

Autotrophic microorganisms carry out a metabolic sequence of reactions known as the **Calvin cycle.** In the Calvin cycle, carbon dioxide is used to form organic matter. The conversion of CO_2 to organic matter requires a significant input of ATP and reduced coenzyme (NADPH).

The Calvin cycle is a complex series of reactions that actually represents three slightly different but fully integrated metabolic sequences (FIG. 6-27). It effectively takes three turns of the Calvin cycle to synthesize one molecule of the organic product of this metabolic pathway, which is glyceraldehyde 3-phosphate. Because glyceraldehyde 3-phosphate contains three carbon atoms, the Calvin cycle is sometimes referred to as a *C_3 pathway.* The glyceraldehyde 3-phosphate molecules that are formed during the Calvin cycle can further react to form glucose and polysaccharides of glucose, such as starch and cellulose. It takes six turns of the Calvin cycle to form a 6-carbon carbohydrate, such as glucose. The net input of energy—as ATP—and reducing power—as NADPH—required for the conversion of carbon dioxide to glucose is 18 ATP and 12 NADPH molecules.

In the Calvin cycle, carbon dioxide is reduced to form organic compounds for glucose synthesis.

In photoautotrophs the ATP and NADPH (energy and reducing power) to drive the Calvin cycle come from the light reactions of photosynthesis. In chemolithotrophs (discussed below) the needed ATP and reduced coenzymes come from inorganic compounds. The Calvin cycle itself is known as a "light-independent" or "dark reaction" because, although it requires ATP and NADPH, it does not require any light reactions.

Chemoautotrophic Metabolism

Some bacteria evolved the metabolic capacity to use inorganic substances as substrates to generate ATP for cellular energy (Table 6-4). For example, some bacteria use reduced sulfur compounds, such as iron sulfide, to generate reducing power and cellular energy. Bacteria that obtain energy in this way are called **chemoautotrophs** or **chemolithotrophs,** from the Greek, meaning obtaining nourishment from stones. These organisms do not require an organic compound or light as a source of energy. They obtain all their energy by oxidizing an inorganic compound. These bacteria have electron transport chains and establish a protonmotive force across membranes, which is used to generate ATP by chemiosmosis. Only a few genera of bacteria obtain their energy from chemoautotrophic metabolism, and all other living organisms depend on them to provide the continuous cycling of materials that are needed for growth.

Chemoautotrophic metabolism, also called chemolithotrophic metabolism, uses inorganic compounds to generate ATP.

Sulfur Oxidation

Various sulfur compounds can be oxidized by chemolithotrophs to meet their energy needs. The chemolithotrophic activities of sulfur-oxidizing microorganisms received considerable attention when it was found that a highly productive submarine area off the Galapagos Islands is supported by the productivity of chemolithotrophs growing on reduced sulfur released from thermal vents in the ocean floor (FIG. 6-28). It is unusual to find an ecological system driven by chemolithotrophic metabolism. Some sulfur-oxidizing chemolithotrophic bacteria, such as *Thiobacillus thiooxidans,* can oxidize large amounts of reduced sulfur compounds with the formation of sulfate. The sulfur-oxidizing activities of this bacterium are detrimental in nature because they result in the formation of acid mine drainage; however, they are beneficial for mineral recovery processes and are used for the recovery of copper and uranium, as well.

TABLE 6-4
Some Examples of Chemolithotrophic Metabolism

REACTION	BACTERIA
$H_2 + \frac{1}{2}O_2 \rightarrow H_2O$	*Alcaligenes eutrophus*
$NO_2^- + \frac{1}{2}O_2 \rightarrow NO_3^-$	*Nitrobacter winogradsky*
$NH_4^+ + 1\frac{1}{2}O_2 \rightarrow NO_2^- + H_2O + 2H^+$	*Nitrosomonas europaea*
$S^\circ + 1\frac{1}{2}O_2 + H_2O \rightarrow H_2SO_4$	*Thiobacillus denitrificans*
$S_2O_3^{2-} + 2O_2 + H_2O \rightarrow 2SO_4^{2-} + 2H^+$	*Sulfolobus acidocaldarius*
$2Fe^{2+} + 2H^+ + \frac{1}{2}O_2 \rightarrow 2Fe^{3+} + H_2O$	*Thiobacillus ferrooxidans*
$CO + O_2 + 2H^+ \rightarrow CO_2 + H_2O$	*Hydrogenomonas carboxydovorans*

FIG. 6-28 The tube worms *(Riftia pachyptila)* that grow extensively near deep sea thermal vents have no gut. They have extensive internal populations of sulfur-oxidizing chemolithotrophic bacteria that produce the nutrients used by these animals for sustenance. The red-brown color of the worms is due to a form of hemoglobin that supplies oxygen and hydrogen sulfide to the chemolithotrophic bacteria within the tissues of the tube worms. Microbial mats of *Beggiatoa* grow between strands of the tube worms at the Guaymas Basin vent site (Gulf of California) at a depth of 2,010 meters.

Nitrification

Nitrifying bacteria oxidize either ammonium or nitrite ions. Bacteria, such as *Nitrosomonas*, oxidize ammonia to nitrite (FIG. 6-29). Other bacteria, such as *Nitrobacter*, oxidize nitrite to nitrate. Because the chemolithotrophic oxidation of reduced nitrogen compounds yields relatively little energy, chemolithotrophic bacteria carry out extensive transformations of nitrogen in soil and aquatic habitats to synthesize their required ATP. The activities of these bacteria are important in soil because the alteration of the oxidation state radically changes the mobility of these nitrogen compounds in the soil column. Nitrifying bacteria lead to decreased soil fertility because positively charged ammonium ions bind to negatively charged soil clay particles, whereas the negatively charged nitrite and nitrate ions do not bind and are therefore leached from soils by rainwater.

Nitrogen Fixation

The evolution of a mechanism for converting atmospheric nitrogen into reduced nitrogen compounds such as ammonia was a major event in the progress and development of cellular metabolism. It is this process, called **nitrogen fixation,** that makes nitrogen available for incorporation into proteins. This is critical because, while carbohydrates and lipids can be synthesized from photosynthetic products based on CO_2 fixation, proteins and nucleic acids cannot be synthesized, because they contain nitrogen. Therefore life could not have persisted and expanded in the early oceans unless a means of replacing organic nitrogen compounds evolved. The process of nitrogen fixation, the incorporation of nitrogen atoms from N_2 gas into protein, requires the breaking of an N≡N triple bond. This is a very strong bond that is extremely difficult to break.

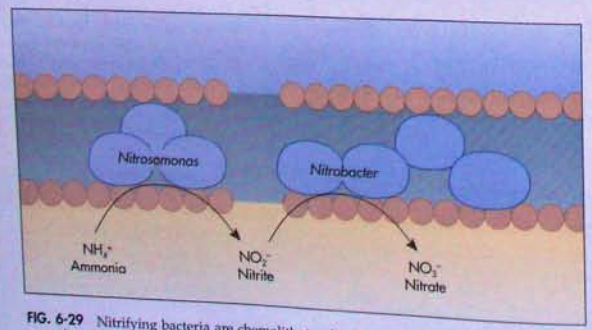


FIG. 6-29 Nitrifying bacteria are chemolithotrophs that oxidize inorganic nitrogen compounds to generate ATP. Some, such as *Nitrosomonas*, oxidize ammonium ions (NH_4^+) to nitrite ions (NO_2^-) *(left)*; others, such as *Nitrobacter*, oxidize nitrite ions to nitrate ions (NO_3^-) *(right)*. These reactions take place within specialized membranes that intrude within the cytoplasm of nitrifying bacteria.

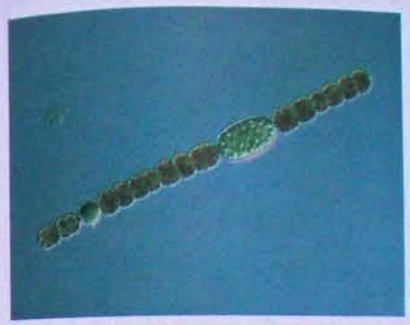
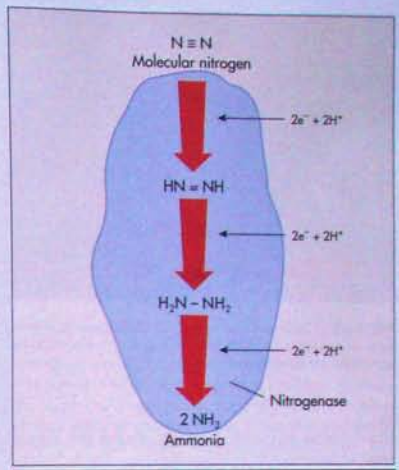


FIG. 6-30 Micrograph of the cyanobacterium *Anabaena cylindrica* showing vegetative cells and a heterocyst (enlarged cell) in which nitrogen fixation occurs. (400 ×).

In the biological fixation of nitrogen, the triple bond of molecular nitrogen is enzymatically broken by **nitrogenase**. This is a complex enzyme system. An iron-containing compound such as *ferredoxin* first obtains electrons from the breakdown of organic molecules or from photosynthetic light reactions and carries them to a protein, *nitrogen reductase*, which channels them to another protein, *dinitrogenase*. With the transfer of six electrons and the use of twelve ATP and four water molecules, nitrogenase converts nitrogen gas into two molecules of ammonia.

Nitrogen-fixing bacteria, called *Rhizobium* and *Bradyrhizobium*, live mutualistically in the nodules on the roots of legume plants (FIG. 6-30). Within the nodule, leghemoglobin, a protein produced by the plant, provides controlled amounts of oxygen so that aerobic energy-yielding metabolism can be carried out without inactivating nitrogenase, which is sensitive to oxygen exposure. When growing alone, *Rhizobium* and *Bradyrhizobium* require oxygen for their metabolism and are unable to fix nitrogen. When they live within root nodules, *Rhizobium* and *Bradyrhizobium* survive in this oxygen-free environment by utilizing the metabolites of the plant.

Other nitrogen-fixing bacteria, such as *Azotobacter* and *Beijerinckia*, are free living. Nitrogen-fixing cyanobacteria have specialized cells, called **heterocysts,** that contain the nitrogenase (FIG. 6-31). The heterocyst provides protection for nitrogenase against molecular oxygen, which is produced photosynthetically by cyanobacteria and which denatures nitrogenase.



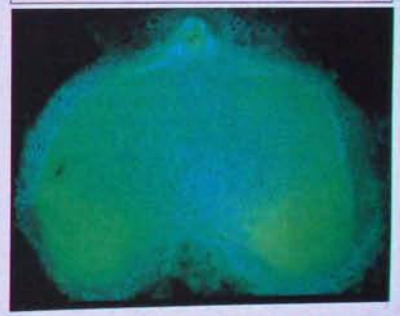


FIG. 6-31 Bacterial cells of *Bradyrhizobium japonicum* within a nodule of a soybean produce nitrogenase, which results in the conversion of molecular nitrogen to ammonia.

Methanogenesis

Some archaebacteria are able to use hydrogen and carbon dioxide to generate the ATP and molecules that compose their cellular structures. The metabolism of these archaebacteria produces methane and they are therefore called **methanogens** (FIG. 6-32). Other methanogens use fatty acids instead of carbon dioxide for the production of methane. Hydrogen gas, carbon dioxide, and fatty acids were available at the time life evolved on Earth. The methanogenic ar-

FIG. 6-32 Colonized micrograph of *Methanospirillum hungatei* cells within a sheath (*orange*). (84,000×). The cells are separated by a cell spacer.

chaebacteria may have been among the first organisms to carry out cellular metabolism. The methanogenic archaebacteria are strict anaerobes. They not only do not use oxygen in their metabolism, they are killed by exposure to oxygen. Methanogens could have grown on the compounds available in the primitive atmosphere of the Earth. Descendants of these archaebacteria still carry out anaerobic methane production today.

The metabolism of methane-producing archaebacteria involves a series of oxidation-reduction reactions. In these reactions, electrons and protons are transferred from one coenzyme to another (FIG. 6-33). The oxidation-reduction reactions of the coenzymes establish an electron chain through which the electrons move. This movement of electrons is coupled with the pumping of protons (hydrogen ions) across a membrane. The return flow of protons by diffusion across the membranes of these archaebacterial cells provides the energy needed to drive the synthesis of ATP by chemiosmosis. This process of chemiosmosis is able to drive the formation of ATP because when protons are pumped out of the cell, a low concentration of protons exists within the cell. This causes diffusion to force the flow of protons from outside the cell back in. The membrane is generally impermeable to protons except that protons can pass through special proton-transporting channels that cross the plasma membrane. The passage of protons through these channels releases energy that results in the synthesis of ATP from ADP and inorganic phosphate (P_i).

Anaerobic methane-producing archaebacteria (methanogens) use chemiosmosis, involving an electron transport chain and proton movement across a membrane, to generate ATP.

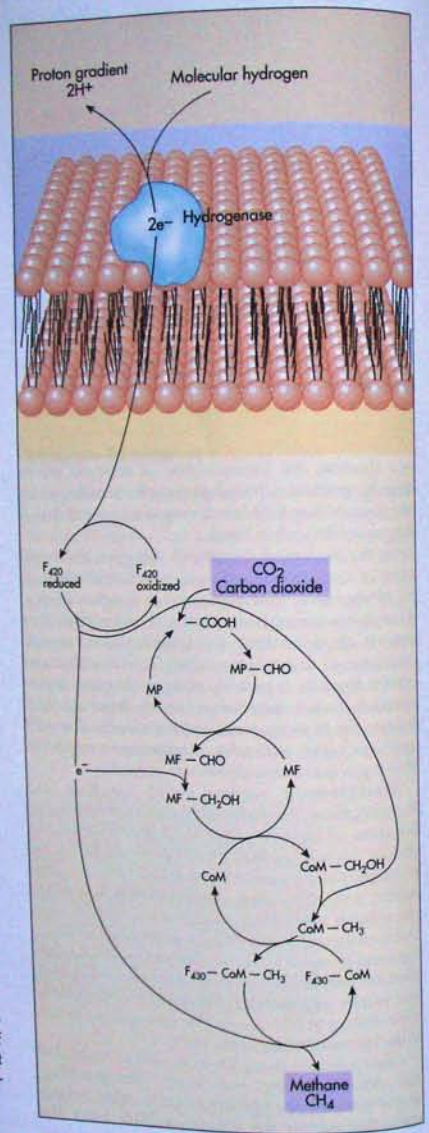


FIG. 6-33 The production of methane by methanogens involves several unique coenzymes and oxidation-reduction reactions to establish a protonmotive force for chemiosmotic ATP generation.

NEWSBREAK

EXPLOSIONS THAT DESTROY HOUSES TRACED TO METHANE FROM LANDFILL

Methane is produced at sites where organic matter decomposes and creates anaerobic conditions. Sanitary landfills represent such sites, and methane production at landfills is so extensive that it may be trapped and used as a fuel. In some cases, however, such methane production represents a serious problem, particularly for nearby houses. Several houses in Kentucky and other states that were located near landfills have been blown off their foundations because methane from a nearby landfill seeped through the ground into their basements. The methane was ignited by the pilot lights of the water heater or furnace systems. The explosions moved the houses several feet away from their original locations. These incidents serve as important reminders to keep houses a safe distance from landfills.

The production of methane by methanogens has several practical consequences. Methane is found in natural gas. It is a flammable gas. Methane seeping from landfills, where it is produced by methanogens degrading the waste deposits, sometimes enters nearby houses and causes explosions. Some sewage treatment facilities collect the methane that is formed during anaerobic decomposition of wastes and use it as a source of fuel for generating heat and electricity. Some communities are supplied with a portion of their natural gas (methane) from this metabolic process. In the future, methane produced by microorganisms may be used as a fuel for automobiles.

SUMMARY

Process of Metabolism Within a Cell (pp. 158-164)

- Cells exhibit various strategies for converting chemical and light energy into the energy stored within ATP, the central currency of energy of the cell. These processes of cellular metabolism also transform starting materials into the organic chemicals that make up the cell's structural and functional components.

Role of Enzymes (pp. 158-159)

- Enzymes are proteins that act as biological catalysts to accelerate the rates of chemical reactions by lowering the activation energy necessary for the reaction to occur. Different enzymes are needed to catalyze different reactions, each cell having thousands of enzymes, each enzyme binding only specific substrates to its active site.

Coenzymes and Oxidation-reduction Reactions (pp. 159-160)

- An oxidation-reduction or redox reaction involves the transfer of an electron from a donor, which is oxidized, to an acceptor, which is reduced. All redox reactions must be balanced. Cellular metabolism generates reducing power to convert substrate materials into the more reduced molecules of the cell by coupling oxidation reactions with the reduction of coenzymes.

ATP and Cellular Energy (pp. 160-161)

- All cells carry out metabolic reactions that transfer energy to ATP. ATP requires energy-releasing metabolic reactions for its formation. ATP's stored energy is used to drive energy-requiring biosynthetic reactions. Breaking an ATP high-energy bond yields 7300 calories per mole of energy. Substrate level phosphorylation is the generation of ATP from ADP + P_i coupled to energetically favorable reactions. The formation of ATP can also be driven by a protonmotive force in chemiosmosis.

Metabolic Pathways and Carbon Flow (pp. 161-162)

- The metabolic pathways utilized in ATP generation involve various intermediary metabolites linked together in a series of small steps to form unified biochemical pathways. In a catabolic pathway, larger molecules are split into smaller ones. Cells form relatively small molecules that can act as the basis for the carbon skeletons of larger macromolecules that are synthesized in anabolic (biosynthetic) pathways.

Autotrophic and Heterotrophic Metabolism (pp. 162-164)

- The synthesis of ATP can be achieved autotrophically—through the oxidation of inorganic substrates or through the conversion of light energy to chemical energy—or may be generated heterotrophically through the utilization of organic substrates.

Metabolic Pathways (pp. 164-187)

Respiration (pp. 165-171)

- The Embden-Meyerhof pathway of glycolysis converts the 6-carbon molecule glucose into two molecules of the 3-carbon molecule pyruvate, plus two molecules of reduced coenzyme and two molecules of ATP.
- Glycolysis is the first step in all pathways of carbohydrate metabolism. Its product, pyruvate, feeds into

the Krebs cycle and is converted to carbon dioxide with a net production of four ATP molecules. The Krebs cycle is not always completed; its intermediary products are siphoned out of the cycle and so must be continuously resynthesized.
- During oxidative phosphorylation, electrons from NADH and $FADH_2$ are transferred through an electron transport chain, which includes a series of oxidation-reduction reactions of membrane-bound carrier molecules and the reduction of a terminal electron acceptor. Chemiosmosis provides the energy for ATP production as a result of this process.
- An external electron acceptor is required to complete respiratory metabolic pathways. In aerobic respiration, oxygen is the terminal electron acceptor. Aerobic respiration is an efficient generator of ATP that comes from chemiosmosis.
- Protonmotive force is the potential energy gradient across a membrane established when protons are pumped across the membrane. Energy released when protons move back across the membrane by diffusion is coupled with the energy-requiring conversion of ADP to ATP. Generation of ATP using protonmotive force is called chemiosmosis.

Lipid and Protein Catabolism (pp. 171-173)
- Lipases break down fats into their fatty acid and glycerol components, which are further metabolized in the cell. Fatty acids are catabolized by beta-oxidation in which carbon fragments of long chains of fatty acids are removed two at a time and acetyl-CoA is formed. ATP is generated chemiosmotically by the reoxidation of reduced coenzymes.
- Proteases break down proteins into short polypeptides and amino acids. Amino acids are enzymatically deaminated, producing carboxylic acid, which can enter either the glycolytic pathway or the Krebs cycle. ATP is generated as a result of the protonmotive force across the plasma membrane of prokaryotes or the mitochondrial membranes of eukaryotes.

Fermentation (pp. 173-179)
- In fermentation, organic substrate molecules are used to generate ATP by substrate level phosphorylation. Organic molecules formed as products of fermentative metabolism serve as terminal electron acceptors. The amount of ATP is limited to that formed during glycolysis, yielding far less ATP per substrate molecule than respiration.
- All fermentation pathways are anaerobic. A complete fermentation pathway begins with a substrate, includes glycolysis and reoxidation of the coenzyme, and terminates in the formation of end products.
- Ethanolic or alcoholic fermentation converts pyruvate to ethanol and carbon dioxide by yeasts, such as *Saccharomyces cerevisiae*. It is used to produce beer, wine, and distilled liquor.
- Lactic acid fermentation produces lactic acid as an end product. Homolactic fermentation uses the Embden-Meyerhof pathway of glycolysis and produces only lactic acid. It is used in the production of dairy products such as cheese and yogurt. It is carried out by streptococci and lactobacilli. Heterolactic fermentation produces ethanol and carbon dioxide and is carried out by *Leuconostoc* and *Lactobacillus* species.
- Propionic acid fermentation is carried out by propionic acid bacteria and produces propionic acid and carbon dioxide. This pathway is used in the production of Swiss cheese, giving it the characteristic holes and flavor.
- Mixed-acid fermentation yields ethanol, acetic acid, formic acid, hydrogen, and carbon dioxide. This pathway is carried out by members of the Enterobacteriaceae, including *E. coli*. It can be detected by the Methyl Red test.
- In the butanediol fermentation pathway, *Klebsiella* species produce butanediol. An intermediary metabolite in this pathway, acetoin, can be detected by the Voges-Proskauer test, which distinguishes *E. coli* from *Enterobacter aerogenes* for water quality testing.
- The butanol fermentation pathway is carried out by members of the genus *Clostridium;* the end products of this pathway can be acetone and carbon dioxide, propanol and carbon dioxide, butyrate or butanol.

Photosynthetic Metabolism (pp. 179-183)
- Photoautotrophs, which include the photosynthetic bacteria, algae, and green plants, use light as their energy source and carbon dioxide as their carbon source. In oxygenic photosynthesis the electrons of water reduce carbon dioxide, and oxygen gas is given off. Chlorophyll and bacteriochlorophyll are the light-trapping pigments.
- Photoheterotrophs use light as the energy source and CO_2 and organic compounds as the carbon source. Green and purple nonsulfur bacteria are photoheterotrophs.
- In photosynthetic microorganisms the flow of electrons—initiated when a chlorophyll molecule is energetically excited by absorbing light energy—establishes a protonmotive force across a membrane during the process of photophosphorylation.
- Photosystems are light-trapping pigments organized into clusters of 200 to 300 molecules. These pigments harvest light energy in their chemical bonds, causing electrons to become excited and reach a higher energy level. That energy is released and transferred to a neighboring pigment when the electron returns to a lower energy level.
- Electron transport systems consist of a series of molecules that alternately accept and donate electrons from and to their neighboring molecules. The electrons are those expelled from a photosystem. These transfers accomplish oxidation-reduction reactions with the release of energy.
- The photosynthetic metabolism of cyanobacteria, algae, and plants releases oxygen atoms split from water into the atmosphere.
- The Calvin cycle is carried out by most autotrophic microorganisms in which carbon dioxide is reduced to form organic matter. This requires reducing power in the form of reduced coenzyme NADPH and ATP. The product of this pathway is glyceraldehyde 3-phosphate.

Chemoautotrophic Metabolism (pp. 183-184)
- Chemoautotrophs (chemolithotrophs) are bacteria that can combine inorganic substances such as sulfur or nitrogen with oxygen to generate ATP for cellular energy via aerobic respiration. Such bacteria play important roles in mineral cycling, for example, the conversion of ammonia to nitrite and nitrate, and hydrogen sufite to sufate.
- Chemoautotrophic microorganisms couple the oxidation of an inorganic compound with the reduction of a suitable coenzyme. They use chemiosmosis to generate ATP. Important mineral cycling reactions are the result of chemoautotrophic metabolism.
- Regardless of the mode of metabolism the strategies are the same: synthesize ATP, reduce coenzyme (NADPH) and small precursor molecules to serve as the building blocks of macromolecules, and then use the energy, reducing power, and precursor molecules to synthesize the macromolecular constituents of the organism.

Nitrogen Fixation (pp. 184-185)
- Nitrogen fixation is the conversion of atmospheric nitrogen into reduced nitrogen-containing compounds such as ammonia. Nitrogen fixation is carried out only by members of a few bacterial genera.
- Nitrogenase is the enzyme that converts molecular nitrogen into reduced nitrogen compounds.
- Some nitrogen fixing bacteria live in symbiotic association with plants in specialized structures called nodules. Others have structures called heterocysts where nitrogen fixation occurs.

Methanogenesis (pp. 185-187)
- Methanogens are strictly anaerobic archaebacteria that produce methane as a result of anaerobic respiration. The oxidation-reduction reactions of the coenzymes in the metabolism of methanogens establishes an electron chain through which electrons move. This movement of electrons is coupled with the moving of protons across a membrane, and the protonmotive force is used for ATP synthesis by chemiosmosis.

CHAPTER REVIEW

Review Questions

1. Define metabolism.
2. What are the differences between catabolism and anabolism?
3. What is an enzyme?
4. What factors influence the activity of enzymes?
5. Describe the chemiosmotic theory of ATP generation.
6. Explain what an oxidation-reduction reaction is.
7. Compare and contrast autotrophy and heterotrophy.
8. Describe what occurs during each of the phases of respiratory metabolism.
9. Describe the similarities and differences between aerobic and anaerobic respiration.
10. What are the starting substrates and end products of glycolysis?
11. What are the light and dark reactions of photosynthesis?
12. What are some commercially useful products of fermentative processes?
13. Compare photoautotrophic and chemolithotrophic metabolism.
14. What is nitrogen fixation? Why is it an important process?
15. What are the differences between fermentation and respiration?

CRITICAL THINKING QUESTIONS

1. What is the significance of the origin of oxygen-producing photosynthesis? How could the origin of this type of metabolism have altered the course of evolution?
2. Lactobacilli and streptococci are microorganisms that normally inhabit the gastrointestinal and vaginal tracts. These organisms typically carry out a homolactic acid fermentation. What is the significance of this observation? How could it relate to resistance to disease? What would happen if these lactic acid fermenters were eliminated from the body?
3. All fermentation reactions have the same common feature with respect to regeneration of oxidation-reduction capability. What is this common feature? How do different fermentation pathways achieve this goal?
4. It is often said that life on Earth depends on the input of solar radiation and that animals and other heterotrophic organisms always depend on green plants. Is this statement accurate? How might you modify this statement to account for the roles of photosynthetic bacteria and algae? How has the discovery of thermal vent regions with biologically productive communities altered the validity of the dependence of life on solar input and photosynthesis?

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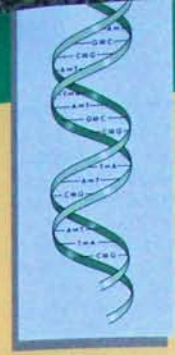

CHAPTER 7

Microbial Genetics: Replication and Expression of Genetic Information

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PREVIEW TO CHAPTER 7

In this chapter we will:

- Discover the underlying mechanisms of heredity and the biochemical events that enable the passage of hereditary information.
- Examine the properties of DNA (deoxyribonucleic acid), the universal master molecule of life that stores genetic information in all cells—bacterial, human, and other.
- See how DNA molecules are replicated so that hereditary information can be passed from one generation to the next.
- Discover how all of the properties of an organism are determined at the molecular level.
- Learn how genetic expression occurs, seeing how information in the DNA is transferred through RNA (ribonucleic acid) molecules to proteins.
- Learn the following key terms and names:

Ames test
anticodon
auxotrophs
base pairing
catabolite repression
codon
diploid
DNA gyrase
DNA polymerase
double helix
exons
frame-shift mutations
genes
genotype
haploid
heterozygous
hnRNA (heterogeneous nuclear RNA)
homozygous
inducible
introns
lethal mutation
mutagens
mutation
nonsense codons
nucleic acid bases
nucleotides
operon model
phenotype
polycistronic
polymerase chain reaction (PCR)
promoter
prototroph
regulatory genes
replica plating
replication fork
ribosomal RNA (rRNA)
RNA polymerase
semiconservative replication
split genes
structural genes
template strand
thymine dimer
transcription
transfer RNA (tRNA)
translation

In 1928 a British microbiologist, Frederick Griffith, was trying to develop a vaccine against pneumonia. He was working with two different strains of the causative bacterium *Streptococcus pneumoniae* (FIG. 7-1). One strain was pathogenic, killing the mice injected with it. The other strain was nonpathogenic. The two strains differed in appearance when viewed under the microscope. The nonpathogenic strain appeared rough and was not surrounded by a capsule. The pathogenic strain appeared smooth, surrounded by a polysaccharide capsule. When Griffith injected heat-killed cells of this smooth, pathogenic strain of *S. pneumoniae* into a mouse, the mouse survived because the dead bacteria were unable to establish an infection in the mouse. However, when he injected a mouse with living cells of the rough nonpathogenic strain, together with dead smooth bacteria, knowing that neither of them could cause disease alone, the mouse died. Unlike the live, rough bacteria he injected, the bacteria he isolated from the dead mouse appeared smooth and surrounded by a capsule.

This was a most puzzling observation. Griffith reasoned that genetic material from the heat-killed bacteria had somehow entered the living nonpathogens and transformed them into pathogenic bacteria. He postulated that heat could kill the pathogenic cells without destroying the substance containing their hereditary information, which included instructions

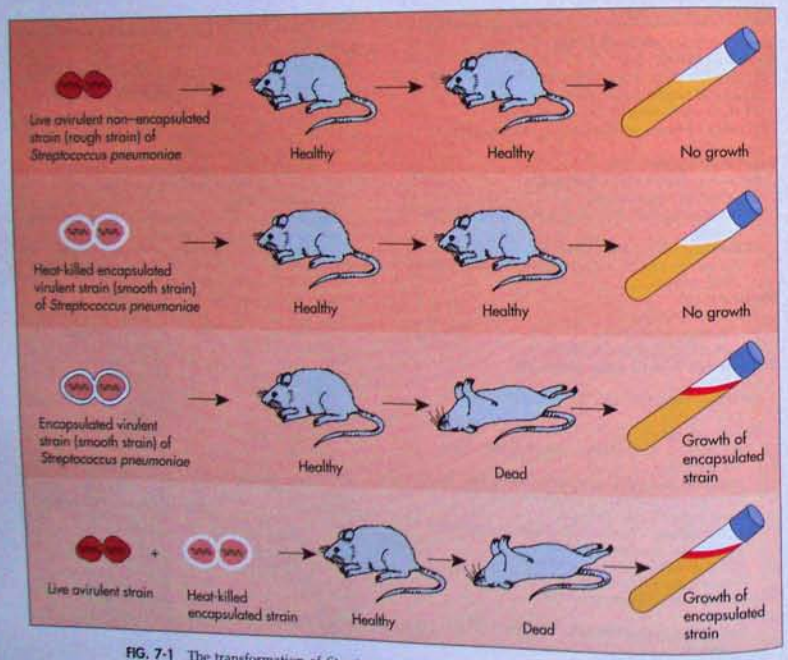


FIG. 7-1 The transformation of *Streptococcus pneumoniae* shows how the properties of a bacterial strain can be altered by a hereditary substance (later identified as DNA). When cells of *S. pneumoniae* are heat killed they leak DNA, which can be picked up by other cells and incorporated into the genetic information of those cells. In this manner, avirulent (nonpathogenic) strains of *S. pneumoniae* that lack the gene for capsule production (virulence factor that contributes to their ability to cause fatal disease) can acquire the gene (DNA) that encodes for capsule production. When this occurs, an avirulent noncapsule-producing strain of *S. pneumoniae* is transformed into a virulent strain that produces a capsule.

on how to cause infection and disease. Griffith had, in fact, observed the movement of hereditary material from one cell to another. The chemical that transmitted the hereditary information for causing disease leaked from the dead pathogens and was picked up by the living bacteria, transforming them into pathogens when it became part of their hereditary material.

Other scientists then began to investigate the specific chemical substance that caused the transformation of a nonpathogen to a pathogen. They were looking for the molecular basis for heredity. Chemical analyses narrowed the possible hereditary molecules to either proteins or nucleic acids. Most scientists hypothesized that proteins were the basis of heredity because their essential roles in metabolism were known. The specific chemical nature of the transforming material observed by Griffith, however, remained a puzzle until 1944 when Oswald Avery and his co-workers were able to demonstrate the chemical nature of the substance that transformed nonpathogenic *S. pneumoniae* to pathogenic *S. pneumoniae*.

Avery hypothesized that a nucleic acid, deoxyribonucleic acid (DNA), rather than protein was the hereditary molecule. He designed experiments to prove this. In Avery's experiments the transforming principle of *S. pneumoniae*, which had been shown to be predominantly DNA with a trace of protein, was treated sequentially with an enzyme that destroys protein and an enzyme that destroys DNA (FIG. 7-2). Avery observed that the protein-destroying enzyme did not affect the ability of the material to transform nonpathogenic *S. pneumoniae* into pathogenic *S. pneumoniae*, whereas treatment with the DNA-destroying enzyme eliminated such transformation. Based on these observations, Avery concluded that the transforming principle must be DNA.

Despite this quite convincing demonstration, the scientific community was not ready to accept that DNA was the universal hereditary molecule. Most scientists remained convinced that proteins would eventually be shown to be the basis of heredity for organisms other than bacteria. Another set of experiments conducted with bacteriophage (viruses that replicate within bacterial cells), however, added convincing evidence that nucleic acids, not proteins, are the source of hereditary information. These experiments, conducted in 1952 by Alfred Hershey and Martha Chase, examined the replication of bacteriophage T2. Although bacteriophage are not living cells, they were known to contain DNA and protein, making them good simple models to examine whether it is protein or DNA that carries hereditary information.

Hershey and Chase used two different radioactive labels to track the movement of protein and DNA separately (FIG. 7-3). Most proteins contain sulfur but none contain phosphorus. Thus the radioactive isotope ^{35}S can be used to label the bacteriophage protein. DNA contains phosphorus but no sulfur, so they used the radioactive isotope ^{32}P to label the bacteriophage DNA. Thus Hershey and Chase cleverly devised a method for following both the DNA and protein components of bacteriophage T2. When they added bacteriophage that had been labelled with ^{35}S to a culture of growing cells of the bacterium *Escherichia coli*, they observed that the ^{35}S label remained outside of the bacterial cells. Thus protein did not enter the bacterial cells. In contrast, when they similarly added ^{32}P-labelled bacteriophages, the ^{32}P label entered the interior of the bacterial cells. This indicated that DNA was the material that entered the cells and therefore must be the substance that carried the hereditary information. The progeny bacteriophages produced from the replication of the original

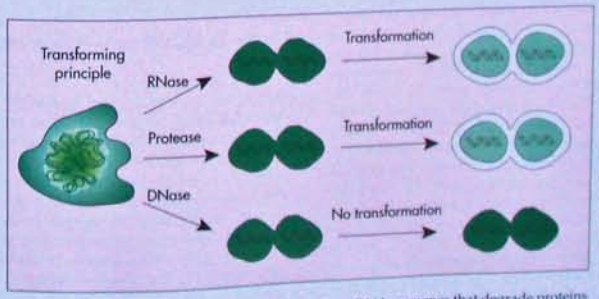


FIG. 7-2 To prove that the hereditary substance was DNA, enzymes that degrade proteins were added to cell extracts. These enzymes did not eliminate transformation, showing that the substance was not a protein. In contrast, the addition of a DNA-destroying enzyme eliminated transformation.

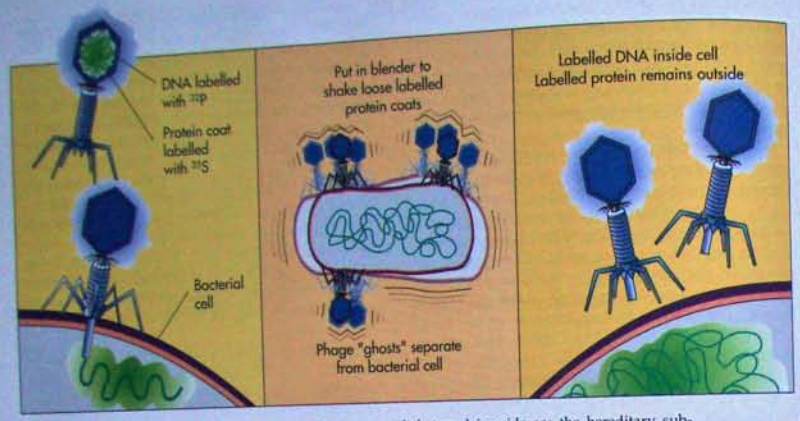


FIG. 7-3 Hershey and Chase demonstrated that nucleic acids are the hereditary substances of viruses. In their experiments ^{32}P was used to label nucleic acids and ^{35}S was used to label proteins. The ^{35}S remained outside of the host cell, whereas the ^{32}P entered the cell. This indicated that the ^{32}P-labelled nucleic acid carried the hereditary information.

bacteriophage contained ^{32}P and not ^{35}S, indicating further that the hereditary material passed from one generation to the next, was, in fact, DNA. Although subsequent experiments have shown that another nucleic acid (ribonucleic acid [RNA]) sometimes is the hereditary substance for viruses, it was now firmly established that DNA is the hereditary molecule for many viruses and all living cells.

DNA is the substance that transmits the hereditary information of many viruses and all cellular organisms.

STRUCTURE OF DNA

Nucleotides—Building Blocks of the Genetic Code

To understand how DNA stores and transmits hereditary information, it is necessary to examine the chemical structure of this molecule. DNA is a large, high-molecular-weight molecule, called a *macromolecule*. It is composed of many subunits called **nucleotides** (FIG. 7-4). Each nucleotide subunit of DNA has three parts: deoxyribose (a 5-carbon sugar), phosphate, and one of four **nitrogenous bases** (sometimes referred to as **nucleic acid bases**). The four different nitrogenous bases that occur in DNA are *adenine* (A), *thymine* (T), *guanine* (G), and *cytosine* (C).

These four nucleotides are like an "alphabet" that makes up the genetic code. They establish the first important property of DNA as the chemical basis for heredity—the ability to encode the genetic information. This is achieved by linking the nucleotides in a specific order—much as the letters of the alphabet are joined to form words.

The hereditary information is coded by the order in which the four different nucleotides occur within the DNA macromolecule.

Chains of Nucleotides—Directionality of DNA

Individual nucleotides are linked to form a long chain consisting of several million nucleotides. The bonds holding the nucleotides together are covalent and hence strong. This is important for the long-term stability of the hereditary macromolecule. Within this chain, nucleotides are locked together in order, thereby establishing the sequences that encode the genetic information. Once encoded in the chain of DNA, the information remains intact unless acted on by a destructive force, such as certain chemicals or radiation.

The chemical bonds holding the chains of nucleotides together are called **3′-5′ phosphodiester bonds** (FIG. 7-5). They are so-named because phos-

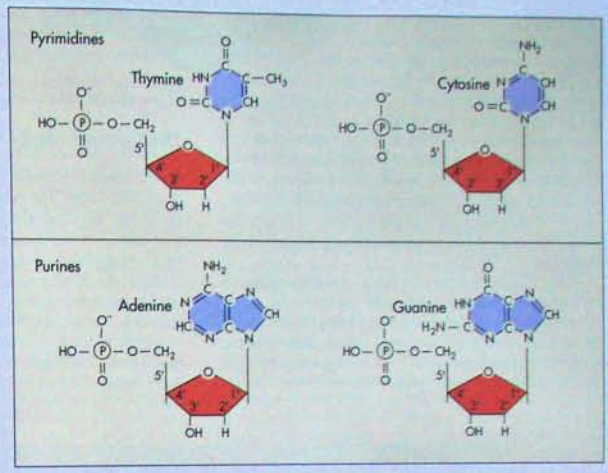


FIG. 7-4 Four different deoxyribonucleotides comprise the subunit molecules of DNA. These have differing nucleic acid bases: thymine, cytosine, adenine, and guanine.

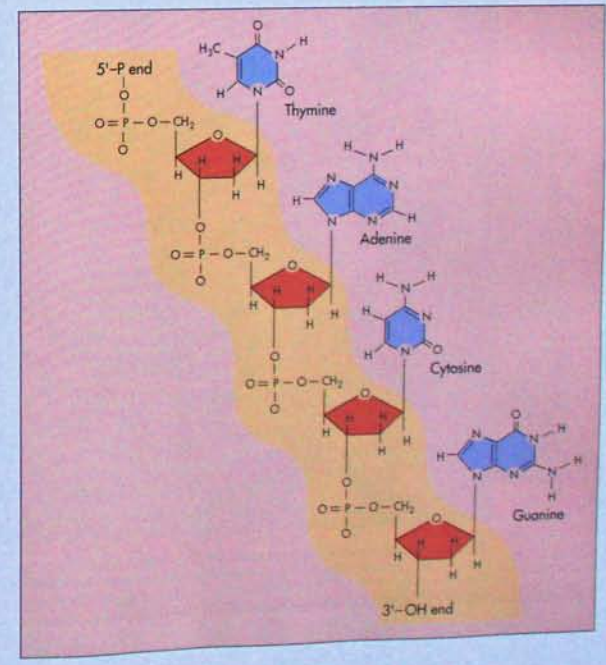


FIG. 7-5 Nucleotides are joined together by phosphodiester bonds between the 3′-OH and 5′-P positions. There is a free 5′-P at one end of the polynucleotide chain and a free 3′-OH at the other end.

phate forms a bridge between the number 3-carbon of one deoxyribose sugar molecule and the number 5-carbon of another. At one end of the chain there is no phosphate bonded to the 3-carbon of a deoxyribose, and at the other end of the molecule, the phosphate attached to the 5-carbon is not involved in forming a phosphodiester linkage. Thus there is a free hydroxyl group at the 3-carbon position at one end of the chain **(3′-OH free end)** and there is a free phosphate group at the 5-carbon position at the other end **(5′-P free end).** This imparts a second important property on the DNA macromolecule—that of directionality.

Having different groups free at the different ends of the molecule distinguishes one end from the other (like left from right) thereby permitting the molecule to be read from a particular direction. This is especially critical for a molecule whose purpose is to store and to transmit genetic information because it establishes the basis for the correct direction for reading the order of nucleotides that encode the genetic information within the DNA macromolecule.

The chains of DNA macromolecules are different at either end, which allows directional recognition.

DNA Double Helix—Complementarity

There are two chains of nucleotides in the DNA macromolecule. The two polynucleotide chains that comprise the DNA double helix run in opposite directions—one chain runs from the 3′-OH to the 5′-P free end and the complementary chain runs from the 5′-P to the 3′-OH free end. These complementary chains twist together to form an arrangement called a **double helix** (FIG. 7-6).

The two chains of the DNA double helix are held together by hydrogen bonding between complementary chains. Two of the nitrogenous bases (C and T)

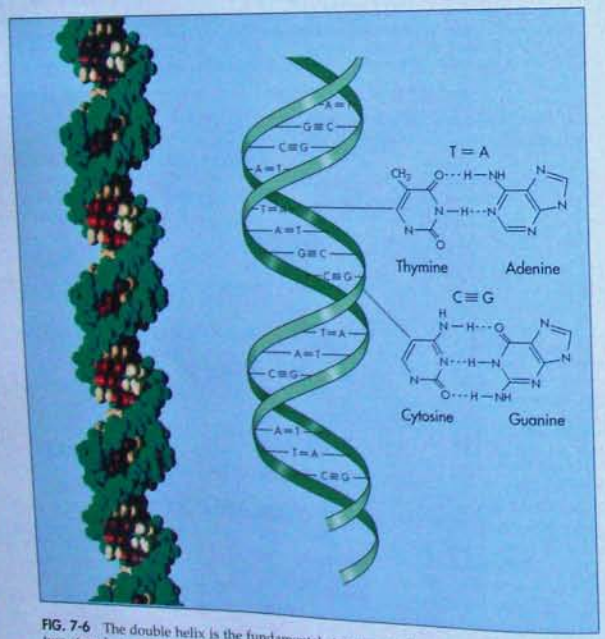


FIG. 7-6 The double helix is the fundamental structure of the DNA macromolecule. The two strands are held together by hydrogen bonding between complementary base pairs. There are three hydrogen bonds between the base pairs guanine and cytosine and two hydrogen bonds between the base pairs adenine and thymine.

HISTORICAL PERSPECTIVE

Discovering the Structure of DNA

In the early 1950s, James Watson, who had only recently received a doctoral degree from Indiana University, teamed with Francis Crick, a Cambridge University researcher, and pieced together the available data to determine the chemical structure of the DNA molecule, showing how DNA could store and transmit hereditary information (see Figure).

Watson and Crick reasoned that the double-ringed adenine and guanine molecules were probably paired with the single-ringed thymine and cytosine molecules along the entire length of DNA. This would fit the observation made by Erwin Chargaff in the United States in the late 1940s that in any given DNA macromolecule the amount of adenine present is always equal to the amount of thymine and the amount of cytosine is always equal to the amount of guanine. It also was consistent with the observations of Maurice Wilkins and Rosalind Franklin at Cambridge University in England, who, using X-ray diffraction methods, showed that DNA was long and thin, with a uniform diameter. If this was not the case, then DNA would bulge where the two double rings were paired and narrow where two single rings were paired, something that was never seen in the X-ray diffraction images of Wilkins and Franklin.

The problem Watson and Crick had was that the structural formulas for the nucleic acid bases guanine and thymine that were in the organic chemistry books at that time were wrong. Because they were trying to build a model with the wrong structural representations for these molecules, they were unable to get the molecules to fit in a way that would be stabilized by hydrogen bonding between their atoms. Only after getting the structural forms of guanine and thymine corrected during a chance meeting with a visiting scientist were Watson and Crick able to build the correct model of DNA. Once they had the accurate structures, they began shifting the bases in and out of various pairing possibilities.

They next used a plumb line and a measuring stick to determine the relative positions of all of the atoms in a single nucleotide. By assuming a helical shape like a spiral staircase, it became clear that the locations of the atoms in one nucleotide would automatically generate the position of the other. They had constructed a model of the DNA double helix. The model Watson and Crick constructed also showed how the chains could be separated and how each could code for a new complementary chain, thereby unraveling the mystery of how a chemical could pass hereditary information to the next generation with such fidelity.

A, James Watson at age 23 and Francis Crick at age 34 developed a model for the structure of DNA while working at the Cavendish Laboratory at Cambridge University, England. The model explained how DNA can transmit hereditary information. They announced their discovery of the molecular structure of DNA in 1953 and shared the Nobel prize for medicine in 1962 along with Maurice Wilkins. **B,** In 1993, on the fortieth anniversary of their discovery, they again posed with their model of DNA, which has proven to be correct.

in DNA are single-ring structures called **pyrimidines** and the other two (A and G) are double-ring structures called **purines.** The charge interactions between purines and pyrimidines allow them to form weak hydrogen bonds (FIG. 7-7). Chemically, the most stable hydrogen bonding occurs when guanine forms three hydrogen bonds with cytosine and when adenine forms two hydrogen bonds with thymine. The proper alignment to form these hydrogen bonds occurs only when the sugar-phosphate backbones of the two DNA chains run in opposing directions and are twisted together to form the double helix.

DNA, which stores and transmits cellular hereditary information, is a double helical molecule.

The hydrogen bonding of A to T and C to G is called **base pairing.** It is this complementarity that establishes the basis for the double helical arrangement of DNA and for the accurate replication of the DNA macromolecule. This is essential for passage of hereditary information from one generation to the next. It also means that in the double helical DNA molecule, the amount of adenine is always the same as the amount of thymine, and the amount of guanine is always the same as the amount of cytosine (A = T and G = C).

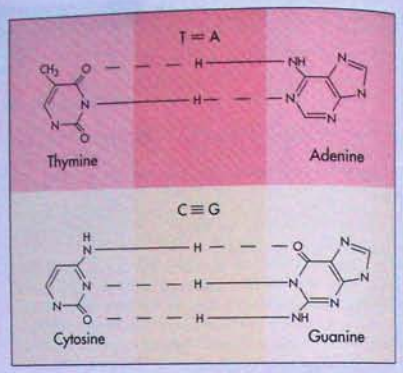


FIG. 7-7 Hydrogen bonding occurs between nucleotide base pairs. Adenine forms two hydrogen bonds with thymine. Guanine forms three hydrogen bonds with cytosine.

Base pairing occurs between complementary nucleotides—adenine pairs with thymine and guanine pairs with cytosine.

REPLICATION OF DNA

When a cell divides, its hereditary information is passed to the next generation. Replication of the hereditary information involves synthesizing new DNA molecules that have the same nucleotide sequences as those of the parental organism. The transfer of hereditary information is possible because DNA has a unique chemical structure in which the two chains of the DNA double helix are **complementary** in nucleotide sequence. Wherever a G is found in one chain, a C is found in the other, and wherever a T is present in one chain, its complementary chain will have an A. A nucleotide sequence of ATCG in one chain has a corresponding sequence of TAGC in the other chain. The nucleotide sequence in one chain specifies the sequence in the other. The information in DNA is, thus, accurately replicated so that an exact copy is passed from one generation to the next.

The order of nucleotides in each chain of a double helical DNA molecule specifies the order of nucleotides in the new complementary chains.

Semiconservative DNA Replication

The process by which a double helical DNA molecule is copied to form a duplicate DNA macromolecule is called **semiconservative replication.** It is so named because during replication each of the chains of nucleotides in the DNA being replicated remains intact. The two chains of nucleotides in the double-stranded DNA molecule are conserved—and a new, complementary chain is assembled for each one. Each of the conserved parental DNA chains serves as the template that specifies the sequence of nucleotides in the newly synthesized strands.

Semiconservative replication was demonstrated experimentally by Matthew Meselson and Franklin Stahl at the California Institute of Technology in 1958 (FIG. 7-8). They grew a culture of *Escherichia coli* in a medium in which the sole source of nitrogen was the heavy isotope ^{15}N. The heavy nitrogen was incorporated into the nucleotides of DNA during bacterial reproduction, so that the DNA of these bacteria became heavier than usual. They then transferred these bacteria to a medium containing the normal lighter isotope ^{14}N. At various time intervals they collected cells and analyzed the DNA to determine if it was "heavy" (^{15}N label), light (^{14}N label), or intermediate (mixture of ^{15}N and ^{14}N label). For these analyses they used an ultracentrifuge—an instrument that spins its contents at high speed—which caused materials to separate out according to their different densities.

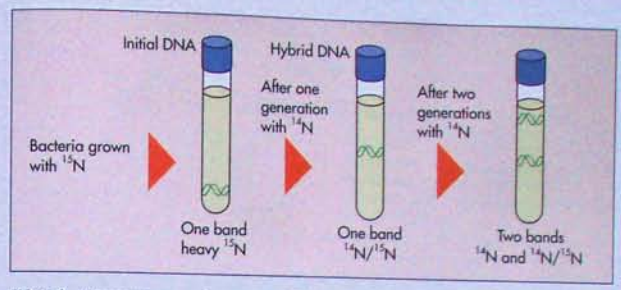


FIG. 7-8 The semiconservative nature of DNA replication was demonstrated by labelling DNA in one generation by the incorporation of heavy nitrogen (^{15}N) and following the fate of this tagged DNA from one generation to the next, using density gradient ultracentrifugation. The location of the bands obtained by ultracentrifugation, that is, the distance that the DNA moves, which is a function of the molecular weight of the DNA, permitted the tracking of the fate of the heavy DNA when the cells were grown in the presence of normal light nitrogen (^{14}N). The banding pattern obtained in these experiments, which is illustrated in the figure, proved that DNA replication occurs by a semiconservative method.

Denser molecules move farther than lighter molecules in cesium chloride density gradient centrifugation, so DNA containing ^{15}N moves a greater distance than DNA containing only ^{14}N. The movement is such that bands of DNA can be distinguished corresponding to light, heavy, and intermediate DNA.

Initially Meselson and Stahl detected only one band. This band corresponded to heavy DNA in which both chains of the DNA contained the ^{15}N label. After sufficient time for one complete round of DNA replication, again only one band of DNA was detected, but now the band was at an intermediate level between all-light isotope and all-heavy isotope DNA. This intermediate band was exactly what was predicted by the hypothesis that DNA replication is semiconservative. Each DNA double helix had one chain from the parental DNA that contained the heavy ^{15}N isotope and one newly synthesized chain that contained only the light ^{14}N isotope. Also as predicted, after sufficient time for a second round of DNA replication, Meselson and Stahl observed two bands of DNA, one intermediate and the other light. This occurred because when the intermediate DNA containing one light and one heavy chain replicated, it contributed one heavy chain to form another intermediate DNA macromolecule and one light chain to form a new all-light DNA macromolecule. This experiment confirmed that DNA replication is semiconservative as suggested by the Watson-Crick model of the DNA double helix.

DNA replication is semiconservative, producing two "half-old, half-new" DNA macromolecules every time the DNA is duplicated.

Steps in DNA Replication

Unwinding the DNA Double Helix—Replication Forks

The first step in semiconservative DNA replication is to pull apart a portion of the DNA helix. This enables each of the chains to act as a template (pattern) to direct the synthesis of a new complementary chain of nucleotides. This can occur because hydrogen bonds are relatively weak. Thus the two chains can separate without breaking apart the covalently linked nucleotides of the chains, which would destroy the information encoded within them. This establishes the basis for one chain serving as a template for the synthesis of a new chain of DNA with a sequence of nucleotides that is exactly complementary.

The chains do not entirely separate before DNA replication. Rather, a localized region of the DNA unwinds because the two parental DNA chains are pulled apart by specific enzymes. This creates a region of two single strands and provides space for individual nucleotides to align opposite their complementary bases for the synthesis of new chains. This region of localized DNA synthesis is called a **replication fork** (FIG. 7-9, p. 201). At the replication fork, enzymes link nucleotides to form a new DNA strand that is complementary to the original template DNA.

The DNA double helix unwinds to form a replication fork where DNA synthesis occurs.

In eukaryotic cells, multiple replication forks form at different locations. Simultaneous synthesis of different portions of the DNA is thus made possible. In a bacterial cell, DNA replication is initiated at only

METHODOLOGY

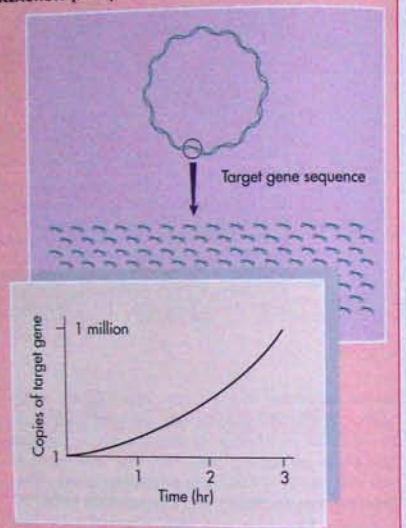
Polymerase Chain Reaction (PCR)

Understanding the mechanism of DNA replication has enabled scientists to develop a method for replicating segments of DNA in the laboratory from slight traces that otherwise might be too small to analyze. The method, called polymerase chain reaction (PCR), has enormous practical importance because it allows rapid amplification of trace DNA by making many additional copies through replication of specific DNA sequences that can then be detected with great sensitivity (see Figure). This permits the detection of even rare genes. Already, PCR has permitted extremely sensitive detection of the AIDS-causing virus in blood, which is essential for the protection of the blood supply. The impact on microbiology and molecular biology is enormous and PCR has become one of the most widely used methods in science. In recognition of the importance of PCR, the 1993 Nobel Prize in chemistry was awarded to Kerry Mullis, who discovered this method.

PCR is based on the following facts about DNA replication: DNA serves as a template for its own replication; the DNA double helix separates into two chains for replication; a pool of free nucleotides provides the nucleotides for the synthesis of new chains; DNA polymerase catalyzes the formation of the new chains; DNA polymerase adds only to the 3′-free OH end of a nucleotide chain; and DNA polymerase requires a short chain of nucleotides (oligonucleotide) to serve as a primer to initiate DNA replication.

To accomplish the replication of DNA outside of living cells by using PCR, a source of template DNA is added along with a pool of free nucleotides and a DNA polymerase. Also added are short oligonucleotide primers that are complementary to the nucleotide sequences flanking the region of the DNA that is to be replicated. These primers define the region of DNA that is replicated by providing the 3′-OH free ends onto which the DNA polymerase can add nucleotides.

PCR procedure uses heat to provide energy for breaking the hydrogen bonds to separate the chains of the DNA double helix. Heating to 95° C will break the hydrogen bonds without breaking the covalent bonds that link the nucleotides in the chains. Once the chains are separated the reaction is cooled, for example, to 40° C, which allows hydrogen bonds to form between the oligonucleotide primers and their complementary regions of the template DNA. The temperature is then raised to approximately 72° C to allow the DNA polymerase to quickly add nucleotides.



The polymerase chain reaction (PCR) is an *in vitro* method for replicating DNA. A target nucleotide sequence is copied repeatedly so that a million copies can be made in less than an hour.

The DNA polymerase used in PCR, called *Taq* polymerase, comes from a bacterium—*Thermus aquaticus*—that lives in hot springs; it is not denatured at high temperatures. Thus this DNA polymerase can withstand repeated exposure to 95° C. This is critical because in PCR the temperature is repeatedly cycled to separate the chains of the DNA double helix, to bind the primers to the template DNA, and to allow the DNA polymerase to synthesize new strands. Each cycle lasts only a few minutes. The effect of repeated cycling is to exponentially increase the number of copies of a defined segment of the DNA. Within an hour a single copy of a gene can be amplified to a million copies. PCR technology has applications for research and diagnosis and is fast becoming a standard procedure in biotechnology and medical diagnostic laboratories.

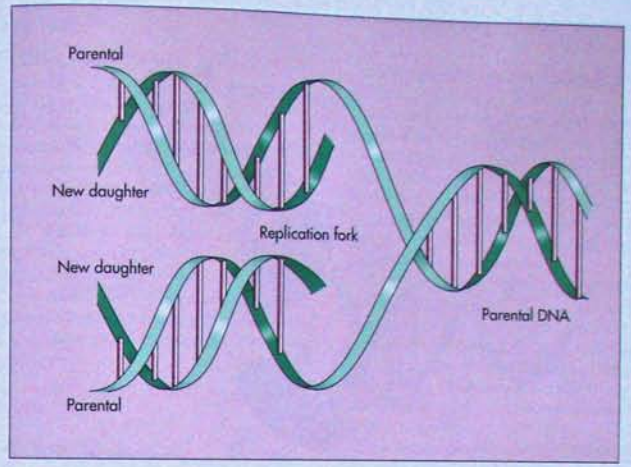


FIG. 7-9 During DNA replication, enzymes separate the two strands of DNA in a localized region called the replication fork. At this site, new nucleotides align opposite base pairs and new strands of DNA are synthesized.

one site, with two replication forks moving from the initiation site in opposite directions around the circular bacterial chromosome. As the replication forks move around the bacterial chromosome, an enzyme—**DNA gyrase**—twists the DNA. This enzyme is unique to bacteria and hence a potential site for the action of an antimicrobial agent. In fact, a new class of antibacterial agents, the **quinolones,** have been discovered that interfere with DNA gyrase. By preventing the formation of replication forks in bacterial cells, quinolones block bacterial reproduction and, hence, can be used to treat bacterial infections. The quinolone ciprofloxacin, for example, is useful in treating *Pseudomonas* infections.

DNA gyrase untwists the DNA of the bacterial chromosome.

Quinolones are antibacterial agents that inhibit DNA gyrase.

Formation of a New Chain of Nucleotides—DNA Polymerase

Free nucleotides within the cell in association with DNA polymerase are positioned opposite their complementary nucleotides in the template. This process of aligning complementary nucleotides (A opposite T and C opposite G) is called base pairing. The order of the nucleotides is specified by the template DNA. After the nucleotides are aligned by base pairing, an enzyme called **DNA polymerase** links the nucleotides by forming phosphodiester bonds. The action of DNA polymerase can be likened to a zipper where the teeth of the zipper are initially aligned and progressively linked together in a continuous motion.

DNA polymerase adds nucleotides to the free 3′-OH end of an existing nucleotide chain of nucleotides (FIG. 7-10). Because DNA polymerase adds nucleotides only to the 3′-OH free end, the direction of DNA synthesis is 5′-P → 3′-OH. Since the two chains of the double helical DNA molecule are antiparallel (one running from the 5′-P → 3′-OH free end and the other running from the 3′-OH → 5′-P free end) this indicates that the synthesis of the two complementary DNA chains must proceed in opposite directions.

One DNA chain can be continuously synthesized. It is the chain that runs in the appropriate direction for the continuous addition of new free nucleotides to the free 3′-OH end. This is the **continuous** or **leading strand of DNA.** Its synthesis occurs simultaneously with the unwinding of the double helical molecule and progresses toward the replication fork.

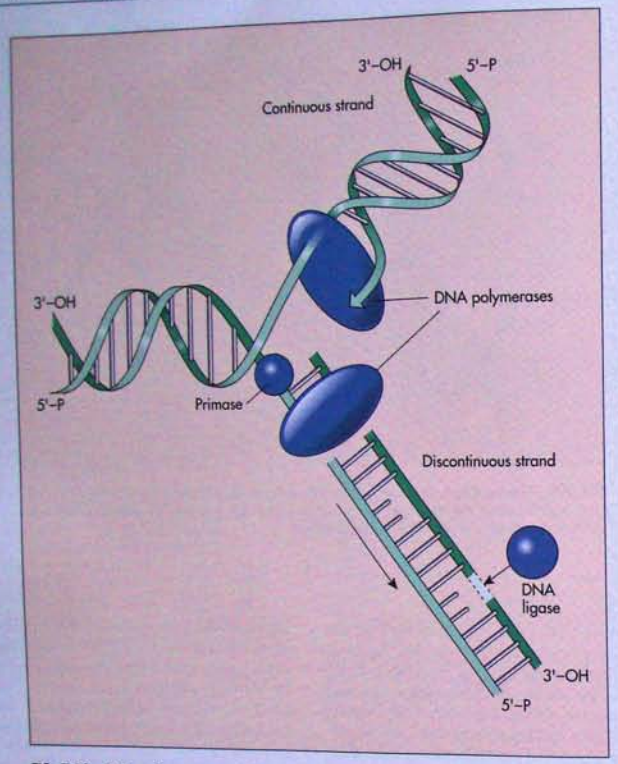


FIG. 7-10 DNA polymerases add nucleotides only to the 3'-OH ends of the newly synthesized DNA polynucleotide chains. One chain is elongated continuously along the direction of formation of the replication fork. The other strand is synthesized as discontinuous segments (Okazaki fragments) that are then joined together by DNA ligase.

The other strand of DNA, however, cannot be synthesized continuously. This is because it runs 3'-OH to 5'-P but DNA polymerase only adds nucleotides in the 5'-P to 3'-OH direction. The initiation of its synthesis can begin only after the double helix has undergone some unwinding. Synthesis of this strand involves formation of short DNA fragments (called Okazaki fragments after the husband and wife team that discovered them) in the direction opposite the direction in which the parent DNA unwinds. Because it is synthesized discontinuously and only after synthesis of the continuous strand has begun, it is called the **discontinuous** or **lagging strand of DNA.** The short DNA fragments of the discontinuous strand are joined together by enzymes called **ligases.** The combined action of DNA polymerase and DNA ligase, thus, accomplishes the synthesis of both complementary strands of DNA during replication.

To make a complementary copy of DNA, the double helix is pulled apart to form a replication fork, complementary nucleotides are aligned by base pairing, and phosphodiester linkages are formed by DNA polymerase.

MUTATIONS

Replication of DNA should always produce exact copies of the hereditary information. Errors, however, sometimes occur. Such errors are called mutations. A **mutation** is any change in the sequence of nucleotides within DNA. Mutations can involve the addition, deletion, or substitution of nucleotides. Even a simple change, such as the deletion or addition of a single nucleotide, can greatly alter the characteristics of an organism. Once they occur, these changes in the DNA are heritable and are passed from one generation to the next. Mutations introduce genetic variability that makes evolutionary change possible. They also sometimes increase the virulence of pathogens and make some microorganisms resistant to antibiotics.

Mutations are stable heritable changes in the nucleotide sequences of DNA.

TYPES OF MUTATIONS

There are several types of mutations (FIG. 7-11). One type of mutation, **base substitution,** occurs when one pair of nucleotide bases in the DNA is replaced by another pair of nucleotides. A **deletion mutation** involves removal of one or more nucleotide base pairs from the DNA. An **insertion mutation** involves the addition of one or more base pairs. Even though they may represent minor changes in the sequence of nucleotides, mutations can have major effects, sometimes proving lethal to the progeny (offspring or descendants) of the organism.

FIG. 7-11 Plate showing growth of *Serratia marcescens.* The wild type colonies are red and the mutant colonies are gray.

Sometimes a mutation results in the death of the microorganism or its inability to reproduce. This is called a **lethal mutation.** In other cases, the mutation alters the nutritional requirements for the progeny of a microorganism. Such a mutation is called a **nutritional mutation.** Often, nutritional mutants will be unable to synthesize essential biochemicals, such as amino acids. **Auxotrophs** are nutritional mutants that require growth factors that are not needed by the parent **(prototroph)** strain.

Replica plating is a method frequently used to detect auxotrophs (FIG. 7-12). In this method, bacterial cells are grown on a master plate and then transferred to sterile plates by repeatedly stamping a pad over the master plate and pressing the pad into plates with media of differing composition. The distribution of microbial colonies should be replicated exactly on each new plate. If a colony is unable to grow on the minimal media, which lacks a specific growth factor, but will grow on the complete medium, this indicates that nutritional mutants, or auxotrophs, are occurring. This method allows an investigator to screen a large number of bacteria for mutations.

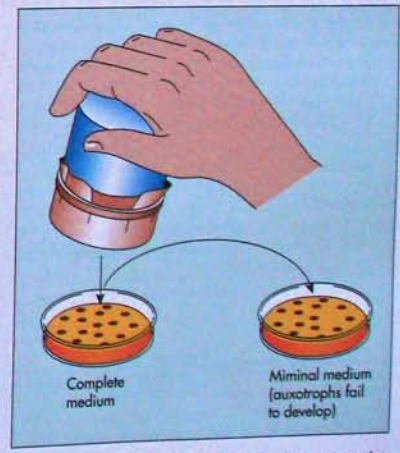


FIG. 7-12 Replica plating is used to identify mutants by transferring identical colonies to different types of media and comparing the colonies that develop on the respective plates. This method is critical in identifying auxotrophic mutants. All colonies develop on a complete medium that satisfies the nutritional needs of both the parental and mutant strains. Colonies of the auxotrophic mutant fail to develop on a minimal medium lacking the specific nutritional growth factors required by the mutant.

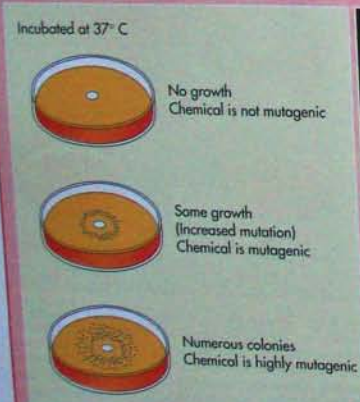
METHODOLOGY

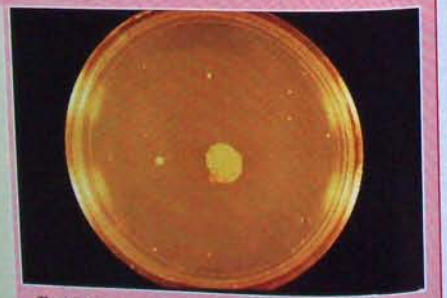
Ames Test

The fact that microorganisms are susceptible to chemical mutagens can be used to determine the ability of various chemicals to increase the rate of mutation, or mutagenicity. In the Ames test procedure, a strain of the bacterium *Salmonella typhimurium* is the test organism for determining chemical mutagenicity (see Figure). This bacterial strain is an auxotroph (nutritional mutant) that requires the amino acid histidine. These organisms are exposed to increasing amounts of the chemical being tested on a solid growth medium that lacks histidine. Normally, the bacteria cannot grow and in the absence of a chemical mutagen no colonies can develop. If the chemical is a mutagen, lethal mutations will occur in the areas of high chemical concentration and no growth will occur in these areas. At lower chemical concentrations along the concentration gradient, however, fewer mutations will occur and some of the cells may revert through mutation to nutritional types that do not require histidine for growth. Such mutants are able to grow and produce visible bacterial colonies on the medium. The appearance of bacterial colonies, therefore, demonstrates that the chemical is a mutagen and the absence of colonies indicates that it is not.

The Ames test procedure also is used to screen chemicals to determine if they are potential cancer-causing agents, or carcinogens. The theoretical basis for this use of the Ames test is that nearly all carcinogens that act directly by attacking DNA are also mutagens. Rather than screening chemicals directly for carcinogenicity, Bruce Ames and his co-workers thought it would be better to test them first for mutagenicity. They recognized, though, that some chemicals are chemically modified in the body and, in particular, some chemicals are inadvertently transformed into carcinogens in the liver in an apparent effort by the body to detoxify these chemicals. Therefore, in testing for potential carcinogenicity, once a chemical has been found to be mutagenic, it is incubated with a preparation of rat liver enzymes to simulate what normally occurs in the liver. Various concentrations of this preparation are then incubated with the *Salmonella* auxotroph to determine whether any products that would cause mutations are formed. The chemicals that do not produce mutations are assumed to be noncarcinogenic or are carcinogens that are not detected by this procedure. Those shown to be mutagenic are subjected to further testing.

Although the Ames test does not positively establish whether a chemical causes cancer, determining whether a chemical has mutagenic activity is useful in screening large numbers of chemicals for potential mutagens, because it is highly probable that a chemical that is a mutagen is also a carcinogen. Since this test can be completed in 24 hours, rapid identification of a mutagen is possible. Today, the use of this bacterial assay greatly simplifies the task of screening many potentially dangerous chemicals, permitting us to recognize potentially carcinogenic compounds. Using bacteria in the Ames assay also allows scientists to avoid animal testing in many cases.





The Ames test procedure is used to screen for mutagens and potential carcinogens. The auxotrophic strain used in this procedure, generally a histidine-requiring mutant of *Salmonella typhimurium*, will not grow on a minimal medium. Mutants that revert to the prototrophic wild type will grow on this medium. The number of colonies that develop after exposure to a chemical indicates the effect of that chemical on mutation rate and therefore its degree of mutagenicity. The development of many colonies indicates that the chemical is highly mutagenic.

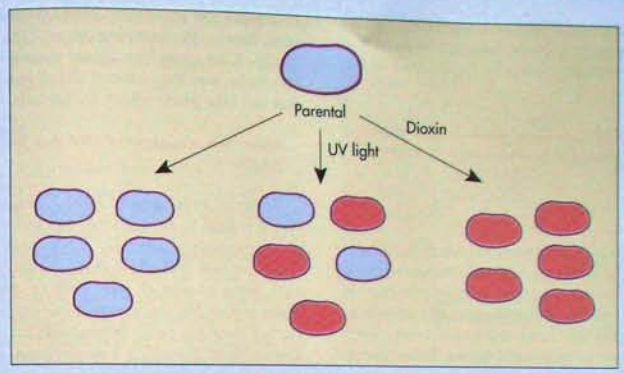


FIG. 7-13 Various chemical and physical agents increase rates of mutation. UV light and dioxin are mutagens that cause formation of mutants *(pink cells)*.

Factors Affecting Rates of Mutation

Naturally occurring rates of mutation are relatively low, about one in a million times the rate of DNA replication. Various physical and chemical agents, however, can modify the nucleotides within DNA, increasing the rate of mutation (FIG. 7-13). Agents that increase the rates of mutation are called **mutagens.**

Exposure to high-energy radiation such as X-rays can cause mutations. Such high-energy ionizing radiation produces breaks in the DNA molecule. The time and intensity of exposure determines the number of lethal mutations that occur. Exposure to gamma radiation, such as that emitted by an isotope of cobalt, ^{60}Co, can be used for sterilizing objects, including plastic Petri plates, because sufficient exposure results in lethal mutations and the death of all exposed microorganisms. Gamma radiation from ^{60}Co has also been used to kill microorganisms on the surfaces of some foods, thereby delaying the spoilage of the food.

Exposure to ultraviolet light also can result in **thymine dimer** formation, that is, the covalent linkage of one thymine to another thymine. Covalent linkages are formed between two thymines on the same strand of DNA. A thymine dimer cannot act as a template for DNA polymerase, and the occurrence of such dimers, therefore, prevents the proper functioning of DNA polymerase. Exposure to ultraviolet light can cause lethal mutations and is sometimes used to kill microorganisms in sterilization procedures. Sometimes ultraviolet light is placed above a work surface when it is not in use to maintain sterility of the surface. Also, air is sometimes passed over ultraviolet lights in hospitals treating patients with respiratory diseases to kill airborne pathogens.

Radiation causes lethal mutations and can be used for sterilization of some materials.

EXPRESSION OF GENETIC INFORMATION

The expression of genetic information involves using information encoded within DNA to direct the synthesis of proteins. In a cell, DNA stores and transmits the complete hereditary information called the **genotype.** Proteins mediate functional activity—called the **phenotype**—that is, the actual appearance and activities of the organism. For example, proteins (enzymes) catalyze all the metabolic activities of cells and produce the observable characteristics that distinguish one microorganism from another.

Proteins mediate functional activity (phenotype); DNA mediates the informational capacity (genotype) of the cell.

The sequence of nucleotides within the DNA molecule ultimately codes for the sequence of amino acids in proteins. Because proteins are the "action" molecules within cells and nucleic acids dictate the formation of proteins, it is the ordering of nucleotides within DNA that provides the information for establishing, controlling, and reproducing cell structure

and function. By specifying and regulating protein synthesis, the genetic informational macromolecules define and control the metabolic capabilities of microorganisms.

The sequence of nucleotides within a cell's DNA determines the sequence of amino acids in its protein molecules.

Genes

DNA is divided into functional sequences known as **genes.** Each gene codes for a specific function. Some genes code for the synthesis of RNA and proteins, respectively determining the sequences of nucleotides and amino acids in these macromolecules. Such genes are known as **structural genes.** Other genes, called **regulatory genes,** act to determine when structural genes are actually expressed. Regulatory genes exert control over the activities of the cell by turning the expression of structural genes on or off. Together, structural and regulatory genes constitute the genotype, that is, the complete genetic informational capacity of an organism. Genes determine all hereditary traits, and they control all the potential activities that can take place within living cells.

Genes are sequences of DNA that have specific functions.

Bacterial cells usually have a single set of genes and are said to be **haploid.** In contrast, eukaryotic microorganisms generally have pairs of matching chromosomes and are **diploid,** having two copies of each gene during at least part of their life cycles. Some eukaryotic microorganisms are haploid during part of their life cycle. When both copies of the gene are identical, the cell is **homozygous.** When the corresponding copies of the gene differ, the cell is **heterozygous.** For example, eukaryotic microorganisms with homozygous genes for color may appear red or white; those with heterozygous genes for red and white may appear red or white if one gene is preferentially expressed (dominant) over the other (recessive) or pink if both genes are simultaneously expressed (codominant).

RNA Synthesis

The transfer of information from DNA to protein is accomplished in two stages (FIG. 7-14). The information in the DNA molecule is initially used to direct the synthesis of ribonucleic acid (RNA) molecules in a process called transcription. Transcription is so named because the information in the DNA is effectively copied or transcribed into RNA. In the second stage, called translation, the RNA directs the synthesis of proteins. Translation is so named because the order of nucleotides in RNA is translated into the order of amino acids of proteins. Transcription and translation occur in tandem in prokaryotic cells.

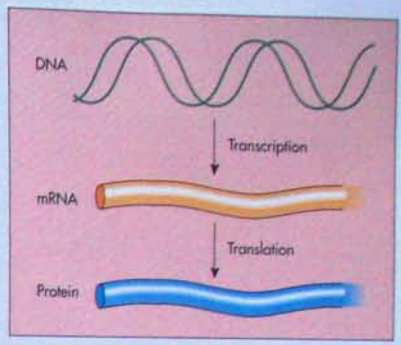


FIG. 7-14 The information in DNA is transferred to RNA during transcription. The RNA then directs the synthesis of proteins during translation.

HISTORICAL PERSPECTIVE

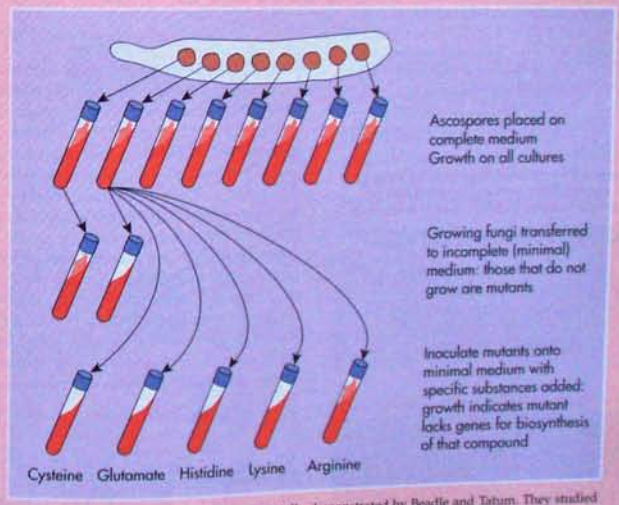
One Gene—One Polypeptide

At the turn of the twentieth century, a physician named Archibald Garrod was studying human metabolic disorders that seemed to run in families. He hypothesized that the specific units of inheritance must function through the synthesis of specific enzymes. This hypothesis received strong support from the studies reported by George Beadle and Edward Tatum in 1941 on the relationship between genes—the units of inheritance—and the metabolism of the bread mold *Neurospora crassa* (see Figure). Prototrophic strains of *N. crassa* can grow on a minimal medium containing only sucrose, mineral salts, and biotin. From these substances, *N. crassa* can synthesize all of its other nutritional requirements, including the amino acids needed for making proteins. This fungus made an excellent choice as the experimental organism to study gene function because much was already known about some of its metabolic pathways. In particular each of the enzymatic steps in the biosynthetic pathway for making the amino acid arginine was known.

Beadle and Tatum designed experiments to detect mutations that produced auxotrophs that could not synthesize arginine. They then analyzed these mutants to see which metabolic step in the synthesis of arginine was affected. To increase the frequency of mutations, Beadle and Tatum X-rayed *Neurospora* spores. The progeny were allowed to grow on a complete medium, which contained all necessary metabolites, including arginine. Next, they tested the abilities of the progeny to grow on a minimal medium lacking amino acids to see if mutations had occurred that resulted in the inability to synthesize arginine. An auxotrophic mutant that could not synthesize arginine would grow on the complete medium but not on the minimal medium.

Beadle and Tatum hypothesized that if genes (hereditary units) controlled the production of specific enzymes, they could detect mutants that could not make different specific enzymes and so could be blocked at different steps in the metabolic pathway for arginine biosynthesis. In fact, Beadle and Tatum identified and isolated many such mutants.

Analysis of cell extracts of mutants revealed a different defective enzyme in each mutant strain. For each enzyme in the arginine biosynthetic pathway, Beadle and Tatum were able to isolate a mutant strain with a defective form of the enzyme needed for that step. They thus provided evidence favoring the "one gene, one enzyme" hypothesis. Other scientists continued to study the relationship between genes and proteins, modifying the original hypothesis slightly, reaching the conclusion that genes code for polypeptides, the chains of amino acids that make up proteins.



The one gene–one hypothesis was experimentally demonstrated by Beadle and Tatum. They studied the fungus *Neurospora*, which forms spores that have single sets of genes. They were able to collect individual spores and to culture them so that they could observe any changes in the genes of the fungus that altered its nutritional requirements. Mutants, which were unable to carry out complete biosynthetic pathways, could grow on complete media but not on minimal media. The minimal media lacked the nutrients required for growth that the mutants could no longer synthesize. Growth on minimal media with specific compounds added, such as vitamins and amino acids, enabled them to determine which compounds the mutant fungi could not synthesize. In this manner they were able to identify the genetic changes that occurred and to associate specific genes with specific metabolic activities.

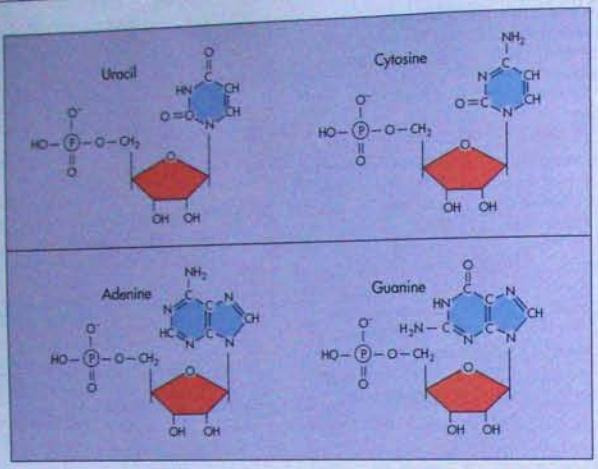


FIG. 7-15 Ribonucleic acid (RNA) is composed of ribonucleotides that have the sugar ribose, a phosphate group, and one of four nucleic acid bases: uracil, cytosine, adenine, or guanine.

However, in eukaryotic cells, transcription occurs in the nucleus and translation subsequently occurs on the ribosomes in the cytoplasm. Therefore, in eukaryotic cells, transcription and translation are separated in time and space.

RNA acts as an informational mediator between the DNA where genetic information is stored and the proteins that functionally express that information.

Ribonucleic Acid (RNA)—Functions and Types

RNA differs from DNA in several respects (FIG. 7-15). The 5-carbon sugar in the RNA nucleotide is ribose, which has one more oxygen atom than the deoxyribose in DNA. One of RNA's nitrogenous bases is uracil instead of thymine. Also, RNA is usually single-stranded.

RNA contains ribose, adenine, uracil, cytosine, and guanine.

Three different kinds of RNA occur in all living cells. They are ribosomal RNA, messenger RNA, and transfer RNA. Each of these RNA molecules has different functions and different physical-chemical properties (Table 7-1). **Ribosomal RNA** (rRNA) is an important structural component of ribosomes, the sites where proteins are synthesized within cells. **Messenger RNA (mRNA)** carries the information from the DNA molecule to the ribosome. At the ribosomes, protein synthesis actually occurs when the information encoded in the mRNA molecule is used to specify the sequence of amino acids that comprise the protein. The mRNA contains the genetic information of DNA in a single-stranded molecule complementary in base sequence to a portion of the base sequence of DNA. The mRNA sequence directs incorporation of amino acids into a growing polypeptide in the process called translation, which takes place on the surface of the ribosome. **Transfer RNA** (tRNA) molecules help align amino acids during protein synthesis in the order specified by an mRNA molecule. Transfer RNAs form a bridge between mRNA and an amino acid, thereby transferring the genetic information carried by the mRNA to the amino acid sequence of the polypeptide. Working together, the three types of molecules convert genetic information from the language of nucleotides to the language of amino acids, the building blocks of proteins.

Three types of RNA molecules (rRNA, mRNA, and tRNA) are involved in the transfer of information from DNA to proteins.

Transcription

Transcription is the process by which information stored in the DNA molecule is used to code for the synthesis of RNA (FIG. 7-16). During transcription, RNA nucleotides in association with RNA polymerase pair with complementary DNA bases. The base pairs between DNA and RNA are: thymine (DNA) and adenine (RNA), adenine (DNA) and uracil (RNA), guanine (DNA) and cytosine (RNA), and cytosine (DNA) and guanine (RNA). Once the RNA bases are properly aligned in the order specified by the DNA molecule, the enzyme **RNA polymerase** links the bases together.

The synthesized molecule of RNA is antiparallel, that is it runs 5'-P to 3'-OH if the chain of DNA that serves as the template runs 3'-OH to 5'-P. Only one chain of the DNA serves as a template for the synthesis of a particular RNA molecule. This DNA chain coding for the synthesis of RNA is known as the **template strand.** Both chains of the DNA can serve as template strands in different regions, and the term *template strand* is applied only to the specific region of the DNA that is being transcribed.

Initiation and Termination of Transcription

The transfer of information from DNA to RNA requires that transcription begin and stop at precise locations. Both prokaryotes and eukaryotes have multiple initiation sites along the DNA molecule for transcription (FIG. 7-17). The specific site where RNA polymerase initially binds to the DNA and, thus,

TABLE 7-1
Characteristics of Various Types of RNA

TYPE OF RNA	ABBREVIATION	SEDIMENTATION COEFFICIENT	FUNCTION
Messenger RNA	mRNA	6—50S	Carries genetic information from DNA to the ribosomes where the information is used to direct the synthesis of polypeptides
Transfer RNA	tRNA	4S	Carries amino acids to the ribosomes and assists in the translation of the information carried by mRNA
Prokaryotic Ribosomal RNA	rRNA	5S 16S 23S	The major structural components of ribosomes that interact with mRNA and tRNA to ensure proper synthesis of polypeptides; there are three major types of rRNAs with differing sedimentation coefficients
Eukaryotic Ribosomal RNA	rRNA	5.8S 18S 28S	The major structural components of ribosomes that interact with mRNA and tRNA to ensure proper synthesis of polypeptides; there are three major types of rRNAs with differing sedimentation coefficients

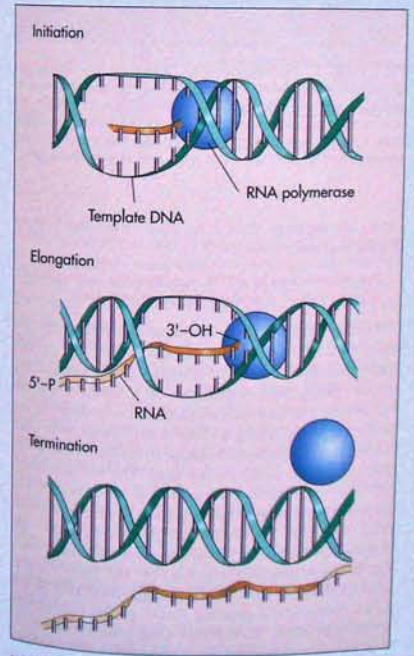


FIG. 7-16 Transcription produces RNA using one of the strands of DNA as a template. The formation of the RNA is catalyzed by RNA polymerase. After initiation the RNA is elongated by this enzyme until termination.

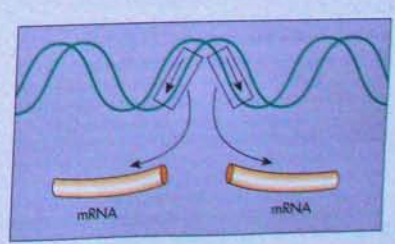


FIG. 7-17 Transcription can occur at multiple sites. This permits the formation of several proteins simultaneously.

where transcription begins is called the **promoter region.** There are different promoters for the initiation of transcription of different genes. The promoter determines the site of transcription initiation and which of the two DNA strands is to serve as the sense strand for transcription in that region.

Transcription begins in the promoter region of DNA where RNA polymerase binds.

The RNA polymerase moves along the DNA template, synthesizing a new strand of RNA until a specific termination sequence is reached. Then the RNA polymerase falls off the sense strand of the DNA and transcription stops.

Synthesis of mRNA in Prokaryotic and Eukaryotic Cells—Split Genes

There is a fundamental difference in how mRNA is formed in prokaryotic and eukaryotic cells. The sequence of nucleotides in the messenger RNA molecule of prokaryotic cells corresponds exactly with the sequence of nucleotides in the DNA. In contrast, the RNA molecules of eukaryotic microorganisms are generally extensively modified after transcription from the DNA to form mRNA (FIG. 7-18).

Unlike the genes of bacterial cells, the DNA sequences coding for RNA molecules in eukaryotic cells is not continuous. There are intervening DNA sequences, called **introns,** between the nucleotide sequences that actually constitute the gene. The sequences that constitute the gene are called **exons.** Hence, the genes of eukaryotic cells are said to be **split genes.** When DNA is transcribed the RNA initially contains both introns and exons. This precursor of messenger RNA in eukaryotes, known as **hnRNA (heterogeneous nuclear RNA),** is then subjected to substantial post-transcriptional modification within the nucleus. This post-transcriptional modification removes the introns and forms a messenger RNA that only has exons.

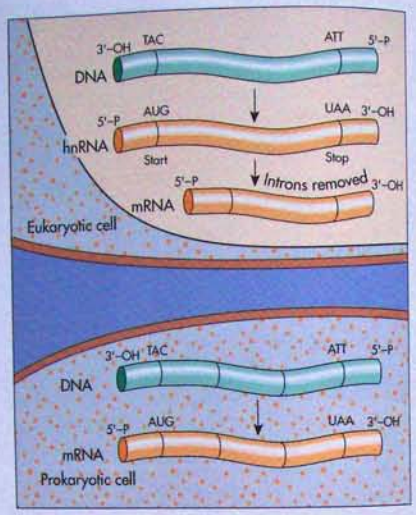


FIG. 7-18 In eukaryotic cells the primary transcript (hnRNA) is extensively modified within the nucleus to produce mRNA. The conversion of hnRNA to mRNA involves the removal of introns. Each mRNA usually encodes only a single gene. In prokaryotic cells the primary transcript serves as the mRNA. It often encodes several genes.

Protein Synthesis—Translation of the Genetic Code

mRNA and the Genetic Code

The information in mRNA specifies the sequence of amino acids in the protein made during **translation.** The translation of the information in the mRNA molecule, that is, reading mRNA, is a directional process. Messenger RNA is read in a 5'-P → 3'-OH direction. The polypeptide that is made is synthesized from the amino terminal to the carboxyl terminal end. Here, we see the importance of having a mechanism for recognizing direction in informational macromolecules. Just as we have the convention for reading the English language from left to right, the correct interpretation of the information stored in the mRNA molecule requires that it be read from the 5'-P → 3'-OH free end.

The information in mRNA specifies the sequence of amino acids in a protein during translation.

Within mRNA, three sequential nucleotides are used to code for a given amino acid. The genetic code, therefore, is termed a **triplet code.** Each of the triplet nucleotide sequences is called a **codon.** Adding or deleting nucleotides causes mutations because the codon changes (FIG. 7-19). The genetic code is said to be degenerate because more than one codon can specify the same amino acid. The mRNA molecule is read one codon at a time. In other words, the three nucleotides that specify a single amino acid are read together. There are no spaces between the codons that are read. Therefore establishing a reading frame is critical for extracting the proper information. Adding or deleting a single base pair in the DNA changes the reading frame of the transcribed messenger RNA. Such **frame-shift mutations** can result in the misreading of large numbers of codons (FIG. 7-20), thus producing proteins that are inactive because they have the wrong amino acid sequence.

Three nucleotides constitute a codon that specifies an amino acid.

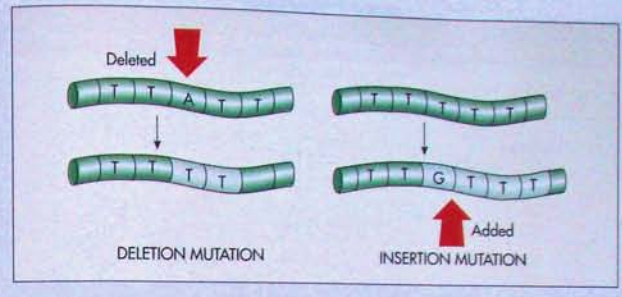


FIG. 7-19 Deletion mutations occur when one or more nucleotides are omitted during DNA replication. Addition mutations occur when one or more nucleotides are added during DNA replication.

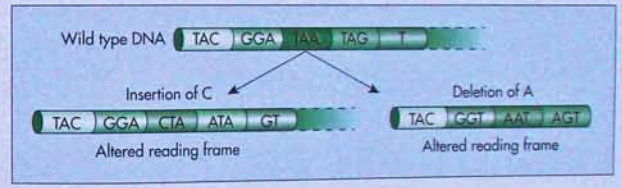


FIG. 7-20 A frame shift mutation results from nucleotide deletions or additions.

Each codon consists of three nucleotides. There are four different nucleotides, making possible 64 codons. The four different nucleotides in different three-base combinations lead to $4 \times 4 \times 4 = 64$ possibilities (Table 7-2, p. 212). The genetic language, which is almost universal, can therefore be said to have four letters in the alphabet and 64 words in the dictionary, each word containing three letters.

Proteins in biological systems normally contain only 20 L-amino acids. Thus there are many more codons than are strictly needed for the translation of genetic information into functional proteins. More than one codon can code for the insertion of the same amino acid into the polypeptide chain. Consequently, a silent mutation can occur in which there is an alteration in the nucleotide sequence without a change in the amino acid sequence. In such cases, changes in genotype would not be reflected in changes in phenotype.

Additionally, there are three codons that do not code for any amino acid. These codons have been referred to as **nonsense codons.** Actually, nonsense codons serve a very important function, acting like the period at the end of a sentence and signalling termination of synthesis of a polypeptide chain.

More than one codon can code for the same amino acid. The genetic code is degenerate.

tRNA and Polypeptide Formation

Translation of the genetic code into protein molecules occurs at the ribosomes. Ribosomes provide the spatial framework and structural support for aligning the translational process of protein synthesis. Distortion of the proper configuration of the ribosome can prevent proper information exchange and expression of the genetic information. This forms the basis for the action of many antibiotics, such as erythromycin.

Transfer RNA (tRNA) attaches to amino acids and brings them to the ribosomes. There is a specific tRNA for each amino acid. In addition to bringing the amino acids to the ribosomes, the tRNA also properly aligns them during translation (FIG. 7-21). Each tRNA molecule contains a specific **anticodon,** a three-base nucleotide sequence that is complementary to the three-base nucleotide sequence of the codon. The pairing of the codons of the mRNA molecules with the anticodons of the tRNA molecules determines the order of amino acid sequence in the polypeptide chain. The third base of the anticodon does not always properly recognize the third base of

TABLE 7-2

Codons of the Genetic Code (mRNA shown in 5′-P → 3′-OH direction)

First Nucleic Acid (5′-P End)	SECOND NUCLEIC ACID: U		SECOND NUCLEIC ACID: C		SECOND NUCLEIC ACID: A		SECOND NUCLEIC ACID: G		Third Nucleic Acid (3′-OH End)
U	UUU	Phenylalanine	UCU	Serine	UAU	Tyrosine	UGU	Cysteine	U
	UUC		UCC		UAC		UGC		C
	UUA	Leucine	UUA		UAA	STOP	UGA	STOP	A
	UUG		UCG		UAG		UGG	Tryptophan	G
C	CUU	Leucine	CCU	Proline	CAU	Histidine	CGU	Arginine	U
	CUC		CCC		CAC		CGC		C
	CUA		CCA		CAA	Glutamine	CGA		A
	CUG		CCG		CAG		CGG		G
A	AUU	Isoleucine	ACU	Threonine	AAU	Asparagine	AGU	Serine	U
	AUC		ACC		AAC		AGC		C
	AUA		ACA		AAA	Lysine	AGA	Arginine	A
	AUG	Methionine	ACG		AAG		AGG		G
G	GUU	Valine	GCU	Alanine	GAU	Aspartate	GGU	Glycine	U
	GUC		GCC		GAC		GGC		C
	GUA		GCA		GAA	Glutamate	GGA		A
	GUG		GCG		GAG		GGG		G

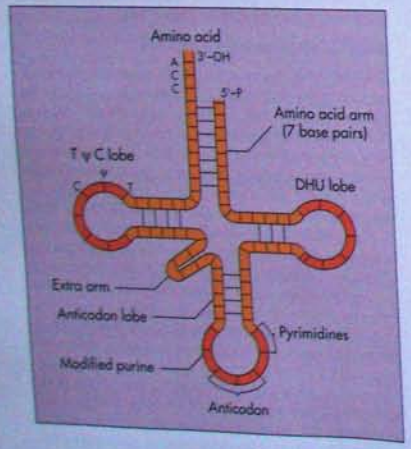


FIG. 7-21 All tRNA molecules have a characteristic four-lobe structure that results from internal base pairing of some of the nucleotides. Each lobe of the tRNA molecule has a distinct function. Several of the lobes are characterized by the inclusion of unusual nucleotides. These nucleotides are formed by enzymatic modification of the nucleotides directly coded for by the DNA; that is, the DNA does not have additional nucleotides that directly call for the insertion of nucleic acid bases other than adenine, uracil, cytosine, and guanine into the RNA. One of the lobes, designated the *DHU* or *D lobe*, contains dihydrouracil (DHU). This lobe binds to the enzyme involved in forming the peptide during translation. The TψC lobe contains the sequence ribothymine (T), pseudouracil (ψ), and cytosine (C). This lobe binds to the ribosome. A third lobe, which also contains modified purines, is designated the *anticodon lobe* because it is complementary to the region of the mRNA, the codon, that specifies the amino acid to be incorporated during protein synthesis. The 3′-OH end always has the terminal sequence ACC, which is where the amino acid binds. This terminal sequence is usually referred to as the *CCA end*, reading from the 5′-P end of the tRNA molecule.

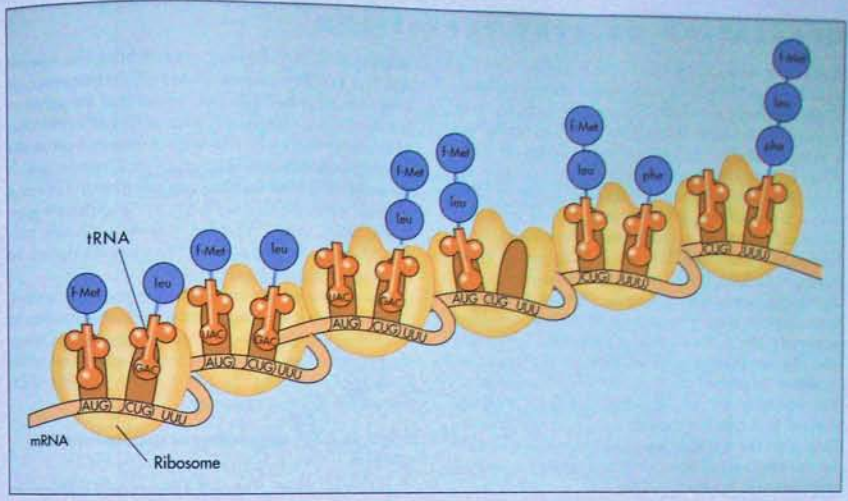


FIG. 7-22 During protein synthesis the codons of the mRNA are translated into an amino acid sequence at the ribosome. Each codon of the mRNA matches an anticodon of a tRNA so that the proper amino acid sequence is formed. The start codon AUG specifies the insertion of formyl methionine (f-Met) at the peptidyl site. A second amino acid is aligned at the aminoacyl site by the pairing of a tRNA with the codon. Formyl methionine is transferred to the amino acid at the aminoacyl site with the formation of a peptide bond. The mRNA then moves along the ribosome so that the tRNA with its two attached amino acids moves to the peptidyl site. A new amino acid is aligned at the aminoacyl site, again by pairing of the appropriate tRNA with the codon. The two amino acids are transferred to the amino acid with the formation of a new peptide bond so that the peptide chain now has three amino acids. The process is repeated over and over to form the long polypeptide chain of amino acids joined by peptide bonds in the sequence specified by the mRNA.

the messenger RNA codon. The first and second bases of the codon sequence are therefore more important in matching the codon with the anticodon. As a result, a codon may pair with more than one anticodon that differs only in the third base position, a phenomenon called **wobble.**

Transfer RNA brings amino acids to the ribosomes and properly aligns them during translation.

Forming the Polypeptide

During translation, transfer RNA molecules bring individual amino acids to be sequentially inserted into the polypeptide chain (FIG. 7-22). The codon of the messenger RNA that specifies where the synthesis of a polypeptide is initiated is called the **start codon.** The tRNAs arrive in the order specified by the codons in the mRNA as the mRNA moves across the surface of a ribosome. When tRNA molecules arrive at the ribosome, the proper anticodon pairs with its matching codon of mRNA. The amino acid is thus aligned so that it can be covalently bound to a growing peptide chain. After a peptide bond is established between amino acids already in the polypeptide chain and the newly aligned amino acid, the messenger RNA then moves along the ribosome by three nucleotides. The movement of messenger RNA, transfer RNA, and the growing polypeptide chain along the ribosome is known as **translocation.** The process is repeated over and over, resulting in the elongation of the polypeptide chain. Eventually, one of the nonsense codons appears on the mRNA as it moves across the ribosome. Since no tRNA molecule pairs with the nonsense codon, the translational process is terminated and the polypeptide is physically released from the ribosome.

Translocation is the movement of messenger RNA, transfer RNA, and the polypeptide chain along the ribosome.

Cells have structural genes that encode the information for specific polypeptide sequences of proteins. Cells also have regulatory genes that code for gene expression. It would be inappropriate and energy depleting for the entire genome to be expressed at one time. By controlling which genes of the organism are to be translated into functional enzymes, the cell regulates its metabolic activities. While some genes are constantly "turned on," others are expressed only in response to the immediate needs of the cell. It is advantageous for a cell to regulate gene expression so that it can conserve its resources. This is important to conserve the supply of energy, as well as to utilize sparingly the limited pool of metabolic intermediates. By regulating gene expression the organism modifies its phenotype to adapt to its environment. For example, the cell does not produce enzymes needed to catabolize lactose unless lactose is available. Also, the cell does not produce enzymes needed for the synthesis of the amino acid tryptophan when tryptophan is available.

Some regions of DNA are specifically involved in regulating transcription. These regulatory genes can control the synthesis of specific enzymes. Sometimes gene expression is not subject to specific genetic regulatory control. In these cases, the enzymes coded for by such regions of the DNA are **constitutive,** that is, they are continuously synthesized. In contrast to constitutive enzymes, some enzymes are synthesized only when the cell requires them. Some such enzymes are **inducible,** that is, made only in response to a specific inducer substance. Others are **repressible,** that is, made unless stopped by the presence of a specific repressor substance.

Operons

In 1961 Francois Jacob and Jacques Monod put forth a hypothesis that induction and repression were under the control of specific proteins. Such proteins would be coded for by regulatory genes. They proposed that regulatory genes were closely associated with the structural genes that code for the enzymes in specific metabolic pathways. Often, several enzymes that have related functions are controlled by the same regulatory gene. Called the **operon model,** the mechanism proposed by Jacob and Monod explains how cells are able to coordinate the expression of genes with related functions.

An **operon** is a cluster of adjacent genes on the chromosome that is controlled by one promoter site. Transcription starting at that promoter site results in the formation of an mRNA coding for several polypeptides. Such an mRNA is said to be **polycistronic,** meaning that it codes for more than one polypeptide. An operator gene within the operon acts like a switch, turning on and off the transcription of structural genes. Either all or none of the genes of the operon are expressed. This is achieved at the level of transcription by controlling the production of the polycistronic mRNA. Induction and repression of genes in an operon are based on whether or not a regulatory repressor protein binds at a regulatory gene of the DNA, called the *operator.* If the repressor protein binds to the operator, it blocks transcription of the succeeding structural genes.

Some operons are regulated by positive control, which involves the binding of a regulator protein to DNA and the stimulation of gene expression. Others are regulated by negative control, which involves binding of a regulator protein to DNA and the shutting down of gene expression.

Regulating the Metabolism of Lactose—the *lac* Operon

The *lac* operon coordinates the expression of three enzymes that are specifically synthesized by *Escherichia coli* for the metabolism of lactose. These enzymes are: β-galactosidase, galactoside permease, and transacetylase. β-galactosidase cleaves the disaccharide lactose into the monosaccharides galactose and glucose. Galactoside permease is required for the transport of lactose across the bacterial plasma membrane. The role of transacetylase is not yet established. The structural genes that code for the production of these three enzymes occur in a contiguous segment of DNA.

The operon for lactose metabolism is called the ***lac* operon** (FIG. 7-23). The *lac* operon includes a promoter region where RNA polymerase binds, an operator region where the repressor protein attaches, and three structural genes that code for three proteins that are involved in lactose metabolism. In addition, there is a regulatory gene at another location that codes for the synthesis of a repressor protein. In the absence of lactose, this repressor protein binds to the operator region of the DNA. The operator region occurs between the promoter and the three structural genes. The binding of the repressor protein at the operator region blocks the transcription of the structural genes. This means that in the absence of lactose, the three structural *lac* genes are not transcribed.

The operator region is adjacent to or overlaps the promoter region. The binding of the repressor protein at the operator region interferes with the binding of RNA polymerase at the promoter region. The inducer binds to the repressor protein so that it is unable to bind at the operator region. Thus in the presence of an inducer that binds with the repressor protein, transcription of the *lac* operon is not blocked and the synthesis of the three structural proteins needed for lactose metabolism proceeds. The *lac* operon is typical of operons that control catabolic pathways; only in the presence of an appropriate inducer is the system turned on.

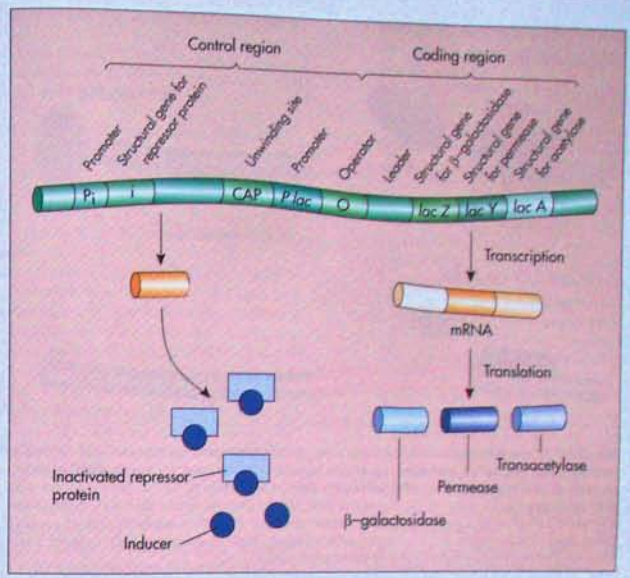


FIG. 7-23 The *lac* operon controls the utilization of lactose. Three structural genes under the control of the *lac* promoter (P *lac*) code for the synthesis of the enzymes needed for lactose utilization. These enzymes are made only when lactose is present.

Catabolite Repression

When more than one carbon source such as glucose and lactose is available at the same time, the cell will use the simpler substance first. Thus glucose is used before lactose. The cell turns on the genes for glucose metabolism and does not turn on (represses) the genes for lactose utilization. This type of repression is called **catabolite repression.** It regulates the expression of multiple genes that are under the control of different promoters. Only some genes are controlled by catabolite repression.

Catabolite repression acts via the promoter region of DNA. This is the region where RNA polymerase binds to initiate transcription (FIG. 7-24). To efficiently bind to the promoter region, RNA polymerase requires a protein called the *catabolite activator protein.* The catabolite activator protein, in turn, cannot bind to the promoter region unless it is bound to cyclic adenosine monophosphate (cAMP).

In the absence of glucose, cAMP is synthesized from ATP by enzymatic action. This maintains an adequate supply of cAMP to permit the binding of RNA polymerase to the promoter region. Thus, when glucose levels are low, cAMP stimulates the initiation of many inducible enzymes.

In the presence of glucose, cAMP levels are greatly reduced. Thus, when glucose is being metabolized, there is not enough cAMP for the catabolite activator protein to bind to promoter region. Consequently, RNA polymerase does not bind to the promoters, and transcription at a number of regulated structural genes ceases in a coordinated manner. Thus, in the presence of an adequate concentration of glucose, a number of metabolic pathways involved in the breakdown of carbohydrates are simultaneously shut off. For example, when glucose is available for catabolism in the glycolytic pathway, disaccharides and polysaccharides are not metabolized because of catabolite repression.

By regulating the metabolism of more complex carbohydrates, the cell conserves its metabolic resources.

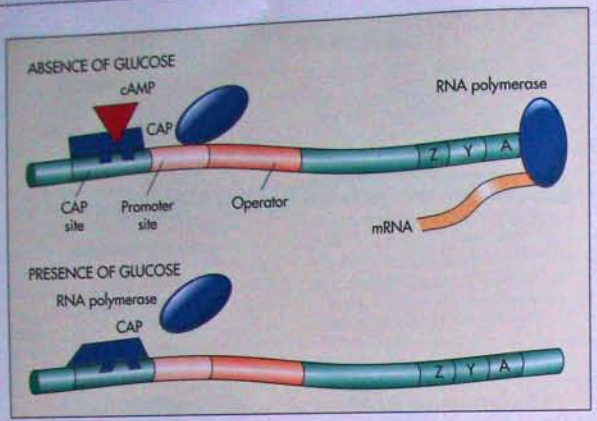


FIG. 7-24 Catabolite repression explains why, in the presence of glucose, several catabolic pathways are shut off. Catabolite repression is based on the need for cyclic AMP (cAMP) to form an activated complex with catabolite activator protein (CAP) at the promoter site that enhances the binding of RNA polymerase. When glucose is metabolized, there is inadequate cAMP to facilitate RNA polymerase binding. Therefore transcription at several promoters ceases. When there is inadequate glucose, there is enough cAMP to bind with CAP and thus transcription occurs at those promoters.

SUMMARY

Molecular Basis of Heredity (pp. 192-194)

- Frederick Griffith, Oswald Avery, Alfred Hershey, and Martha Chase made important contributions to the discovery that the genetic information of a cell is stored within its DNA macromolecules.

Structure of DNA (pp. 194-198)

Nucleotides—Building Blocks of the Genetic Code (p. 194)

- DNA is composed of nucleotides that are linked together. A nucleotide consists of a nucleic acid base, a deoxyribose sugar, and a phosphate group.
- Four nucleic acid bases occur in DNA: cytosine, guanine, adenine, and thymine. The nucleotides are linked by strong covalent bonds. Nucleotides are linked by 3′-5′ phosphodiester linkages. At the ends of the DNA strand, there are no linkages and free hydroxyl groups are present. One end has a free hydroxyl group at the 3-carbon position of the monosaccharide (3′-OH end); the other end of the strand has a free phosphate group at the 5-carbon position of the monosaccharide (5′-P free end). This gives DNA directionality.

Chains of Nucleotides—Directionality of DNA (pp. 194-196)

- DNA is a double helix molecule composed of two polynucleotide chains. The chains are held together by hydrogen bonding between complementary nucleotide bases.

DNA Double Helix—Complementarity (pp. 196-198)

- The complementary base pairs are adenine and thymine, which are held together by two hydrogen bonds, and guanine and cytosine, which are held together by three hydrogen bonds. This complementarity establishes the basis for the double helix and the accurate replication of DNA.

Replication of DNA (pp. 198-202)

- Replication of the hereditary information involves synthesizing new DNA molecules that have the same nucleotide sequences as those of the parent organism. The two chains of the DNA double helix are complementary and the nucleotide sequence in one chain specifies the sequence in the other.
- DNA chains are complementary and antiparallel; one chain has the 3′-OH free end and its complementary chain has the 5′-P free end.

Semiconservative DNA Replication (pp. 198-199)

- DNA replication is semiconservative, that is, a parent chain remains intact and a new complementary chain is assembled for each one. Thus each new DNA macromolecule is half old and half new.

Steps in DNA Replication (pp. 199-201)

- DNA replication begins when the double helix unwinds to form a replication fork, separating the chains to serve as templates.
- The parental DNA is pulled apart at the replication fork, providing space for free nucleotides to align opposite their complementary bases for the synthesis of new chains.
- DNA replication begins at only one site in bacteria. DNA gyrase twists the DNA as the replication fork moves around the bacterial chromosome.

Formation of a New Chain of Nucleotides—DNA Polymerase (pp. 201-202)

- DNA polymerase links the nucleotides by forming phosphodiester bonds after the nucleotides are aligned by base pairing. DNA polymerase adds nucleotides to the free 3′-OH end of an existing nucleotide chain.
- The continuous, or leading, chain of DNA is the DNA chain that can be continuously synthesized because it runs in the appropriate direction for the continuous addition of free nucleotides to the free 3′-OH end. The lagging, or discontinuous, strand of DNA cannot be synthesized continuously because initiation of its replication can begin only after the double helix has already unwound somewhat. Short DNA fragments are synthesized in the direction opposite the direction in which the parent DNA unwinds. These fragments are joined together by ligases.

Mutations (pp. 203-205)

Types of Mutations (p. 203)

- A mutation is a change (addition, deletion, or substitution) in the nucleotide sequences of DNA. A lethal mutation results in the death of a microorganism or in its inability to reproduce; a conditionally lethal mutation exerts its effect only under certain environmental conditions; an unconditionally lethal mutation is lethal regardless of environmental conditions. Temperature-sensitive mutations alter the range of temperatures over which the microorganisms may grow. Nutritional mutations alter the nutritional requirements for the progeny; nutritional mutants (auxotrophs) require growth factors not needed by the parental (prototrophic) strain.

Factors Affecting Rates of Mutation (p. 205)

- Mutagens are chemicals that increase the rate of mutation. High-energy ionizing radiation causes mutation and can be used for sterilizing objects. Ultraviolet light can cause mutations by producing thymine dimers.

Expression of Genetic Information (pp. 205-213)

- The genotype represents the total informational capacity of the cell. It is mediated by DNA. The phenotype, the discernible characteristics of an organism, including the functional appearance and activities of the cell, is mediated by proteins.
- The sequence of nucleotides within the DNA determines the sequence of amino acids in the protein molecules of the cell.

Genes (pp. 206-207)

- A gene is a segment of the genetic material that has a specific function. Structural genes code for the synthesis of RNA and proteins, respectively determining the sequences of nucleotides and amino acids in these macromolecules. Regulatory genes control cell activity by specifying when particular structural genes are actually expressed.
- Prokaryotic cells have a single chromosome and therefore are haploid. Eukaryotic cells generally have pairs of matching chromosomes, making them diploid. In homozygous cells the genes at a locus are identical copies; in heterozygous cells the genes differ.

RNA Synthesis (pp. 207-210)

- Protein synthesis involves two stages: transcription to form RNA and translation of the RNA to form a polypeptide chain.
- RNA contains ribose, phosphate, adenine, uracil, cytosine, and guanine. There are three types of RNA. Ribosomal RNA is a structural component of ribosomes. Messenger RNA carries the information from the DNA to the ribosome. Transfer RNA helps align amino acids during protein synthesis in the order specified by mRNA.
- In transcription, the information in the DNA is transferred to RNA. During transcription, DNA serves as a template that determines the order of the bases in the RNA. The RNA that is formed by transcription is complementary to the DNA. RNA polymerase links the bases, forming 3′-5′ phosphodiester bonds. The template strand is the DNA chain that codes for the synthesis of RNA.
- Transcription begins at specific promoter regions where RNA polymerase binds.
- The sequence of nucleotides in prokaryotic mRNA corresponds exactly with the sequence of nucleotides in DNA. Eukaryotic genes are split genes, that is, the sequence of nucleotide bases in the mRNA is not complementary to the specific contiguous linear sequence of bases in the DNA. Eukaryotic RNA (heterogeneous nuclear RNA) must be extensively modified after transcription from DNA to form mRNA.

Protein Synthesis—Translation of the Genetic Code (pp. 210-213)

- In translation, mRNA is used to establish the sequence of amino acids that make up the protein. Translation occurs at the ribosomes.
- Translation is a directional process. mRNA is read in a 5′-P to 3′-OH direction. Polypeptides are synthesized from the amino terminal to the carboxyl terminal end.
- The genetic code has 64 possible codons; each codon is a triplet containing three nucleotides. There is more than one codon for most amino acids, and different codons can specify the same amino acid.
- Nonsense codons are ones for which there are no amino acids; the nonsense codons signal termination of synthesis of a polypeptide chain.
- The ribosome moves along the mRNA, exposing one codon at a time. As each triplet is exposed by the ribosome, a transfer RNA (tRNA) brings the specified amino acid to the ribosome; the tRNA has an anticodon region that is complementary to the codon and is responsible for bringing the correct amino acid specified by the codon. The ribosome moves to the next triplet and the process is repeated.

- Translocation is the movement of mRNA, tRNA, and the polypeptide chain along the ribosome.

Regulation of Gene Expression (pp. 214-216)

- The expression of genetic information can be regulated at the level of transcription.
- Constitutive enzymes are continuously synthesized at a constant rate and are not regulated. Inducible enzymes are made only at appropriate times, e.g., when synthesis is induced by appropriate factors.

Operons (pp. 214-215)

- The operon model of gene control explains the basis of control of transcription. An operon consists of structural genes that contain the code for making proteins; an operator region, which is the site where repressor protein binds and prevents RNA transcription; and a promoter region, which is the site where RNA polymerase binds. It is also controlled by a regulatory gene, which codes for the repressor protein.
- The *lac* operon regulates the utilization of lactose. In the presence of lactose an inducer binds to a repressor protein, preventing it from binding to the operator region of the operon; this results in derepression of *lac* operon, and structural genes needed for the utilization of lactose are transcribed until the lactose has been broken down.

Catabolite Repression (pp. 215-216)

- Catabolite repression is a generalized type of repression. Catabolite repression supersedes the control exerted by the operator region. Catabolite repression acts via promoter region of DNA by blocking the normal attachment of RNA polymerase; a catabolite activator protein is needed to bind RNA polymerase to promoter region and cAMP is required for efficient binding to occur. In the presence of glucose, the amount of cAMP is reduced; therefore the catabolite activator protein cannot bind to promoter, and transcription is unable to occur.

CHAPTER REVIEW

Review Questions

1. Explain the difference between a gene and a chromosome.
2. What is the difference between genotype and phenotype?
3. What is the relationship between DNA and heredity?
4. What is the genetic code?
5. What is a mutagen?
6. What is a mutation?
7. How is DNA replicated in bacterial cells?
8. Describe the process of protein synthesis.
9. Define induction and explain how it regulates gene expression in bacteria.
10. Define catabolite repression and explain how it regulates gene expression in bacteria.
11. What are the different types of mutations?
12. Describe how the Ames test is used to detect carcinogens.
13. Compare and contrast the storage of genetic information in a prokaryotic and a eukaryotic cell.
14. Compare and contrast the expression of genetic information in a prokaryotic and a eukaryotic cell.
15. What is DNA gyrase and what role does it play in DNA replication?
16. How could DNA gyrase be used as a target for an antimicrobial agent for the treatment of disease?
17. How would you go about increasing the rate of mutations?
18. How could you recognize the occurrence of a mutant?
19. How could you design an experiment to select mutants?

CRITICAL THINKING QUESTIONS

1. How does the structure of DNA relate to the ability of this molecule to serve as the universal hereditary molecule of all living organisms? Why is fidelity essential for replication of DNA? How does a bacterial cell replicate its DNA and make very few errors in the process? What is the consequence of making an error during DNA replication?
2. Why is it so important for the bacterial cell to regulate the expression of its genes? What are the advantages and disadvantages of bacterial genes being organized into operons? Why are operons for catabolic pathways normally inducible (turned on by an inducer) and those for biosynthetic pathways normally repressible (turned off by a repressor).
3. Why can eukaryotic cells have split genes? What roles might introns play in the eukaryotic cell?
4. DNA has the sugar deoxyribose and RNA has the sugar ribose. Compared to deoxyribose, ribose has an extra hydroxyl group. The extra hydroxyl group tends to help break phosphate bonds. How would this affect the relative stability of RNA and DNA in a cell? How would this difference in stability be related to the different functions of RNA and DNA in a cell? What are the essential functions of DNA and RNA in a cell?
5. Do all substances that cause mutations in bacteria cause cancer in humans? How could you go about determining whether foods you eat contain substances that might be mutagenic or carcinogenic?

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CHAPTER 8

Genetic Recombination and Recombinant DNA Technology

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  - Recombinant Human Growth Hormone
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- Safety of Genetic Engineering
- *Highlight:* Tracking and Containing Genetically Engineered Microorganisms

PREVIEW TO CHAPTER 8

In this chapter we will:

- Learn that genes can be transferred from one DNA macromolecule to another, recombining to form new genetic combinations.
- Examine the recombinational processes by which genes are exchanged.
- Study the natural ways by which DNA is transferred from one cell to another.
- Discover how scientists are able to manipulate DNA to form new combinations of genes through recombinant DNA technology.
- See examples of how recombinant DNA technology has been used to genetically engineer new organisms.
- Learn the following key terms and names:

alleles
cloning
cloning vector
complementary DNA (cDNA)
conjugation
conjugative plasmids
defective phages
donor strain
endonuclease
generalized transduction
genetic engineering
homologous recombination
human gene therapy
lysogenic conversion
R (resistance) plasmids
rec (recombination) genes
recombination
restriction enzyme
reverse transcriptase
specialized transduction
temperate phages
Ti (tumor-inducing) plasmid
transduction
transformation
transgenic organisms
transposase
transposons

RECOMBINATION

Genetic recombination occurs when genes move from one location to another. Recombination can occur between DNA molecules in the same cell or between DNA molecules from different cells. In some cases, recombination involves the movement of genes to new locations within the DNA macromolecule. Such changes in the locations of genes can alter which regulatory genes control particular genes. In other cases, recombination involves exchanging and/or combining genetic information. In these cases, DNA from two different sources comes together in a new DNA macromolecule. Such recombinational events result in an exchange of different forms of genes that can produce new combinations of genes.

Recombination provides a mechanism for the redistribution of the changes that occur in DNA as a result of mutation. Like mutation, recombination is a source of *genetic diversity*. Recombination is more likely to produce beneficial genetic combinations than mutations because it is less likely to destroy the function of a gene. It also may produce combinations of genes that can provide the organism with a valuable new function. Recombination thus provides a mechanism for generating diversity within the gene pool of a microbial population.

Recombination can produce numerous new combinations of genes, greatly altering the inherited genetic information.

HOMOLOGOUS RECOMBINATION

Homologous recombination occurs when the DNA that is exchanged comes from corresponding genes. An exchange of allelic forms of genes is the result (FIG. 8-1). **Alleles** are alternative forms of genes concerned with the same trait or characteristic. Alleles occur as multiple forms of a gene located at the same *locus* (location) of *homologous chromosomes* (corresponding pairs of chromosomes). Recombination can produce new combinations of alleles.

Homologous recombination, an exchange of segments of DNA with similar nucleotide sequences, produces new combinations of alleles.

Pairs of chromosomes exchange corresponding portions of the DNA in a process called **crossing over** (FIG. 8-2). This is the process that occurs in meiosis when sex cells—such as sperm and egg cells—are formed for reproduction. When chromosomes cross over, a bridge is enzymatically formed between the two DNA strands. The chromosomes then rotate so that the two strands no longer cross each other. At this point they are still held together by covalent linkages. An endonuclease cleaves the DNA strands. A **restriction endonuclease**, or **restriction enzyme**, is an endonuclease enzyme that cleaves a DNA macromolecule at a specific site by breaking bonds within

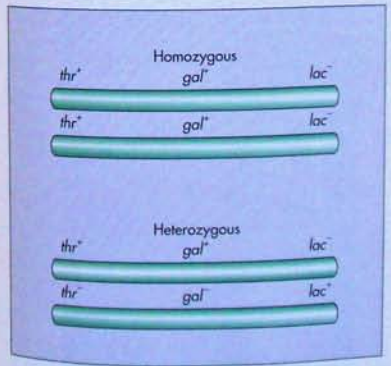


FIG. 8-1 Diploid cells have two sets of genes that may be identical (homozygous alleles) or different (heterozygous alleles).

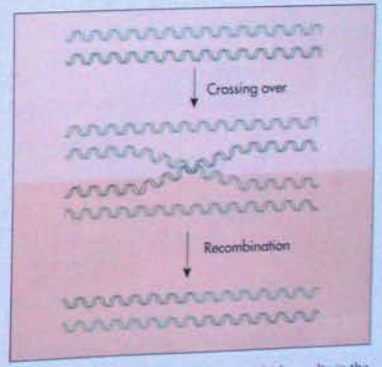


FIG. 8-2 Chromosomal crossing over, which results in the recombination of genes, is a classic example of homologous recombination.

the molecule. Two independent chromosomes are formed. This results in the formation of a new combination of genes, that is, a *recombinant DNA macromolecule*.

Portions of a bacterial chromosome can also be transferred from a donor to a recipient bacterium by homologous recombination. In bacteria, specific recombination enzymes and single-stranded regions of DNA are involved in such recombination. An endonuclease can nick one DNA strand, forming a free 3'-OH end within a DNA macromolecule. The free 3'-OH end acts as a primer for DNA synthesis so that one strand of the DNA is copied. The single strand of DNA that is synthesized pairs with the corresponding region of the homologous chromosome. This establishes a union called a **heteroduplex** (FIG. 8-3). The term ***heteroduplex*** indicates that the two strands of DNA are not fully complementary so that there is a mixture of single- and double-stranded regions of DNA. The heteroduplex forms because the two paired strands of DNA are not exactly complementary. The nucleotide sequences that are complementary form a duplex (a double-stranded complementary segment), while the noncomplementary regions remain unpaired and single stranded. The formation of the heteroduplex is catalyzed by enzymes coded for by ***rec*** **(recombination) genes.**

Homologous recombination is catalyzed by recombination (*rec*) gene products.

Nonhomologous Recombination

Nonhomologous recombination does not involve rec enzymes. It permits the joining of DNA molecules from different sources that have very little similarity of nucleotide sequences (little homology). Through this process, segments of DNA can move from one location to another, permitting the insertion of plasmids (extrachromosomal genetic elements) into the bacterial chromosome and the transfer of genes (such as antibiotic resistance genes) from one plasmid location to another (FIG. 8-4). Nonhomologous recombination also permits the incorporation of viral DNA into the DNA of a host bacterial or eukaryotic cell (FIG. 8-5). The process of incorporating viral DNA into host cell bacterial DNA is called **lysogenic conversion.** When this occurs, certain viral genes can be expressed by the bacterial host cells, resulting in bacterial production of proteins coded for by the viral genes.

Nonhomologous recombination can occur between dissimilar segments of DNA.

Insertion Sequences

There are several types of genetic elements that can move from site to site within a bacterial chromosome and undergo nonhomologous recombination. These are called **transposable genetic elements. Insertion sequences** (ISs) are small transposable genetic elements containing about 1,000 nucleotides (FIG. 8-6). The nucleotide bases in the IS regions do not appear

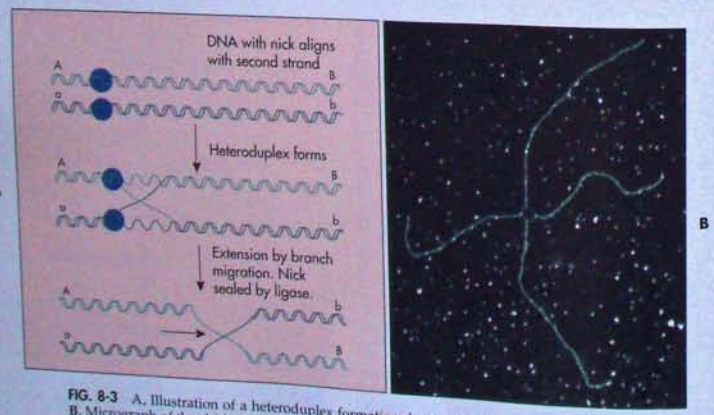


FIG. 8-3 A, Illustration of a heteroduplex formation during homologous recombination. B, Micrograph of the chi form of a heteroduplex during homologous recombination.

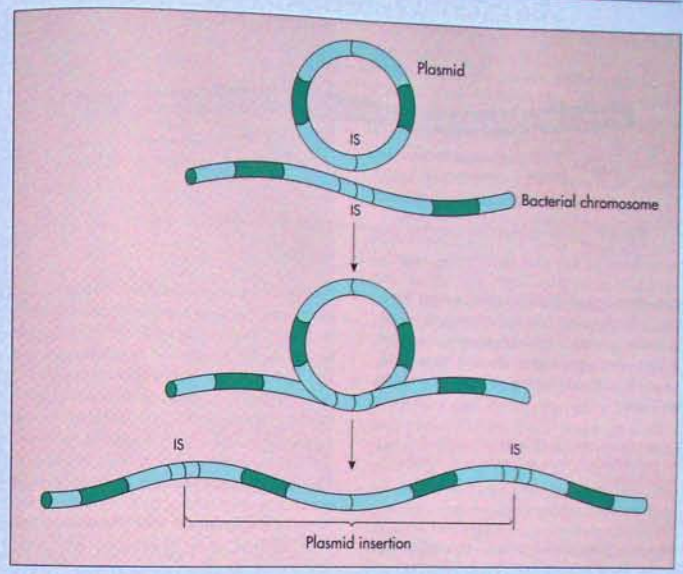


FIG. 8-4 Plasmids can insert into a bacterial chromosome by recombination.

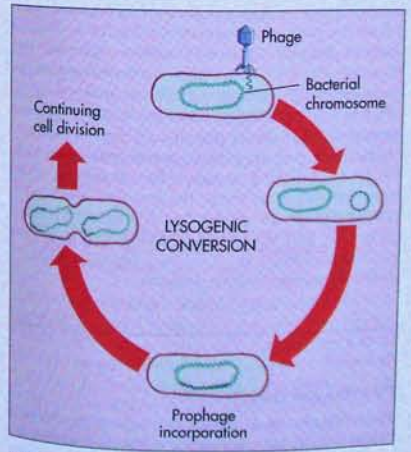


FIG. 8-5 Lysogenic conversion occurs when a temperate phage transfers bacterial DNA that it has acquired, and that DNA recombines with the DNA of the host cell. The bacterial cell then replicates the phage DNA along with the bacterial chromosome DNA.

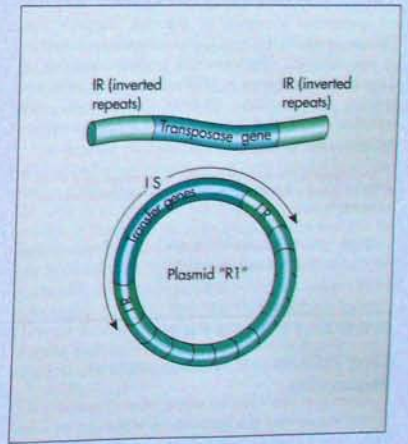


FIG. 8-6 An insertion sequence (IS) has inverted repeats that facilitate its nonreciprocal recombination; an IS may also code for a transposase.

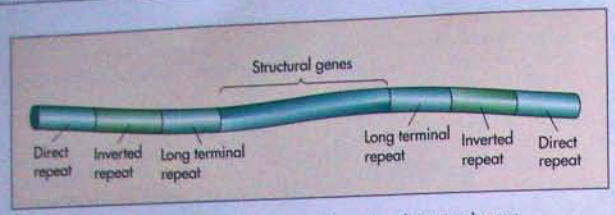


FIG. 8-7 A transposon has repeat sequences and a series of structural genes.

to code for proteins but may have a regulatory function. They may be involved in specifying the locations at which site-specific recombination occurs. ISs, for example, can alter a promoter site and thereby affect the expression of nearby genes. ISs can also insert into structural genes.

Insertion sequences are small transposable elements that can move from one location to another on the chromosome.

Transposons

Transposons are transposable genetic elements that contain genetic information for the production of structural proteins (FIG. 8-7). They are larger than IS elements. Transposons encode several genes, including an enzyme that brings about transposon insertion called a **transposase.** Transposase does not recognize any particular sequence on the host chromosome. The site at which it acts to insert a transposon is more or less random. Therefore the genetic locations of these elements change at random, jumping from one location to another. Barbara McClintock was awarded the Nobel prize for her work on transposons in corn plants.

Transposons contain insertion sequences and structural genes; they can move genes more or less at random.

More and more examples are being discovered of novel functions made possible by the ability of organisms to use transposition to move genes to new locations. Many transposons code for antibiotic resistance and their transposition can be important for determining the properties of pathogens and the drugs that can be used to treat microbial infections.

A medically important instance of gene mobilization by transposons is exhibited by African trypanosomes and *Plasmodium*. These protozoa cause African sleeping sickness and malaria, respectively. The surface glycoproteins of these protozoa are important in establishing their infective properties. The hosts of trypanosome and *Plasmodium* infections defend against these organisms by producing substances specifically directed against these surface glycoproteins. Trypanosomes and *Plasmodium* overcome this defense mechanism by transposition. The trypanosome DNA, for example, contains a cluster of several thousand different glycoprotein-encoding sequences. These sequences cannot be transcribed as they are because the cluster lacks a promoter. The promoter site is located within a transposon that periodically jumps randomly from one position to another within the cluster. Changes in the cluster sequence result in the appearance of new surface glycoproteins before the host's defense system is able to kill all the infecting protozoa. By the time new defense substances are made, the protozoa again transpose the genes for production of surface glycoproteins. Host defenses again are foiled. The result is a persistent infection in which the protozoa are able to defeat the human defense systems.

GENE TRANSFER

Gene Transfer and Recombination in Prokaryotes

There are several mechanisms by which genes naturally move from one bacterial cell to another. These methods of gene exchange differ in the way the DNA is transferred between a donor and a recipient cell.

Plasmids and Gene Transfer

Plasmids are small extrachromosomal genetic elements that permit microorganisms to store genetic information in addition to that contained in the bacterial chromosome (FIG. 8-8). Plasmids do not normally contain the genetic information for the essential metabolic activities of the microorganism. They generally contain several genes that code for specialized features, such as antibiotic resistance. Plasmid transfer allows the hereditary information encoded within the plasmid DNA to move from one bacterial population to another. The acquisition or loss of a plasmid alters the genotype of a bacterial strain. Individuals in a bacterial population that possess plasmids contain genetic information different from individuals in the population that lack plasmids.

FIG. 8-8 Colorized transmission electron micrograph of plasmid DNA *(green)* from two bacteria. Micrograph of plasmids pBF4 *(larger molecules)* from *Bacillus fragilis* and pSC101 *(smaller molecules)* from *Escherichia coli.*

Plasmids, which are extrachromosomal genetic elements that store additional genetic information, are capable of self-replication and can transfer genes from one bacterium to another.

Although plasmids contain only a very small portion of the bacterial genetic information, they are important. Plasmids can contain (1) genetic information that determines the ability of bacteria to mate and whether a bacterial strain acts as the donor during mating, (2) information that codes for resistance to antibiotics and other chemicals, such as heavy metals, that are normally toxic to bacteria, (3) genetic information for the degradation of various complex organic compounds, such as the aromatic hydrocarbons found in petroleum, and (4) genetic information for toxin production that renders some bacteria pathogenic to humans.

Bacterial cells can contain more than one plasmid. Certain pairs of plasmids cannot be stably replicated within the same bacterial cell. Incompatible plasmids that cannot exist in the same cell are said to belong to the same incompatibility group (*Inc* group). The genes that prevent co-existence are encoded within the incompatible plasmids themselves.

There are several types of plasmids that serve different functions (FIG. 8-9). The ability of plasmids to transfer genes from one cell to another was discovered in the late 1950s by Joshua Lederberg and Edward Tatum in studies on a particular plasmid of *Escherichia coli*, called the **F plasmid,** for fertility factor. Only cells containing the genes of the F plasmid act as plasmid donors. F plasmids are **conjugative plasmids,** meaning they contain several genes that promote their transfer to other cells. Other nonconjugative plasmids lack such genes and do not readily transfer from one cell to another. In particular, the F plasmids contain a site at which DNA polymerase binds and initiates DNA so that, once transferred, F plasmids can replicate within the recipient cell. The F plasmid can replicate in Gram-negative enteric bacteria—such as *E. coli*. Other conjugative plasmids are capable of moving into numerous other bacterial species.

F plasmids are conjugal transfer plasmids that permit transfer of genes from donor to recipient bacterial cells.

The **colicinogenic plasmids** of *E. coli* carry the genes for a protein, called a colicin. Colicin is toxic to only closely related bacteria. A strain of *E. coli* with a colicinogenic plasmid produces colicins that allow it to fight off competition by other *E. coli*.

Of greater significance are the **R (resistance) plasmids** that carry genes that code for antibiotic resistance. R plasmids can be passed not only from one strain to another but also from one bacterial species to another, such as from *Escherichia coli* to pathogenic strains of *Shigella* or *Salmonella*. Antibiotic-resistant strains of bacteria have become a serious health problem because R plasmids can occur in pathogenic bacteria and the treatment of human bacterial diseases is complicated by the occurrence of these pathogens that are resistant to multiple antibiotics.

R plasmids transfer genes for antibiotic resistance.

Transformation

In **transformation,** a free (naked) DNA molecule is transferred from a donor to a recipient bacterium, fol-

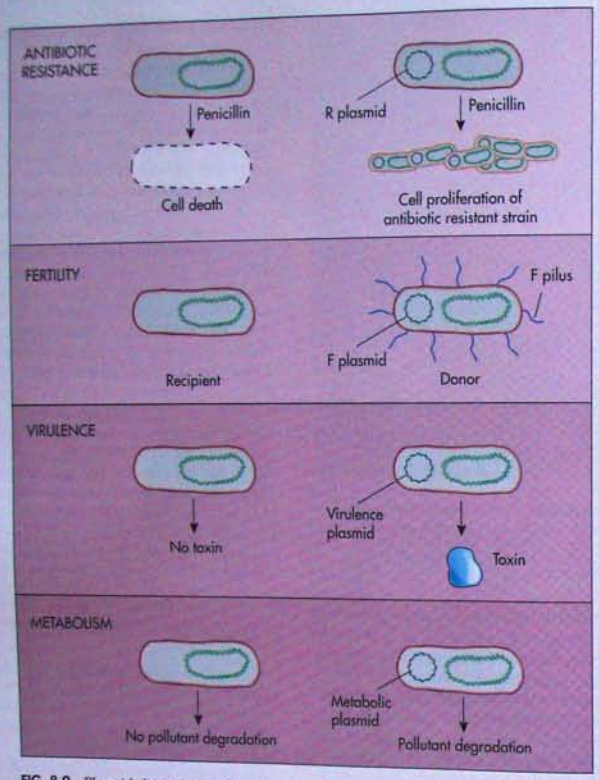


FIG. 8-9 Plasmids have different functions, including resistance to multiple antibiotics.

lowed by recombination within the recipient bacterial cell (FIG. 8-10). The donor bacterium leaks its DNA, generally as a result of lysis and death of the bacterium. The recipient bacterium takes up the leaked free DNA and incorporates it by recombination into its DNA. The classic example of transformation, involving the change of nonpathogenic *Streptococcus pneumoniae* to pathogenic *S. pneumoniae*, was critical in the discovery that DNA is the hereditary macromolecule of cells (see Chapter 7). This discovery of transformation also showed the importance of transformation, since nonpathogenic *S. pneumoniae* were transformed into deadly pathogens.

Transformation involves the transfer of naked DNA, followed by recombination.

For free DNA to be picked up by a recipient cell, the recipient cell must be **competent.** Competency means that the cell must be capable of transporting the donor DNA across its plasma membrane or have specific receptor sites on its surface. Competence probably depends on properties of the cell wall that permit the binding of DNA and its transport into the recipient cell. The recipient cell also must be **compatible,** meaning that once inside the donor, the DNA must not be enzymatically destroyed by the endonucleases of the recipient cell. The highest frequencies of transformation occur when donor and recipient are closely related. If recombination occurs, the progeny are called *recombinants* and are said to be *transformed*. Such recombinants typically have properties different from the original recipient cell.

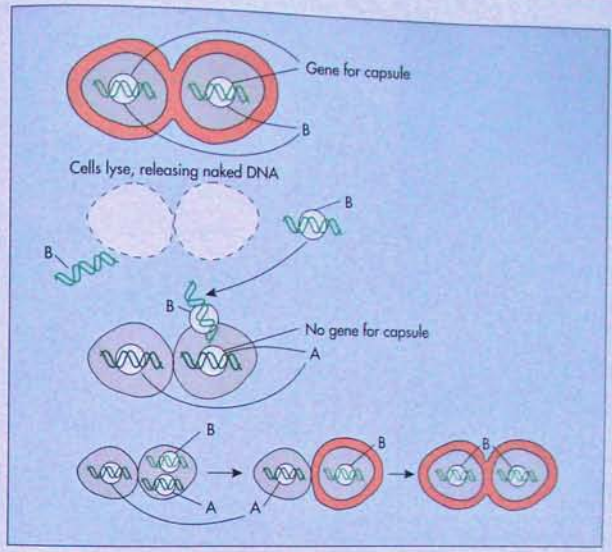


FIG. 8-10 The transformation of *Streptococcus pneumoniae* is the classic example of change in the properties of a bacterial strain due to the transfer of naked DNA. The gene for capsule production (designated B) is released from cells that lyse and taken up by nonencapsulated bacterial cells that have an inactive gene (designated A). Recombination results in the replacement of the inactive A form of the gene with the active B form so that the recombinant cells acquire the ability to produce capsules and, in this case, induces virulence.

Only a few genera of bacteria exhibit natural transformation. These include *Acinetobacter, Bacillus, Haemophilus, Neisseria, Rhizobium, Staphylococcus,* and *Streptococcus*. Some of the genes that are transferred among strains of these bacteria effect pathogenicity and the ability to treat diseases they cause. As examples, genes for capsule production, which make strains more resistant to host defenses and hence increase pathogenicity, and genes for antibiotic resistance, which make infections difficult to treat, are transferred by transformation.

Transduction

In **transduction,** DNA is transferred from a donor to a recipient cell by a viral carrier, followed by recombination within the recipient bacterial cell. Recombination within the recipient cell follows (FIG. 8-11). Viruses normally replicate within a host cell. During this process, the host cell DNA is enzymatically cleaved into pieces, and occasionally some DNA fragments can be accidentally packaged within viral protein coats. Thus a virus sometimes acquires a portion of the DNA of the host cell in which it replicates. When the virus leaves the host cell it carries a segment of the host cell DNA that is protected within the protein coat of the virus. When the virus infects a new host cell, the DNA that was acquired from the earlier host cell is carried along and can recombine with the DNA of the new host cell.

Transduction involves recombination of bacterial DNA after transfer by a virus.

Phages are viruses that replicate within bacterial cells. Phages that acquire bacterial DNA during replication within a host cell retain their ability to infect new host cells. However, they often lose their ability to complete phage replication because some bacterial genes have replaced essential phage genes. These phages are called **defective phages** because they have lost the ability to kill the host cell. The bacterial DNA they acquire replaces some phage genes that are essential for completion of phage replication. Defective phages can carry DNA from a donor bacterial cell and inject it into a recipient bacterial cell. They

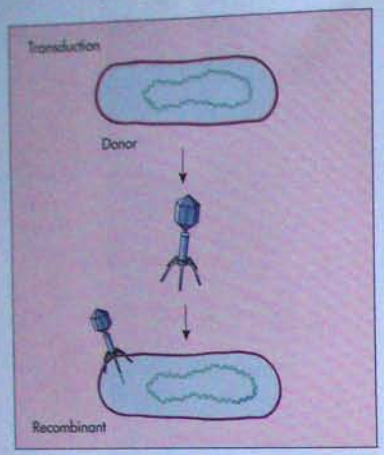


FIG. 8-11 Transduction transfers bacterial DNA via a phage. Bacterial DNA is packaged together with phage DNA during phage replication within a bacterial host cell. This occurs when fragments of bacterial DNA are joined with phage DNA in the phage capsid during phage replication. The phage carries that bacterial DNA along with phage genes to another host cell, where recombination (transduction) occurs.

can randomly carry any host cell genes. If the genes that are transferred by these phages are homologous to ones in the newly infected cell, homologous recombination can occur. This process, called **generalized transduction,** can result in the exchange of any homologous alleles. If recombination occurs, the recombinants are said to be *transduced*. Bacterial genes can be transferred to newly infected cells at low frequency by generalized transduction (FIG. 8-12).

Generalized transduction results in transfer of alleles by homologous recombination.

Other phage are called **temperate phages** because they don't always cause the lysis of the host cell. Instead, the DNA of a temperate phage may become integrated into the bacterial chromosome at specific sites. Such sites are where phage DNA can undergo nonhomologous recombination with host bacterial cell DNA. Sometimes the phage DNA comes out of integration and carries with it bacterial genes that flanked the integration site so that they acquire and transfer only certain host cell genes that are located near a specific site (FIG. 8-13). Because only a limited number of specific genes can be transferred by these phages, the process is called **specialized transduction.**

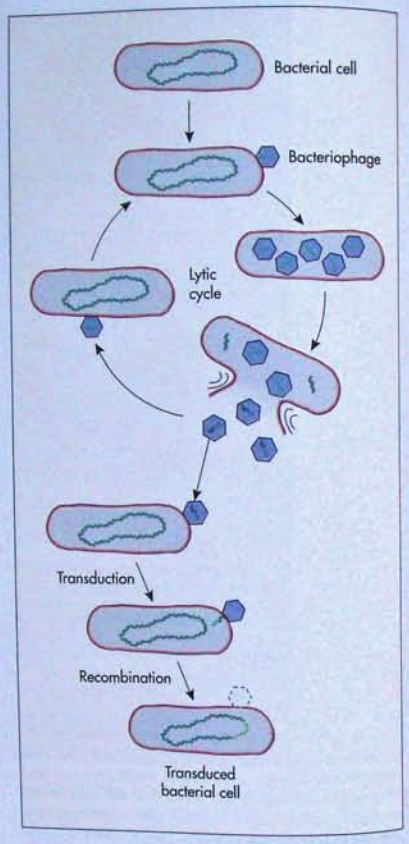


FIG. 8-12 In generalized transduction, various genes are transferred from a donor to a recipient cell via a bacteriophage. Homologous recombination occurs after the exchange.

Specialized transduction results in the transfer of specific genes by nonhomologous recombination.

Conjugation

Conjugation, or **mating,** involves the transfer of DNA from a donor to a recipient by cell-cell contact between the donor and recipient bacterial cells, followed by recombination within the recipient bacterial cell. Preventing physical contact precludes transfer of DNA by conjugation. The necessary physical contact between mating bacteria is established

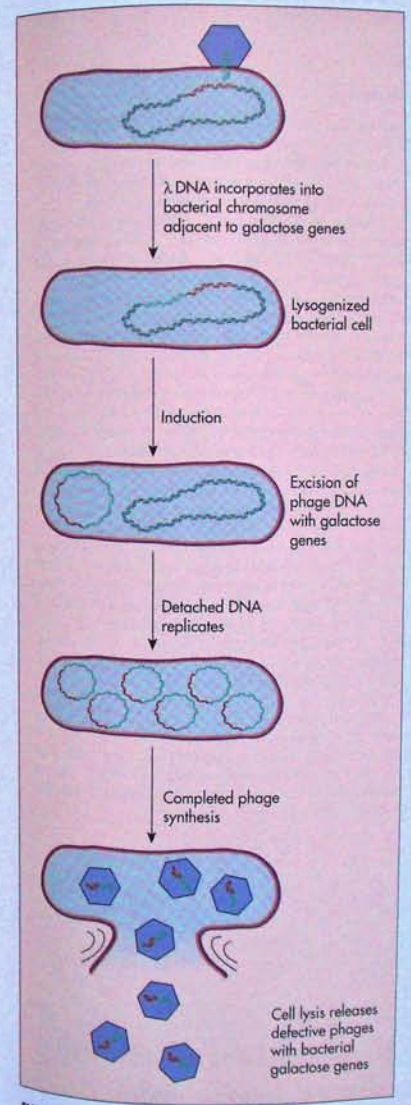


FIG. 8-13 Specialized transduction by lambda (λ) phage results in the transfer of a limited number of specific genes by nonhomologous recombination.

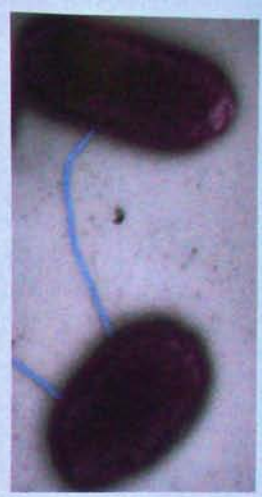


FIG. 8-14 Micrograph of mating cells of *Escherichia coli*. The cells are joined by the F pilus *(blue)* of the donor strain.

through the **F (fertility) pilus** (FIG. 8-14). As discussed in Chapter 5, pili are filamentous appendages of Gram-negative bacteria that project from the cell's surface and are involved in attachment processes. F pili specifically join mating bacteria. When an F pilus establishes a bridge between two bacterial cells, there is a change in plasma membrane permeability so that DNA can move from one cell to another.

In conjugation, physical contact between bacterial cells established by an F pilus is needed for DNA transfer from donor to recipient cell.

Bacterial strains that produce F pili act as donors during conjugation. Donor strains are designated **F⁺ strains** if the F plasmid, which codes for F pilus production, is independent, that is, free in the cytoplasm. Strains are **Hfr (high frequency recombinant)** if the F plasmid DNA is incorporated into the bacterial chromosome. Strains lacking F pili are recipient strains and are designated **F⁻ strains.**

During bacterial mating, a single strand of donor DNA is replicated. The single-stranded copy of the DNA is transferred to the recipient where the complementary strand is synthesized. The precise portion of the DNA that is transferred depends on the time of mating, that is, how long the F pilus maintains contact between the mating cells.

METHODOLOGY

Genetic Mapping

Mating of different strains of bacteria that have different allelic forms of multiple genes can be used for genetic mapping, that is, for determining the relative positions of those genes in the bacterial chromosome. The occurrence of recombinants that result from mating often is used to map the order and, thus, to determine the relative locations (loci) of genes.

In the case of *E. coli*, which has been well studied, mating between Hfr and F⁻ strains has been used to map large sections of the bacterial chromosome. By vibrating a culture of mating bacteria, the cell-to-cell contact is broken and further transfer of DNA ceases. Such interruption of mating can be done at various times after conjugational cell-cell contact begins. The order of genes on the bacterial chromosome can be determined by examining the times at which recombinants for given genes are found.

In mating experiments aimed at mapping the order of genes, the recovery of recombinants of marker genes is normally used as a reference point for establishing the fine structure of the genome. If a gene of unknown location shows a high frequency of recombination along with the marker gene, it is likely that the marker and unknown genes are closely associated in the chromosome. If, however, the genes are far apart, it is unlikely that recombinants of both the marker gene and the gene of unknown location will occur in the progeny.

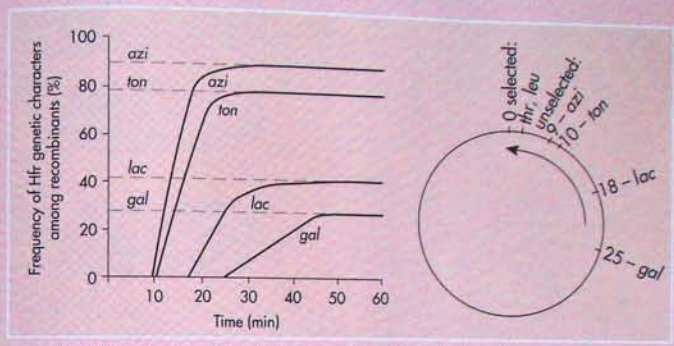
Let us consider an example of how the locations of genes are determined by the transfer times for recombination as determined by interrupted mating (FIG. *A*). Suppose the genetic markers are threonine biosynthesis *(thr)*, leucine biosynthesis *(leu)*, azide sensitivity *(azi)*, phage T1 sensitivity *(ton)*, lactose utilization *(lac)*, galactose utilization *(gal)*, and streptomycin sensitivity *(str)*. In this example an Hfr that is $thr^+, leu^+, azi^s, ton^r, lac^+, gal^+, str^r$ is mated with an F⁻ strain that is $thr^-, leu^-, azi^r, ton^s, lac^-, gal^-, str^s$. The superscript $^+$ indicates that the organism has the genes for biosynthesis or utilization, whereas the superscript $^-$ indicates that the organism lacks functional copies of these genes; the superscript ^{r} indicates resistance, whereas the superscript ^{s} indicates sensitivity.

Mating of the Hfr and F⁻ strains is initiated by mixing the two cultures at time t=0. After mating for 10, 15, 20, 25, 30, 40, 50, and 60 minutes, a portion of the mixed culture is removed and agitated in a blender to interrupt mating and the cells are then plated on a medium containing glucose and streptomycin. On this medium, recombinants that are $thr^+ leu^+ str^s$ will grow because they can synthesize threonine and leucine and are resistant to streptomycin. Recombinants that are $thr^+ leu^+ str^r$ are selected in this manner. The original strains do not have this combination of genes and will not grow. Azide sensitivity *(azi)*, phage T1 sensitivity *(ton)*, lactose utilization *(lac)*, and galactose utilization *(gal)* are unselected markers because this medium does not specifically detect the different alleles of these genes. The $thr^+ leu^+ str^r$ recombinants that form colonies are scored for the alleles of the unselected markers that are present in the selected recombinants by replica plating on media that individually select for *azi*, *ton*, *lac*, and *gal*. The frequencies of unselected markers among $thr^+ leu^+ str^r$ selected recombinants are plotted as a function of time until mating is physically interrupted (FIG. *B*). Extrapolation of the frequency of each unselected marker to zero indicates the earliest time at which markers become available for recombination with the chromosome of the F⁻ cell. These times permit the ordering of genes, that is, construction of a genetic map, with the assignment of distances between genes based on the time (in minutes) elapsed from the initiation of conjugation until the earliest time at which a marker from the Hfr strain is detected as a recombinant with the F⁻ strain (FIG. C).

A

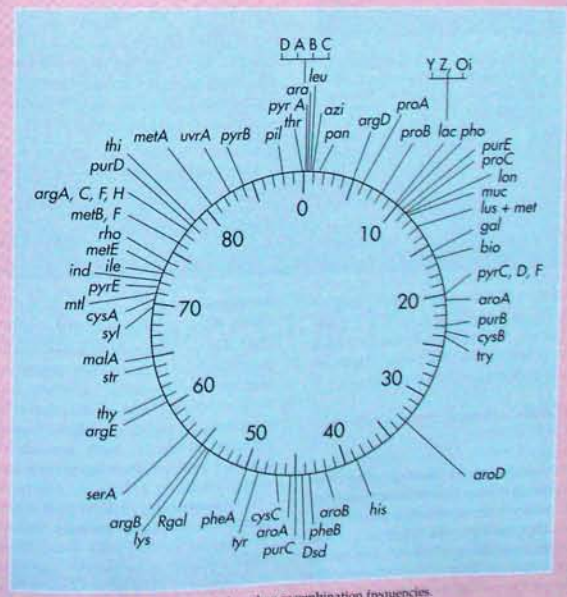
The relative positions of the genes can be established by mating bacteria for varying times and interrupting the mating to halt further gene transfer. The number of recombinants increase with time. Each experiment is assessed by comparison of the number of colonies on a complete medium and a medium restricted to growth of an auxotrophic mutant.

B



The transfer times of genes and the frequency of recombination indicates the relative positions of genes. Graphs like this are used for gene mapping.

C



A genetic map is based on recombination frequencies.

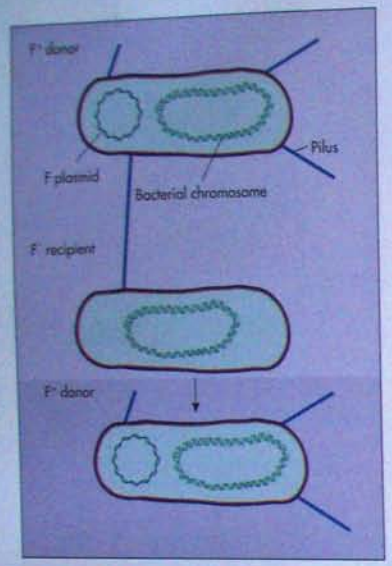


FIG. 8-15 Donor bacteria that have the fertility gene (F gene) produce F pili, which establishes contact for DNA transfer. In F⁺ strains the F gene is on a plasmid. The mating of a donor F⁺ strain with a recipient F⁻ strain *(top)* results in transfer of the F plasmid and the production of F⁺ progeny; there is relatively little recombination.

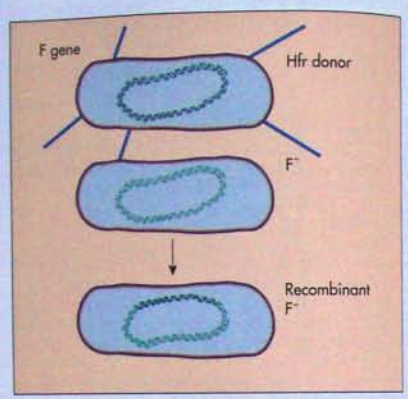


FIG. 8-16 The F gene can be incorporated into the bacterial chromosome to produce donor strains, designated Hfr (high frequency recombination strains). The mating of an Hfr strain with an F⁻ strain *(bottom)* results in transfer of many genes from the bacterial chromosome and a high frequency of recombination; the F gene is not transferred, so that the progeny are F⁻ recipient strains.

When an F⁺ cell is mated with an F⁻ cell, the F plasmid DNA is copied and usually transferred from the donor to the recipient (FIG. 8-15). The F plasmid also stays with the donor. Therefore the offspring of such a mating are mostly donor strains. The F plasmid confers the genetic information for acting as a donor strain. Few if any genes other than those on the F plasmid are transferred in such matings.

Mating of an F⁺ strain with an F⁻ strain results in the production of F⁺ strains.

When an Hfr strain is mated with an F⁻ strain, the genes of the bacterial chromosome are transferred to the recipient cell before those for F pilus production. The F plasmid is often not near the beginning of the DNA that is transferred (FIG. 8-16). Since only rarely is there sufficient mating time to accomplish the complete transfer of the complete bacterial chromosome, the recipient cell normally remains F⁻. However, a relatively large portion of the bacterial chromosome is transferred from the donor to the recipient. Thus there is a relatively high frequency of recombination of genes of the bacterial chromosome when Hfr strains are mated with F⁻ strains.

Mating of an Hfr strain with an F⁻ strain results in the production of F⁻ strains and a high frequency of recombination.

Genetic Exchange in Eukaryotes

In eukaryotic microorganisms, genetic exchange normally occurs during the sexual reproduction phase of the life cycle (FIG. 8-17). Sexual reproduction involves the recombination of DNA from two parents. The DNA comes together when specialized sexual reproductive cells unite. The *vegetative cells* (growing nonreproductive cells) of many eukaryotic organisms are *diploid*, that is, they have two sets of chromosomes. To exchange genetic information these organisms normally form specialized reproduction haploid gametes that have only one copy of each chromosome.

The conversion of a diploid to a haploid state occurs in the process of **meiosis.** Meiosis is also known as *reduction division* (FIG. 8-18). Meiosis begins after DNA replication, so that the starting cell actually has four copies of each gene. During this tetraploid state the chromosomes are aligned side by side. Recombination can occur by crossing over between homologous chromosomes. The chromosomes are pulled apart by spindle fibers. A second meiotic division then occurs without further DNA replication, forming four nuclei. Each nucleus contains a haploid number of chromosomes.

Homologous recombination occurs during meiosis, the process that results in the reduction of the number of chromosomes and the conversion of a diploid cell into a haploid cell.

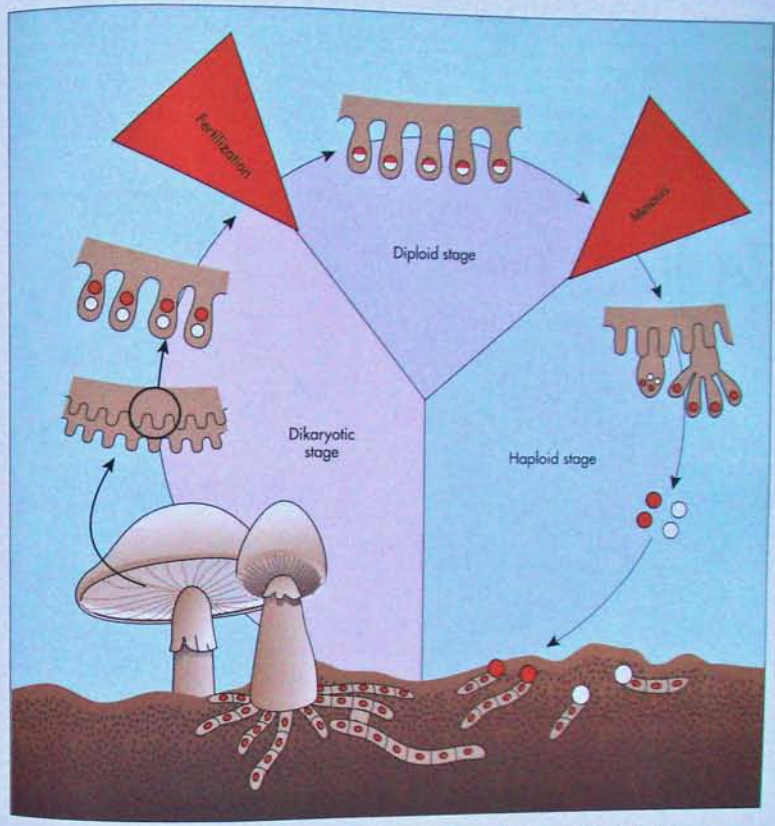


FIG. 8-17 In sexual reproduction, gametes from two parental cells join to form a progeny zygote. The sexual basidiospores *(brown* and *white)* germinate to form mycelia that are dikaryotic. Subsequent fusion of mycelia in basidiomycetes gives rise to the diploid stage.

The haploid nuclei of these reproductive cells can later fuse with the nuclei of reproductive cells. The union of the haploid nuclei during the fusion of reproductive cells **(syngamy)** reestablishes the diploid state (FIG. 8-19). One of the haploid sets of chromosomes comes from a **donor strain** (male) and the other haploid set comes from a **recipient strain** (female). The process of gene exchange establishes a life cycle with haploid, dikaryotic, and diploid stages.

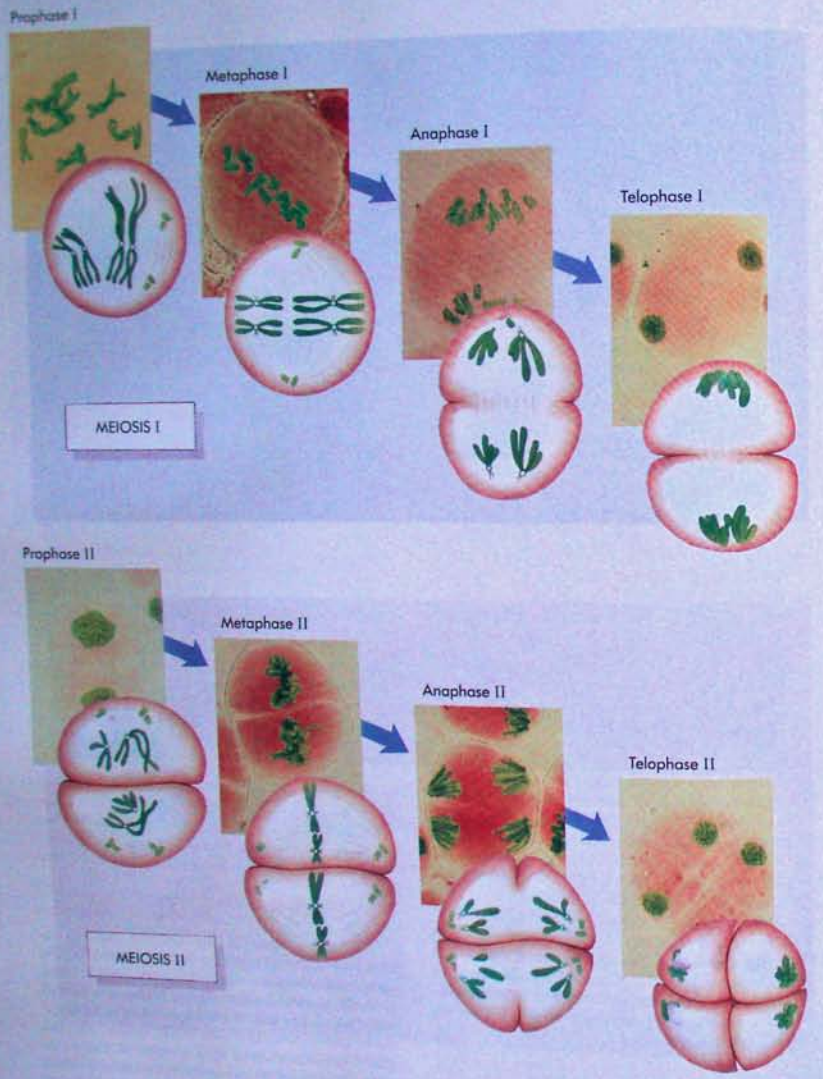


FIG. 8-18 During meiosis, a diploid parent cell (cell with pairs of chromosomes) undergoes reductive division to produce four haploid daughter cells (cells with individual sets of chromosomes). In the first stage of meiosis (prophase), chromosome cross-over occurs between the pairs of homologous chromosomes. Then during metaphase, microtubular spindles form and align the pairs of chromosomes. Next, during anaphase, the chromosomes are pulled apart to opposite poles so that one set of each of the chromosomes is located at each pole at telophase. Cytokinesis occurs so that two cells are formed. These phases are repeated, resulting in the formation of four haploid cells in the second stage of meiosis.

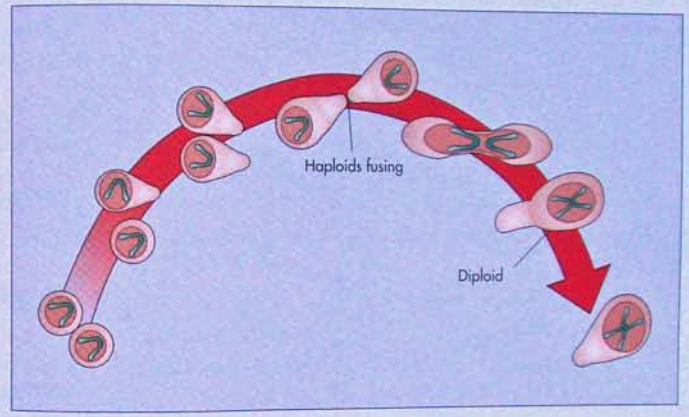


FIG. 8-19 In syngamy haploid gametes join to restore a diploid state.

RECOMBINANT DNA TECHNOLOGY

Formation of Recombinant DNA

Based on an understanding of the natural recombination processes, scientists have learned how to recombine DNA from different sources *in vitro*, that is, in the laboratory outside of living cells. This is the foundation of recombinant DNA technology, the deliberate union of specific genes by scientists to make recombinant DNA macromolecules. Recombinant DNA technology promises to be a powerful tool for understanding basic genetic processes and has tremendous potential industrial applications. DNA macromolecules formed in this manner have a combination of genes from a donor source and from a recipient. The source DNA can be human, plant, bacterial, viral, or even chemically synthesized DNA. The recipient can be the DNA of any organism. Organisms can thus be formed that contain genes from more than one species. Such organisms are called **transgenic organisms.**

Recombinant DNA technology is the purposeful union of DNA from different sources in the laboratory outside of living cells.

The enzymes involved in the normal recombination and replication of DNA are frequently used to join DNA from different sources. Restriction endonucleases are used to cut DNA into specific fragments. Some endonucleases cut the DNA at a **palindromic sequence.** A palindrome is a symmetrical sequence of nucleotide bases that can be read identically in one direction on one strand and in the other direction on the opposite strand. For example, [illegible] is a palindromic sequence. When an endonuclease cuts a

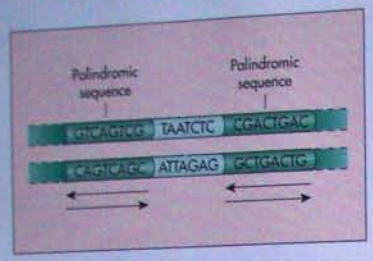


FIG. 8-20 A palindromic sequence has the same genetic information regardless of the direction of reading.

palindromic sequence, DNA with staggered single-stranded ends is thus produced (FIG. 8-20). These ends of the cut DNA can act as cohesive or sticky ends during recombination. This makes them amenable for splicing with segments of DNA from a different source that has been excised by using the same endonuclease. Ligases are used to splice the pieces of DNA. For example, a fragment of DNA

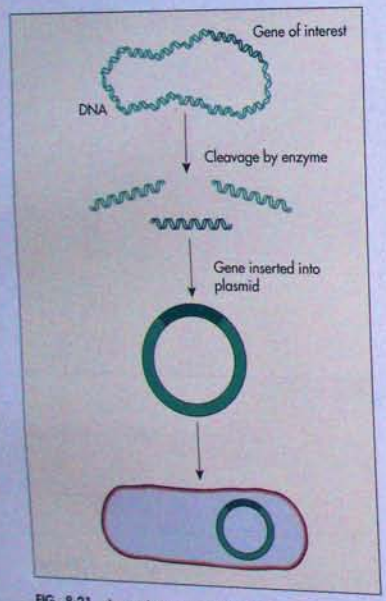


FIG. 8-21 An endonuclease cuts DNA at specific sites within the double helix. There are many different endonucleases that recognize different nucleotide sequences. Ligases join fragments of DNA. Using endonucleases and ligases, specific fragments of DNA can be cut from a chromosome or plasmid and joined together (ligated) to form a recombinant DNA molecule. This is the key to genetic engineering.

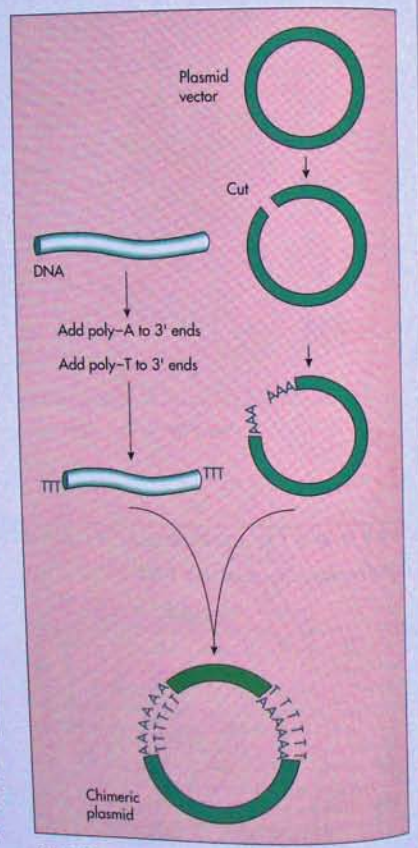


FIG. 8-22 Poly-A and poly-T tails can be added to fragments of DNA to establish homology for recombination.

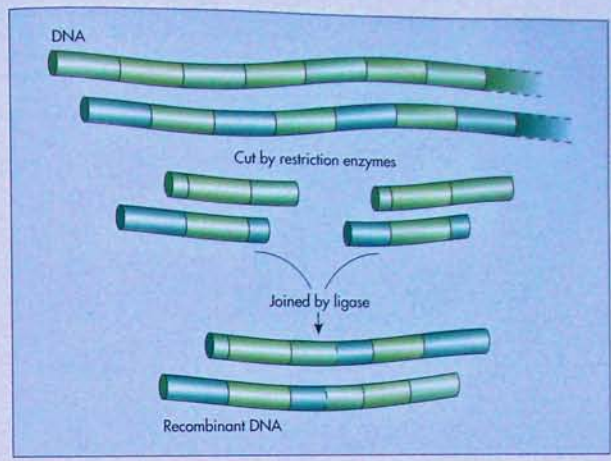


FIG. 8-23 Having homologous ends allows DNA fragments to join. A ligase can join DNA when the ends are homologous.

produced by using endonucleases can be added to a plasmid carrier (FIG. 8-21).

If two different endonucleases are used, one to cut open the recipient DNA and another one to excise a segment of donor DNA, it often is necessary to establish an artificial homology at the terminal ends of the donor and recipient DNA macromolecules (FIG. 8-22). This can be accomplished by using an enzyme that adds adenine to the plasmid DNA to create a polyA (poly-adenine) tail and a different enzyme that adds thymidine to the donor DNA to form a polyT (poly-thymine) tail. Pairing occurs between homologous regions of complementary bases. Ligase enzymes are used to seal the circular plasmid. The tails left by the action of the endonuclease are cleaved *in vitro*, using exonuclease enzymes. Virtually any source of DNA can be used as a donor, including human DNA. By adding a polyT tail to the donor DNA after its excision with an endonuclease, the donor DNA can be made complementary to the polyA tails of the recipient DNA, permitting the formation of a recombinant DNA macromolecule.

If the same endonuclease is used to cut both the donor and recipient plasmid DNA, the strands will have homologous ends. Then it is not necessary to add polyA and polyT tails. The ends of the DNA molecules are sealed by ligases, thus creating a recombinant DNA macromolecule that contains a foreign segment of DNA (FIG. 8-23).

Short nucleotide sequences can also be synthesized chemically. This artificial synthesis of a short DNA segment can be accomplished by using an automated DNA synthesizer. The DNA that is produced in this manner can be enzymatically joined to DNA from other sources to create recombinant DNA.

The problem of the discontinuity of the split genes of eukaryotic cells can be overcome by using an mRNA molecule and a **reverse transcriptase.** Reverse transcriptase is an enzyme that uses an RNA template to synthesize a DNA macromolecule. This will produce a DNA macromolecule with a contiguous sequence of nucleotide bases containing the complete functional gene (FIG. 8-24). The single-stranded DNA molecule formed in this procedure is complementary to the complete mRNA molecule and hence is called **complementary DNA** (cDNA). The RNA can be removed by using a nuclease. A complementary strand of DNA can then be synthesized opposite the cDNA strand. The double-stranded DNA derived from a eukaryotic cell can be joined with DNA obtained from a bacterial cell. In this way recombinant DNA can be made that contains eukaryotic and prokaryotic genes.

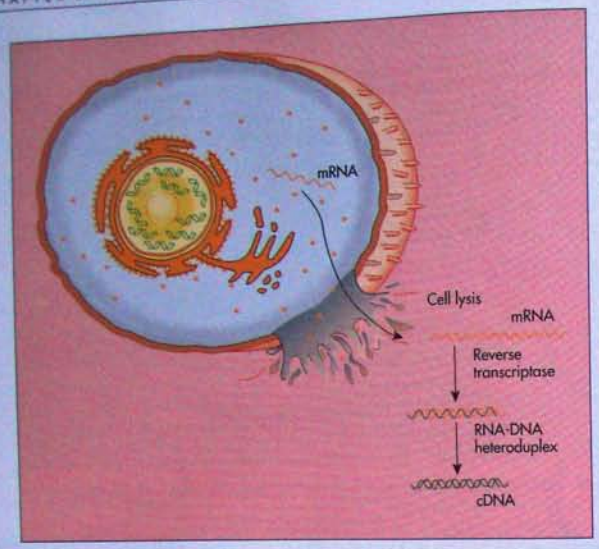


FIG. 8-24 cDNA can be produced from an RNA template using reverse transcriptase. This is useful for genetic engineering of eukaryotic genes because it eliminates introns (intervening sequences) that are present in eukaryotic genes. The cDNA can then be cloned and expressed in prokaryotic cells.

Cloning Recombinant DNA

Once a recombinant DNA molecule is formed it can be transferred to living cells where it can be replicated. When the cells divide, the recombinant DNA is passed to the progeny cells. Genes contained in recombinant DNA can be **cloned** (asexually reproduced to form copies that are identical with the original) in this manner. Various methods are used to add recombinant DNA to cells for cloning. Transformation and transduction are used to facilitate the transfer of recombinant DNA into living cells for cloning. Although most bacterial cells, including those of *E. coli*, are not naturally competent for transformation, scientists can treat cells in the laboratory with agents such as detergents so that they will take up DNA. Thus *E. coli* and most other bacteria can be transformed with recombinant DNA.

Recombinant DNA can be transferred to living cells where it can be replicated and passed onto progeny cells.

Plasmids often are used as vectors (carriers) for the cloning of recombinant DNA. Plasmids can be isolated, and the plasmid DNA can be cut open by using a site specific endonuclease, commonly called a **restriction enzyme.** This creates sites where foreign donor DNA can be inserted. Once the plasmid containing the desired DNA segments is formed, it can be added to a culture of a suitable recipient bacterium that will incorporate the plasmid. Because plasmids will transfer DNA into cells, recombinant plasmids are extremely useful for cloning recombinant DNA. Plasmid DNA, including the foreign DNA segments, can be replicated and passed from one generation to another. Bacteria containing recombinant plasmids act as factories to produce multiple copies of identical cloned genes.

Recombinant plasmids are used for cloning recombinant DNA. Bacteria containing recombinant plasmids produce multiple copies of identical cloned genes.

Plasmid pBR322 was created specifically for use in cloning DNA. It is frequently used for cloning because it has unique sites where restriction enzymes will cut so that new sequences of DNA can be introduced and because it can replicate in *E. coli* (FIG. 8-25). Such a plasmid that is used for the replication of recombinant genes in a host cell (in this case in *E. coli*) is called a **cloning vector.** The use of plasmid pBR322 as a cloning vector is advantageous because it also contains genes for ampicillin and tetracycline resistance and has multiple specific restriction enzyme sites. The presence of antibiotic resistance markers permits its detection using selective media containing these antibiotics. Plasmid pBR322 contains single restriction sites for several endonucleases, including sites within the genes coding for antibiotic resistance. Fragments of foreign DNA can, therefore, be inserted into specific sites. If the insertion occurs at the antibiotic resistance site, resistance is lost. The nucleotide sequence of the antibiotic resistance gene is disrupted. This *insertional inactivation* is useful for detecting the presence of foreign DNA within a plasmid.

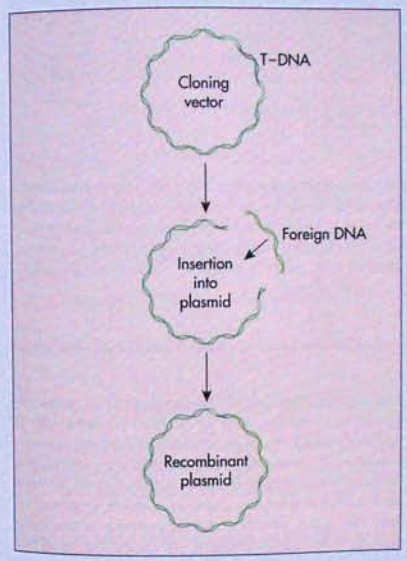


FIG. 8-25 Plasmid pBR322 is often used as a cloning vector because it has a number of unique sites for different endonucleases. This permits the insertion of foreign DNA at specific sites.

Insertional inactivation can detect the presence of foreign DNA in a plasmid.

Protoplast fusion can also be used to transfer DNA from one cell to another so that recombination can occur (FIG. 8-26). Protoplasts are cells that have had their walls removed by enzymatic and/or detergent treatment. They are protected against lysis due to osmotic shock by suspension in a buffer containing a high concentration of a solute, such as sucrose. Protoplast fusion is a particularly useful technique for achieving gene transfer and genetic recombination in organisms with no efficient natural gene transfer mechanism. Interestingly, more than two strains can be combined in one fusion. Recombinants that have inherited genes from all parents in the fusion are generated. The basic procedure involves polyethylene glycol-induced fusion of protoplasts followed by the regeneration of normal cells. An important feature of bacterial protoplast fusion is that it enables establishment of a transient quasi-diploid state during fusion. This permits recombination between complete bacterial chromosomes, as opposed to fragments of the donor bacterial chromosome and the recipient bacterial chromosome.

DNA can be transferred by protoplast fusion.

Regardless of the mechanism of gene transfer, for the information encoded in the cloned DNA sequence to be expressed, it must be transcribed and

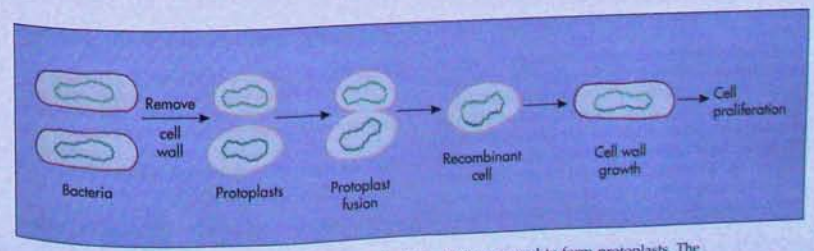


FIG. 8-26 Cell walls of Gram-positive bacteria can be removed to form protoplasts. The fusion of protoplasts permits gene transfer and recombination.

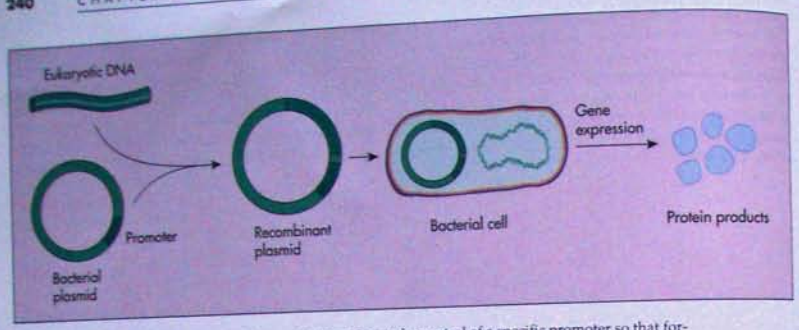


FIG. 8-27 An expression vector has genes under control of a specific promoter so that foreign genes added through recombination can be expressed.

translated to form an active protein molecule. The expression of the foreign genetic information requires that the appropriate reading frame be established and that the transcriptional and translational control mechanisms be turned on to permit the expression of the DNA. In particular, the cloned genes must be under the control of a promoter that permits transcription (FIG. 8-27). With the knowledge of what has to be accomplished to achieve cloning and gene expression, numerous recombinant organisms have been created and used for scientific studies and for practical applications.

GENETIC ENGINEERING

Because the underlying genetic code—specified by the sequences of nucleotides in DNA—is almost always the same in the cells of all organisms, scientists can create cells with entirely new properties using recombinant DNA technology. The use of recombinant DNA technology for this purpose is called **genetic engineering.** Although recombinant DNA technology theoretically can be used to genetically engineer the evolution of entirely new organisms, it is used to add only one or a few genes to an organism.

The use of recombinant DNA technology permits the purposeful manipulation of DNA to create organisms with new or different characteristics through genetic engineering.

Through genetic engineering, scientists can artificially direct recombinant processes for beneficial purposes. Until the development of recombinant DNA technology, bacteria could produce only substances from their own bacterial genes. Now, however, bacteria that have had plant and animal genes added to their DNA by genetic engineers can produce plant and animal proteins such as insulin and human growth hormone (Table 8-1). New vaccines for preventing human diseases have been developed using recombinant DNA technology. Genetically engineered yeast cells produce a vaccine used to prevent hepatitis B infection, which is especially important to health care workers who are at high risk of contracting this disease. Such genetically engineered bacteria are revolutionizing the economics of the pharmaceutical industry and providing new ways of treating and preventing human diseases. Genetic engineering also has great potential for improving agricultural productivity. Gene therapy, the insertion of genes into human chromosomes, may be useful in treating some human diseases. Experiments are underway at the National Institutes of Health with human cells that have added genes that code for a factor that causes shrinkage of tumors. The aim of these experiments is to determine whether genetically engineered human cells can be used to treat cancer.

Genetic engineering has opened up many new possibilities for employing microorganisms to produce substances of economic importance and for genetically modifying plants and animals.

RECOMBINANT PLANTS WITH BACTERIAL GENES

Recombinant Nitrogen Fixers

One of the greatest benefits that may be realized through genetic engineering is the introduction of the capacity to fix nitrogen into plants, such as wheat and rice, that are not able to utilize atmospheric nitrogen. For years scientists have been exploring the relationships between *Rhizobium* and the plants with which this nitrogen-fixing bacterium can establish symbiotic (mutually beneficial) relationships. Microbiologists are studying the genetics and biochemistry of infection by *Rhizobium* with the aim of employing recombinant DNA techniques to genetically engineer plants containing the bacterial genes for nitrogen fixation (*nif* genes).

In one series of studies, the genes for nitrogen fixation were inserted into the genome of a eukaryotic cell, a yeast (FIG. 8-28). Plasmids from *E. coli* and a yeast were cleaved and then fused to form a single hybrid plasmid. This plasmid could be recognized by the yeast cell and integrated into its chromosomal DNA. The genes to be introduced into the yeast were then isolated from the chromosome of *Klebsiella pneumoniae*, a nitrogen fixer. The *nif* genes code for some 17 proteins. Another *E. coli* plasmid was cleaved and the isolated *nif* genes were introduced to form a second hybrid plasmid. Because bacterial DNA had previously been inserted into one of the yeast chromosomes, the yeast cell recognized the hybrid *E. coli* plasmid. The second plasmid was then integrated into the yeast chromosome. Although the insertion of the prokaryotic *nif* genes into the eukaryotic yeast cell demonstrates that genetic material can be transferred between different biological systems, unfortunately the nitrogen-fixing genes were not expressed in the yeast. More studies are needed to elucidate the factors controlling expression of the *nif* genes before success is obtained.

It is increasingly apparent that the ability to engineer organisms depends on developing a thorough understanding of the molecular biology of gene expression. Once we understand the mechanisms of gene regulation in eukaryotes, we will be able to apply this knowledge through genetic engineering to create crop plants that are able to fix their own nitrogen. This will greatly enhance the ability to produce the world's food supply without the environmental problems caused by the use of chemical fertilizers.

TABLE 8-1
Some Human Proteins Produced by Recombinant Microorganisms

PROTEIN	PRODUCT NAME	FUNCTION AND USE
Insulin	Humulin, Novolin	Hormone that regulates sugar levels in blood; used in treatment of diabetes
Human growth hormone	Protropin, Humatrope	Hormone that stimulates growth of human body; used in treatment of dwarfism
Bone growth factor	—	Stimulates growth of bone cell; used in treatment of osteoporosis
Interferon Alpha	Berofor, IntronA, Wellferon, Roferon-A , human recombinant alpha interferon	Used in treatment of cancer and viral diseases
Interferon Beta	Frone, Betaseron, human recombinant beta interferon	Used in treatment of cancer and viral diseases
Interferon Gamma	Actimmune	Used in treatment of cancer and viral diseases
Interleukin-2	Proleukin, human recombinant interleukin-2	Used in treatment of immunodeficiencies and cancer
Tumor necrosis factor (TNF)	—	Used in treatment of cancer
Tissue plasminogen activator (TPA)	Actilyse	Dissolves blood clots; used in treatment of heart disease and during heart surgery
Food clotting factor VIII	Recombinate	Stimulates blood clot formation; used in treatment of hemophiliacs
Epidermal growth factor	—	Regulates calcium levels and stimulates growth of epidermal cells; used in treatment of wounds to stimulate healing
Granulocyte colony stimulating factor	Filgrastin, Neupogen	Regulates production of neutrophils in bone marrow; used to prevent infections in cancer patients
Erythropoietin (EPO)	Procrit Epogen	Stimulates red blood cell production; used in treatment of anemia in dialysis patients

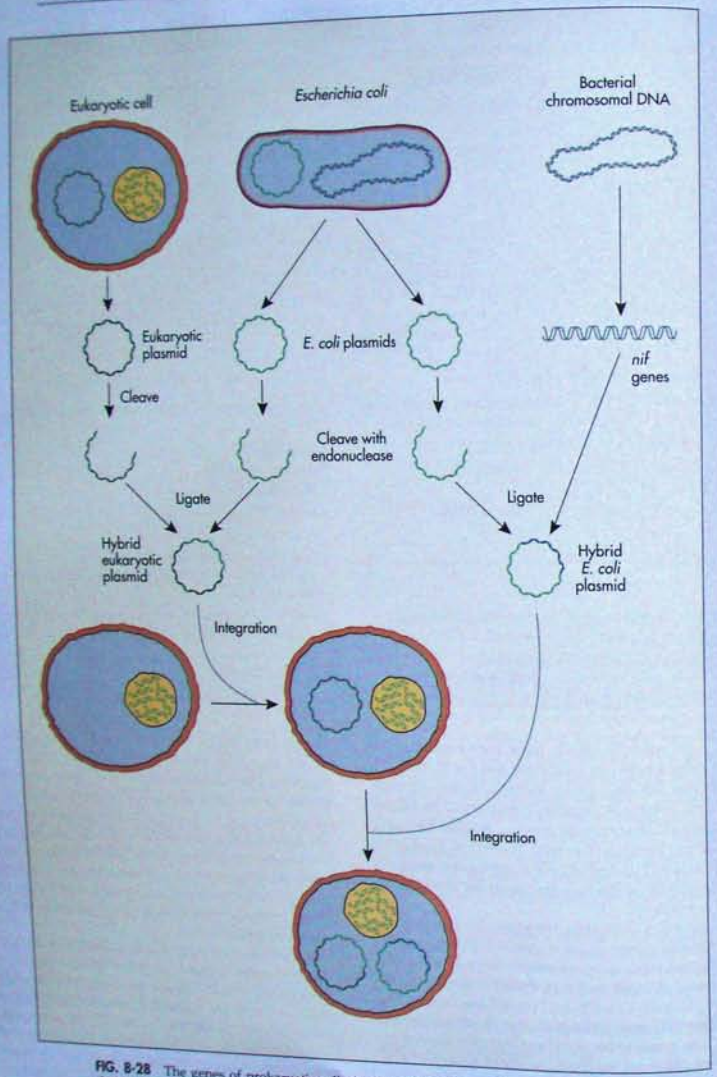
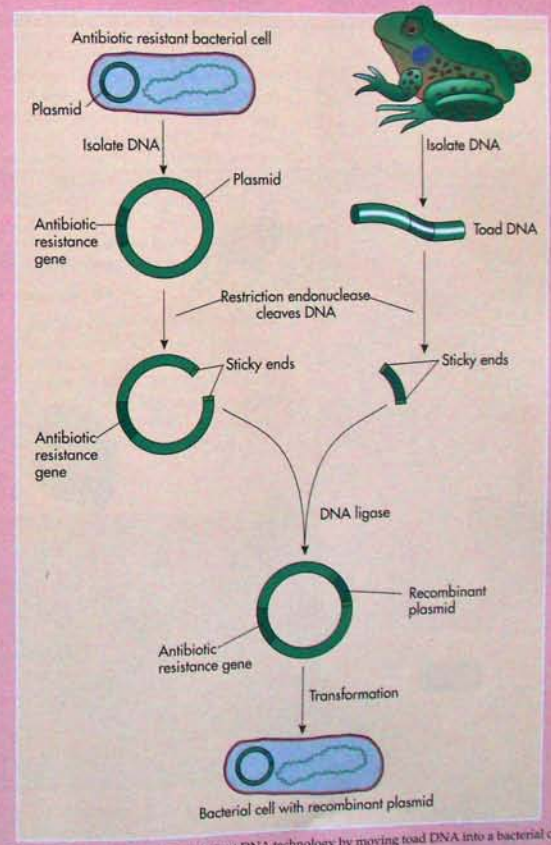


FIG. 8-28 The genes of prokaryotic cells for nitrogen fixation (*nif* genes) can be moved through recombinant DNA technology into eukaryotic DNA, for example, of a yeast. If this could also be accomplished for corn, wheat, and rice, greater food production without chemical fertilizers could be achieved.

HISTORICAL PERSPECTIVE

First Demonstration of Genetic Engineering

Stanley Cohen and Herbert Boyer in 1973 were the first to demonstrate that eukaryotic genes could be genetically engineered into a bacterial cell (see Figure). Cohen and Boyer used a restriction endonuclease—called *Eco*R1 (*Escherichia coli* restriction enzyme number 1)—to cut a plasmid of *Escherichia coli*. They isolated a DNA fragment 9,000 nucleotides long that contained both the nucleotide sequence necessary for replicating the plasmid and a gene that conferred resistance to the antibiotic tetracycline. In the presence of a ligase, this DNA fragment could form a new circular plasmid, which Cohen named pSC101.



Cohen and Boyer pioneered recombinant DNA technology by moving toad DNA into a bacterial chromosome.

Continued.

First Demonstration of Genetic Engineering—cont'd

Cohen and Boyer then used *Eco*R1 to cut up DNA isolated from a toad cell. They then mixed the toad-DNA fragments with opened-circle molecules of pSC101. They used a ligase to join the bacterial pSC101 DNA with the toad DNA. They then allowed cells of a tetracycline sensitive strain of *E. coli* to take up DNA from the mixture. They plated the cells on a medium containing tetracycline so that only cells that had become resistant to tetracycline would grow. From among these pSC101-containing cells they were able to isolate ones containing the toad DNA. These bacteria had the toad gene spliced into the bacterial pSC101 plasmid.

The pSC101 containing the toad gene is the first product of recombinant DNA technology. It does not exist naturally. It is a form of recombinant DNA, a DNA macromolecule created in the laboratory by molecular geneticists who joined eukaryotic and bacterial DNA. Thus the first recombinant genome produced by genetic engineering was a bacterial plasmid into which a toad gene was inserted.

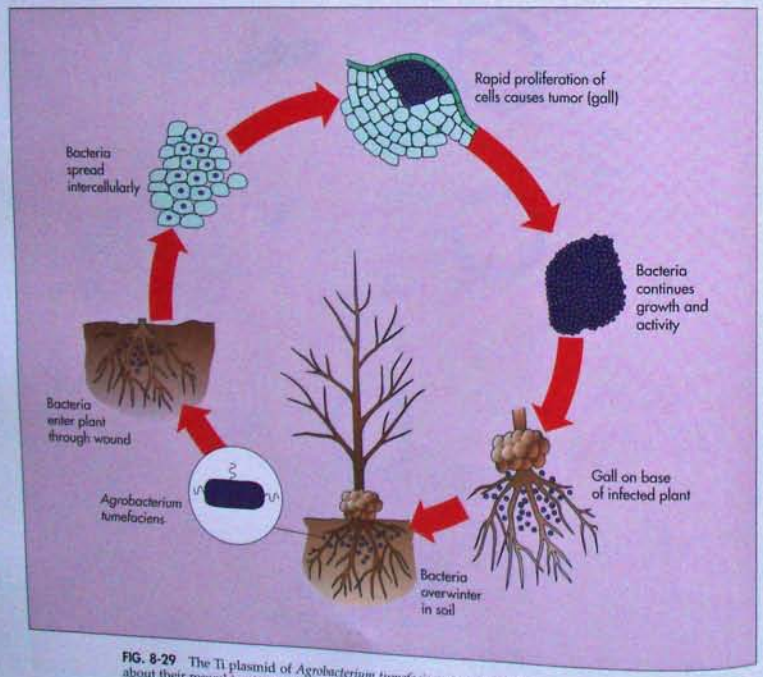


FIG. 8-29 The Ti plasmid of *Agrobacterium tumefaciens* can carry foreign genes and bring about their recombination into plant cells when they are infected with this bacterium.

Gene Transfer Using *Agrobacterium tumefaciens* Ti Plasmid

One approach for improving agricultural crops that holds great promise is to use bacterial plasmids as vectors. The vectors move genetic information from one plant to another. The **Ti (tumor-inducing) plasmid** of *Agrobacterium tumefaciens* appears to be suitable for such a process (FIG. 8-29). *A. tumefaciens* causes crown gall tumors in most dicotyledonous plants. The Ti plasmid of *A. tumefaciens* induces infected plant cells to synthesize nitrogen compounds called opines. During the normal infectious process, a section of the plasmid combines with chromosomal DNA in the nucleus of the plant cell. It may therefore be possible to employ the Ti plasmid as a vector for inserting foreign DNA into the DNA of plant cells. To do so the plasmid would be cut open at a site within the Ti plasmid DNA and the foreign gene spliced into it. When tumor cells are grown in tissue culture, they would continue to carry and replicate Ti-DNA during the normal divisional process. If foreign genes inserted into Ti-DNA are also transmitted to plant progeny, new plant strains can be genetically engineered.

Recombinant Bacteria with Human Genes

Recombinant Human Insulin

Human insulin, a protein produced in the pancreas, is necessary for the regulation of carbohydrate metabolism. The active form of insulin consists of two polypeptides connected by disulfide bridges. The polypeptides are coded by separated parts of an insulin gene, which also codes for additional products. Diabetes, a disease characterized by a shortage of insulin, is treated by insulin injections. The source of this insulin traditionally has been commercially produced insulin isolated from beef or pork pancreas. The insulin of most mammals is similar in structure. However, the immune systems of some diabetics can recognize beef or pork insulin as a foreign (nonhuman) protein and destroy the insulin. For this reason, it is preferable to treat diabetics with human insulin.

Human insulin can be produced by using recombinant *E. coli* (FIG. 8-30). The two polypeptides of insulin are made separately and then linked chemically. A nucleotide triplet coding for methionine is

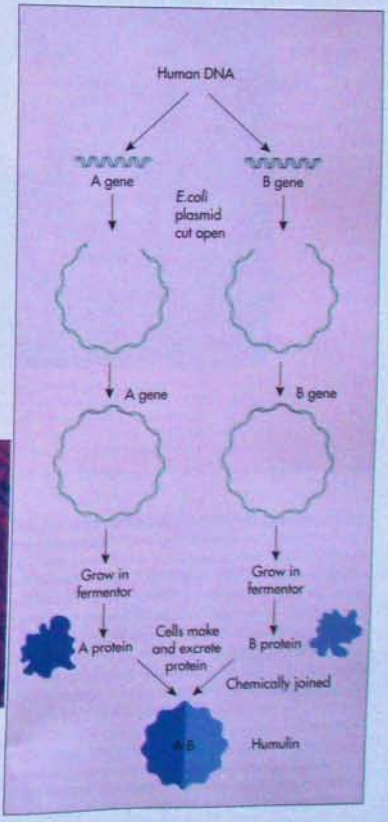


FIG. 8-30 **A**, Crystals of humulin. **B**, Human insulin (humulin) is produced by recombinant strains of *Escherichia coli*. One recombinant strain is genetically engineered to produce the A protein and a second recombinant strain produces the B protein. The two proteins are then chemically combined to produce commercial humulin.

HIGHLIGHT

First Environmental Release of Genetically Engineered Bacteria

In 1987 Steven Lindow, a professor at the University of California, released a genetically engineered bacterium into the environment in an experiment (FIG. *A*). The release came after years of debate among scientists about the potential ecological consequences of such releases, extensive governmental oversight, and a court battle with environmentalists. The bacterium that Lindow released is a genetically engineered strain of *Pseudomonas syringae*, designed to reduce frost damage to agricultural crops.

A

Ice-minus bacteria have been formed by recombinant DNA technology and applied to crops to protect them against frost damage. Here a field test is underway in which the ice-minus bacteria are applied at planting to potato plants. The naturally occurring bacteria on plant surfaces contribute to plant damage because they produce surface proteins that catalyze the formation of ice crystals. The ice-minus bacteria lack the surface proteins that initiate ice crystal formation leading to frost damage.

B

The ice-minus bacteria on the plant surface prevent ice crystal formation at temperatures where ice crystals otherwise would form and damage the plant. The bean leaf with the wild-type bacteria on its surface freezes (*right*). The bean leaf with the ice-minus bacteria does not freeze at the same temperature (*left*).

Normally, bacteria living on the surfaces of plants produce proteins that serve as nuclei around which ice condenses. This allows the formation of frost. The genetically engineered strain of *P. syringae*, created by using recombinant DNA methods to delete a specific segment of nucleotides, lacks the gene for producing the protein that initiates ice crystal formation. It is therefore called an ice-minus strain of *P. syringae*. If the normal microbial populations on the plant surface can be replaced by the genetically engineered ice-minus bacteria, the temperature would have to be several degrees lower before ice crystals form and damage the plant (FIG. *B*). This would provide the necessary margin for the survival of many crops. The field test conducted by Lindow demonstrated that application of the recombinant *P. syringae* can prevent ice crystal formation and hence protect plants from frost damage. Application of ice-minus *P. syringae* to plants can potentially save millions of tons of crops that are destroyed annually by frost because genetically engineered ice-minus *P. syringae* can protect plants against frost damage.

placed between the *lac* promoter genes of *E. coli* and a human insulin polypeptide gene. A ligase is used to attach the nucleotide sequence to plasmid pBR322, which was cloned in *E. coli*. The *E. coli* expresses the genes in the recombinant DNA and produces the polypeptide of human insulin. Different strains are used to produce the two polypeptides needed for insulin, and chemical treatment is then used to link them together. The final product—**humulin**—is identical to insulin purified from the human pancreas. Humulin is commercially produced by the Eli Lily Company.

Through genetic engineering, recombinant strains of *E. coli* have been created that produce human insulin.

Recombinant Human Growth Hormone

Through a series of novel procedures involving a combination of chemical synthesis and isolation of the natural molecules, a gene for a human growth hormone has been constructed. It was placed into *E. coli*. Human growth hormone is a polypeptide that is 191 amino acid units long elaborated by tissues of the pituitary gland. Its absence leads to a form of dwarfism but it can be cured by administration of the hormone. The segment of the gene that codes for the first 34 amino acids of the peptide was constructed chemically from nucleotides. Such short nucleotide sequences are often easy to make using automated DNA synthesizers.

The remainder of the gene was constructed enzymatically. Because the genes that code for human growth hormone production in eukaryotic cells are split, reverse transcriptase was employed to copy the gene for the hormone from mRNA obtained from human pituitary tissues. The use of reverse transcriptase simplified the job of cutting and splicing because the DNA produced in this way is colinear with the sequence of nucleotides in the mRNA. Restriction endonucleases cut out the needed fragment. DNA ligase was then used to join the natural and synthetic fragments. The complete gene produced in this manner has been inserted into a modified version of plasmid pBR322 incorporating the *lac* operon. The hormone could therefore be produced independently in bacterial cells. Clinical trials have shown that children who receive injections of bacterially produced human growth hormone for a few months approach normal height for their age group, with no significant side effects.

Recombinant bacteria are used to produce human growth hormone.

Human Gene Therapy

Recombinant DNA technology can be used to move genes into human cells to cure a disease condition. This potential application of genetic engineering—known as **human gene therapy**—has raised many scientific and ethical questions. On one hand, medical science attempts to alleviate disease conditions, increasing longevity and improving the health and well-being of individuals. On the other hand, science should not try to genetically engineer a new human

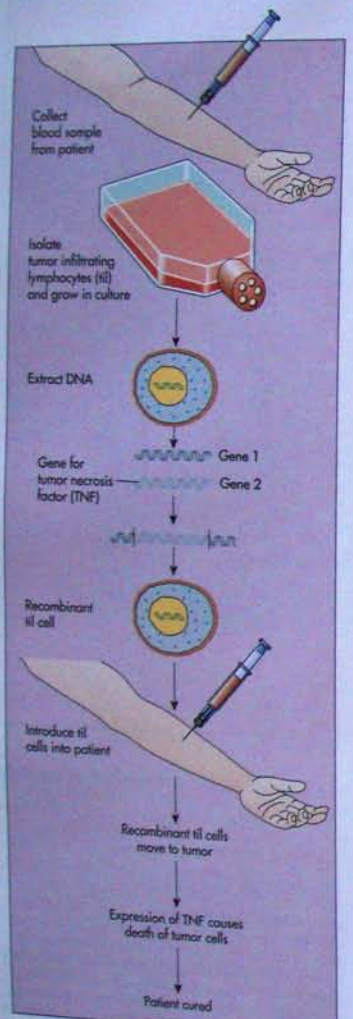


FIG. 8-31 Genes can be added to tumor infiltrating lymphocytes (til cells) through recombinant DNA technology that may help remove malignant tumors.

race. Scientists do not fully understand the regulation of human genes and how specific genes relate to disease conditions, and more needs to be learned.

Human gene therapy is the use of recombinant DNA technology to treat disease.

To help understand the nature of human chromosomes, the Department of Energy and the National Institutes of Health (NIH) have undertaken a major scientific investigation project, called the **Human Genome Project,** to determine the nucleotide sequences of all human genes. This project is aimed at developing an understanding of how human genotype relates to human phenotype. This research project will provide important insights into how gene therapy might be applied to correct certain disease conditions. At present, the policy under the guidelines of the Recombinant Advisory Committee of NIH is that no human gene therapy can be considered if it allows the use of recombinant genes that can be passed on to the next generation. Therefore gene therapy may not be applied to reproductive cells involved in the passage of heredity information. Gene therapy, however, may be considered on a case-by-case basis when it is to be applied to the somatic cells of an individual.

In the first approved "gene therapy" experiment, Stephen Rosenberg, Michael Blaese, and French Anderson at the NIH inserted the bacterial gene for tetracycline resistance into the DNA of certain human blood cells called tumor infiltrating lymphocytes (til cells) (FIG. 8-31). They did so to track the fate of til cells introduced into the body as part of a therapy for treating advanced forms of cancer. Specifically, these scientists developed a cancer treatment in which they isolate til cells from a cancer patient, grow the til cells in the laboratory, and reinject large numbers of til cells into the patient. In some cases, the til cells attack the tumors (destroying the malignant tumors), but, in other cases, til cell treatment fails. To determine why the treatment succeeds in some cases and fails in others, they used recombinant til cells containing the bacterial tetracycline resistant gene to monitor their fate.

Rosenberg, Blaese, and Anderson next proposed inserting the gene for tumor necrosis factor into the DNA of til cells. This gene codes for a substance that causes tumors to shrink. They inject the special recombinant til cells containing the tumor necrosis factor gene into patients with tumors in an attempt to increase the effectiveness of their cancer treatment. These experiments represent the first attempts to use recombinant DNA technology to directly alter the genetic instructions of human cells within the body.

Recombinant tumor infiltrating lymphocytes have been used to develop new methods for cancer treatment.

SAFETY OF GENETIC ENGINEERING

The potential of genetic engineering to create new gene combinations that have not evolved naturally raises ethical and safety questions. The scientists who discovered that natural restrictions on the transfer of genes between different species could be bypassed by recombinant DNA technology were uncertain about the safety of such procedures, that is, whether recombinant DNA technology would produce organisms dangerous to human health or to the environment. The public feared that scientists would create an Andromeda strain, a fictional but powerful microorganism that would cause widespread disease and wreak havoc on human health.

The capability to create genetically engineered microorganisms to be released into the environment has sparked concern among the public and scientific communities.

In February 1975, scientists gathered at a conference in Asilomar, California, to consider the safety issues related to the construction of genetically engineered microorganisms. Initially it was thought that engineered microorganisms should be contained within laboratories. Methods, therefore, were sought to ensure that microorganisms could not escape containment. Scientists declared a moratorium on the construction of such organisms until a fail-safe strain could be constructed that could not survive outside of special laboratory conditions. Only after scientists had constructed a strain *E. coli* K12 that was so demanding in its nutritional requirements that it could not live except in a specialized growth medium did scientists resume their work. Also, special facilities were constructed with elaborate air filtration and water treatment systems to ensure that the genetically engineered microorganisms could not escape.

Scientists designed methods for containing genetically engineered microorganisms and for ensuring that if one escaped laboratory containment it could not survive in the environment.

Additionally, governmental guidelines and regulations for preventing accidental release of genetically engineered microorganisms into the environment were established. The NIH formed the Recombinant Advisory Committee (RAC) to oversee the safe use of genetic engineering technology. Despite the extensive controversy about the safety of genetically engineered organisms, experience has shown that the risks are low and manageable and that the benefits are great.

While the initial thrust was to try to contain genetically engineered microorganisms in the laboratory, it soon became clear that great benefits could be realized in constructing genetically engineered organisms to deliberately be released into the environment. Microorganisms to degrade toxic pollutants, to be used as vaccines in humans and other animals, and to enhance agricultural productivity would have to be released into the environment to realize the beneficial uses of genetic engineering for environmental applications. Additional governmental regulations are aimed at ensuring the safety of environmental applications of genetically engineered organisms.

HIGHLIGHT

TRACKING AND CONTAINING GENETICALLY ENGINEERED MICROORGANISMS

Among the major concerns about releasing genetically engineered microorganisms into the environment are the need to track the movement of such organisms and to contain microorganisms that may accidentally cause undesirable side effects. Recognizing these concerns, I initiated two lines of research in my laboratory, one to develop sensitive methods for monitoring genetically engineered microorganisms and the other to develop containment systems that could be used to mitigate any undesirable impact that a deliberate release of such organisms might have.

After several years of work, Robert Steffan, working in my laboratory, developed methods for extracting DNA from environmental samples and using the polymerase chain reaction and gene probes for detecting genetically engineered microorganisms. The combination of PCR and gene probes provides the necessary sensitivity and specificity for tracking genetically engineered microorganisms in the environment. As few as one genetically engineered microorganism per gram of soil can be detected and positively identified. Thus our work provides an essential tool for tracking the spread of genetically engineered microorganisms with sufficient precision to allay fear that unseen microorganisms were spreading beyond control.

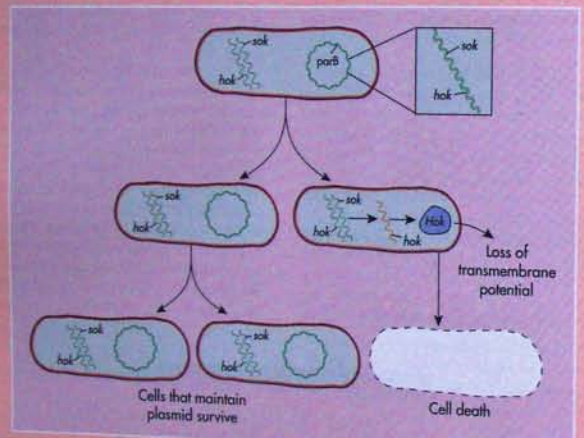
With regard to containment of genetically engineered microorganisms, I decided to use recombinant DNA technology to create suicide vectors, that is, genetic elements that could be placed into genetically engineered microorganisms that would cause such organisms to kill themselves under specific environmental conditions. Soren Molin in Denmark had shown that some naturally occurring genes code for suicide functions and potentially could be used to contain genetically engineered microorganisms. In the studies conducted in my laboratory, Asim Bej, with the collabora-

Continued

tion of Michael Perlin, Soren Molin, and the late Stephen Cuskey created suicidal microorganisms in which the *lac* operon was used to control expression of a gene called *hok* that codes for a polypeptide that destroys the essential functioning of the cell's plasma membrane. By chemically signalling the *lac* operon to turn on or off, we were able to show that, if necessary, we could cause a genetically engineered microorganism with the suicide vector to kill itself (see Figure). Thus we have engineered a way of containing a genetically engineered microorganism and preventing harmful effects that it might cause.

The use of suicide vectors is appealing to environmentalists and the industrial companies that may produce genetically engineered microorganisms. Industrial companies can use suicide vectors to ensure limited environmental survival and hence the sale of new genetically engineered microorganisms for reuse at the same time that they offer a means for safeguarding against untoward environmental effects. Environmental groups such as the National Audubon Society see the advantage of containment for ensuring environmental safety.

Thus the work undertaken in my laboratory and in Soren Molin's laboratory has gone a long way in developing the methods necessary for reducing risk associated with the deliberate release of genetically engineered microorganisms into the environment.



The parB locus of a plasmid in *Escherichia coli* acts as a suicide gene so that the plasmid must be maintained for the cells to survive. One strand of the DNA has the *hok* gene that encodes a polypeptide that kills the cell; if *hok* is expressed the cells die. The other strand of the DNA has the *sok* gene, which produces an antisense mRNA. If the *sok* RNA binds to the *hok* mRNA, the Hok polypeptide is not produced. If *sok* RNA is not there, *hok* is translated. Because the *sok* RNA has a shorter-half life than the *hok* mRNA, if the plasmid with parB is lost, Hok polypeptide is produced and the cell dies. As long as the cell maintains the plasmid with parB, the cell production of *sok* RNA blocks the translation of *hok* mRNA.

SUMMARY

Recombination (pp. 221-224)

- Genetic recombination occurs whenever genes move from one location to another. Movement can be to a new location within a single DNA macromolecule or exchanging or combining DNA from two different sources. Such changes can result in the alteration in the expression of genes or new combinations of genes. Recombination redistributes the changes that result from mutation.

Homologous Recombination (pp. 221-222)

- Homologous recombination occurs when the exchanged DNA comes from corresponding genes and produces new combinations of alleles. Pairs of chromosomes contain the same gene loci pair and exchange corresponding portions of the same chromosomes.

Nonhomolgous Recombination (pp. 222-224)

- Nonhomologous recombination can occur between different DNA segments, even from different species. It permits the insertion of plasmids into bacterial chromosomes, the transfer of genes, and the incorporation of viral DNA into prokaryotic and eukaryotic cells.

Insertion Sequences (pp. 222-224)

- Insertion sequences are small transposable genetic elements composed of about 1,000 nucleotides that can move from site to site within a bacterial chromosome. ISs move by nonhomologous recombination.

Transposons (p. 224)

- Transposons are genetic elements that code for the production of structural proteins. They encode transposase that catalyzes nonhomologous recombination. Tranposase inserts transposons at random within a chromosome. The protozoans that cause African sleeping sickness and malaria have their genes mobilized by transposons, producing random changes in the surface polysaccharides that the host defense system cannot defeat.

Gene Transfer (pp. 224-235)

Gene Transfer and Recombination in Prokaryotes (pp. 224-232)

Plasmids and Gene Transfer (pp. 224-225)

- Plasmids are small extrachromosomal genetic elements that store genetic information that code for specialized functions. Plasmids can replicate and can transfer genes among bacteria that can have more than one plasmid.
- F plasmids are conjugative plasmids that contain special genes that promote their transfer to other cells, where they can replicate because they carry a DNA replication origin. Colicinogenic plasmids code for a protein that is toxic to closely related bacteria. R plasmids carry genes that code for resistance to antibiotics and can be passed not only among strains but also among bacterial species.

Transformation (pp. 225-227)

- Free DNA is transferred from a donor to a recipient bacterium and then is recombined during transformation. The recipient cell must be competent and compatible.

Transduction (pp. 227-228)

- In transduction, DNA is transferred from a donor to a recipient cell by a viral carrier, followed by recombination within the recipient cell. During viral replication within a host cell, some host DNA may break off and be packaged in viral protein coats and passed along as the virus infects a new cell.
- Temperate phage have acquired bacterial DNA and do not kill their host cells because the bacterial DNA replaces genes that code for the completion of phage replication.
- Some temperate phage can carry almost any host cell gene. Generalized transduction results in the transfer of alleles by homologous recombination. Some temperate phage acquire and transfer only certain host cell genes from specific sites. This process is called specialized transduction. Specialized transduction results in the transfer of genes by nonhomologous recombination.

Conjugation (pp. 228-232)

- During conjugation, DNA is transferred from a donor to a recipient cell via an F pilus. The F pilus is coded for by an F plasmid. If the F plasmid is free in the cytoplasm the donor is an F^+ strain. If it is incorporated into the bacterial chromosome, the donor is a high frequency recombinant (Hfr) strain. Recipient strains do not have F plasmids and do not have F pili.
- During bacterial conjugation, a single strand of donor DNA is replicated and transferred to the recipient where the complementary DNA strand is synthesized. The amount of DNA that is transferred, and hence the number of genes transferred, depends on how long the F pilus maintains contact between the mating cells.
- When F^+ and F^- strains mate, the resultant strains are F^+. When Hfr and F^- strains mate, the recipient strains remain F^- but there is a high frequency of recombination of genes of the bacterial chromosome.

Genetic Exchange in Eukaryotes (pp. 232-235)

- Genetic exchange in eukaryotes usually occurs during sexual reproduction and involves the recombination of DNA from two parents. Diploid eukaryotic vegetative cells form haploid gametes or haploid spores to exchange genetic information. Conversion from diploid to haploid occurs during meiosis. Haploid nuclei of reproductive cells later fuse with nuclei of reproductive cells of an appropriate mating type in syngamy, reestablishing the diploid state.

Recombinant DNA Technology (pp. 235-240)

Formation of Recombinant DNA (pp. 235-237)

- Recombinant DNA technology is the deliberate union of genes to make recombinant DNA macromolecules. Both donor and recipient DNA can be from any source.
- Restriction endonucleases cut the DNA at a palindromic sequence of bases, producing DNA with staggered single-stranded ends that can be spliced with DNA from other sources if excised with the same endonuclease. Ligases splice the pieces of DNA. If different endonucleases cut the DNAs, an artificial homology must be established at the terminal ends of the donor and recipient DNAs by adding a polyA tail to the plasmid and a polyT tail to the donor DNA.
- Reverse transcriptase and an mRNA can produce a DNA macromolecule with a contiguous sequence of nucleotide bases containing complete functional genes to overcome the problem of discontinuity of eukaryotic split genes. Therefore recombinant DNA can be made containing both eukaryotic and prokaryotic genes.

Cloning Recombinant DNA (pp. 238-240)

- Cloning is the asexual reproduction of genes contained in recombinant DNA. Plasmids are often used as carriers for such cloning. Bacteria containing re-

combinant plasmids produce multiple copies of identical cloned genes.
- Insertional inactivation can detect the presence of foreign DNA within a plasmid. It is caused by the disruption of the nucleotide sequence of a gene by the insertion of foreign DNA.
- Protoplast fusion can transfer DNA from one cell to another.

Genetic Engineering (pp. 240-250)

Recombinant Plants with Bacterial Genes (pp. 240-245)

Recombinant Nitrogen Fixers (pp. 240-244)
- The genetics and biochemistry of infection with the nitrogen-fixing *Rhizobium bacterium* are being studied to try to use recombinant DNA techniques to genetically engineer plants that will fix their own nitrogen.

First Demonstration of Genetic Engineering (pp. 243-244)
- Genetic engineering is the use of recombinant DNA technology for the creation of cells with entirely new properties. In 1973, Cohen and Boyer proved that eukaryotic genes could be genetically engineered into a bacterial cell by inserting a toad gene into a bacterial plasmid.

Gene Transfer Using Agrobacterium tumefaciens *Ti Plasmid (p. 245)*
- The tumor-inducing plasmid of *Agrobacterium tumefaciens* can be used as a vector for moving genetic information from one plant to another.

Recombinant Bacteria with Human Genes (pp. 245-247)

Recombinant Human Insulin (pp. 245-246)
- Human insulin can be produced by using recombinant *E. coli*.

First Environmental Release of Genetically Engineered Bacteria (pp. 246-247)
- The first genetically engineered bacteria to be released into the environment was an ice-minus strain of *Pseudomonas syringae* designed to protect crops from frost damage.

Recombinant Human Growth Hormone (pp. 246-247)
- A gene coding for the production of human growth hormone has been created and placed into *E. coli*. Injections of bacterially produced human growth hormone are successful in allowing children suffering from a form of dwarfism to grow.

Human Gene Therapy (pp. 247-248)
- Human gene therapy is the use of recombinant DNA technology to move genes into human cells to cure disease. The human genome project is designed to determine the nucleotide sequences of all human genes in order to understand how human genotype relates to human phenotype.

Safety of Genetic Engineering (p. 249)
- Scientists have developed methods for safely handling genetically engineered microorganisms. Government regulations have been established to oversee the development and applications of genetic engineering.

CHAPTER REVIEW

REVIEW QUESTIONS

1. Define genetic recombination.
2. By what mechanisms are genes transferred between bacteria?
3. What is a plasmid and what is its function?
4. What is a transposon and what is its function?
5. How is recombinant DNA produced *in vitro*?
6. Compare general and specialized transduction.
7. How could you distinguish between transformation, transduction, and conjugation?
8. What is a gene probe?
9. What are the biological functions of reverse transcriptase, DNA ligase, transposase?
10. What is the difference between transposons and insertion sequences?
11. How can recombination alter the ability to use specific antibiotics in treating disease?
12. How can a bacterium be genetically engineered to produce a human protein?

CRITICAL THINKING QUESTIONS

1. Why is the general public frightened of genetically engineered microorganisms? Could a scientist really create the "Andromeda strain" or the dinosaurs of Jurassic Park? Why has the scientific community lowered its level of concern about the release of genetically engineered microorganisms into the environment? What safeguards have been developed to protect the public and the environment from "potential new pathogens"? What safeguards are needed?
2. How can recombination lead to the evolution of new organisms? How could you go about creating a photosynthetic fungus? How could you improve the agricultural productivity through genetic engineering of plants and microorganisms?
3. What are some of the potential uses for recombinant DNA? How will recombinant DNA technology alter the practice of medicine in the next decade? How can recombinant DNA technology be used to improve environmental quality?

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UNIT
THREE

Microbial Growth and Its Control

CHAPTER 9

Viral Replication

CHAPTER OUTLINE

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Double-stranded RNA Viruses
Single-stranded RNA Viruses
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Retroviruses
Transformation of Animal Cells
Historical Perspective: Discovery of Oncogenes

PREVIEW TO CHAPTER 9

In this chapter we will:

- Learn how viruses replicate within host cells, using their nucleic acids (RNA or DNA) to direct the formation of viral progeny.
- Examine the general replication strategy used by all viruses for their replication and the specific steps used by different bacterial, plant, and animal viruses to accomplish this task.
- See why viruses have tremendous replication potential.
- Learn that viral DNA can be incorporated into host cells, changing the properties of the host cells.
- Learn the following key terms and names:

adsorption
animal viruses
assembly
bacteriophages
budding
burst size
capsid
early protein synthesis
eclipse period
envelope
late protein synthesis
latent period
lysogeny
lytic phages
nucleocapsid
oncogenes
one-step growth curve
penetration
phages
plant viruses
plus RNA strand
prophage
release
reverse transcriptase
reverse transcription
temperate phage
uncoating
viral maturation
virions

GENERAL ASPECTS OF VIRAL REPLICATION

Viruses are acellular, nonliving microorganisms that are totally dependent on host cells for replication. They are incapable of independent metabolism, growth, or reproduction. Some microbiologists view viruses as parasites of the host cells within which they replicate. Others consider viruses to be genetic extensions of their host cells. Supportive of both viewpoints is the high degree of specificity necessary between a particular virus and the specific host cell within which that virus replicates. Specificity between virus and host cell is based on the ability of the virus to physically attach to the host cell and the ability of the viral nucleic acid to direct viral replication within that host cell. For a virus to replicate within a host cell, the host cell must be permissive, meaning that the host cell must allow entry and must not degrade the viral nucleic acid genome. The virus also must be compatible with the host cell, meaning that the virus must be capable of using and directing the metabolism of the host cell for viral replication.

To replicate, a virus must be able to enter a compatible host cell and, using the host cell's metabolism, the viral nucleic acid must be able to direct viral replication within the host cell.

Viruses have two essential components: a nucleic acid and a wall-like structure—called the **capsid.** The nucleic acid is either RNA or DNA and carries the hereditary information. The capsid is composed of protein and surrounds the nucleic acid. Together the capsid and enclosed nucleic acid are called the **nucleocapsid.** The replication of a virus involves making a copy of the nucleic acid and capsid protein to construct a new nucleocapsid. Some viruses also have an **envelope** that is acquired mainly from the host cell and composed largely of nuclear or cytoplasmic membrane. In some cases, viral proteins are added to the envelope.

Within a host cell, the viral nucleic acid genome directs the formation of new viruses. It uses the structures and metabolism of the cell to make viral protein and nucleic acid. In many cases, the viral nucleic acid genome actually codes for the shutdown of the metabolic activities normally involved in the host cell's reproduction. The virus then uses the host's biochemical components and anatomical structures for the production of new viruses. In particular, the virus employs the host cell's ribosomes for producing viral proteins and ATP for carrying out biosynthesis. Viral replication results in changes within the host cell, often causing its death.

Viral replication occurs only within compatible host cells.

Viral replication requires that a virus gain entry to a host cell and take control of that cell's metabolism.

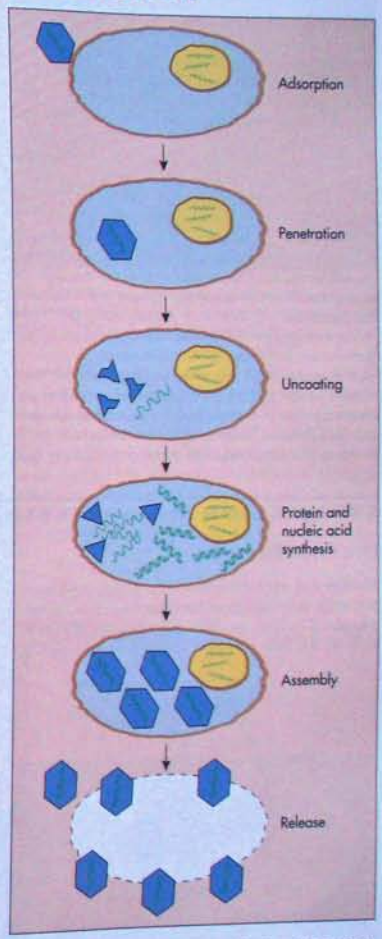


FIG. 9-1 Viral replication begins with adsorption to a host cell. Most animal and plant viruses then penetrate the cell and the viral coat (capsid) is rapidly degraded so that the viral nucleic acid is uncoated and is free within the host cell. Within the host cell the viral nucleic acid directs the synthesis of viral proteins and nucleic acids. Viral progeny (complete virions) are assembled from the synthesized viral nucleic acid and capsid components and subsequently released from the host cell.

Stages of Viral Replication

The replication of a virus occurs in a series of stages. Viral replication begins with **adsorption** when a virus attaches to the outer surface of a suitable host cell (FIG. 9-1). Generally, adsorption of a virus to a host cell involves specific binding sites on the cell surface, which explains in part the high degree of specificity between the virus and the host cell. The second stage of viral replication, called **penetration,** occurs when the virus or its nucleic acid genome crosses the plasma membrane and enters the host cell. In the third stage, **uncoating,** the viral nucleic acid is released from the capsid. In viruses that replicate within bacterial cells, penetration and uncoating usually occur simultaneously so that the viral nucleic acid genome enters the bacterial host cell. In viruses that replicate in plant and animal cells, the entire virus normally penetrates the host cell and uncoating then occurs in the cytoplasm or nucleus.

Within the host cell, the viral nucleic acid takes control of the cell's metabolism. First, the viral nucleic acid directs the synthesis of proteins **(early protein synthesis).** These early proteins include polymerases that are needed to make copies of the viral nucleic acid. Then multiple new copies of the viral nucleic acid genome are produced **(nucleic acid synthesis).** New proteins needed for capsid production are made last **(late protein synthesis).**

The nucleic acid genome is then packaged into the capsid. This process is called **assembly** or **viral maturation.** During the assembly process the capsid can first be constructed and then the viral nucleic acid packed into the capsid. Alternatively the capsid may be built around the viral nucleic acid to form the nucleocapsid.

Finally, **release** occurs when the assembled viruses leave the host cell. Often, many viruses are released simultaneously and the host cell is killed in the process. The released viruses, called **virions,** are infectious and when they encounter a suitable living host cell the entire process of viral replication begins anew.

The stages of viral replication are: adsorption to a host cell, penetration, uncoating, early protein synthesis, nucleic acid replication, late protein synthesis, assembly, and release.

REPLICATION OF BACTERIOPHAGES

Lytic Phage Replication

Viruses that replicate only within specific host bacterial cells are known as **bacteriophages** or, simply, **phages.** The replication of most bacteriophages results in the lysis (rupture) of the host bacterial cell when new phages are released. Therefore these bacteriophages are referred to as **lytic phages.** Because release is by lysis, the replication of lytic phages always results in the death of the host cell.

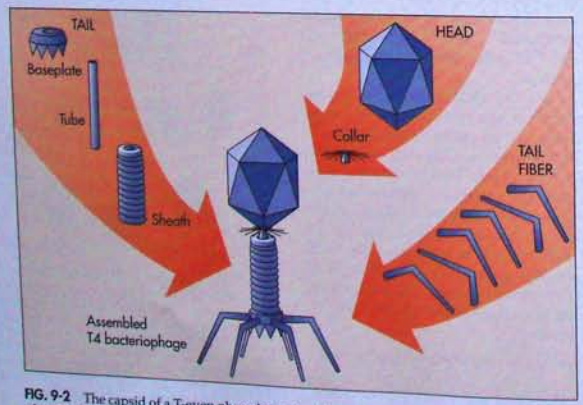


FIG. 9-2 The capsid of a T-even phage has a head that contains the nucleic acid and a complex tail that attaches to the host cell and accomplishes the injection of the nucleic acid.

T-even Phage Replication

One type of bacteriophage, the T-even phage (phage with tails), have a capsid that has distinct head and tail structures (FIG. 9-2). These phage are designated by even numbers, for example, T2, T4, and T6. The DNA genome is contained within the head structure and the tail is involved in adsorption to the host cell. The genomes of T-even phages are double-stranded DNA—like that of cellular organisms.

Replication of a T-even phage begins with the adsorption of a T-even phage tail to a bacterial host cell (FIG. 9-3). There are specific receptor sites on the bacterial cell surface where the phage tail may attach. The bacteriophage's tail releases lysozyme, which breaks down a portion of the bacterial cell wall. This allows the phage tail to penetrate the cell wall but not the plasma membrane. The phage tail then contracts, forcing the phage DNA into the periplasmic space (the region between the outer membrane of the bacterial cell wall and plasma membrane of a Gram-negative bacterial cell). A pore probably is formed in the plasma membrane, allowing the phage DNA to enter the cell. Penetration and uncoating, thus, occur simultaneously. The phage DNA subsequently migrates across the plasma membrane and into the cell.

The entire T-even phage does not penetrate the bacterial cell, but rather, the phage injects only its DNA into the bacterium.

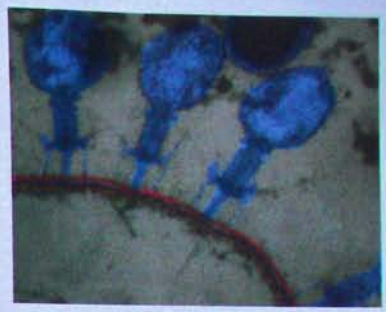


FIG. 9-3 Colorized micrograph showing the attachment of T-even phage to a cell of *Escherichia coli* and the extension of the tail tube as an injection needle.

When the phage DNA enters a compatible bacterial cell, it is not degraded by the nucleases that otherwise would destroy it. This is because the phage DNA is resistant to the nucleases of a compatible cell. The viral DNA is transcribed within the host cell to make viral mRNA. The mRNA is subsequently translated at the ribosomes of the cell to make phage proteins (early protein synthesis). Early phage proteins cause stoppage of normal host cell biosynthesis, that

METHODOLOGY

Assaying for Lytic Phage

Numbers of bacteriophage can be determined by inoculating a suspension of host bacterial cells with the phage. The suspension of host bacterial cells is spread over a solid nutrient growth medium and various dilution of phage are then spread over the same surface. In the absence of lytic bacteriophage, the bacteria form a confluent lawn of growth. Lysis by bacteriophage is indicated by the formation of a zone of clearing or plaque within the lawn of bacteria (see Figure). Each plaque corresponds to the site where a single bacteriophage acted as an infectious unit and initiated its lytic replication cycle. The spread of infectious phage from the initially infected bacterial cell to the surrounding cells results in the lysis of the bacteria in the vicinity of the initial phage particle and hence this zone of clearing. Plaques do not continue to spread indefinitely. With T4 phage, for example, the plaque size is limited because heavy reinfection of a host cell before the time of normal lysis extends the period of synthesis of viral protein and nucleic acid, thereby preventing completion of the lytic replication cycle. This phenomenon is known as lysis inhibition. The number of plaques that develop and the appropriate dilution factors can be used to calculate the number of bacteriophages in a sample.

Replication of lytic bacteriophage causes the formation of plaques in a lawn of bacterial cells growing on an agar surface.

is, the bacterial cell ceases making structures for new bacterial cells. Under the direction of the viral nucleic acid genome, the bacterial cell begins to synthesize proteins involved in the replication of phage nucleic acid (nucleic acid synthesis) and then the various proteins that make up the capsid of the phage (late protein synthesis). The head and tail structures of the phage are made up of proteins coded for by different phage genes, with at least 32 genes involved in the formation of the tail structure and at least 55 genes involved in the formation of the head structure of the phage.

After the production of the individual components of the virus, the virus is assembled by packing the nucleic acid genome into the protein capsid (assembly). The assembly of the T-even phage capsid is a complex process. Assembly of the head and tail structures requires several enzymes that are coded for by the phage nucleic acid. The head and tail units of the T-even phage capsid are assembled separately and later combined. The phage DNA is packed into the head structure, and when the head structure is completely filled with DNA, any extra DNA is cleaved by a nuclease.

Release of the assembled T-even phage occurs because one of the late proteins coded for by the phage is lysozyme. Lysozyme catalyzes the breakdown of the bacterial peptidoglycan wall structure. Lysozyme causes sufficient damage to the cell wall so that the wall is unable to protect the cell against osmotic shock. This results in the lysis of the bacterial cell and the release of the phage into the surrounding medium.

Lysozyme, a product of late protein synthesis, damages the cell wall, leaving it susceptible to osmotic shock and lysis. Thus new phage are released.

Replication of RNA Bacteriophage

There are some RNA phage, such as Qβ and f2, that carry out a lytic replication cycle (FIG. 9-4). The hereditary information for these phage is contained in a single strand of RNA. This RNA, designated as a **plus RNA strand,** contains the viral hereditary information and acts as the nucleic acid genome of the phage. The designation of the RNA genome as a plus strand indicates that it can also serve as an mRNA molecule. The plus strand RNA can be translated at the ribosomes of the bacterial cell to produce the proteins for making the viral capsid.

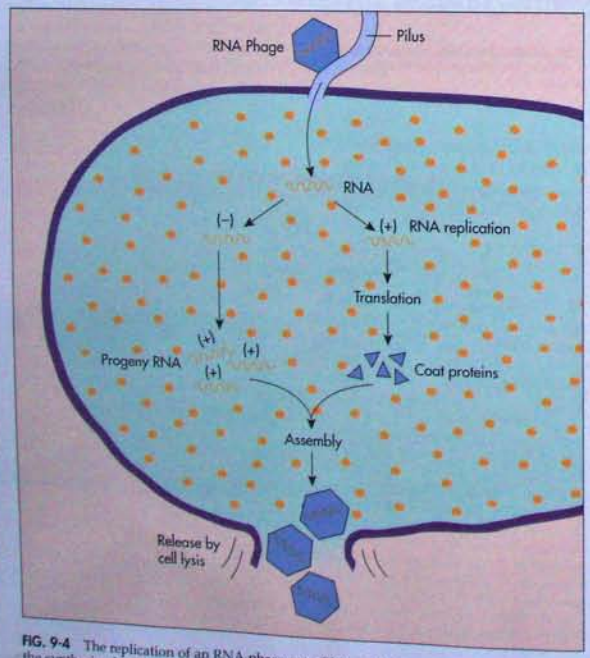


FIG. 9-4 The replication of an RNA phage uses RNA as the hereditary molecule to direct the synthesis of protein for capsid and new RNA genomes.

The RNA also must be replicated so that it can serve as the hereditary macromolecule of the virus. To form new copies of this RNA for viral progeny, the plus strand is used as a template for forming a double-stranded RNA replicate that has both plus and complementary minus RNA strands. To make this replication form, RNA nucleotides in association with RNA polymerase pair with the complementary nucleotides in the plus strand and RNA polymerase links them together. Then the minus RNA strand is used as a template for transcription to produce new plus RNA copies. A copy of plus RNA is then packaged into a capsid and a new phage is assembled. After assembly, the host cell is lysed by an enzyme coded for by the phage. This releases the progeny phage.

The replication of single-stranded RNA phage involves the formation of a double-stranded RNA macromolecule that serves as a template for transcription of new plus RNA.

Bacteriophage Growth Curve

Lysis of a bacterial cell releases a large number of phage simultaneously. Consequently, the lytic replication cycle exhibits a **one-step growth curve** (FIG. 9-5). The growth curve for lytic bacteriophage begins with an **eclipse period,** which is the time period from penetration until assembly. During the eclipse period there are no complete infective phage particles because once the phage nucleic acid genome is uncoated it is unable to infect another cell to initiate a new replication cycle. Only complete phage with a capsid can adsorb to host cells and initiate infection of another cell. Until new phage are assembled, therefore, there are no infective phage within the host cell. The eclipse period ends only when an average of one completely assembled infectious phage has been produced for each host cell.

The eclipse period is the time between entry of the phage nucleic acid into a host cell and the formation of a completely assembled phage within that cell.

The **latent period** is longer than the eclipse period, beginning when the phage injects its nucleic acid into a host cell but not ending until the first assembled virus appears outside the host cell. The latent period, thus, starts with penetration and ends after release. The latent period for a T-even phage typically is about 15 minutes. During the time between the end of the eclipse period and the end of the latent period, assembled phage accumulate within the bacterial cell.

The latent period is the time between entry of the viral nucleic acid and the release of viral progeny.

Completely assembled phage continue to accumulate within the bacterial cell until they reach a number known as the **burst size** (FIG. 9-6). The burst size,

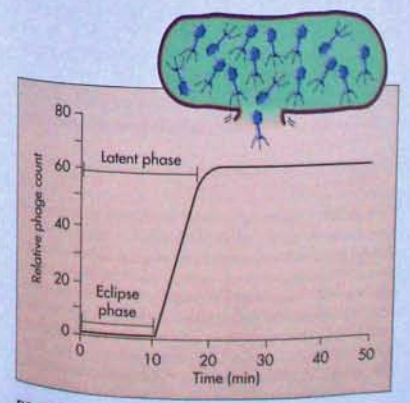


FIG. 9-5 Because many viruses are released simultaneously when a host cell lyses, lytic viruses exhibit a one-step growth curve.

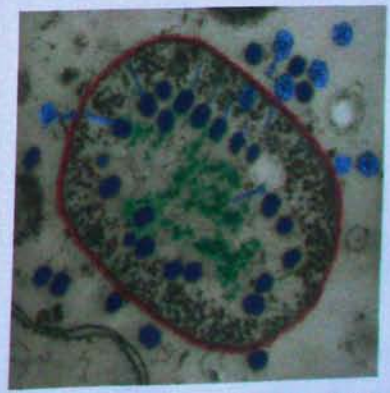


FIG. 9-6 Colorized micrograph of *Escherichia coli* with assembled bacteriophage *(blue)* inside the bacterial host cell.

which varies from cell to cell, represents the average number of infectious viral units that are present when a particular type of host cell lyses or bursts. A typical burst size for a T-even phage is 200. When the cell lyses, the viruses are released into the extracellular fluid. As a result of the simultaneous release of a number of infective phage, the number of phage that can initiate a lytic replication cycle increases greatly in a single step. The entire lytic growth cycle for some T-even phage can occur in less than 20 minutes under optimal conditions so that phage have a tremendous replicative capacity.

The burst size is the number of phage released with lysis of the host cell.

Temperate Phage—Lysogeny

Some DNA bacteriophage are called **temperate phage** because they can infect host cells without replicating the entire phage and without causing lysis of the bacterial host cell (FIG. 9-7). Temperate phage, such as the well-studied phage lambda (λ), do not always cause lysis of the host cell. Rather, these temperate phage often establish a state of **lysogeny** in which a portion of the viral nucleic acid is incorporated into the bacterial chromosome or a bacterial plasmid. This occurs when the phage DNA, having entered a host cell, undergoes recombination with the host cell DNA. Once incorporated into the bacterial DNA, the viral DNA is referred to as a **prophage**. The prophage is replicated with the bacterial DNA during normal host cell DNA replication and the host cell survives and reproduces.

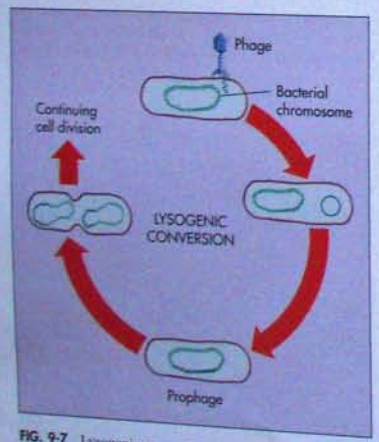


FIG. 9-7 Lysogenic conversion occurs when a temperate phage transfers bacterial DNA that it has acquired and that DNA recombines with the DNA of the host cell. The bacterial cell then replicates the phage DNA along with the bacterial chromosome DNA.

Temperate phage can incorporate their DNA into the DNA of the bacterial host cell to establish a state of lysogeny.

The incorporated phage DNA, called a prophage, is replicated with host cell DNA.

The presence of a prophage prevents reinfection of the host cell by the same phage. The host cell is thus protected against lysis due to phage infection. Many of the genes of the integrated viral DNA are repressed. Regulatory genes prevent transcription of most of the phage genes and therefore few viral proteins are made. Complete phage are not made and the phage genes for cell lysis are not expressed so that the host cell is not killed.

Some phage genes, however, may be expressed. The proteins that are made can greatly alter the properties of the host cell, sometimes accounting for the ability of the cell to cause human disease. For example, cells of the bacterium *Corynebacterium diphtheriae* produce a protein, called diphtheria toxin, when they are in a state of lysogeny because one of the prophage genes that is expressed codes for this protein toxin. It is the production of diphtheria toxin during an infection with *C. diphtheriae* that causes the disease diphtheria. Strains of *C. diphtheriae* that do not carry the prophage are harmless nonpathogens. Similarly the protein toxin produced by *Clostridium botulinum* (botulinum toxin) causes botulism when ingested; the protein toxin produced by *Streptococcus pyogenes* that causes symptoms of scarlet fever and some of the protein toxins produced by *Staphylococcus aureus* that cause toxic shock syndrome are all coded for by prophage genes. Only strains of these bacteria carrying prophage produce the protein toxins responsible for these diseases.

The presence of a prophage can alter the properties of a host cell.

Protein toxins coded for by prophage are responsible for some human diseases.

A prophage can be passed from one generation of host bacteria to the next indefinitely. The prophage, however, can be excised from the bacterial chromosome. This may occur when the bacterial cell is stressed by an environmental factor, such as exposure to ultraviolet light. Once excised from the host cell DNA, the regulatory genes may no longer repress the expression of all phage genes. In this case, the phage can reestablish a lytic replication cycle, with production of phage progeny and lysis of the host cell.

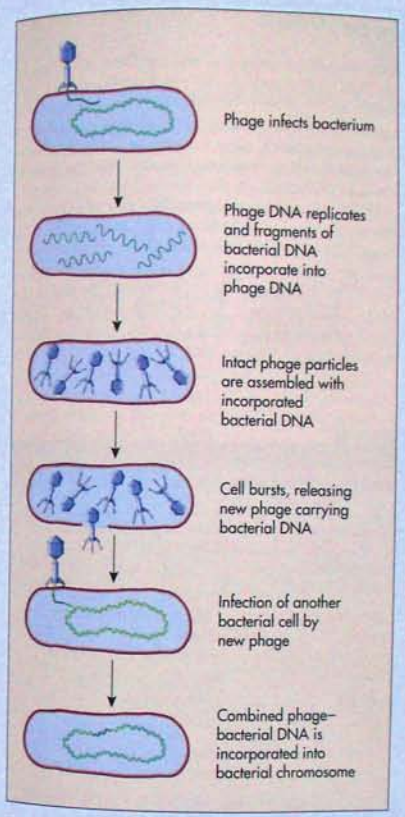


FIG. 9-8 Some viruses can carry bacterial genes. This forms the basis for transduction.

In some cases, an excised prophage carries with it some bacterial DNA (FIG. 9-8). Thus bacterial genes coded for by regions of the bacterial DNA adjacent to the site where the prophage had been inserted into the host cell DNA can be incorporated into phage progeny. When these phage infect new host cells they carry the bacterial genes they acquired. Thus these phage transfer bacterial genes from a donor cell to a recipient cell.

Such phage-mediated transfer of bacterial DNA is a form of transduction called specialized transduction, discussed in Chapter 8. Specialized transduction is so named because only certain specific genes

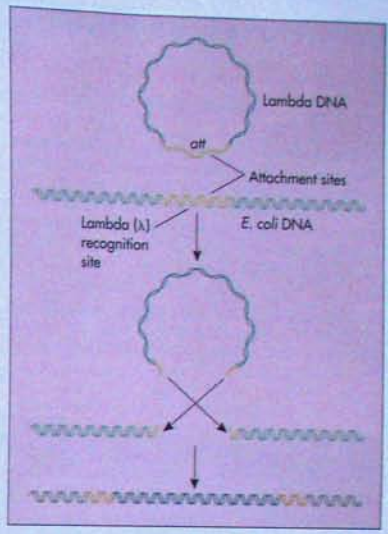


FIG. 9-9 The genome of bacteriophage lambda (λ) can integrate into the bacterial chromosome of *Escherichia coli* at specific sites. The insertion of lambda DNA always occurs at the same site of the *E. coli* genome, between the genes for galactose utilization and biotin synthesis. The lambda phage DNA has a site specific attachment gene (*att*), and a site specific attachment enzyme (integrase) is involved.

that are located adjacent to the specific site where prophage DNA is incorporated into a host bacterial cell can be transferred. For example, bacteriophage lambda (λ) can establish a state of lysogeny in *Escherichia coli* (FIG. 9-9). Lambda phage have a gene that codes for an integrase. This enzyme recognizes specific nucleotide sequences of the bacterial chromosome and inserts the lambda DNA at sites where those nucleotide sequences occur. The prophage is incorporated into the bacterial chromosome of *E. coli* at the site where the genes for biotin synthesis and galactose utilization are located. When a lambda prophage is excised it can carry with it the adjacent bacterial genes, that is, the *gal* gene for galactose utilization and/or the *bio* gene for biotin synthesis. Such lambda phage can transfer these bacterial genes, changing strains of *E. coli* that require biotin for growth into ones that do not and strains of *E. coli* that cannot utilize galactose into ones that can.

REPLICATION OF PLANT VIRUSES

Specialized transduction transfers certain specific host cell genes located adjacent to the site of the prophage to a recipient cell.

Viruses that replicate within plant cells, called **plant viruses**, follow the same basic steps described earlier for the general replication of viruses. Entry of a plant virus into a susceptible plant often involves abrasions or insect bites. The virus then adsorbs to a susceptible host cell. Both the viral capsid and the viral nucleic acid genome cross the plasma membrane of a plant cell by endocytosis if the plant cell plasma membrane engulfs the viral particle and brings it within the cell. Uncoating of the plant viral nucleic acid then occurs within the plant cell. The viral nucleic acid takes control of the synthetic activities of the host cell, directing the production of viral proteins (early protein synthesis), viral nucleic acid genomes (nucleic acid synthesis) and capsid (late protein synthesis). New viruses are assembled and the assembled viruses are released with the lysis of the host plant cell.

Plant viruses cause various diseases of plants and the viruses are often named after the disease they cause (Table 9-1). Tobacco mosaic virus (TMV), for example, causes a disease of tobacco plants characterized by the occurrence of patchy regions where the plant cells have died due to viral infection. Replicated tobacco mosaic viral particles form crystalline cytoplasmic inclusions within infected plant cells

TABLE 9-1

Some Examples of Plant Viruses and the Diseases They Cause

PLANT VIRUS	DESCRIPTION OF VIRUS	PLANT DISEASE
Cauliflower mosaic virus	Double-stranded DNA virus; reproduces in cytoplasm	Mosaic disease of cauliflower; characterized by localized patches of yellow, dying cells (due to loss of chlorophyll-containing functional photosynthetic cells) or black, dead cells
Cucumber mosaic virus	Naked, icosahedral, RNA virus	Mosaic disease of cucumber; characterized by localized patches of yellow, dying cells (due to loss of chlorophyll-containing functional photosynthetic cells) or black, dead cells
Barley yellow dwarf virus	Small isometric, RNA virus	Dwarfism of barley; characterized by lack of stem development
Tobacco ringspot virus	Polyhedral, RNA virus; transmitted by nematodes	Ringspots on tobacco; characterized by circular patches where plant cells are dying or have died
Tobacco necrosis virus	Isometric RNA virus	Tobacco rot; characterized by softening of plant structures in the regions of viral infection and by blackening of those regions due to death of plant cells
Tobacco mosaic virus	Rod-shaped, helically symmetrical, single-stranded RNA virus	Mosaic disease of tobacco; characterized by localized patches of yellow, dying cells (due to loss of chlorophyll-containing functional photosynthetic cells) or black, dead cells
Tomato bushy stunt virus	Small, cubically symmetrical, RNA virus; resistant to elevated temperatures and organic solvents	Stunting of tomato; characterized by limited growth
Turnip yellow mosaic virus	Icosahedral RNA virus; transmitted by flea beetles	Mosaic disease of turnip, characterized by localized patches of yellow, dying cells (due to loss of chlorophyll-containing functional photosynthetic cells) or black, dead cells
Watermelon mosaic virus	Flexible, rod-shaped, RNA virus; 700-950 nm in size	Mosaic disease of watermelon; characterized by localized patches of yellow, dying cells (due to loss of chlorophyll-containing functional photosynthetic cells) or black, dead cells

HISTORICAL PERSPECTIVE

PLANT VIRUSES SHOW RNA CAN SERVE AS THE HEREDITARY MACROMOLECULE

In 1935, Wendell Stanley purified and partially crystallined tobacco mosaic virus (TMV). Although it was first thought that TMV crystals were pure protein, later studies showed that they also contained RNA. Experiments with TMV were very important in establishing nucleic acids as the informational molecules in viruses and that RNA could act as the viral hereditary macromolecule .

In 1957, Heinz Fraenkel-Conrat and co-workers isolated TMV from tobacco leaves. From a common weed, they isolated a second, rather similar kind of virus, Holmes ribgrass virus (HRV). Both TMV and HRV consist of protein and a single strand of RNA. Both are plant RNA viruses that infect tobacco plants, causing lesions on the leaves. The two viruses produce different kinds of lesions, however, so that the source of a particular infection can be identified.

In Fraenkel-Conrat's experiment, TMV and HRV were each separated into their protein and RNA components. Hybrid viruses were produced by mixing the HRV RNA and the TMV protein and allowing virus particles to form by self-assembly from the RNA and protein. To determine if viral protein or viral RNA was the hereditary substance, Fraenkel-Conrat now infected healthy tobacco plants with a hybrid virus composed of TMV protein capsids and HRV RNA (see Figure).

When the reconstituted virus particles were added to tobacco leaves, the lesions that developed were of the HRV type. Normal HRV viruses were found in high numbers in the lesions. Thus the RNA from the HRV and not the protein from the TMV contained the information necessary to specify the production of the viruses. Clearly, the hereditary properties of the virus were determined by the RNA and not the protein.

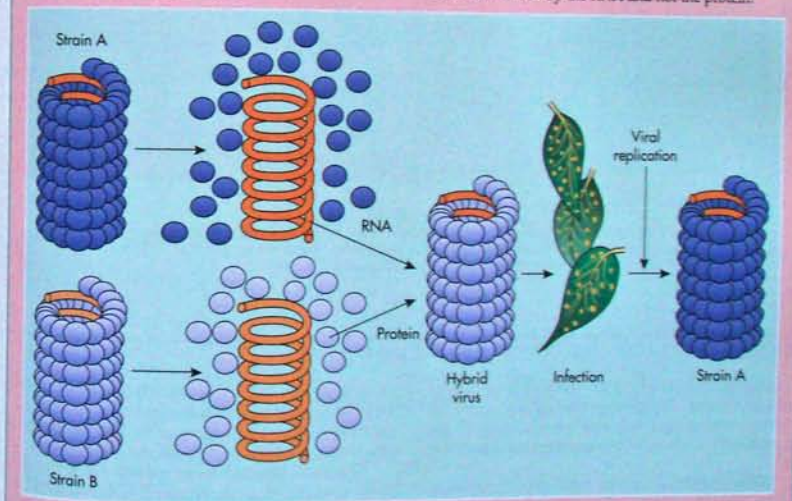


Diagram of Fraenkel-Conrat's experiment (*Strain A*, TMV; *Strain B*, HRV).

(FIG. 9-10). The chloroplast of a tobacco mosaic virus infected leaf becomes chlorotic (yellow due to loss of chlorophyll), leading to the death of the plant cell because it can no longer carry out photosynthesis to supply its cellular energy. The death of the plant cell releases completely assembled TMV progeny and viral nucleic acid that has not been packaged with the protein capsids. Within plants, both completely assembled viral particles and viral RNA can move from one cell to another, establishing new sites of infection. As a consequence of the replication of the viruses within the plant cells, the plant develops characteristic disease symptoms, which include the appearance of a mosaic pattern of chlorotic spots on the leaves that gives the disease and the virus their names.

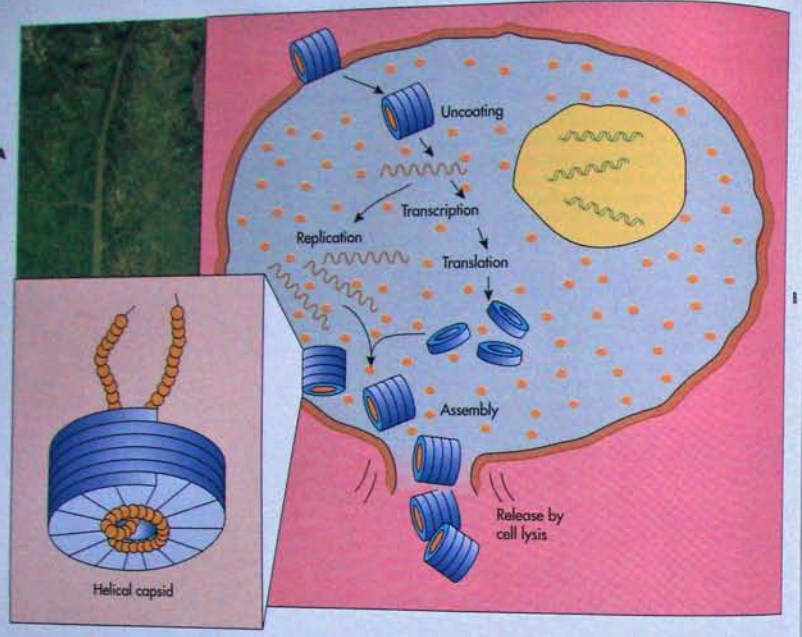


FIG. 9-10 **A,** Leaf infected with tobacco mosaic virus. **B,** The assembly of tobacco mosaic virus involves sequential addition of protein discs to surround the single-stranded viral RNA genome (the ends are shown at the top of the insert).

REPLICATION OF ANIMAL VIRUSES

Viruses that replicate within animal cells are called **animal viruses**. There are many different types of animal viruses; replication of these viruses within human cells can result in numerous diseases (Table 9-2).

The essential steps in the replication cycle of animal viruses, like those of other viruses are: (1) the adsorption of the virus to the surface of the animal cell, (2) penetration so that the intact virus or the viral nucleic acid enters the host cell, (3) uncoating so that the viral nucleic acid is released from the capsid within the host cell, (4) early protein synthesis involving transcription to form viral mRNA and translation using viral-coded messenger RNA to form proteins, (5) replication of viral nucleic acid to form copies of viral nucleic acid genome, (6) late protein synthesis to form late proteins needed for capsids, (7) assembly of complete viral particles, and (8) release of new viruses. While these essential stages for replication are the same for all viruses, animal viruses are diverse and exhibit variations in the specific details of the stages involved in replication.

The initial adsorption of animal viruses to host cells typically depends on specific chemicals on the surfaces of the virus and the host cell. These surface receptors often help establish the specificity between virus and host cell. Some viruses, such as adenoviruses—which cause upper respiratory infections—have protein spikes that project from the surface of the capsid. These spikes act as the binding sites that permit the virus to adsorb to the surface of a compatible host cell. In this way, adenoviruses attach to the cells lining the upper respiratory tract.

Adsorption of animal viruses to host cells depends on chemicals on the viral surface.

TABLE 9-2
Some Examples of Human Viruses and the Diseases They Cause

GROUP AND TYPE OF VIRUS	DESCRIPTION OF VIRUS	DESCRIPTION OF DISEASE
DOUBLE-STRANDED DNA VIRUSES		
Papilloma virus	Small virus that causes tumors	Replication of the virus within epithelial cells causes warts, which are rough, elevated benign tumors on the skin, urinary tract, or genitals
Herpesvirus	Medium to large size enveloped virus	
Herpes simplex virus (human herpes virus—HHV)		Replication of the virus within epithelial cells causes fever blisters, which are painful or itchy recurrent vesicular lesions usually of the lips or genitals; the virus goes into a dormant stage by entering nerve cells so that the body retains the virus; recurrent outbreaks of symptoms occur; genital herpes is a sexually transmitted disease
Varicella-zoster virus (VZV)		Replication of the virus causes chickenpox, which is characterized by an itchy rash on the scalp or trunk that spreads to the face and leads to vesicle formation and scabs; shingles can occur years later and is characterized by painful, nodular or vesicular lesions that appear in patches on the skin
Epstein-Barr virus (EBV)		Replication of the virus causes infectious mononucleosis, which is characterized by sore throat, swollen lymph nodes, temperature and fatigue; the virus often is transmitted through exchange of saliva
Cytomegalovirus (CMV)		Replication of the virus causes generalized salivary gland disease, which is characterized by sore throat, swollen lymph nodes, fever, and fatigue
Poxvirus	Very large, enveloped, brick-shaped viruses	
Variola virus		Replication of the virus causes smallpox, a once deadly disease that has been eliminated through an effective program of vaccination
SINGLE-STRANDED (PLUS-STRAND) RNA VIRUSES		
Picornavirus	Very small, nonenveloped virus that infects the respiratory tract or gastrointestinal tract	
Poliovirus		Replication of the virus can occur in various body tissues; replication within the nervous system can cause poliomyelitis (commonly called polio or infantile paralysis) that is characterized in some cases by loss of motor function (paralysis); in some cases, viral replication within the spinal column causes meningitis; the virus is often transmitted via contaminated food or water
Rhinovirus		Replication of the virus within the respiratory tract causes the common cold, bronchitis, and croup, which usually are characterized by congestion, coughing, sneezing, and a mild fever
Hepatitis A virus (HAV)		Replication of the virus within the liver causes infectious hepatitis (hepatitis A), which is characterized by a high fever and jaundice (yellowing of the skin due to loss of liver function); the virus is often transmitted via contaminated food or water
Coxsackievirus		Replication of the virus within the respiratory tract causes the common cold, and replication within the oral cavity causes herpangina, which is characterized by lesions at the back of the mouth

Continued.

TABLE 9-2—cont'd

Some Examples of Human Viruses and the Diseases They Cause

GROUP AND TYPE OF VIRUS	DESCRIPTION OF VIRUS	DESCRIPTION OF DISEASE
SINGLE-STRANDED (PLUS-STRAND) RNA VIRUSES—cont'd		
Togavirus	Small, enveloped virus; some transmitted by insects	
St. Louis encephalitis		Replication of the virus within brain cells causes encephalitis, which is characterized by coma and often is fatal; the virus is transmitted via mosquitoes
Rubella virus		Replication of the virus causes German measles, which is characterized by the eruption of a skin rash
Hepatitis C virus (HCV)		Replication of this virus within the liver causes hepatitis C, which is also called non-A, non-B hepatitis; hepatitis C is characterized by a high fever and jaundice (yellowing of the skin due to loss of liver function); the disease is transmitted via blood
Flavivirus	Small, enveloped viruses	
Yellow fever virus*		Replication of the virus within the liver causes yellow fever, which is characterized by a high fever and jaundice (yellowing of the skin due to loss of liver function); the virus is transmitted via mosquitoes
SINGLE-STRANDED (MINUS-STRAND) RNA VIRUSES		
Orthomyxovirus	Medium to large size, enveloped virus	
Influenza A, B, and C viruses		Replication of these viruses within the respiratory tract causes influenza, which is commonly called flu; influenza is characterized by a high fever, cough, malaise, and body ache; the virus is transmitted through the air; secondary infections can cause fatalities due to pneumonia; susceptible high-risk individuals (elderly and immunocompromised individuals) should be vaccinated to prevent this disease
Paramyxovirus	Medium to large size, enveloped virus	
Measles virus		Replication of this virus causes measles, which is characterized by a red skin rash, high fever, cough, and malaise
Mumps virus		Replication of the virus causes mumps, which is characterized by swelling of one or both salivary glands
Rhabdovirus	Medium-size, enveloped, bullet-shaped virus	
Rabies virus		Replication of the virus within the nervous system causes rabies, which is initially characterized by sensitivity to stimuli such as light and noise, difficulty in swallowing, a fear of water (hydrophobia), followed by delirium, coma, and death; the disease is usually transmitted via the bites of infected animals or in some cases by inhalation and is prevented by vaccination
DOUBLE-STRANDED RNA VIRUSES		
Reovirus		
Rotavirus	Small, naked virus	Replication of the virus within the gastrointestinal tract causes acute infantile gastroenteritis characterized by severe diarrhea that is a common disease in children
Reovirus		Replication of the virus in humans does not cause a recognized disease

*Yellow fever virus is a flavivirus, a separate group of viruses (flavi means yellow).

TABLE 9-2—cont'd

Some Examples of Human Viruses and the Diseases They Cause

GROUP AND TYPE OF VIRUS	DESCRIPTION OF VIRUS	DESCRIPTION OF DISEASE
DOUBLE-STRANDED RNA VIRUSES CONTAINING REVERSE TRANSCRIPTION		
Retroviruses	Medium-size, enveloped virus	
Human immunodeficiency virus (HIV)		Replication of the virus within T lymphocytes of the immune defense system causes acquired immunodeficiency syndrome (AIDS), which is characterized by a loss of immune defenses against infections, leading to opportunistic infections by various pathogens and eventual death
Human T cell leukemia virus (HTLV-I and HTLV-II)		Replication of the virus within T lymphocytes causes leukemia, which is characterized by the malignant growth of infected cells

Some animal viruses are uncoated outside of the host cell so that only the viral nucleic acid enters the host cell. This occurs in enteroviruses, such as polioviruses, which are single-stranded RNA viruses. Most animal viruses, however, enter the host cell by endocytosis and uncoating occurs within the host cell (FIG. 9-11). The plasma membrane of the host cell surrounds the adsorbed virus, forming a membrane-bound vesicle. This vesicle is released within the cell. In some cases the vesicle containing the virus fuses

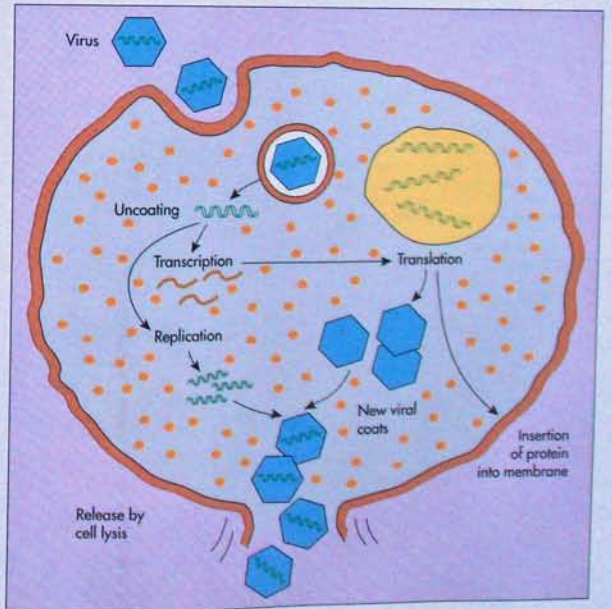
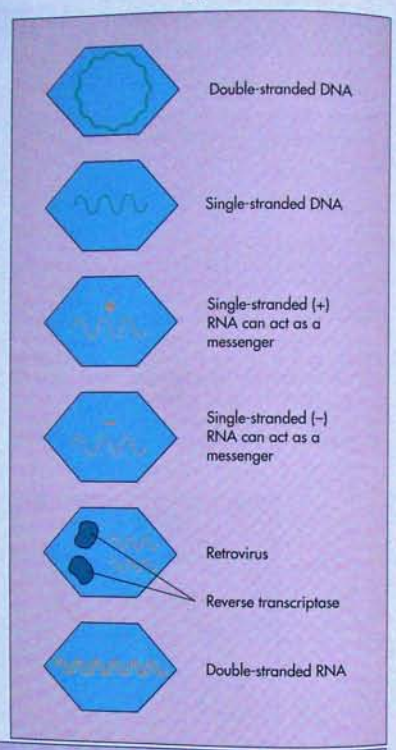


FIG. 9-11 Viruses can enter host cells in several ways. Some animal and plant viruses enter the host cell via endocytosis and subsequently are released into the cytoplasm.

with a lysosome. A lysosome is an organelle that contains digestive enzymes. Lysosomal enzymes degrade the capsid, releasing the viral nucleic acid. In other cases, such as for herpesvirus, the virus is released within the cytoplasm where uncoating occurs when enzymes attack the capsid.

Some animal viruses contain enzymes as well as nucleic acid within the capsid. These viral enzymes, which are released during uncoating, can initiate the synthesis of viral nucleic acid and proteins. The subsequent process of protein synthesis and nucleic acid replication varies greatly, depending on the nature of the nucleic acid acting as the hereditary macromolecule. Some animal viruses are single-stranded DNA, others double-stranded DNA, others single-stranded RNA, and yet others double-stranded RNA. Animal viruses are further distinguished based on the relationship between the nucleic acid of the virus and the viral mRNA (FIG. 9-12). A viral mRNA is designated as a plus strand and its complementary sequence that cannot be used as an mRNA is called a minus strand. Similarly, a DNA strand complementary to a viral mRNA is designated as a minus strand. Thus it is the minus strand of DNA or RNA that serves as the template for the formation of viral mRNA.



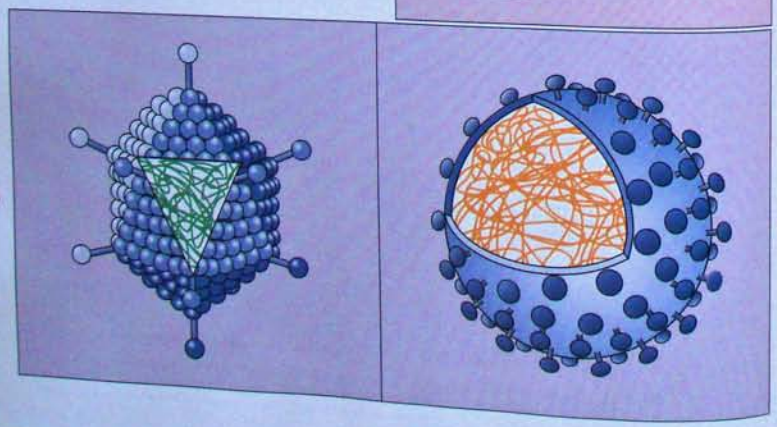


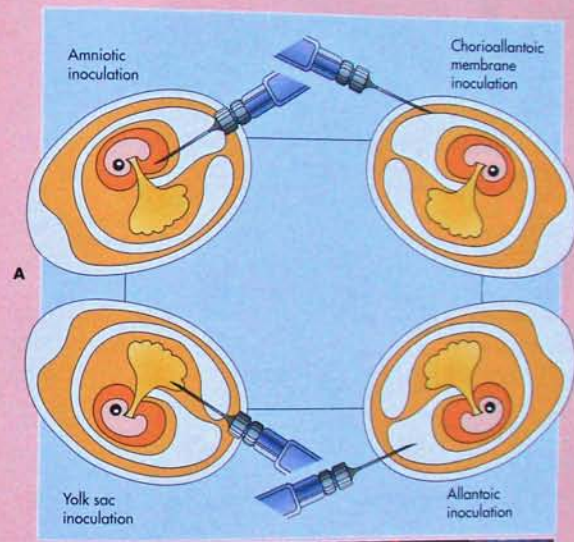
FIG. 9-12 **A,** Viruses have a central nucleic acid core, which may be RNA *(gold)* or DNA *(green)* surrounded by a protein coat called a capsid *(blue)*. **B,** Some animal viruses, such as adenovirus, have isometric symmetry and a DNA genome. **C,** Other viruses, such as coronavirus, have complex capsids and an envelope with protruding proteins surrounding an RNA genome.

METHODOLOGY

Laboratory Culture of Animal Viruses

Because viruses only replicate within host cells, the laboratory culture of animal viruses requires that living animal cells be used. In some cases, whole live animals such as mice, rabbits, guinea pigs, and monkeys are used. Some animal viruses can be cultured only in such living animals.

In other cases, animal viruses are cultured in embryonated eggs (fertilized eggs in which an embryo is developing) (FIG. *A*). Most often, embryonated chicken and duck eggs are used. A hole is made through the egg shell and a suspension containing the virions is injected. The cultured viruses can later be harvested and used for

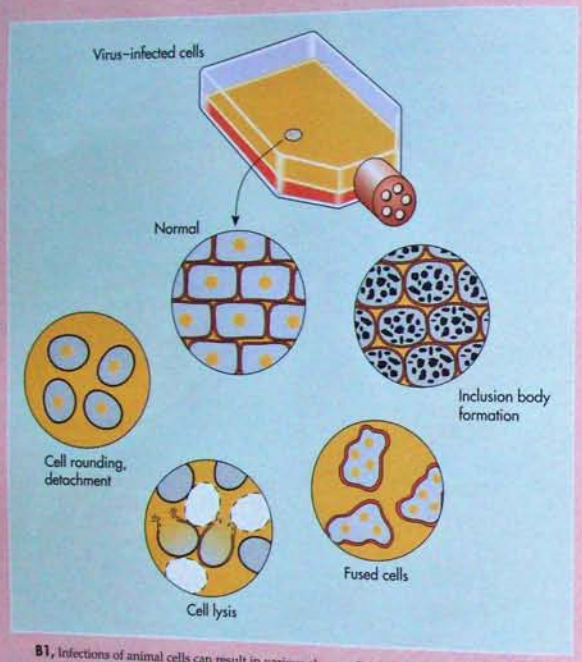


A, Inoculation of eggs to grow viruses. **B,** Viruses being cultured for influenza vaccine production.

Continued

Laboratory Culture of Animal Viruses—cont'd

B1



B1, Infections of animal cells can result in various abnormalities known as cytopathic effects.

scientific studies or for other uses, such as vaccine production.

It is possible to grow animal cells in a fluid broth, called tissue cultures. Viruses can then replicate within the laboratory grown animal cells. Cultivation of viruses in tissue cultures has largely replaced culture in embryonated eggs. For example, rabies vaccine, which used to be made by culturing rabies viruses in embryonated duck eggs, is now made by cultivating rabies viruses in tissue cultures of human fibroblast cells.

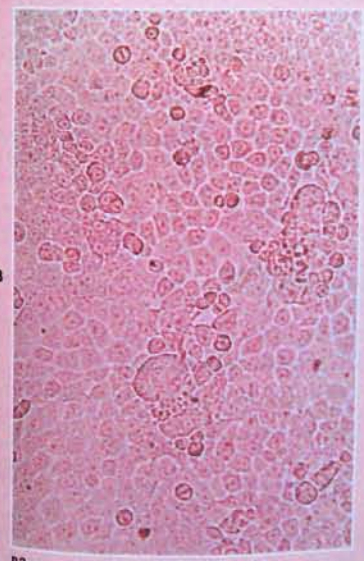
Cultivation of animal viruses in tissue culture can be used to determine the numbers of viruses in a suspension, in a method analogous to the plaque assay for enumeration of bacteriophage (FIG. B). In a typical procedure, a tissue culture monolayer of animal cells (single layer of animal cells) growing on a plate surface is inoculated with dilutions of a viral suspension and incubated for various periods of time. Viral infection of the animal tissue culture cells may result in plaque formation, indicative of localized death of animal cells, which can be observed microscopically, or, more commonly, with the naked eye. The number of plaques that form and the dilution factors are employed to determine the concentration of viruses in the sample.

Additionally, virus-infected animal cells often develop abnormally. Such abnormalities are visible as a change in appearance, known as the cytopathic effect (CPE). For example, inclusion bodies may form within a cell infected with a virus, the size or shape of the nucleus may change in a cell infected with a virus, or other visible changes may occur. It is possible to observe CPE in animal cell cultures and to determine the number of viruses by counting the number of cells exhibiting the characteristic morphological changes.

B2

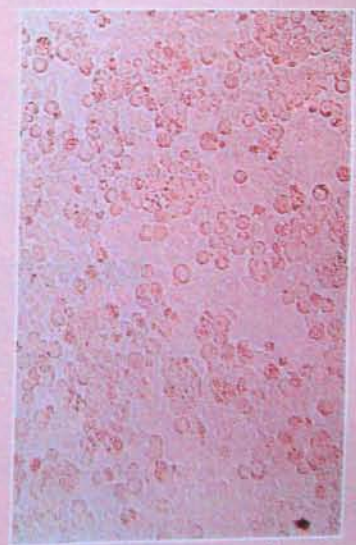
B2, Viruses being grown in tissue culture and observed with an inverted microscope to detect cytopathic effects (CPEs) in the cell culture.

B3



B3, Light micrograph showing the cytopathic effect on HEp-2 cells grown in tissue culture by an infection with adenovirus.

B4



B4, Light micrograph showing the cytopathic effect on HEp-2 cells grown in tissue culture by an infection with respiratory syncytial virus.

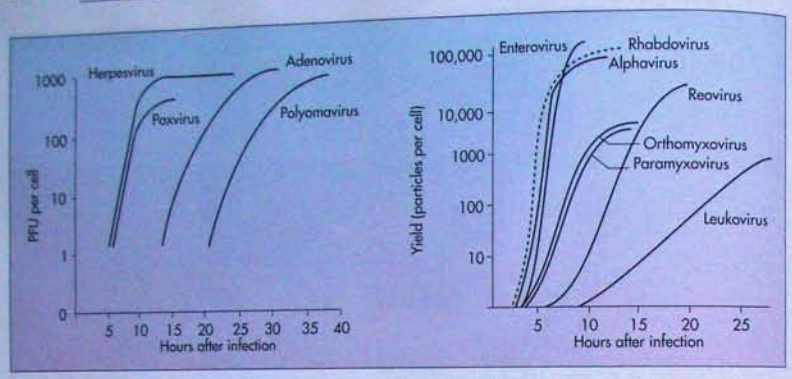


FIG. 9-13 Replication of animal viruses often takes hours, as shown as the time needed to increase the plaque forming units (PFU), or yield, of viral particles.

The DNA of animal viruses generally enters the nucleus. There it is replicated, whereas RNA animal viruses need only enter the cytoplasm of the animal cell to be replicated. In some cases, viral assembly occurs within the cytoplasm, whereas other animal viruses are formed within the nucleus of the host cell.

Once assembled, the virions can be released in one of two ways. Some viruses, such as the rhinoviruses that cause the common cold, are released by lysis and hence death of the host cell. Such viruses exhibit a replication cycle, which closely resembles that of lytic bacteriophage. In such instances there is a step-wise growth curve, with a burst of a large number of viruses released simultaneously. Unlike bacteriophage, however, the single-step growth curve for animal viruses occurs in hours rather than minutes (FIG. 9-13).

Other viruses are released gradually from living host cells by a process called **budding** (FIG. 9-14). Viruses released in this manner include the AIDS-causing human immunodeficiency virus (HIV) and the herpesviruses. Budding is a form of exocytosis in which the plasma membrane of the host cell engulfs an assembled virus, forming a vesicle that is transported outside of the cell. Thus the viruses released are enclosed within a membrane that comes from the host cell and are said to be enveloped. Budding release generally results in a protracted infection in which the host cell may be debilitated.

Budding releases enveloped viruses without killing the host cell.

NEWSBREAK

Controversy Over Plans to Destroy Remaining Smallpox Virus

Although the disease smallpox has been eliminated through an extensive worldwide immunization program, a few stock cultures of the smallpox virus are maintained in the United States and Russia for scientific studies. A bilateral agreement to destroy these last remaining smallpox viruses, after their DNA sequences have been determined, has sparked controversy within the scientific community. Some scientists feel that biodiversity must be preserved and the stocks of smallpox virus should be maintained. These scientists point to the fact that the stock cultures were deposited in culture collections with the expectation that they would be maintained forever. They argue that as long as such cultures remain in secure collections there is no public health danger. Other scientists feel that smallpox viruses should be further studied as a model of how viruses cause disease. These scientists argue that alternate animal hosts should be developed to permit such studies, even though it raises the risk of reestablishing smallpox as an infectious disease. Public health officials argue that scientific curiosity doesn't warrant the health risk. They point out that the last case of smallpox resulted from an accidental release of the smallpox virus from a laboratory in England where the virus was being studied. The American Society for Microbiology supports destruction of the smallpox virus, to be considered as a special case in the interest of protecting human health. A panel of scientists is expected to meet to read a final decision on the fate of the last remaining smallpox viruses.

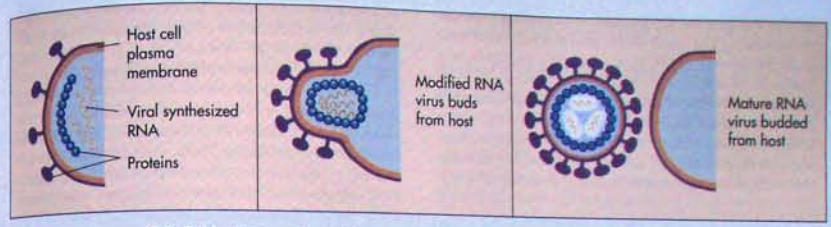


FIG. 9-14 Some enveloped viruses are released from the host cell by budding.

Replication of DNA Animal Viruses

Double-stranded DNA Viruses

The replication of **adenoviruses**, which often causes respiratory tract infections, is representative of the replication of the double-stranded DNA animal viruses. Following penetration of the virus, the host cell continues its normal metabolic activities for a short period of time (FIG. 9-15). It may take several hours before uncoating of the virus occurs. The uncoated viral nucleic acid genome enters the nucleus

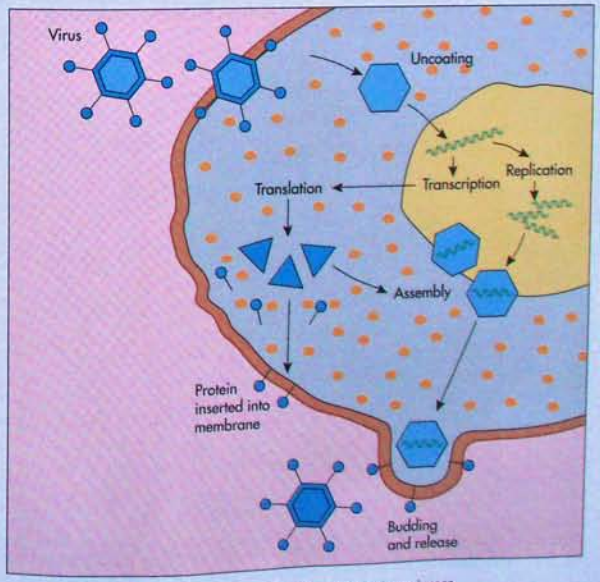


FIG. 9-15 The replication cycle of adenoviruses.

where it codes for the inhibition of normal host cell metabolism. The viral DNA genome acts as a template for its own replication, directing enzymes normally involved in cellular metabolism to make viral nucleic acids and proteins. Viral proteins produced at the ribosomes in the cytoplasm of the host cell move into the nucleus where assembly of adenoviruses occurs. Accumulation of adenoviruses within the nucleus produces inclusion bodies consisting of crystalline arrays of densely packed adenoviruses (FIG. 9-16). The accumulation of adenoviruses leads to lysis and death of the host cell. With lysis of the host cell, numerous adenoviruses are released.

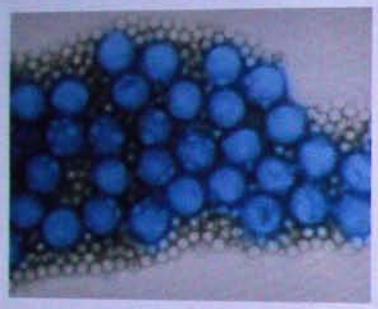


FIG. 9-16 Colorized micrograph of densely packed adenoviruses. (120,000 ×).

Herpesviruses, which include those that cause genital herpes and infectious mononucleosis, are similarly assembled within the nuclei of the host cells in which they replicate. These viruses acquire an envelope of host cell membrane lipids when the assembled viruses bud through the nuclear membrane. Thus herpesviruses are enveloped viruses that are surrounded by a portion of host cell nuclear membrane. Enveloped herpesviruses are released slowly from the host cell by exocytosis. Once a herpesvirus infection occurs, the host cells remain infected and a latent herpesviral infection persists for the entire life of the host. During latency, the viral nucleic acid persists within the neurons of the nerve ganglia. There is no release of viruses during this period.

Poxviruses, such as the smallpox virus, use a somewhat different replication strategy (FIG. 9-17). Poxviruses are large double-stranded DNA viruses that carry their own RNA polymerase protein, which they use to make mRNAs. These viruses are able to replicate within the cytoplasm of an infected host cell. They are released when there are sufficient assembled viruses to initiate host cell lyses. These released viruses are enveloped; they acquire the envelope from the nuclear membrane.

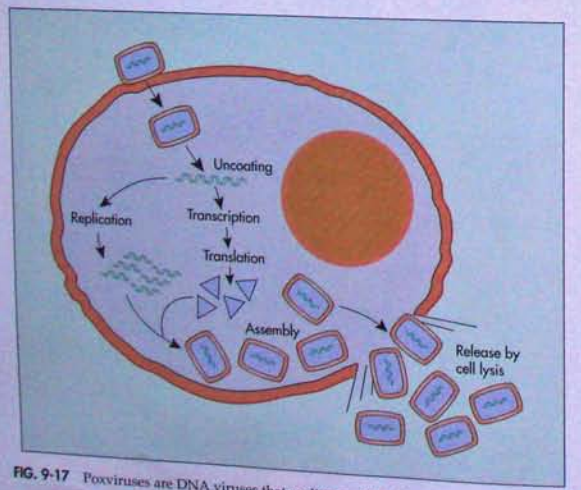


FIG. 9-17 Poxviruses are DNA viruses that replicate within the cytoplasm of a host cell.

Single-stranded DNA Viruses

Parvoviruses contain a single strand of DNA. Many pet owners worry about parvovirus infections that can cause lethal infections of dogs. Some populations of parvoviruses contain only a minus strand or a plus strand of DNA, whereas other populations have roughly equal numbers of viruses with plus and minus strands of DNA. Within a host cell the single strand of DNA of a parvovirus is copied to make a double-stranded DNA macromolecule. Replication of this double-stranded DNA can produce plus and minus strands of DNA for incorporation into the virions.

Replication of RNA Animal Viruses

Double-stranded RNA Viruses

Reoviruses (respiratory enteric orphan viruses) are double-stranded RNA viruses that carry an RNA polymerase that is used for the synthesis of new viral RNA molecules. Reoviruses, such as rotavirus that causes diarrhea in children, contain several different double-stranded RNA molecules. Each of the RNA molecules codes for the production of a different protein. The proteins are assembled into the viral capsid and the viral RNA produced during replication is inserted into the capsid before release of the completed reoviruses (FIG. 9-18).

Single-stranded RNA Viruses

The single-stranded RNA **picornaviruses,** such as poliovirus, are among the smallest viruses (pico means small). Because poliovirus is a plus strand virus, the RNA genome can function as an mRNA. The poliovirus RNA is translated into proteins that inhibit the host cell's synthesis of RNA and protein. The RNA genome also codes for the production of a large polypeptide that is subsequently cleaved to form several different proteins, including an RNA polymerase and the proteins used to make the viral capsid (FIG. 9-19). The RNA polymerase is used to produce a complementary **replicative minus RNA strand,** which then can serve as a template for the synthesis of new plus strand viral RNA genomes. The assembly of the capsid and insertion of the RNA is followed by the release of a large number of viral particles. Release of the poliovirus occurs because blockage of cellular protein synthesis by the poliovirus leads to breakdown of lysosomes. Lysosomal digestive enzymes lyse the host cell.

In the case of **influenza viruses,** the individual single strands of viral RNA are minus strands that

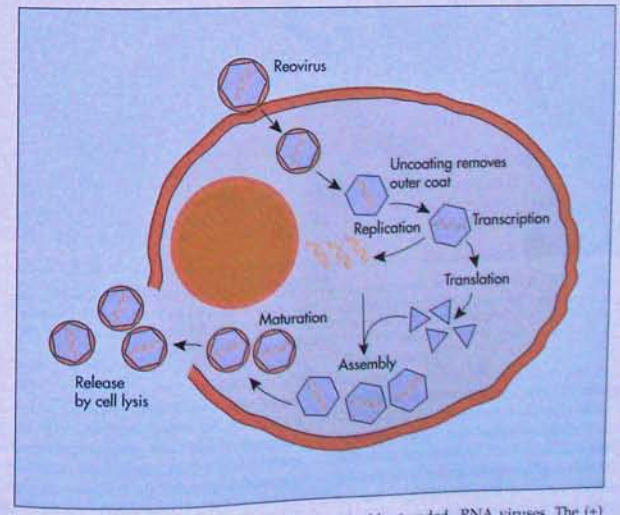


FIG. 9-18 Replication of reoviruses, which are double-stranded RNA viruses. The (+) RNA molecules are placed into capsids, and integral capsid-RNA replicase uses this as a template to form the (−) strand of the double-stranded RNA genome.

HIGHLIGHT

How New Viral Diseases Originate

Periodically, new infectious viral diseases emerge, challenging scientists and physicians to find their origins. In some cases, these diseases may have existed for some time but have gone undetected. In other cases, genetic changes due to mutation or recombination may have led to new strains with altered virulence. Periodic outbreaks of influenza are due to such genetic changes that result in the evolution of new strains of influenza viruses. Other theories are needed to explain the emergence of seemingly new pathogenic viruses, such as the human immunodeficiency virus that causes AIDS in the late 1970s and the hantavirus that killed over 20 people in the summer of 1993 (mostly Navajos) in the southwest United States.

In recent years, scientists have found evidence that changing environments is a major cause of emerging infectious diseases. Construction of roadways through jungles and rain forests may allow pathogens to spread rapidly to huge numbers of people. One of the most dramatic indications that humans were disrupting the balance between pathogens and humans came when Brazil built a highway deep in Amazonian jungle to its new capital, Brasilia. Soon after construction of the highway in the 1950s, viruses, some of which were unknown, were found in the blood of highway workers. One of these viruses, the Oropouche virus, also was found in the blood of a sloth dead at the side of the highway. Oropouche virus was not known to be responsible for epidemics in humans or animals before 1960. In 1961, the Oropouche virus was identified as the cause of a flu-like epidemic in Brazil that afflicted 11,000 people.

While it was clear that Oropouche was to blame for the epidemic, it was not clear how a virus never seen in human beings before had emerged to cause a new disease. Finding the answer took scientists almost two decades. In 1980, the Oropouche virus was isolated from biting midges. During construction of the highway through the Amazonian jungle, the midges had undergone a population explosion. This led to a huge increase in vectors carrying the Oropouche virus.

Similar environmental changes may underlie the emergence of the new viruses that cause AIDS, Ebola hemorrhagic fever, Marburg hemorrhagic fever, and yellow fever—where the viruses probably initially occurred in monkeys; Rift Valley fever—where the viruses probably initially occurred in cattle, sheep, and mosquitoes; and Hantaan virus—where the viruses probably initially occurred in rodents.

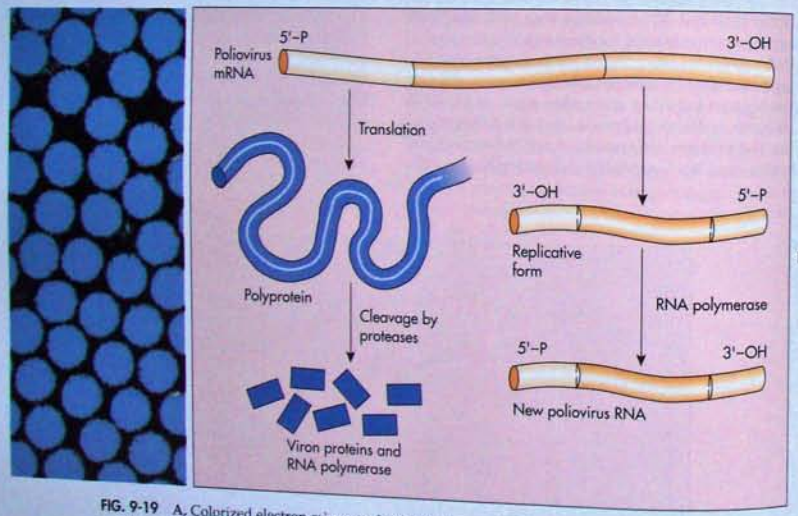


FIG. 9-19 **A**, Colorized electron micrograph of polioviruses. **B**, Poliovirus RNA serves as a messenger for production of a polyprotein that is then cleaved to form capsid proteins and RNA polymerase. The RNA also serves as the template for producing a replicative form that, in turn, is the template for new poliovirus genomic RNA.

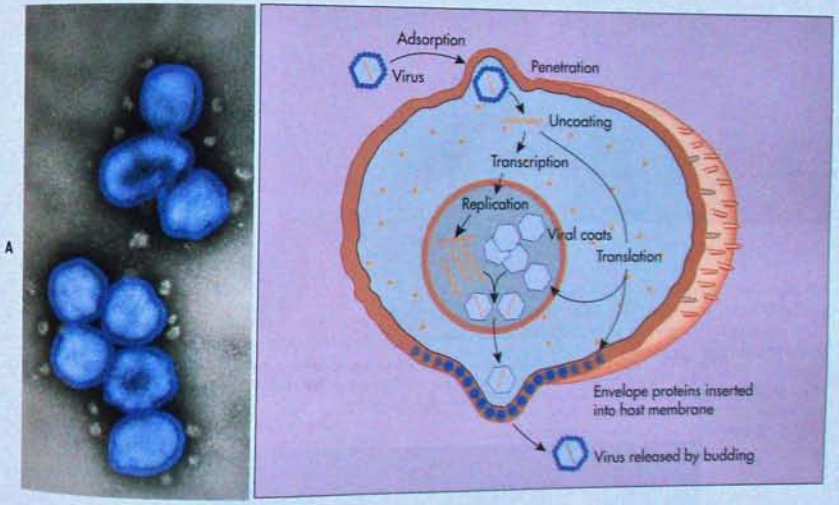


FIG. 9-20 **A**, Colorized electron micrograph of influenza viruses (72,000 ×). **B**, Sequencce of events during influenza virus replication.

serve as templates for the transcription of mRNAs. Since it is a minus strand it does not directly act as the mRNA. An influenza virus has eight different RNA molecules, each of which codes for a different mRNA. One of these RNA molecules codes for the RNA polymerase required for the production of mRNA. Replication of the minus strand of viral RNA involves the production of a complementary RNA strand that then serves as a template for the synthesis of new minus RNA. Release of influenza virions occurs by budding and the virions are thus enveloped in host cell plasma membrane. One of the proteins on the surface of the virus, neuramidase (N), protrudes through the surrounding membrane that becomes the viral envelope and facilitates the release of the influenza virus. Another protein, hemagglutinin (H), also projects from the surface of an influenza virus through the envelope. Hemagglutinin is important in the ability of the released viruses to adsorb and enter into new host cells (FIG. 9-20). There are several chemically different H and N proteins and different influenza viruses are designated by these proteins, for example, as H_2N_2. Each year as the flu season approaches, the Centers for Disease Control release information on the strains of influenza viruses that are anticipated to cause major outbreaks of influenza. These strains are designated by the H and N proteins and these designations are generally reported by the local news media.

Retroviruses

Retroviruses are RNA viruses that use **reverse transcriptase** to produce a DNA molecule within the host cell (FIG. 9-21). Reverse transcriptase is an RNA-directed DNA polymerase. The process of making DNA using an RNA template is called **reverse transcription.** The retroviruses use their RNA as a template for producing a complementary DNA that is then integrated into the host genome. The information in the DNA molecule is used to direct the synthesis of RNA, which is accomplished by transcrip-

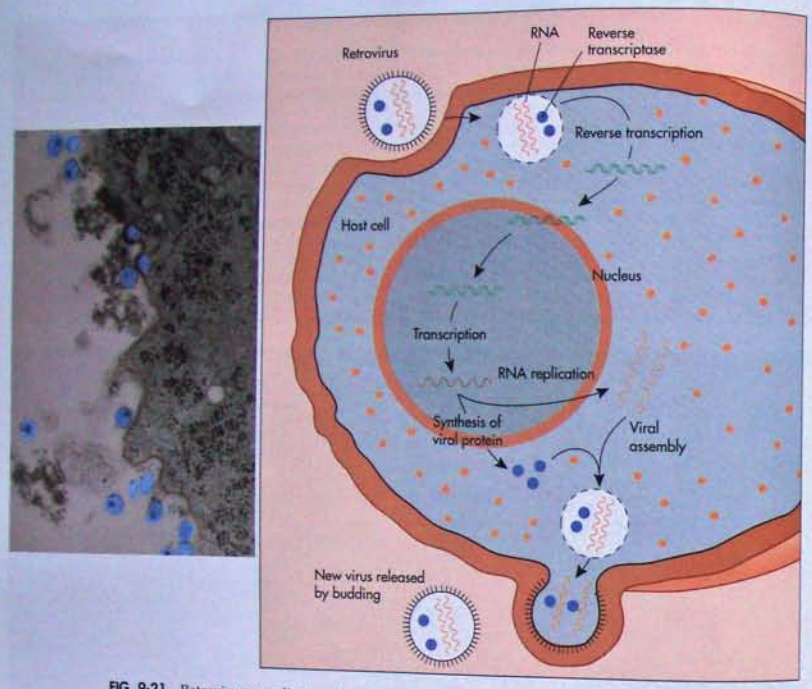


FIG. 9-21 Retroviruses replicate using reverse transcriptase to form DNA that is used to produce viral proteins and RNA genomes for viral progeny. The electron micrograph *(left)* shows the viruses *(blue)* at the surface of a cell.

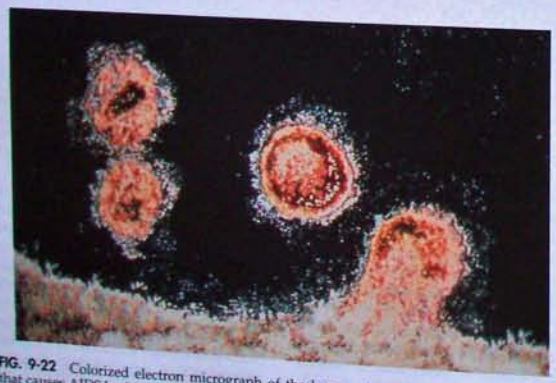


FIG. 9-22 Colorized electron micrograph of the human immunodeficiency virus (HIV) that causes AIDS budding from the host cell that produced it.

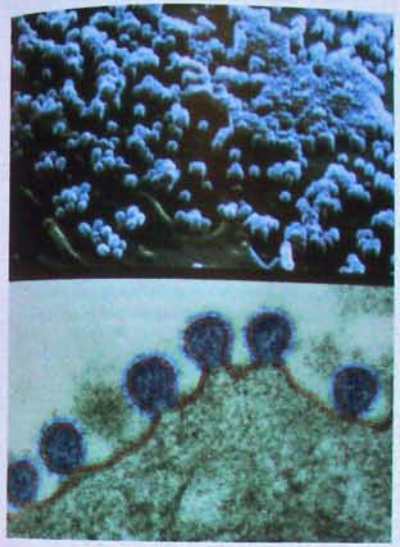


FIG. 9-23 **A,** Colorized scanning electron micrograph showing budding release of human immunodeficiency virus. **B,** Colorized transmission electron micrograph showing budding release of human immunodeficiency virus.

tion. Some of the RNA acts as mRNA for synthesizing viral proteins, and some of the RNA is put into the RNA of the viral progeny.

Retroviruses carry out reverse transcription, using reverse transcriptase to make DNA from an RNA template.

Retroviruses are released from host cells by budding. This replication does not result in cell lysis and immediate cell death, therefore these viruses can be released slowly and continuously from infected host cells. Eventually, however, the siphoning of cellular resources for viral replication can cause the death of the host cell. The AIDS-causing human immunodeficiency virus (HIV) is a retrovirus that replicates in this manner (FIG. 9-22). Infections with HIV are persistent because of the budding release of assembled viruses from the host cells (FIG. 9-23). The replication of HIV within T lymphocytes, which are essential cellular components of the body's immune defense system, causes a great decrease in the ability of the body to defend itself against microbial infections. Individuals with AIDS thus are susceptible to various opportunistic infections and eventually succumb to the onslaught of such infections.

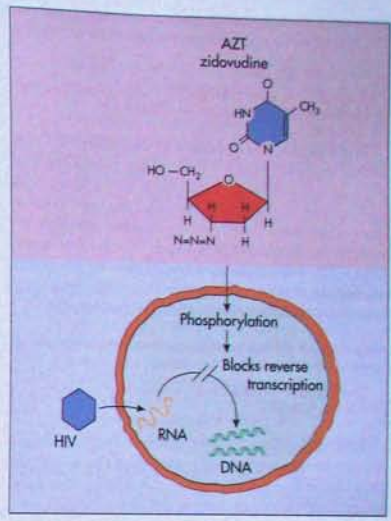


FIG. 9-24 Illustration of action of AZT in blocking HIV replication.

The progress of AIDS can be slowed by using the drug azidothymidine (AZT), also called zidovudine. AZT is incorporated instead of thymidine nucleotides when DNA is made by reverse transcription (FIG. 9-24). The presence of AZT can block replication of HIV and therefore is useful in treating individuals with HIV infections. It does not, however, eliminate all infected cells and is not a cure for AIDS.

Transformation of Animal Cells

In some cases, infection with an animal virus does not lead to viral replication. Rather, the viral DNA is incorporated into the chromosomal DNA of the host cell. The DNA produced by reverse transcription during the replication of retroviruses, as well as the DNA of some other viruses such as herpes and papillomaviruses, can be incorporated into the host cell's chromosomes. This situation is analogous to that of temperate phage, where the phage DNA is incorporated into the DNA of a host bacterial cell as a prophage. In animal cells the integrated viral DNA is called a **provirus.** Like the prophage, such incorporated viral DNA can be passed from one generation of animal cells to another. It is therefore possible for animals to inherit viral genes. Within the chromosomes of the host cell the viral DNA can be transcribed, resulting in the production of viral-specific

RNA and viral proteins. Animal cells carrying viral DNA may have different properties than uninfected cells.

A provirus is viral DNA that is incorporated into the chromosomal DNA of the host cell.

In particular, the presence of viral-derived DNA within the host cell can transform the animal cell into a malignant (cancerous) cell. Such transformed cells have altered surface properties and continue to grow even when they contact a neighboring cell. This results in the formation of a tumor. Viruses that transform cells and cause cancerous growth are called **oncogenic viruses.** The genes that actually induce cancerous transformations are called **oncogenes.**

Oncogenic viruses are viruses that transform cells so as to cause cancerous growth.

Oncogenes are genes that induce such cancerous transformations.

Viral oncogenes are very similar in nucleotide sequence to oncogenes that occur naturally in animal cells. Animal cells have proto-oncogenes that code for genes involved in normal cellular growth. If activated, these proto-oncogenes act to cause transformation of the cell so that it begins to grow malignantly and rapidly and no longer stops growing when it contacts a neighboring cell (loss of contact inhibition). A viral oncogene that becomes integrated into the DNA of an animal host cell causes transformation only when it is expressed. If a viral oncogene is inserted next to an active promoter (site where RNA synthesis begins), a high level of transcription of the viral oncogene ensues and mRNA is produced. Translation of these mRNAs forms proteins that appear to be involved in transforming a normal cell into a malignant one.

Many oncogene-coded proteins are kinases, which are enzymes that are involved in the transfer of phosphate from ATP to various organic compounds. One normal function of a kinase is to add phosphate to either the amino acid serine or threonine. In normal (noncancerous) cells, phosphate is not added to tyrosine, but in transformed (cancerous) cells, phosphate is added to tyrosine by a kinase. The presence of phosphorylated tyrosine within a protein alters the function of that protein. Apparently the normal regulatory functions of the cell are thus altered. These proteins often become concentrated in the cell nucleus, suggesting that they act as regulator proteins to alter gene expression. Some proteins coded for by oncogenes are known to enhance transcription of a number of genes. Such genes may be central in cell growth and division.

The activation of multiple oncogenes may be necessary to actually cause cancer. In many cases such activation does not involve viruses. Such activity can be the result of exposure to mutagenic agents that alter gene regulation so that the oncogenes are expressed. **Carcinogens** are agents that cause gene mutations that lead to cancer. Known carcinogens include numerous natural and synthetic compounds such as asbestos, benzene, substances in cigarette smoke, X-rays, gamma rays, and ultraviolet radiation.

Carcinogens are natural or synthetic compounds that cause genetic mutations that lead to cancer.

Additionally, various viral infections may lead to specific forms of cancer. Papillomaviruses, which cause genital warts, frequently are found in cancerous cervical cells. This suggests that these viruses have oncogenes that can cause human cervical cancer. The Epstein-Barr virus, a herpesvirus that causes infectious mononucleosis, appears to be the cause of Burkitt's lymphoma and nasopharyngeal carcinoma, a cancer of the nose and throat. Burkitt's lymphoma is a rare cancer of the lymphatic system that mostly affects children in Africa. Among the RNA viruses, only the retroviruses form DNA during their replication that can be integrated into the host cell's chromosomes. Hence, it is not surprising that the only RNA viruses that are oncogenic are retroviruses. Human T cell leukemia viruses (HTLV 1 and HTLV 2) are examples of such retroviruses that have been shown to cause some types of human leukemia. Leukemias are cancers affecting the white blood cells, and the human T cell leukemia viruses specifically cause transformation of one type of white blood cell: the T cells that are involved in protecting the body against infections.

HISTORICAL PERSPECTIVE

Discovery of Oncogenes

Oncogenes were discovered in 1975 by J. Michael Bishop and Harold Varmus. They found noncancerous uninfected chicken cells that contained a gene almost identical to the *src* oncogene of Rous sarcoma virus. This is an RNA retrovirus that causes malignancies in chickens. When Rous sarcoma virus infects chickens, the *src* gene escapes normal gene regulation, but it is transcribed and proteins are synthesized that are coded for by the viral RNA. Transcription of the *src* gene results in excessive cell growth and malignancy development. Since then, more than 30 viral oncogenes have been discovered that have gene sequences nearly identical to the normal DNA of the animal cells.

SUMMARY

General Aspects of Viral Replication (pp. 257-258)

- Viruses are composed of a nucleic acid, either RNA or DNA, and a capsid made of protein that surrounds the nucleic acid.
- Within host cells, viral nucleic acid directs the formation of new viruses, using the host cell's ribosomes for producing viral proteins and ATP for carrying out synthesis of viruses.
- A high degree of specificity is required between a virus and the host cell within which it can replicate.

Stages of Viral Replication (p. 257)

- The stages of viral replication generally include adsorption of the virus to specific binding sites on the host cell's surface, penetration of the cell's cytoplasmic membrane by viral nucleic acid, control of the cell's metabolic activities by the viral nucleic acid, use of the host's biochemical components and anatomical structures for production of viral replicates, and release of new viruses.

Replication of Bacteriophages (pp. 258-263)

Lytic Phage Replication (pp. 258-261)

- Bacteriophages are viruses that replicate only within specific host bacterial cells. They are called lytic phages if their replication results in the lysis of the host bacterial cell when the new phages are released.

T-even Phage Replication (pp. 259-260)

- T-even phages are double-stranded DNA viruses whose capsids have distinct head and tail structures. The tail attaches to specific receptor sites on the host and releases lysozyme that breaks down the cell wall, allowing the tail to penetrate. Contraction of the tail forces the phage DNA from the head into the periplasmic space. Normal host cell biosynthesis ceases as the synthesis of proteins for new phage nucleic acids and capsids is directed by the viral nucleic acid. The head and tail units are assembled separately and later combined. Phage DNA is packaged in the head structure. The phage codes for lysozyme, which helps break down the bacterial peptidoglycan wall structure. Lysis of the cell wall releases new phages.

Replication of RNA Bacteriophage (pp. 260-261)

- In RNA phages, a plus RNA strand contains the viral hereditary information and is used as a template for a double-stranded RNA replicate that has both plus and complementary minus RNA strands during its lytic replication cycle. The new plus RNA serves as the hereditary molecule for incorporation into new viruses.

Bacteriophage Growth Curve (pp. 261-262)

- Lytic replication cycles have a one-step growth curve that begins with an eclipse period, which is part of the latent period, the time between the entry of the viral nucleic acid and the release of viral progeny. New phages are released simultaneously when their number reaches the burst size and the cell lyses.

Temperate Phages—Lysogeny (pp. 262-263)

- Temperate phages establish a state of lysogeny in which a portion of the viral nucleic acid is incorporated into a bacterial chromosome or plasmid. The incorporated prophage is replicated with the host cell DNA and passed to later generations. The phage genes usually are not expressed, but when they are, they can greatly alter the properties of the host cell.
- Specialized transduction is the phage-mediated transfer of bacterial DNA. Specific genes located adjacent to the specific site where prophage DNA is incorporated into the host cell can be transferred to a recipient cell.

Replication of Plant Viruses (pp. 264-266)

- Replication of the viruses that infect plant cells is similar to the lytic replication cycle of bacteriophages. It begins with the adsorption of the virus onto a susceptible plant cell; next the viral nucleic acid penetrates into the plant cell; then the viral DNA assumes control of the host cell's biosynthesis activities; the viral nucleic acid codes for synthesis of viral nucleic acid and capsid components; and the viral particles are assembled and released by lysis of the host cell.

Replication of Animal Viruses (pp. 266-282)

- The steps in the replication of animal viruses are: attachment (adsorption of the virus to the surface of the animal cell), penetration (entry of the intact virus or viral nucleic acid into the host cell), uncoating (release of viral nucleic acid from the capsid), transcription (formation of viral mRNA), translation of early proteins (using viral-coded mRNA), replication of viral nucleic acid, translation of late proteins, assembly of complete viral particles, and release of new viruses.
- Newly formed animal viruses can be released by lysis or budding.

Replication of DNA Animal Viruses (pp. 275-277)

Double-stranded DNA Viruses (pp. 275-276)

- Uncoating of a double-stranded DNA virus takes several hours, during which the host cell continues its normal metabolic activities. Once within the nucleus the viral DNA codes for the inhibition of normal host biosynthesis. The viral DNA acts as a template for its own replication. Viral mRNA produced by transcription is translated at host cell ribosomes, producing proteins for assembly of the viral capsid. The viruses are assembled in the nucleus and released by lysis.

Single-stranded DNA Viruses (p. 277)

- Single-stranded DNA viruses copy the single strand within a host cell to make a double-stranded DNA macromolecule. mRNA is produced by transcription to code for the production of capsid proteins. Single-stranded DNA is also produced for viral genomes.

Replication of RNA Animal Viruses (pp. 277-281)

Double-stranded RNA Viruses (p. 277)

- Some double-stranded RNA viruses carry RNA polymerase to synthesize new viral nucleic acid molecules.

Single-stranded RNA Viruses (pp. 277-279)

- In the single-stranded RNA polioviruses, the viral RNA acts as an mRNA and codes for the production

of a single large polypeptide when it enters the host cell. The polypeptide is cleaved, forming RNA polymerase and the proteins used to make the viral capsid. The RNA polymerase is used to produce a complementary replicative minus RNA strand that acts as a template for the synthesis of new plus strand viral RNA.

- In some single-stranded RNA viruses, the RNA within the virion can act as mRNA and therefore is a plus strand. In others, the single strand of viral RNA is a minus strand that serves as a template for the transcription of mRNAs rather than acting as the mRNA.

Retroviruses (pp. 279-281)

- Reverse transcription is the process of making DNA using an RNA template. Single-stranded RNA retroviruses use reverse transcriptase to do this.

Transformation of Animal Cells (pp. 281-282)

- Under certain conditions, animal viral DNA is incorporated as a provirus into the chromosomal DNA of the host cell and is passed on with animal cell reproduction. A provirus can transform the animal cell into a malignant cell and cause the formation of a tumor.
- Viruses that transform cells and cause cancerous growth are called oncogenic viruses. Oncogenes are genes that induce these cancerous transformations. Carcinogens are agents that cause gene mutations that lead to cancer.
- An integrated viral oncogene only causes host cell transformation when it is expressed. Many oncogenes code for the production of kinases, which in transformed cells add phosphate to the amino acid tyrosine rather than serine or threonine. Phosphorylated tyrosine alters the regulatory functions of the cell.

CHAPTER REVIEW

REVIEW QUESTIONS

1. Describe a typical virus.
2. Describe the similarities and differences between the replication of bacteriophage, animal viruses, and plant viruses.
3. Describe the steps in the lytic replication of a T-even bacteriophage.
4. What are the differences between the replication of DNA and RNA animal viruses?
5. What is lysogeny?
6. What is a transformed cell?
7. What is an oncogene?
8. What is a retrovirus and how does its replication differ from other types of viruses?
9. How does viral replication differ from the reproduction of cellular organisms?
10. Compare the budding and lytic modes of animal virus replication.
11. What is the burst size?
12. Describe the "growth curve" for a lytic bacteriophage.
13. What is meant by virus host cell specificity?
14. What advantages does a temperate bacteriophage have over a lytic bacteriophage?

CRITICAL THINKING QUESTIONS

1. Why are viruses obligate intracellular parasites? Why do they need host cells? Why do viruses normally replicate only within certain host cells? What would the consequence be if viruses did not need specific receptors to attach to host cells?
2. Since the smallpox virus only replicates within human host cells, why has it been possible to eliminate smallpox? There are some vials containing smallpox viruses stored in the United States and Russia. Some scientists want these stocks of smallpox virus destroyed. Others want them preserved. Should the virus be destroyed or maintained?
3. Some scientists who argue that the smallpox virus should be preserved want to develop an alternate animal host system so they can study how the smallpox virus causes disease. What would be the benefits and risks of developing an alternate host system?
4. One hypothesis about the origins of the human immunodeficiency virus (HIV) that causes AIDS is that it originated from another virus in African green monkeys. How could a virus that originally infected only monkeys have become infectious for humans? Could other viruses evolve the ability to cause human diseases?
5. What is the significance in finding that several viruses have oncogenic potential? How are these viruses related to cancer?
6. Why are infections with viruses that replicate with budding release, such as herpes viruses and HIV, persistent?
7. How does the replication of HIV differ from the replication of the common cold virus? Why is the body able to eliminate the common cold virus but not HIV? Why are there no useful drugs for controlling or eliminating the common cold? Why can physicians use drugs such as AZT to treat, but not to cure, AIDS.

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CHAPTER 10

Bacterial Reproduction and Growth of Microorganisms

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PREVIEW TO CHAPTER 10

In this chapter we will:

- Study the reproduction of bacteria.
- See that bacterial reproduction results in a characteristic growth curve.
- Learn that a consequence of bacterial reproduction by binary fission is a high reproductive capacity.
- Examine the factors that influence bacterial growth rates.
- Learn the following key terms and names:

acidophiles
alkalophiles
barophiles
barotolerant
batch culture
binary fission
budding
chemostat
colony forming units (CFUs)
continuous culture
cysts
death phase
direct counting procedures
doubling time
exponential phase
facultative anaerobes
generation time
growth curve
halophiles
lag phase
log phase
mesophiles
microaerophiles
most probable number enumeration procedure
obligate aerobes
obligate anaerobes
optimal growth temperature
osmophilic
osmotolerant
psychrophiles
salt tolerant
stationary growth phase

BINARY FISSION

Most bacteria reproduce by **binary fission.** Each bacterial cell divides exactly in half to form two equal-size progeny (daughter) cells (FIG. 10-1). Since bacteria typically are single celled, the reproduction of a single cell accomplishes the reproduction of the entire organism. Binary fission is an asexual process—meaning that a single cell divides to form genetically identical progeny and that genetic recombination does not occur in the process.

During the reproduction of a bacterial cell, the parent cell elongates and the cell wall grows inward, dividing the cell in half. This establishes two progeny cells, each surrounded by a cell wall and a plasma membrane. Each of the progeny cells receives a complete set of hereditary information. Replication of the bacterial chromosome is a prerequisite for reproduction of a duplicate bacterial cell.

Binary fission is the most common means of bacterial reproduction.

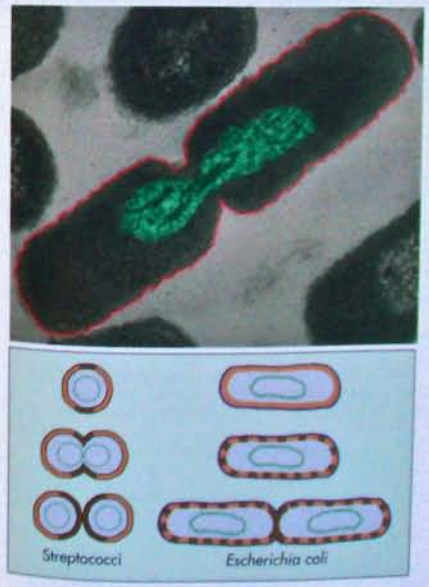


FIG. 10-1 **A,** Colorized micrograph of *Escherichia coli* dividing by binary fission. **B,** Cell growth occurs at specific sites so that the cell elongates prior to division. The cell wall and plasma membrane are growing inward to separate the cells and the replicated bacterial chromosomes.

During cell division the bacterial chromosome appears to be attached to the plasma membrane and cell wall. Formation of a crosswall or **septum** by the inwardly moving cell wall and plasma membrane physically separates the bacterial chromosomes and distributes them to the two daughter cells. Septum formation pinches off and separates the two complete bacterial chromosomes, providing each progeny cell with a bacterial chromosome (genome) containing a complete set of genetic information. This process requires active protein synthesis to move the bacterial chromosomes to the proper positions. On completion of the crosswall there are two equal-size cells that can separate. Repeating the process results in the multiplication of the bacterial population.

ALTERNATE MEANS OF BACTERIAL REPRODUCTION

Binary fission is the most common mode of bacterial reproduction. Some bacteria multiply by other means. The various modes of replication differ in how the cellular material is apportioned between the daughter cells and whether the cells separate or remain together as part of a multicellular aggregation. For example, bacteria in the genus *Hyphomicrobium* attach to solid surfaces in fresh and saltwater environments and reproduce by budding. **Budding** is a type of division characterized by an unequal division of cellular material. Similarly, *Caulobacter* cell division is unequal; cell division is by elongation of a stalked cell, followed by fission (FIG. 10-2). The daughter cell develops when a crosswall forms, segregating a small portion of the cytoplasm containing

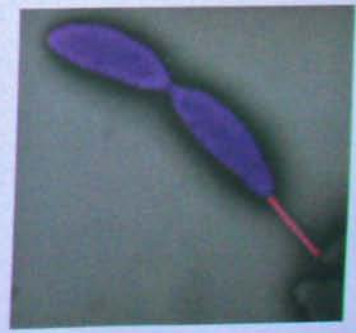


FIG. 10-2 Colorized electron micrograph of *Caulobacter crescentus* showing mother cell with stalk *(pink)* and daughter cell forming by fission.

a duplicate genome. In the case of the actinomycetes, such as *Streptomyces*, reproduction involves the formation of hyphae. In this mode of reproduction the cell elongates, forming a relatively long and generally branched filament, or **hypha**. Regardless of the mode of reproduction, bacterial multiplication requires replication of the bacterial chromosome and synthesis of new boundary layers, including cell wall and plasma membrane structures. All these modes of reproduction are asexual, like binary fission.

Bacterial Spore Formation

Spores are specialized cells produced by some bacteria that are involved in survival or reproduction. The production of spores represents an interesting deviation from vegetative cell reproduction. Some types of spores, including **endospores** (heat resistant spores formed within the cell) and **cysts** (resting or dormant cells sometimes enclosed in a sheath) are not reproductive structures and their production does not increase the number of living cells. In contrast, **arthrospores** (spores formed by the fragmentation of hyphae) are produced by different bacteria as part of their reproductive cycles. The fragmentation of hyphae to produce arthrospores forms numerous progeny cells. Additionally, myxobacteria form reproductive structures, called fruiting bodies, within which numerous spores, called **myxospores** (resting cells of the myxobacteria formed within a fruiting body), are formed. Myxospores are the progeny that result from reproduction of myxobacteria; they are able to survive transport through the air and increase the survival capacity of myxobacteria by permitting dissemination to areas with adequate supplies of nutrients to support bacterial growth and reproduction.

Spores are specialized resistant resting cells produced by bacteria. Endospores are involved in survival, but other types of spores, such as arthrospores, are involved with reproduction.

BACTERIAL GROWTH

Generation Time

Bacterial growth is synonymous with bacterial cell reproduction. Growing bacterial cells increase in biomass through their metabolism in which they convert compounds containing carbon, nitrogen, phosphorus, and other elements into the components of the cell. Most then divide into progeny of equal biomass through binary fission. By its very nature, bacterial reproduction by binary fission results in doubling of the number of viable bacterial cells. Therefore, during active bacterial growth, the size of the microbial population is continuously doubling. Once cell division begins, it proceeds exponentially as long as growth conditions permit. One cell divides to form two, each of these cells divides so that four cells form, and so forth in a geometric progression. The time required to achieve a doubling of the population size, known as the **generation time** or **doubling time**, is the unit of measure of microbial growth rate (FIG. 10-3).

METHODOLOGY

Logarithms

To conveniently represent large numbers, particularly when the numbers may range over many orders of magnitude (multiples of 10)—as is the case for numbers of bacterial cells that may occur as a few or millions of cells—scientists use a mathematical transformation called the logarithmic transformation. The logarithm to the base 10 ($\log_{10}$) of a number is the exponent indicating the power of 10 to which a number must be raised to produce a given number. Thus the $\log_{10}10 = 1$ because the exponent to which 10 must be raised to equal 10 is 1 (10^1). Similarly the $\log_{10}100 = 2$ because $10^2 = 100$, and thus the exponent of 10 needed to equal 100 = 2. In a similar fashion the $\log_{10}1{,}000 = 3$, $\log_{10}10{,}000 = 4$, $\log_{10}100{,}000 = 5$, $\log_{10}1{,}000{,}000 = 6$, and so forth. Obviously in plotting the growth of a bacterial cell over the range of generations that takes 1 cell ($\log_{10}1 = 0$) to 1 billion cells ($\log_{10}1{,}000{,}000{,}000 = 9$), it is far easier to plot the logarithm of the cell number using a scale of 0 to 9 than it would be to try to plot the cell numbers on an arithmetic scale of 1 to 1,000,000,000 (see Figure).

GENERATION	GENERATION NUMBER	NUMBER OF CELLS	LOGARITHM OF NUMBER OF CELLS
0		1	0
5	2^5	32	1.51
10	2^{10}	1,024	3.01
15	2^{15}	32,768	4.52
20	2^{20}	1,048,576	6.02
25	2^{25}	33,554,432	7.53
30	2^{30}	1,073,741,824	9.03

Comparison of arithmetic number of cells and logarithmic increase in numbers during bacterial growth.

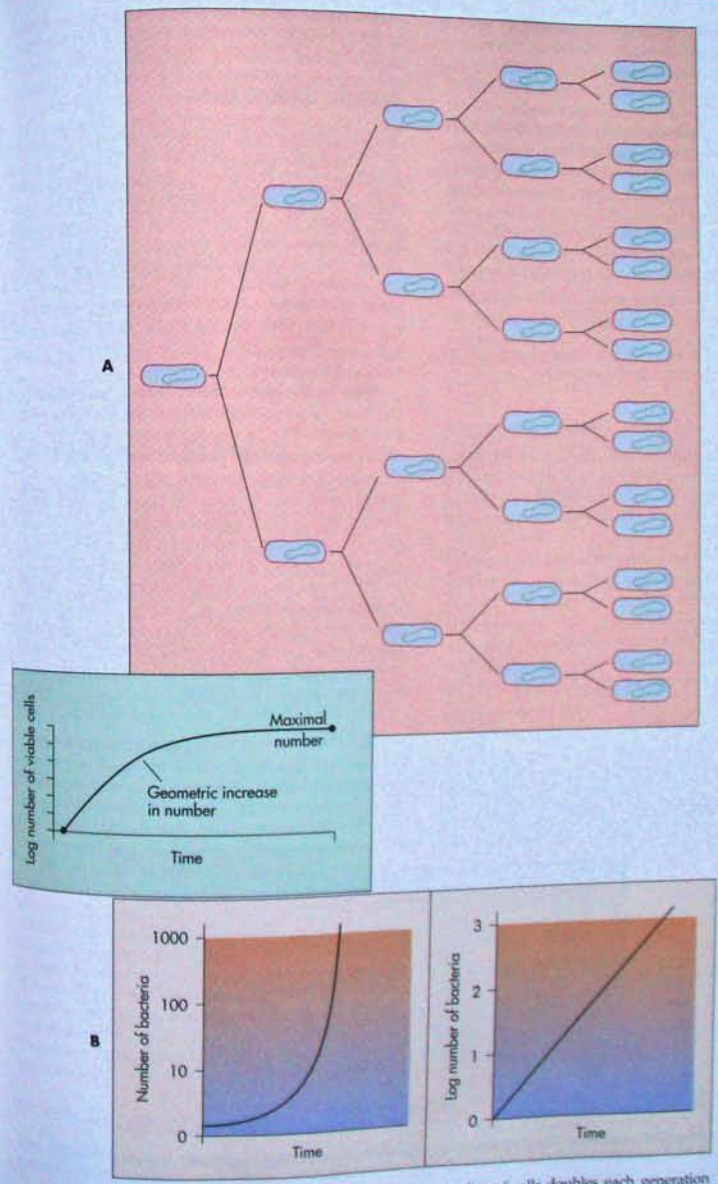


FIG. 10-3 **A,** During exponential growth the number of cells doubles each generation time. **B,** A graph of the log number of bacteria versus time *(right)* compared with number of bacteria versus time *(left)*.

For a growing bacterial culture, the generation time can be expressed as:

$$g = \frac{t}{n}$$

where g is the generation time, t is time, and n is the number of generations. The number of cells of a growing culture are expressed by the equation:

$$N_t = N_0 \times 2^n$$

where N_0 is the number of cells at time 0, N_t is the number of cells at any time (t) after time 0, and n is the number of generations. Rearranging this formula:

$$n = \frac{\log_{10} N_t - \log_{10} N_0}{\log_{10} 2}$$

Since $1/\log_{10}2$ is equal to 3.3, the original equation for the generation time can be written as:

$$g = \frac{t}{3.3\,(\log_{10} N_t - \log_{10} N_0)}$$

where g is the generation time, $\log N_t$ is the logarithm to the base 10 of the number of bacteria at time t, $\log N_0$ is the logarithm to the base 10 of the number of bacteria at the starting time, and t is the time period of growth.

By determining cell numbers during the period of active cell division, the generation time can be estimated. A bacterium such as *Escherichia coli* can have a generation time as short as 20 minutes under optimal conditions. Considering a bacterium with a 20-minute generation time, one cell would multiply to 1,000 cells in 3.3 hours and to 1,000,000 cells in 6.6 hours.

Generation time or doubling time is the unit of measure of bacterial growth; it is the time it takes for the size of a bacterial population to double.

Bacterial Growth Curve

When a bacterium is inoculated into a new culture medium, it exhibits a characteristic pattern or change in cell numbers. This pattern is called a **growth curve** (FIG. 10-4). The normal growth curve of bacteria has four phases, the lag phase, the log or exponential growth phase, the stationary phase, and the death phase. During the **lag phase** there is no increase in cell numbers. Rather, the lag phase is a period of adaptation during which bacteria are preparing for reproduction: synthesizing DNA, RNA, other structural macromolecules, and the various enzymes needed for cell division.

After the lag phase, the bacteria begin to multiply by binary fission, doubling in number every time they divide. This is the **log phase** of growth, also called the **exponential phase.** It is so named because the logarithm of the bacterial cell numbers increases linearly with time. During this phase, bacterial reproduction occurs at a maximal rate for the specific set of growth conditions. It is during this period that the generation time of the bacterium is determined.

After some period of exponential growth, the **stationary growth phase** is reached. The stationary phase often occurs when the maximum population density that can be supported by the available resources is reached. Once the stationary growth phase is reached, there is no further net increase in bacterial cell numbers. During the stationary phase the growth rate is exactly equal to the death rate, and cell numbers therefore remain constant. A bacterial population may reach stationary growth when a required nutrient is exhausted, when inhibitory end products accumulate, or when physical conditions do not permit a further increase in population size. The duration of the stationary phase varies, with some bacteria exhibiting a very long stationary phase.

Eventually the number of viable bacterial cells begins to decline. This signals the onset of the **death phase.** During the death phase the number of living bacteria decreases because the rate of cell death exceeds the rate of new cell formation.

A bacterial growth curve has four phases: (1) lag phase during which bacteria prepare to divide, (2) log or exponential growth phase during which cell numbers increase with regular doublings of viable cells, (3) stationary phase during which cell numbers remain constant, and (4) death phase during which viable cell numbers decline.

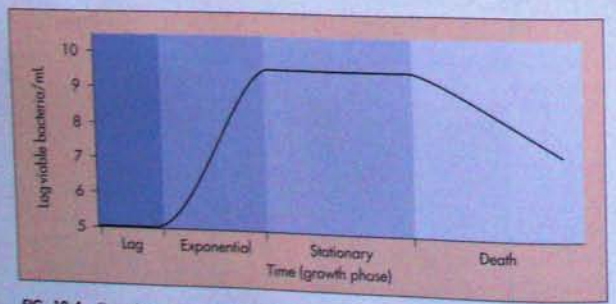


FIG. 10-4 Growth curve for bacteria has four distinct phases: lag, exponential (log), stationary, and death.

Batch and Continuous Growth

The normal bacterial growth curve is characteristic of bacteria in **batch culture.** In batch culture, growth occurs in a closed system with fresh sterile medium simply inoculated with a bacterium to which new materials are not added. A flask containing a liquid nutrient medium inoculated with the bacterium *E. coli* is an example of such a batch culture. In batch culture, growth nutrients are expended and metabolic products accumulate in the closed environment. The batch culture models situations such as occur when a canned food product is contaminated with a bacterium.

Bacteria may also be grown in **continuous culture.** In continuous culture, nutrients are supplied and end products continuously removed so that the exponential growth phase is maintained. Because end products do not accumulate and nutrients are not completely expended, the bacteria never reach the stationary phase. A **chemostat** is a continuous culture device in which a liquid medium is continuously fed into the bacterial culture (FIG. 10-5). The liquid medium contains some nutrient in growth-limiting concentration, and the concentration of the limiting nutrient in the growth medium determines the rate of bacterial growth. Even though bacteria are continuously reproducing, a number of bacterial cells are continuously being washed out and removed from the culture vessel. Thus cell numbers in a chemostat reach a plateau.

Bacterial Growth on Solid Media

The development of bacterial colonies on solid growth media follows the basic normal growth curve. The dividing cells do not disperse and the population is densely packed. Under these conditions, nutrients rapidly become limiting at the center of the colony. Microorganisms in this area rapidly reach stationary phase. At the periphery of the colony, cells can continue to grow exponentially even while those at the center of the colony are in the death phase. Bacterial colonies generally do not extend indefinitely across the surface of the media but have a well-defined edge. Therefore individual well-isolated colonies can develop from the growth of individual bacterial cells. The fact that the bacteria have reproduced asexually by binary fission means that all the bacteria in the well-isolated colony should be genetically identical; that is, each colony should contain a clone of identical cells derived from a single parental cell.

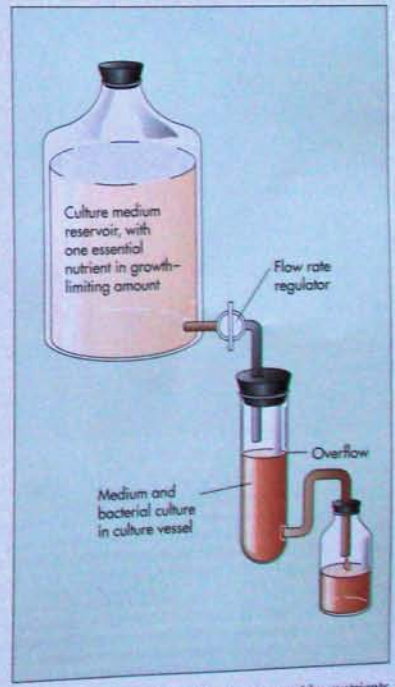


FIG. 10-5 A chemostat continuously provides nutrients with a growth rate limiting factor to a flow-through culture chamber in which bacteria grow.

ENUMERATION OF BACTERIA

To assess rates of bacterial reproduction, it is necessary to determine numbers of bacteria. Various methods can be employed for enumerating bacteria. These include viable plate count, direct count, and most probable number (MPN) determinations.

VIABLE COUNT PROCEDURES

The **viable plate count** method is one of the most common procedures for the enumeration of bacteria. In this procedure, serial dilutions of a suspension of bacteria are plated onto a suitable solid growth medium and after a period of incubation (during which single cells multiply to form visible colonies) the number of colonies are counted or enumerated (FIG. 10-6).

Frequently, the suspension is spread over the surface of an agar plate containing growth nutrients **(surface spread technique)** (FIG. 10-7). Alternatively, it can be mixed with the agar while it is still in a liquid state and poured into the plate **(pour plate technique)** (FIG. 10-8). The plates are incubated to allow the bacteria to grow and form colonies. The formation of visible colonies generally takes 16 to 24 hours.

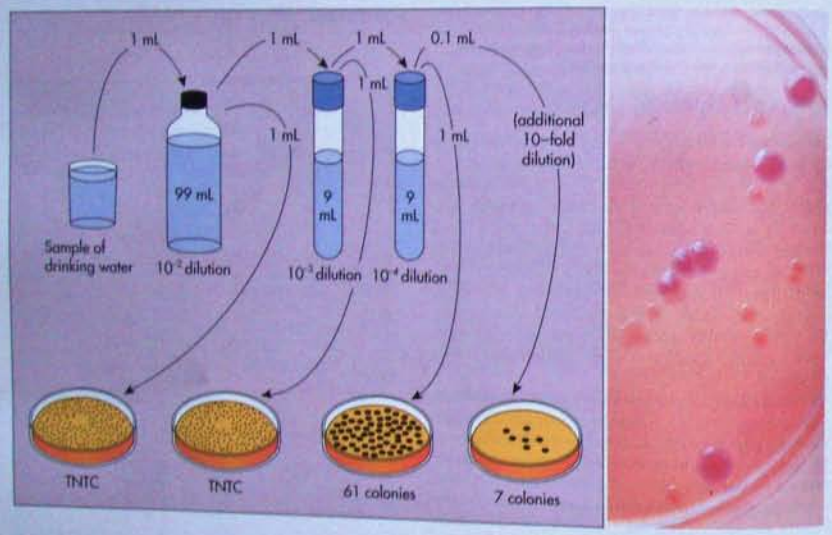


FIG. 10-6 **A**, The plate count procedure is used to determine the viable population in a sample containing bacteria. Dilutions are achieved by adding an aliquot of the specimen to a sterile water dilution tube. If 1 mL of a sample is added to 99 mL of sterile water, the dilution is 1:100 (10^{-2}). (The same dilution could also have been achieved by adding an 0.1 mL sample to 9.9 mL of sterile water). Greater dilutions are achieved by sequentially diluting the sample in series. Adding 1 mL from the first dilution to 9 mL of sterile water achieves an additional tenfold dilution so that the total dilution is 1:1000 (10^{-3}). Adding 1 mL from that second dilution to 9 mL of sterile water achieves a further tenfold dilution so that the total dilution is 1:10000 (10^{-4}). Transferring 1 mL samples from each tube to agar media maintains these dilution factors. Transferring 0.1 mL samples increases the dilution by a factor of 10. After incubation the number of colonies are counted. Counts on the plates in the range of 30 to 300 colonies are used to calculate the concentration of bacteria. The standard notation "TNTC" means too numerous to count (greater than 300 colonies). In this example the plate with 61 colonies would be used to calculate the number of bacteria in the original water sample. Because these colonies developed on a plate in which 1 mL from a 1:10000 dilution was added, the number of bacteria per mL in the original sample is calculated as 6.1 $\times 10^5$ (61×10^4). **B**, Colonies of lactose fermenting bacteria growing on MacConkey agar.

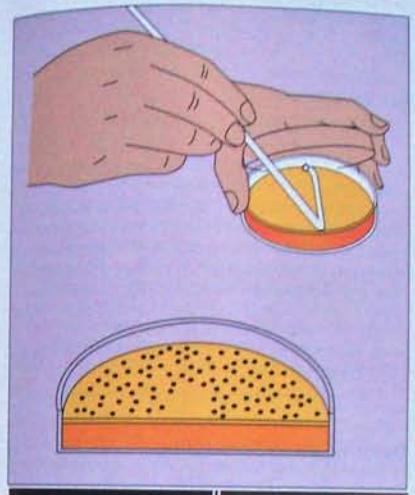


FIG. 10-7 The spread plate technique for isolating and enumerating microorganisms.

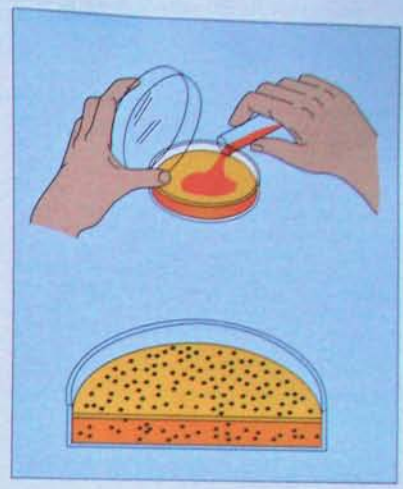


FIG. 10-8 The pour plate technique for isolating and enumerating microorganisms.

It is assumed that each colony arises from an individual bacterial cell. By counting the number of colonies that develop, the **colony forming units** (CFUs), and by taking into account the dilution factors, the concentration of bacteria in the original sample can be determined. Preferably two or three plates are counted to determine numbers of bacteria in a sample. Each plate counted should have 30 to 300 colonies. If bacterial numbers in a sample are low, it is sometimes necessary to filter the suspension to concentrate the bacterial cells by collecting the cells on a membrane filter. The typical membrane filter for collecting bacterial cells is made of nitrocellulose or cellulose acetate and has a pore size of 0.2 to 0.45 µm, which is small enough to trap most bacterial cells. The membrane filter with the trapped bacteria is then placed onto a suitable medium so that bacterial reproduction can occur, and the colonies that develop on the filter are counted. Countable plates are those having between 30 and 300 colonies. Less than 30 colonies is not acceptable for statistical reasons and more than 300 colonies on a plate are likely to produce colonies too close to distinguish as individual CFUs. Such samples are noted as TNTC (too numerous to count).

A major limitation of the viable plate count procedure is that it is selective. There is no single combination of incubation conditions and medium composition that permits the growth of all bacterial types. The nature of the growth medium and the incubation conditions determine which bacteria can grow and thus be counted. Viable counting measures only cells that are capable of growth on the given plating medium under the set of incubation conditions that are used. Sometimes cells are viable but nonculturable unless rigorous steps are taken to acclimate the microorganisms to laboratory culture conditions. The viable plate

NEWSBREAK

Unsafe Ice Cream Goes Undetected

An ice cream manufacturing plant in New York City in the early 1970s, in compliance with the required testing procedures to ensure the microbiological safety of its food product, routinely sent samples of the ice cream to a local quality control microbiology laboratory. The laboratory performed viable plate counts to detect coliform bacteria. The presence of coliform bacteria indicates contamination with human fecal matter, making the ice cream unsafe for consumption. The plates were overgrown with coliform bacteria, and the technician at the testing laboratory recorded TNTC, the standard notation for too numerous to count. Records were compiled indicating unsafe levels of contamination but no action was taken. This was because the Board of Health inspector who examined the records did not recognize the abbreviation TNTC and was looking for a number greater than 10 per 100 mL to signal a contamination problem. The inspector did not inquire as to the meaning of TNTC, and it was not until another inspector visited the facility and viewed the records that the problem was detected. The underlying problem with the ice cream was in the plumbing of the building, which had connected the effluent from the restrooms directly to the influent for water used in the manufacture of the ice cream.

count relies on the reproduction of individual bacterial cells to form visible colonies, which are counted to enumerate numbers of bacteria in a sample. Another problem and source of possible error associated with this technique is in the enumeration of bacteria that grow in chains or clumps that are hard to disperse. For example, a chain containing ten attached cells will grow into one colony instead of ten. Therefore using the viable plate count method to measure numbers of bacteria that tend to remain attached to one another can lead to erroneously low values.

The viable plate count procedure is selective because no one combination of incubation conditions and media allows all types of bacteria to grow.

Direct Count Procedures

Bacteria can also be enumerated by **direct counting procedures.** In this procedure, counting is done without the need to first grow the cells in culture. In one direct count procedure, dilutions of samples are observed under a microscope, and the numbers of bacterial cells in a given volume of sample are counted. These numbers are used to calculate the concentration of bacteria in the original sample (FIG. 10-9). Special counting chambers, such as a hemocytometer or Petroff-Hausser chamber, are sometimes employed to determine the number of bacteria. These chambers are ruled with squares of a known area and are so constructed that a film of liquid of known depth can be introduced between the slide and the cover slip. Consequently, the volume of the sample overlying each square is known.

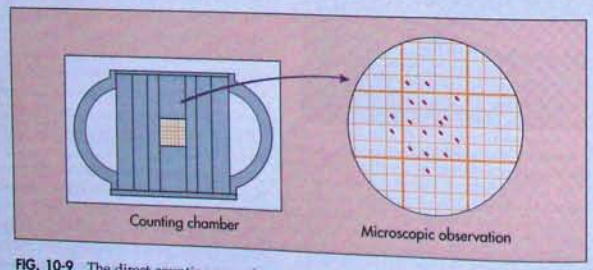


FIG. 10-9 The direct counting procedure using a Petroff-Hauser counting chamber. The sample is added to a counting chamber of known volume. The slide is viewed and the number of cells determined in an area delimited by a grid. In the counting chamber shown, the entire grid has 25 large squares for a total area of 1 mm^2 and a total volume of 0.02 mm^3, formed by the spacing of an overlying coverslip. There are 12 cells within the single large grid (composed of 16 smaller boxes) in this example. Assuming the number of cells in this single grid is representative of all the grids, the number of cells within the total area under the grid is 12 cells. The concentration of cells is therefore 300/0.02 mm^3.

It is often desirable to stain the cells. This helps in visualizing bacterial cells. Alternatively, a known volume of a sample containing a suspension of bacteria is poured through a filter, such as a nitrocellulose 0.2 μm pore size filter. The bacteria are stained on this filter, often using a fluorescent stain, and counted under a microscope. Many fluorescent dyes, such as acridine orange, stain all cells, making it impossible to differentiate living from dead bacteria. The difficulty in establishing the metabolic status of the observed bacteria is a major limitation of this procedure.

Bacterial cells can be enumerated by direct microscopic count procedures.

Instruments also are available for direct counting of bacterial cells. Particle counters, such as a Coulter particle counter, permit the discrimination of particles based on size so that particles the size of bacteria are counted automatically. As long as there are no nonliving interfering particles in the same size range of bacteria, this is a rapid counting method.

Most Probable Number (MPN) Procedures

Another approach to bacterial enumeration is the determination of the most probable number (MPN). MPN is a statistical method based on probability theory. In a **most probable number enumeration procedure,** multiple serial dilutions are performed to reach a point of extinction. The point of extinction is the dilution level at which not even a single cell is deposited into one or more multiple tubes (FIG. 10-10). A criterion is established for indicating whether a particular dilution tube contains bacteria. Such a criteria can be development of cloudiness or turbidity in a liquid growth medium or gas production (detected by its accumulation in an inverted tube). The pattern of positive and negative test results are then used to estimate the concentration of bacteria in the original sample, that is, the most probable number of bacteria, by comparing the observed pattern of results with a table of statistical probabilities for obtaining those results (Table 10-1). Often a 95% confidence limit is given in MPN tables.

The most probable number procedure is a statistical method based on probability theory used to determine bacterial populations by diluting bacterial samples to the point of extinction.

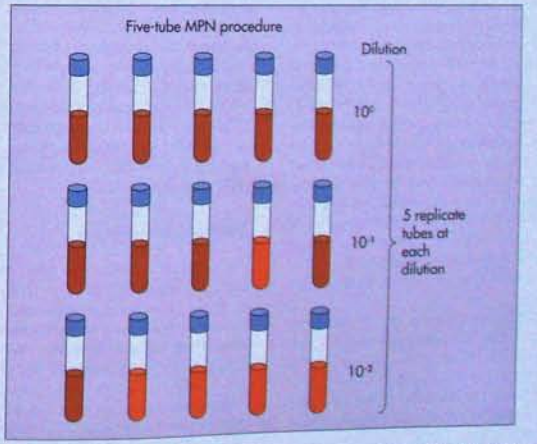


FIG. 10-10 The most probable number (MPN) procedure involves inoculation of multiple tubes with replicate samples of dilutions. The pattern of tubes that show growth *(brown)* and those that do not *(orange)* are compared with a statistical table to calculate the MPN of bacteria in the original sample. In this example all 5 tubes at the 10^0 show growth, 4 of 5 tubes at the 10^{-1} dilution show growth, and only 1 of 5 tubes at the 10^{-2} dilution show growth. Therefore the MPN bacteria in the original sample is 170 per 100 mL (see Table 10-1).

TABLE 10-1

Table of Most Probable Numbers (MPN)

NUMBER OF POSITIVE TUBES AT THE STATED DILUTION				NUMBER OF POSITIVE TUBES AT THE STATED DILUTION			
10^0	10^{-1}	10^{-2}	MPN/100mL	10^0	10^{-1}	10^{-2}	MPN/100mL
0	1	0	0.18	5	0	0	2.3
1	0	0	0.20	5	0	1	3.1
1	1	0	0.40	5	1	0	3.3
2	0	0	0.45	5	1	1	4.6
2	0	1	0.68	5	2	0	4.9
2	1	0	0.68	5	2	1	7.0
2	2	0	0.93	5	2	2	9.5
3	0	0	0.78	5	3	0	7.9
3	0	1	1.1	5	3	1	11.0
3	1	0	1.1	5	3	2	14.0
3	2	0	1.4	5	4	0	13.0
4	0	0	1.3	5	4	1	17.0
4	0	1	1.7	5	4	2	22.0
4	1	0	1.7	5	4	3	28.0
4	1	1	2.1	5	5	0	24.0
4	2	0	2.2	5	5	1	35.0
4	2	1	2.6	5	5	2	54.0
4	3	0	2.7	5	5	3	92.0
				5	5	4	160.0

FACTORS INFLUENCING BACTERIAL GROWTH

The rates of bacterial growth and death are greatly influenced by environmental parameters. Some environmental conditions favor rapid bacterial reproduction, whereas others do not permit any bacterial growth. Not all bacteria can grow under identical conditions. Each bacterial species has a specific tolerance range for specific environmental parameters. Outside the range of environmental conditions under which a given bacterium can reproduce, it may either survive in a relatively dormant state or may lose viability. Loss of viability means the loss of the ability to reproduce, which leads to death. The effects of environmental factors on bacterial growth and death rates can be seen as differences in rates of reproduction or death of a culture under varying environmental conditions.

It is a particular environmental parameter or interaction of environmental parameters that controls the rate of growth or death of a given bacterial species. Bacteria have particular physiological properties that determine the conditions under which they can grow. In nature, conditions cannot be controlled and many species co-exist; fluctuating environmental conditions favor population shifts because of the varying growth rates of individual microbial populations within the community of a given location. In the laboratory it is possible to adjust conditions to achieve optimal growth rates for a given microorganism. Many laboratory and industrial applications use pure cultures of microorganisms. This facilitates the adjustment of the growth conditions so that they favor optimal growth of the particular bacterial species. Similarly, in industrial fermentors, which are large growth chambers (often thousands of liters) used to grow bacterial cultures, conditions can be adjusted to optimize bacterial growth rates, thereby maximizing the production of desired bacterial metabolic products.

TEMPERATURE

Temperature is one of the most important factors affecting rates of microbial growth. The temperatures at which specific enzymes and cellular structures function varies from one microbial species to another, depending on the specific chemical compositions specified by the genome of the organism. The minimum and maximum temperatures at which a microorganism can grow establish the **temperature growth range** for that microorganism (FIG. 10-11). Within this growth range there will be an optimal growth temperature at which the highest rate of reproduction occurs.

The temperature growth range is defined by the minimum and maximum temperatures at which e microorganism can grow.

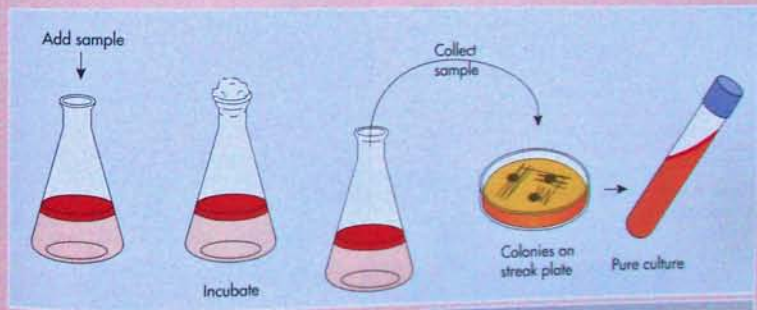
METHODOLOGY

ENRICHING FOR SPECIFIC BACTERIA

By taking into account the physiological characteristics of specific bacterial species or types, it is possible to design conditions that favor the growth of those bacteria. This is the basis of enrichment culture technique, a method used to isolate specific groups of bacteria based on designing the culture medium and incubation conditions to preferentially support the growth of a particular bacterial type. Liquid enrichment media tend to select the bacteria that are able to grow best among all the bacteria introduced into the media. For example, to isolate bacteria capable of metabolizing petroleum hydrocarbons, one can design a culture medium containing a hydrocarbon as the sole source of carbon and energy (see Figure). By doing so, one establishes conditions whereby only bacteria that are capable of metabolizing hydrocarbons can grow. Because other bacteria cannot reproduce in this medium, hydrocarbon-utilizing bacteria are thereby selected, resulting in enrichment (increased proportions) of the selected bacteria. Similarly, a culture medium that favors the growth of autotrophic microorganisms could be designed by providing ammonium ions and carbonate as the sole source of carbon in the medium.

The design of an enrichment procedure takes into consideration the composition of the medium and also environmental factors, such as temperature, aeration, pH, and so forth. For example, temperature can be adjusted to 5° C to favor the growth of microorganisms that live at low refrigerator temperatures or to 37° C to enrich for microorganisms that are capable of growth at the temperature of the human body. Cultures may be aerated by shaking or by bubbling with air to favor the growth of aerobes, or oxygen may be totally excluded to enrich for anaerobes. The enrichment culture technique mimics many natural situations in which the growth of a particular microbial population is favored by the chemical composition of the system and by environmental conditions.

A



B

A, To establish an enrichment, a medium is inoculated with a sample, for example, soil or water, that may contain microorganisms with specific characteristics. The medium and the incubation conditions are designed to favor the growth of the microorganisms. for example, microorganisms capable of degrading petroleum hydrocarbons. The desired microorganisms should be able to outcompete others in the sample and increase in number so they then can be isolated and pure cultures established. **B**, Enrichment cultures are designed to selectively support the growth of specific microorganisms. In a medium with petroleum hydrocarbon as the sole source of carbon and energy, hydrocarbon-degrading microorganisms are selectively enriched (*left flask*). Control showing oil slick and lack of enrichment for hydrocarbon degraders (*right flask*). Growth of the hydrocarbon-degrading microorganisms emulsifies the oil so that it disperses through the medium in the flask. A pure culture of the hydrocarbon degrader can be isolated from the enrichment culture.

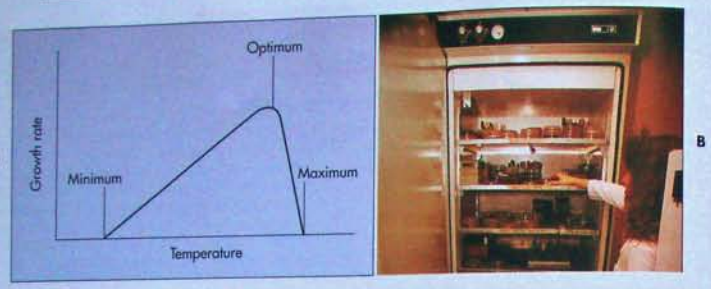


FIG. 10-11 **A,** Microorganisms exhibit specific temperature growth ranges. There is a minimal and a maximal temperature for growth. **B,** An incubator is used to control temperature for culturing microorganisms.

The **optimal growth temperature** is defined as the temperature at which the maximal growth rate occurs. This is the temperature that corresponds to the shortest generation time (Table 10-2). The ability of a microbial species to compete for survival in a given system is favored when temperatures are near its optimal growth temperature. It is not surprising that the optimal growth temperature for most human pathogens is 37° C, the temperature of the human body.

Optimal growth temperature is the temperature at which the generation time is shortest and therefore at which the maximal growth rate occurs.

Different microorganisms have different optimal growth temperatures (FIG. 10-12). Some microorgan-

TABLE 10-2

Growth Rates for Some Representative Bacteria Under Optimal Conditions

ORGANISM	TEMPERATURE (° C)	GENERATION TIME (MIN)
	Thermophile	
Bacillus stearothermophilus	60	11
	Mesophile	
Escherichia coli	37	20
Bacillus subtilis	37	27
Staphylococcus aureus	37	28
Streptococcus lactis	37	30
Pseudomonas putida	30	45
	Psychrophile	
Vibrio marinus	15	80

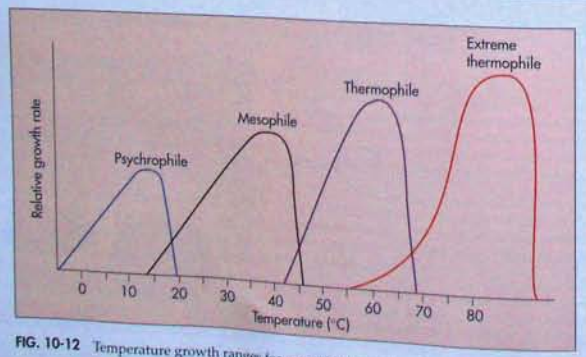


FIG. 10-12 Temperature growth ranges for mesophiles, psychrophiles, and thermophiles.

HISTORICAL PERSPECTIVE

Deep Sea Thermal Vent Bacteria

The work of Holger Jannasch established that microbial activities occur at very low rates in the deep oceans; deep ocean sediments, in effect, are biological deserts because of their low temperatures, high pressures, and low inputs of organic matter. These rates are so low that bologna sandwiches accidentally submerged inside the submersible Alvin were not decomposed during several months of exposure to microorganisms of the deep sea.

What a surprise when investigators found an area of extremely high biological productivity at a depth of 2,550 m in a region of thermal vents (subsea volcanoes) off the Galapagos Islands. The thermal vents warmed the waters, but what was the source of food supporting the growth of worms several feet long and clams several feet across? There was no light to support photosynthesis, and transport of organic matter from the surface was unlikely. The most likely explanation was that chemolithotrophic metabolism by autotrophic bacteria based on oxidation of hydrogen sulfide from the vents was providing the organic matter to support the growth of other organisms (see Figure). Establishing that bacterial chemolithotrophy was the source of organic matter would be difficult; to reach the vents in the Alvin would take hours, time on the bottom to carry out experiments would be extremely limited, and working Alvin's mechanical arms would be difficult. Nevertheless, this was the task undertaken by Holger Jannasch and Carl Wirsen of the Woods Hole Oceanographic Institution.

Jannasch and his associates were able to collect samples using specialized pressurized chambers and return living bacteria from the thermal vents to the laboratory for study. These investigators found that all surfaces intermittently exposed to H_2S-containing hydrothermal fluid were covered with mats composed of layers of prokaryotic, Gram-negative cells interspersed with amorphous manganese-iron metal deposits. Enrichment cultures using thiosulfate as the energy source made from mat material resulted in isolations of different types of sulfur-oxidizing bacteria, including the obligately chemolithotrophic genus *Thiomicrospira*. These studies established that chemolithotrophic bacteria supported the productivity of the thermal rift region.

Jannasch and other scientists then asked about the maximal temperature at which bacteria in thermal vents could grow. Bacteria were observed in waters coming from the vents with temperatures well in excess of 100° C. Could bacteria actually grow there or had the bacteria grown elsewhere at lower temperatures? What was the upper temperature limit at which bacteria can reproduce? Some scientists hypothesized that, since there was liquid water because of the high pressures, bacteria could grow at temperatures of even 500° C.

Experiments were conducted by John Baross and Jody Deming who incubated bacterial samples from the thermal vents in chambers under very high pressures at temperatures of 250° C. Because the chambers had to remain sealed under pressure to maintain the tempera-

A, Photograph of the deep sea submarine ALVIN.

Continued

DEEP SEA THERMAL VENT BACTERIA—CONT'D

B

B, Colorized micrograph of deep sea thermal vent bacterial community; the filaments of *Beggiatoa* are abundant.

ture and prevent water from turning to steam, it was impossible to sample the chambers and culture bacteria. Deming and Baross therefore measured protein and nucleic acid content at the end of the experiment, both of which appeared to increase. Based on these observations they reported that bacterial growth occurred in the chambers incubated at 250° C. Their results were immediately questioned by many scientists. Holger Jannasch could repeatedly grow some of the bacteria from the thermal vents at temperatures of 100° to 110° C, but not at higher temperatures. No one was able to repeat the experiments that purportedly demonstrated bacterial growth at 250° C. Independent confirmation is critical in science. Eventually it was shown by Art Yayanos at the Scripps Institute of Oceanography that the results reported by Deming and Baross could be explained by abiotic changes that occur at high temperature and pressure. Bacterial growth apparently had not occurred at 250° C. The initial report had not met the essential test of the scientific method—that of repeatability by others. The upper demonstrated growth temperature remains about 110° C.

isms grow best at low temperatures. Such organisms, known as **psychrophiles,** have optimal growth temperatures of under 20° C. As long as liquid water is available, some psychrophilic microorganisms are capable of growing below 0° C. Psychrophilic microorganisms are commonly found in the world's oceans and are also capable of growing in a household refrigerator, where they are important agents of food spoilage.

Mesophiles are microorganisms that have optimal growth temperatures in the middle temperature range between 20° and 45° C. Most of the bacteria grown in introductory microbiology laboratory courses are mesophilic. Many mesophiles have an optimal temperature of about 37° C. Many of the normal resident microorganisms of the human body, such as *Escherichia coli*, are mesophiles. Similarly, most human pathogens are mesophiles and thus grow rapidly and establish an infection within the human body.

Thermophilic microorganisms are organisms with high optimal growth temperatures. Thermophiles, such as *Bacillus stearothermophilus*, grow at relatively high temperatures, often growing only above 40° C. The upper growth temperature for extreme thermophilic microorganisms, such as those found in deep thermal rift regions of the areas where volcanic activity heats the ocean water under very high pressure, is about 110° C. Water will remain in a liquid state at temperatures above 100° C when it is subjected to high pressure. Thermophiles have optimal growth temperatures above 45° C and many thermophilic microorganisms have optimal growth temperatures of about 55° to 60° C. One finds thermophilic microorganisms in such exotic places as hot springs and effluents from laundromats. However, many thermophiles can survive very low temperatures, and viable thermophilic bacteria are routinely found in frozen antarctic soils.

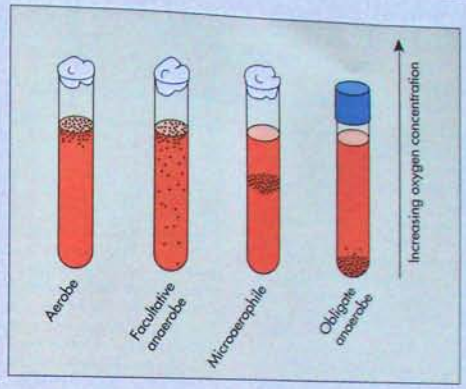


FIG. 10-13 Drawing showing oxygen growth relationships for aerobic, anaerobic, and facultative bacteria in tubes of nutrient media. The growth of aerotolerant anaerobes (obligately fermentative) would be the same as that of facultative anaerobes (organisms capable of fermentation and respiration).

Psychrophiles have optimal growth temperatures of under 20° C; mesophiles grow best between 20° and 45° C; and thermophiles grow best at higher temperatures above 45° C, with 55° to 60° C often being optimal.

OXYGEN

Another factor that greatly influences bacterial growth rates is the concentration of molecular oxygen. Bacteria are classified as aerobes, anaerobes, facultative anaerobes, or microaerophiles, based on their oxygen requirements and tolerances (FIG. 10-13). **Aerobic bacteria (obligate aerobes)** grow only when oxygen is available to support their respiratory metabolism. In laboratory cultures and industrial batch cultures, oxygen is often supplied by forced aeration or mixing (for example, on a rotary shaker) to support the growth of aerobes. **Anaerobic bacteria (obligate anaerobes)** grow in the absence of molecular oxygen. Anaerobic bacteria may carry out fermentation or anaerobic respiration to generate ATP. Some anaerobes have very high death rates in the presence of oxygen, and such organisms are termed **strict anaerobes.** Even the briefest exposure to air can kill strict anaerobes. Other obligately anaerobic bacteria, although unable to grow, have low death rates in the presence of oxygen.

While obligate anaerobes grow only in the absence of molecular oxygen, **facultative anaerobes** such as *E. coli* can grow with or without oxygen. Many facultative anaerobes are capable of both fermentative and respiratory metabolism. Some are capable of both aerobic and anaerobic respiration.

Aerobes need oxygen to support their respiratory metabolism and anaerobes grow in the absence of molecular oxygen, carrying out fermentation or anaerobic respiration; facultative anaerobes grow aerobically or anaerobically.

Although oxygen is required for the growth of many microorganisms, it can also be toxic. Some microorganisms grow only over a very narrow range of oxygen concentrations. Such microorganisms are known as **microaerophiles.** Microaerophiles require oxygen but exhibit maximal growth rates at reduced oxygen concentrations because higher oxygen concentrations are toxic to these organisms.

Oxygen can exist in several energetic states, some of which are more toxic than others. One of these energetic states, called singlet oxygen, is a chemically reactive form that is extremely toxic to living organisms. Phospholipids in bacterial plasma membranes can be oxidized by singlet oxygen, leading to a disruption of membrane function and the death of bacterial cells. Peroxidases in saliva and phagocyte cells (blood cells involved in the defense mechanism of the human body against invading microorganisms) generate singlet oxygen, accounting in part for the antibacterial activity of saliva and the ability of phagocytic blood cells to kill invading microorganisms.

Singlet oxygen is chemically reactive and extremely toxic to living organisms.

The conversion of oxygen to water occurs when oxygen serves as a terminal electron acceptor in respiration pathways. This involves the formation of an

METHODOLOGY

Growing Cultures of Aerobic and Anaerobic Bacteria

Under controlled laboratory conditions, it is possible to adjust oxygen concentrations to maximize the growth rate of a particular bacterial species. Because oxygen diffuses only slowly into liquid, the concentration of oxygen frequently limits the growth rate of aerobic and facultatively anaerobic bacteria in liquid culture. To supply oxygen for the growth of aerobic microorganisms and overcome the growth rate limitations caused by low oxygen concentrations, liquid cultures can be agitated at high speed on a shaker table or by an impeller within the culture vessel, or oxygen can be supplied to the culture vessel through forced aeration (FIG. *A*). Interrupting the supply of oxygen to an actively growing culture for even a brief period of time can lead to anaerobic conditions, in some cases causing a rapid die-off of the bacteria. Some microbial populations can lose viability if a rotary shaker is turned off for only a few minutes, such as may occur when changing flasks on the shaker table.

Whereas aeration enhances the rates of aerobic growth, oxygen must be excluded from the growth medium to permit the growth of obligate and strict anaerobes. This can be accomplished by adding chemicals that react with and remove molecular oxygen from the growth medium. For example, sodium thioglycollate is frequently added to liquid culture media for the growth of anaerobes because it reacts with molecular oxygen, removing free oxygen from solution. Similarly, the amino acid cysteine and other compounds containing sulfhydryl groups can also be used to scavenge molecular oxygen from a growth medium. For liquid cultures, nitrogen may be bubbled through the medium to remove air and traces of oxygen, and then the culture vessel is sealed tightly to prevent oxygen from reentering.

There are many types of anaerobic culture chambers that can be employed to exclude oxygen from the atmosphere (FIG. *B*). Common forms of anaerobic chambers, such as the Gas Pak system, generate hydrogen, which reacts with the oxygen as a catalyst within the chamber to produce water. Carbon dioxide is also generated in this system to replace the volume of gas depleted by the conversion of oxygen to water. It is also possible to combine several approaches to ensure absolute anaerobic conditions. In the Hungate roll tube method, after sterilization of a prereduced medium (a medium from which oxygen is excluded by the incorporation of a chemical that scavenges the free oxygen) within a sealed test tube, the medium is rolled during cooling so that the medium covers the inside of the test tube; the medium is then inoculated with a microorganism under a stream of carbon dioxide or nitrogen and tightly sealed with butyl rubber stoppers to keep oxygen out; the development of microbial colonies can be seen on the tube surface, and individual cultures can be observed without disturbing other cultures.

A

A rotary shaker is used to maintain aerobic conditions in liquid cultures.

B

An anaerobic glove box like this may be used to culture anaerobes.

FIG. 10-14 The action of catalase is readily visualized when hydrogen peroxide is added to cells that have produced this enzyme.

intermediary form of oxygen known as the superoxide anion (O_2^-), in addition to forming singlet oxygen. The superoxide anion is converted to hydrogen peroxide and oxygen by the action of the enzyme **superoxide dismutase,** which is produced by most aerobic and facultatively anaerobic bacteria. Superoxide dismutase removes the toxic superoxide anion but forms hydrogen peroxide (H_2O_2), which is also toxic. The reaction that describes the action of superoxide dismutase is:

$$2O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2$$

Hydrogen peroxide is frequently used to kill bacteria, for example, when hydrogen peroxide is applied to a cut to prevent infection. Some bacteria produce enzymes that destroy hydrogen peroxide. **Catalase** converts hydrogen peroxide to water and oxygen. The reaction that describes the action of catalase is:

$$2H_2O_2 \longrightarrow 2H_2O + O_2$$

Bacterial production of catalase can be demonstrated by adding a loopful of a microbial culture to a 3% solution of hydrogen peroxide. The evolution of gas bubbles, oxygen, is evidence of the action of the catalase (FIG. 10-14).

TABLE 10-3

Bacterial Enzymes that Protect the Cell Against Toxic Forms of Oxygen

MICROORGANISM	CATALASE	SUPEROXIDE DISMUTASE
Aerobe	+	+
Facultative anaerobe	+	+
Microaerophile	–	+
Obligate anaerobe	–	–

Obligate aerobes and facultative anaerobes usually produce both catalase and superoxide dismutase (Table 10-3). These enzymes permit such microorganisms to grow without accumulating toxic forms of oxygen. In contrast, obligate anaerobes, such as *Clostridium* species, generally lack these enzymes. The inability of these organisms enzymatically to remove toxic forms of oxygen probably accounts for the fact that they are obligately anaerobic and sensitive to oxygen.

Salinity

Halophiles are bacteria that specifically require sodium chloride for growth (FIG. 10-15). Moderate halophiles, which include many marine bacteria, grow best at salt (NaCl) concentrations of about 3% NaCl. Extreme halophiles exhibit maximal growth rates in saturated brine solutions. These organisms grow quite well in salt concentrations of greater than 15% NaCl and can grow in places like salt lakes and pickle barrels (FIG. 10-16). High salt concentrations normally disrupt membrane transport systems and denature proteins. Extreme halophiles must possess physiological mechanisms for tolerating high salt concentrations. For example, *Halobacterium*, possesses an unusual plasma membrane and many unusual enzymes that require a high salt concentration for activity.

Halophiles require a high salt concentration for growth.

Most bacteria, however, do not possess these physiological adaptations and cannot tolerate high

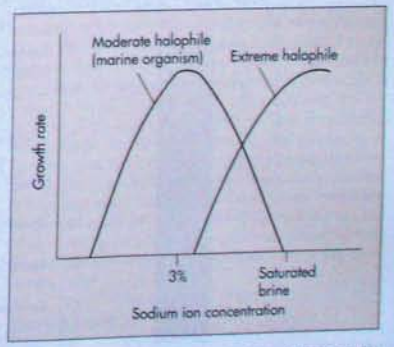


FIG. 10-15 The halophiles require sodium chloride (NaCl) for growth. Marine bacteria typically grow best near 3% NaCl. Some extreme halophiles grow best near 15% NaCl.

FIG. 10-16 Halophiles growing within salt lakes often turn the water pink; this sometimes occurs in Great Salt Lake, Utah.

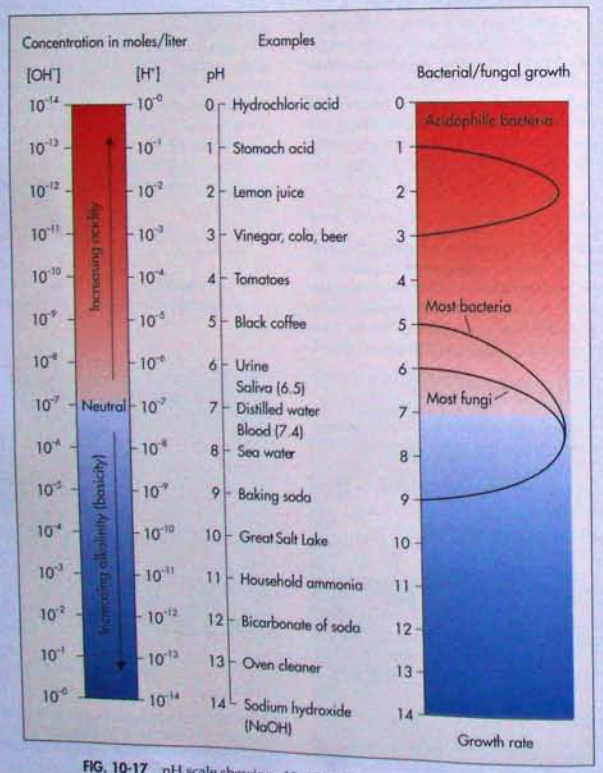


FIG. 10-17 pH scale showing pH values of some common substances.

salt concentrations. The degree of sensitivity to salt varies for different bacterial species. Many bacteria will not grow at a salt concentration of 3%. Some strains of *Staphylococcus*, however, are **salt tolerant** and grow at concentrations greater than 10% NaCl. This physiological adaptation in *Staphylococcus* is important because some members of this genus grow on skin surfaces where salt concentrations can be relatively high.

Acidity and pH

The pH of a solution describes the **hydrogen ion concentration** ($[H^+]$). When bacteria are cultured in the laboratory, they produce acids that can interfere with their own growth. To neutralize the acids and maintain the proper pH, chemicals called **buffers** are included in the growth medium. Peptones and amino acids in some media act as buffers. Many media also contain phosphate salts, which exhibit their buffering effect in the pH growth range of most bacteria. They are also nontoxic and even provide phosphorus, an essential nutrient element.

Buffers neutralize acids and maintain the proper pH.

Microorganisms vary in their pH tolerance ranges (FIG. 10-17). The pH is equal to log $[H^+]$ or 1/log $[H^+]$. A neutral solution has a pH of 7.0; acidic solutions have pH values less than 7; and alkaline or basic solutions have pH values greater than 7. Most micro-organisms grow best at near neutral pH. These microorganisms are called **neutralophiles or neutrophiles.** Fungi generally exhibit a wider pH range, growing well over a pH range of 5 to 9, compared to most bacteria, which grow well over a pH range of 6 to 9 (Table 10-4).

Although most bacteria are unable to grow at low pH, there are some exceptional cases. Some bacteria tolerate pH values as low as 0.8. There are even some bacteria, called **acidophiles,** that are restricted to growth at low pH values. Some members of the genus *Thiobacillus* are acidophilic and grow only at pH values near 2; they can grow in sulfuric acid.

Alkalophiles are microorganisms that prefer to grow at high pH values. These microorganisms are found in alkaline environments that are high in sodium such as salt lakes and soils rich in sodium carbonate. The alkalophile *Bacillus alkalophilus* grows best at pH 10.5.

TABLE 10-4
Table of pH Tolerances of Various Bacteria

ORGANISM	MINIMUM pH	OPTIMUM pH	MAXIMUM pH
		ACIDOPHILE	
Thiobacillus thiooxidans	1.0	2.0-2.8	4.0-6.0
Lactobacillus acidophilus	4.0-4.6	5.8-6.6	6.8
		NEUTRALOPHILE	
Escherichia coli	4.4	6.0-7.0	9.0
Clostridium sporogenes	5.0-5.8	6.0-7.6	8.5-9.0
Pseudomonas aeruginosa	5.6	6.6-7.0	8.0
Erwinia carotovora	5.6	7.1	9.3
		ALKALOPHILE	
Bacillus alcalophilus	8.0-8.5	10.5	11.0

Pressure

The solute concentration of a solution affects the osmotic pressure that is exerted across the plasma membrane. The cell walls of bacteria make them relatively resistant to changes in osmotic pressure, but extreme osmotic pressures can result in the death of bacterial cells. In **hypertonic** solutions, bacterial cells may shrink and become desiccated; in **hypotonic** solutions, the cell may burst (FIG. 10-18). Bacteria that can grow in solutions with high solute concentrations are called **osmotolerant.** These bacteria can withstand high osmotic pressures. Some fungi, such as *Xeromyces*, are actually **osmophilic**.

Hydrostatic pressure is another type of pressure that can influence bacterial growth rates. **Hydrostatic pressure** refers to the pressure exerted by a water column as a result of the weight of the water column. Each 10 meters of water depth is equivalent to approximately 1 atmosphere pressure. Most bacteria are relatively tolerant to the hydrostatic pressures in most natural systems but cannot tolerate the extremely high hydrostatic pressures that characterize deep ocean regions. High hydrostatic pressures of greater than 200 atmospheres generally inactivate enzymes and disrupt membrane transport processes. However, some bacteria—referred to as **barotolerant**—can grow at high hydrostatic pressures, and there even appear to be some bacteria—referred to as **barophiles**—that grow best at high hydrostatic pressures.

Light Radiation

Photosynthetic microorganisms require light in the visible spectrum to carry out photosynthesis. The rate of photosynthesis is a function of light intensity. At some light intensities, rates of photosynthesis

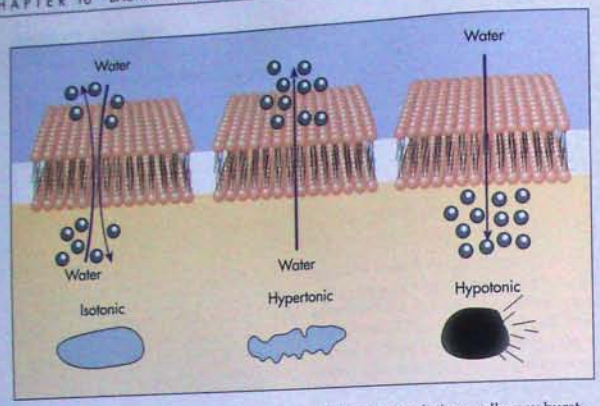


FIG. 10-18 Cells respond to osmotic pressure. In hypotonic solutions, cells may burst.

reach a maximum. Although light intensities above this level do not result in further increases in the rates of photosynthesis, light intensities below the optimal level result in lower rates of photosynthesis.

The wavelength of light also has a marked effect on the rates of photosynthesis. Different photosynthetic microorganisms use light of different wavelengths. For example, anaerobic photosynthetic bacteria use light of longer wavelengths than eukaryotic algae are capable of using. Many photosynthetic microorganisms have accessory pigments that enable them to use light of wavelengths other than the absorption wavelength for the primary photosynthetic pigments. The distribution of photosynthetic microorganisms in nature reflects the variations in the ability to use light of different wavelengths and the differential penetration of different colors of light into aquatic habitats.

The rate of photosynthesis is a function of light intensity and wavelength.

Exposure to visible light can also cause the death of bacteria (FIG. 10-19). Exposure to visible light can lead to the formation of singlet oxygen, which can result in the death of bacterial cells. Some bacteria produce pigments that protect them against the lethal effects of exposure to light. For example, yellow, orange, or red carotenoid pigments interfere with the formation and action of singlet oxygen, preventing its lethal action. Bacteria possessing carotenoid pigments can tolerate much higher levels of exposure to sunlight than nonpigmented microorganisms. Pigmented bacteria often grow on surfaces that are exposed to direct sunlight, such as on leaves of trees. Many viable bacteria found in the air produce colored pigments.

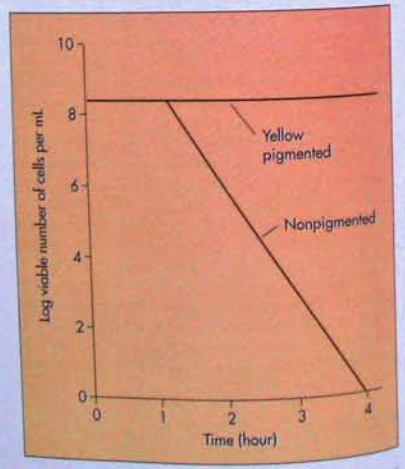


FIG. 10-19 Pigmentation is important in the ability of bacteria to survive exposure to light.

SUMMARY

Bacterial Reproduction (pp. 287-288)

Binary Fission (p. 287)

- Binary fission is the normal form of asexual bacterial reproduction and results in the production of two equal-size daughter cells. Replication of the bacterial chromosome is required to give each daughter cell a complete genome.
- A septum or crosswall is formed by the inward movement of the plasma membrane, separating the two complete bacterial chromosomes in an active protein-requiring process, physically cutting thechromosomes apart and distributing them to the two daughter cells. Cell division is synchronized with chromosome replication.

Alternate Means of Bacterial Reproduction (pp. 287-288)

- Other modes of bacterial reproduction are predominantly asexual, differing in how the cellular material is apportioned between the daughter cells and whether the cells separate or remain together as part of a multicellular aggregation. Budding is characterized by an unequal division of cellular material. In hyphae formation the cell elongates, forming relatively long, generally branched filaments; crosswall formation results in individual cells containing complete genomes.

Bacterial Spore Formation (p. 288)

- Sporulation results in the formation of specialized resistant resting cells or reproductive cells called spores. Endospores are heat-resistant nonreproductive spores that are formed within the cells of a few bacterial genera; cysts are dormant nonreproductive cells sometimes enclosed in a sheath. Myxobacteria form fruiting bodies within which they produce progeny myxospores that can survive transport through the air. Arthrospores are spores formed by hyphae fragmentation that permit reproduction (increase in cell number) of some bacteria.

Bacterial Growth (pp. 288-291)

- Binary fission leads to a doubling of a bacterial population at regular intervals. The generation or doubling time is a measure of the growth phase.

Generation Time (pp. 288-290)

- The time required to achieve a doubling of the population size is known as the generation time or doubling time.
- The generation time is a measure of bacterial growth rate.
- The generation time of a bacterial culture can be expressed as:

$$g = \frac{t}{3.3\,(\log_{10} N_t - \log_{10} N_0)}$$

Bacterial Growth Curve (pp. 291-292)

- The normal growth curve for bacteria has four phases: lag, log or exponential, stationary, and death. During the lag phase, bacteria are preparing for reproduction, synthesizing DNA and enzymes for cell division; there is no increase in cell numbers. During the log phase the logarithm of bacterial biomass increases linearly with time; this phase determines the generation or doubling time. Bacteria reach a stationary phase if they are not transferred to new medium and nutrients are not added; during this phase there is no further net increase in bacterial cell numbers and the growth rate is equal to the death rate. The death phase begins when the number of viable bacterial cells begins to decline.

Batch and Continuous Growth (p. 291)

- In batch cultures, bacteria grow in a closed system to which new materials are not added. In continuous culture, fresh nutrients are added and end products removed so that the exponential growth phase is maintained.

Bacterial Growth on Solid Media (p. 291)

- On solid media, bacteria do not disperse and so nutrients become limiting at the center of the colony; bacterial colonies have a well-defined edge. Each colony is a clone of identical cells derived from a single parental cell.

Enumeration of Bacteria (pp. 292-296)

Viable Count Procedures (pp. 292-294)

- Numbers of bacteria are determined by viable plate count, direct count, and most probable number determinations. In viable plate counts, serial dilutions of bacterial suspensions are plated on solid growth medium by the pour plate or surface spread technique, incubated, and counted. Since each colony comes from a single bacterial cell, counting the colony forming units, taking into account the dilution factors, can determine the original bacterial concentration.

Direct Count Procedures (pp. 294-295)

- Bacteria enumerated by direct counting procedures do not have to be grown first in culture or stained. Special counting chambers are often used.

Most Probable Number (MPN) Procedures (pp. 295-296)

- In most probable number enumeration procedures, multiple serial dilutions are performed to the point of extinction. Cloudiness or turbidity can be the criterion for existence of bacteria at any dilution level; gas production or other physiological characteristics can be used to determine the presence of specific types of bacteria.

Factors Influencing Bacterial Growth (pp. 296-306)

- Environmental conditions influence bacterial growth and death rates. Each bacterial species has a specific tolerance range for specific environmental parameters. Changing environmental conditions cause population shifts. Laboratory conditions can be manipulated to achieve optimal growth rates for specific organisms.

Temperature (pp. 296-301)

- There are maximum and minimum temperatures at which microorganisms can grow; these extremes of

temperature at which growth occurs establish the temperature growth range.
- Several categories of bacteria are defined based on optimal growth temperatures: psychrophiles have optimal growth temperatures of under 20° C; mesophiles have optimal growth temperatures in the middle range (20° to 45° C); and thermophiles grow optimally at higher temperatures, above 45° C.

Oxygen (pp. 301-303)
- Aerobic microorganisms grow only when oxygen is available (respiratory metabolism). Anaerobic microorganisms grow in the absence of molecular oxygen by fermentation or anaerobic respiration. Obligate anaerobes grow only in the absence of molecular oxygen. Facultative anaerobes can grow with or without oxygen and are usually capable of both fermentative and respiratory metabolism. Microaerophiles grow only over a very narrow range of oxygen concentrations; they require oxygen, but high concentrations are toxic.
- Microorganisms possess enzyme systems for detoxifying various forms of oxygen; catalase is involved in the destruction of hydrogen peroxide; superoxide dismutase destroys the toxic superoxide radical.

Salinity (pp. 303-305)
- Most microorganisms cannot tolerate high salt concentrations, but some salt-tolerant bacteria, such as *Staphylococcus*, will grow at high salt concentrations. Halophiles require sodium chloride for growth and extreme halophiles can grow at very high salt concentrations.

Acidity and pH (p. 305)
- The pH of a solution describes its hydrogen ion concentration. Microorganisms vary in their pH tolerance ranges, with fungi generally exhibiting a wider pH range (5 to 9) than bacteria (6 to 9).
- Neutralophiles grow best at near neutral pH. Acidophiles are restricted to growth at low pH values. Some acidophiles grow only at pH 1-2. Alkalophiles grow best at high pH values.

Pressure (p. 305)
- Extreme osmotic pressures can result in microbial death because cells shrink and become desiccated in hypertonic solutions; in hypotonic solutions, cells may burst. Osmotolerant microorganisms can grow in solutions with high solute concentrations. Osmophilic microorganisms require high solute concentrations.
- Hydrostatic pressure is the pressure exerted by a column of water as a result of the weight of the water column (10 meters water = 1 atmosphere of pressure). Most microorganisms are relatively tolerant to hydrostatic pressures in most natural systems, except deep ocean regions.

Light Radiation (pp. 305-306)
- Exposure to visible light can cause death of some microorganisms; some microorganisms produce pigments (often yellow-orange) that protect them against the lethal action of light radiation. Photosynthetic microorganisms require visible light to carry out metabolism and the rate of photosynthesis is a function of light intensity.

CHAPTER REVIEW

Review Questions

1. Define bacterial growth.
2. What is binary fission?
3. How are microorganisms classified based on optimal growth temperature?
4. Define pH and explain its relation to microbial growth.
5. Define osmotic pressure and explain its relation to microbial growth.
6. How are microorganisms classified based on oxygen requirements?
7. How can oxygen be toxic to cells? How do cells protect themselves from these toxic molecules?
8. What are the phases of microbial growth?
9. What is generation time?
10. Describe some direct and indirect measures of microbial growth.
11. What are the similarities and differences between bacteria growing in the environment and those in a continuous culture?
12. What are the advantages and disadvantages of the viable plate count method to assess bacterial numbers?
13. What are the advantages and disadvantages of the direct microscopic count method to assess bacterial numbers?
14. What special requirements do bacteria need to survive in very hot environments? In very cold environments?
15. Describe the different parts of the bacterial growth cycle. What is happening in the cell and in the population of cells in each phase?
16. What does exponential growth mean? What is happening during the exponential growth phase?
17. How long would it take a single bacterial cell to form 1,000,000 cells if it had a generation time of 30 minutes?

CRITICAL THINKING QUESTIONS

1. Suppose you wanted to isolate a microorganism that was a mesophilic, degraded cellulose and was microaerophilic. What conditions would you have to provide to isolate such a microorganism in the laboratory? Where would you obtain the inoculum for establishing the culture?
2. Some bacteria that live in deep ocean waters are obligate barophiles that tend to lyse or rupture when brought to normal atmospheric pressures. What special requirements would these bacteria need to survive in their high pressure environment? Why can't they survive at the ocean surface? How can they be cultured in the laboratory?
3. Why would you want to distinguish between the numbers of live bacteria and dead bacteria in a population? How would you go about doing this? How would you deal with viable nonculturable bacteria?
4. It takes about 60 minutes to replicate the bacterial chromosome. Given that every daughter cell formed by binary fission must have a complete bacterial chromosome, how can some bacteria reproduce every 30 minutes?
5. Why does the clinical microbiology laboratory employ so many different methods for isolating and identifying pathogenic microorganisms? Why can't one set of standardized conditions be employed?

Readings

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CHAPTER 11

Control of Microbial Growth and Death

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  - Detergents
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  - Aldehydes
  - Acids
  - Ethylene Oxide
  - Hydrogen Peroxide
  - Ozone
  - Dyes
  - Heavy Metals
- Antibiotics

PREVIEW TO CHAPTER 11

In this chapter we will:

- Learn that microbial populations can be controlled by limiting growth or increasing death rates.
- Examine the factors that control rates of microbial growth and death.
- See how physical environmental conditions can be modified to control microbial populations.
- Study the chemical approaches for killing or preventing microbial growth.
- Review various types of chemicals used to control microorganisms, including pathogens.
- Learn the following key terms and names:

algicides
antibiotics
antimicrobial agents
antiseptics
autoclave
bactericides
bacteriostatic
chloramination
decimal reduction time (D value)
desiccation
disinfectant
dry heat sterilization
ethylene oxide sterilization
fungicides
fungistatic
germicides
high efficiency particulate air filters (HEPA filters)
high temperature-short time or HTST process
incineration
infrared radiation
low temperature-hold (LTH process)
ozonation
pasteurization
preservative
quaternary ammonium compounds (quats)
sanitizer
shelf life
sporicidal
sterilization
thermal death point (TDP)
ultra high temperature process (UHT process)
virucides

CONTROL OF MICROORGANISMS BY PHYSICAL ENVIRONMENTAL FACTORS

Rates of microbial growth and death are greatly influenced by several environmental factors. Some environmental conditions favor rapid microbial reproduction; others preclude microbial growth or even result in microbial death. Each microorganism has a certain tolerance range for specific environmental parameters. Outside the range of environmental conditions under which a given microorganism can reproduce, it may either survive in a relatively dormant state or may lose viability. Loss of viability means that it will lose the ability to reproduce and consequently die.

By adjusting environmental conditions, one can increase the death rate of microorganisms. This is an important consideration when trying to kill microorganisms. The ability to kill microorganisms is very important in many instances, such as when trying to reduce the numbers of microorganisms in foods so that they do not spoil, and when it is necessary to totally eliminate microorganisms from pharmaceuticals and medical instruments to make them sterile (free of living organisms) and safe for use with patients. Microbial populations also may be physically removed or excluded so as to limit the numbers of microorganisms that can multiply.

Microbial populations can be controlled by modifying environmental conditions.

It is also possible to alter environmental conditions so that microorganisms do not die but also do not reproduce. This method is used for the preservation of microorganisms, such as in culture collections and food preservation, and for preventing spoilage. Many times the conditions needed to heat sterilize a product alter the texture and color of the desired product. It is for this reason that we use freezing to preserve many foods whose taste and textural qualities are destroyed if sterilized at high temperatures.

There are many factors that determine the effectiveness of a particular agent in controlling microorganisms. These factors include the type of microorganism, the amount and type of material to be treated, the duration of the treatment, the concentration or intensity of the agent, and environmental factors such as pH, temperature, and water availability.

PHYSICAL EXCLUSION OR REMOVAL OF MICROORGANISMS

An effective method for controlling microorganisms is by physically excluding them. **Filtration** can be used to remove microorganisms from liquids and gases. Generally, filtration is accomplished by passage of the substance through a filter with 0.2 to 0.45 µm diameter pores. Many pharmaceuticals, such as solutions that are administered intravenously to patients, are sterilized by passage through such filters. Bacteria and other living organisms are eliminated from the solution by trapping them on the filter, but viruses and some very small bacteria may pass through the filter.

Microorganisms can also be removed from air by passage through **high efficiency particulate air filters** (HEPA filters), which remove particulate material larger than about 0.3 µm. Clean rooms, such as operating theaters and rooms where drugs are packaged, often employ HEPA filters. Many microbiology laboratories also have laminar flow hoods in which air that is filtered through a HEPA filter is blown across the work area to prevent contamination during culturing of microorganisms. While not nearly as effective as a HEPA filter, wearing a face mask helps decrease the exchange of microorganisms between people. Surgical staff wear face masks to prevent exhaling microorganisms into the open surgical wound. Staff and visitors wear masks when they are with patients who have infections that may be transmitted through the air. These precautions generally are adequate. However, greater precaution may be warranted for contact with patients with tuberculosis, and it is now required in many situations that a respirator with a HEPA filter be worn rather than a simple surgical mask.

Regardless of whether filtration or other methods, discussed below, are used to eliminate microorgan-

NEWSBREAK

BRINGING SANITARY CONDITIONS TO HOSPITALS

During the Crimean War in the 1850s, poor sanitary conditions led to the rapid spread of typhus. More soldiers were dying of disease than as a direct result of warfare. Florence Nightingale was called on to help in the military hospitals. She attempted to create sanitary conditions, believing in the healing power of pure air. Her reforms in hospital sanitation markedly improved the survival rate of patients and limited the further spread of disease. Although Nightingale had only contempt for the germ theory of disease, the sanitary conditions she fostered worked because she was stemming the spread of disease-causing microorganisms.

isms, many items that are free of microorganisms are kept sterile by wrapping them in paper, plastic, or foil. Such wrappings are impermeable to microorganisms and prevent recontamination. For example, foods are packaged in cans, jars, or other containers that prevent contamination with microorganisms that could contaminate the foods, causing them to spoil and rendering them a threat to human health. Similarly, syringes, needles, scalpels, and other medical instruments are sealed in sterile packages, as are Petri dishes, pipettes, and many other items used in the microbiology laboratory. Surgical gloves and gowns help prevent microorganisms from passing from one person to another, especially during surgical procedures or where a hospital worker comes in contact with a patient that has a contagious disease.

High Temperatures

Temperature is one of the most important environmental factors affecting the rates of microbial growth and death. Temperature influences the rates of chemical reactions by altering the three-dimensional shapes of proteins, thereby affecting the rates of enzymatic activities. Heat can kill microorganisms by denaturing their enzymes. Consequently, high temperatures can be used to kill microorganisms in order to control their proliferation. At temperatures exceeding the maximal growth temperature, the death rate exceeds the growth rate. The higher the temperature above the maximal growth temperature, the higher the death rate for that microorganism.

Temperature influences the rates of chemical reactions by affecting the three-dimensional configuration of proteins and thus enzymatic activity; high temperatures kill microorganisms by denaturing their enzymes.

High temperatures can be used to reduce the numbers of microorganisms or to eliminate all viable microorganisms. When using heat to kill microorganisms, the degree of the microorganism's heat resistance must be considered so that the correct exposure times and temperatures are used. Heat resistance varies among different microorganisms; these differences can be expressed through the concept of thermal death point. **Thermal death point** (TDP) is the lowest temperature required to kill all of the microorganisms in a liquid suspension in 10 minutes. Heat is the most widely applicable and effective agent for killing microorganisms and also the most economical and easily controlled.

The heat killing of microorganisms can be described by the **decimal reduction time (*D* value)** (FIG. 11-1). *D* is defined as the time required for a ten-fold reduction in the number of viable cells at a given

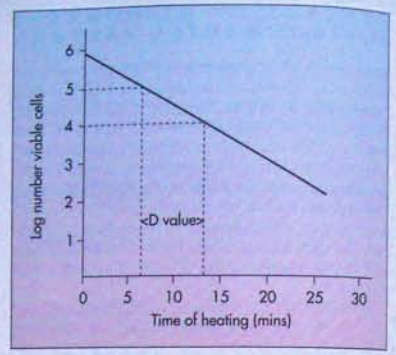


FIG. 11-1 The D value is the time in minutes needed to reduce the number of viable microorganisms by a factor of ten (one log unit).

temperature, that is, the time required for a log reduction in the number of microorganisms. This is the time required to bring about a 90% reduction in the numbers of viable microorganisms. As the temperature is increased above the maximal growth temperature for a microorganism, the decimal reduction time is shortened. The decimal reduction time varies for different microorganisms. In the food industry, the decimal reduction time is important in establishing appropriate processing times for sterilizing food products.

The *D* value describes the rate of death at a given temperature.

Pasteurization

Pasteurization is a process that uses relatively brief exposures to moderately high temperatures to reduce the numbers of viable microorganisms and to eliminate human pathogens (FIG. 11-2). Such procedures prolong **shelf life** and ensure safety of the food as long as human pathogens are eliminated. A pasteurized food, however, retains viable microorganisms, which means that additional preservation methods are needed to extend the shelf life of the product. These other preservation methods, such as refrigeration, are used to reduce the growth rates of the surviving microorganisms. Pasteurization of milk, for example, is required by law in the U.S. to eliminate pathogenic bacteria, namely *Brucella* sp., *Coxiella burnetii*, and *Mycobacterium tuberculosis*. These bacteria are associated with the transmission of disease via contaminated milk. They are relatively sensitive to elevated temperatures, and pasteurization is, therefore, normally achieved by exposure of milk to 62.8° C for 30 minutes **(low temperature-hold or LTH process)** or 71.7° C for 15 seconds **(high temperature-short time or HTST process)**. Milk produced in the United States is normally preserved by pasteurization and requires refrigeration to extend its shelf life.

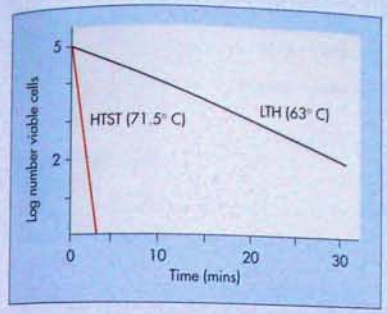


FIG. 11-2 Pasteurization reduces the numbers of viable microorganisms and eliminates human pathogens. The same reduction of viable microorganisms is achieved by heating to 63° C for 30 minutes as is accomplished by exposure to 71.5° C for only 0.33 minutes. The long time hold (LTH) pasteurization process uses 63° C and the high temperature-short time (HTST) pasteurization process uses 71.5° C.

Pasteurization uses brief exposures to moderately high temperatures to reduce numbers of viable microorganisms and eliminate human pathogens but does not eliminate all viable microorganisms.

Sterilization

Sterilization is the complete elimination of living organisms. In many microbiological procedures, high temperatures are used to kill all viable microorganisms, that is, to sterilize materials. As long as there are no endospore-forming bacteria, boiling at 100° C for 10 minutes is adequate to eliminate microorganisms from water. When the potential for the contamination of water supplies with enteric pathogens exists, boiling ensures the bacteriological safety of the water. In many cases, higher temperatures are needed to ensure that all microorganisms, including endospore producers, are killed.

Sterilization eliminates all living organisms.

Excessive heat destroys the quality of milk, for example, boiling alters the smell and taste of milk, but brief exposure to high temperatures kills all the microorganisms in milk without destroying its quality. In the **ultra high temperature** or **UHT process,** exposure to 141° C for 2 seconds is used to sterilize milk. Sterilized milk is currently marketed in several European countries and has been introduced in the United States. It has an indefinite shelf life, provided the container remains sealed.

The heat used in sterilization can be moist or dry heat. Dry heat kills by oxidation. This is, for example, what happens when paper slowly chars in a heated oven when the temperature is below the ignition point of paper. Moist heat kills microorganisms more quickly because the water hastens the breaking of hydrogen bonds that hold proteins in their three-dimensional structure.

Dry heat sterilization requires higher temperatures for much longer exposure periods to kill all the microorganisms in a sample. Exposure in an oven for 2 hours at 170° C (328° F) is generally used for the dry heat sterilization of glassware and other items. The longer period of time and higher temperature (relative to moist heat) are required because heat in water is more readily transferred to a cool body than heat in air. Heat sterilization is very important in medical microbiology where sterile instruments are required for surgical procedures and where contaminated materials must be sterilized before they can be reused or safely discarded.

We routinely sterilize transfer loops by flaming them red hot before aseptically transferring a culture from one site to another. In this case we use a very high temperature for a short time. A similar principle is used in **incineration**, an effective way to sterilize and dispose of contaminated paper cups, bags, and dressings. Medical wastes are safely disposed by using incineration to kill any viable microorganisms.

Dry heat sterilization uses higher temperatures and often takes longer than moist heat sterilization.

Moist heat is far more penetrating than dry heat and, hence, more effective for killing microorganisms. Steam under pressure is frequently used in sterilization procedures. Steam at 121° C for 15 minutes is at least as effective as a dry oven treatment at 170° C for 2 hours and kills all microorganisms, including endospores (FIG. 11-3). Culture media in bacteriological laboratories are normally prepared by heat sterilization in an **autoclave,** an instrument that permits exposure to steam under pressure. The autoclave is basically a chamber that can withstand pressures of greater than two atmospheres. The materials to be sterilized are placed in a chamber, and the chamber is sealed. Steam then is transferred from a jacket into the chamber, forcing out all the air. The steam is held in the chamber for the necessary time and then vented from the chamber. In the normal heat steril-

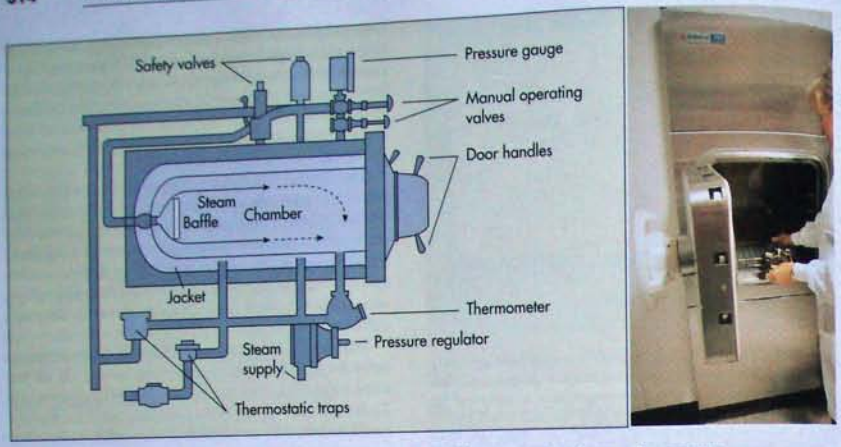


FIG. 11-3 An autoclave is used to sterilize materials by exposure to steam under pressure.

METHODOLOGY

Sterility Testing

Testing of sterility is an important quality control procedure. Autoclaves have pressure gauges and thermometers that monitor the sterilization operation. These monitors are supplemented with internal monitors. Chemicals that darken when exposed to specific heat treatments are sometimes included with each sterilization batch. These chemicals are often impregnated into a tape or wrapping material so that, after adequate exposure to elevated temperature, the darkened chemical spells out the word STERILE or AUTOCLAVED (see Figure). In this way the proper operation of the sterilizer is monitored and, simultaneously, the sterilized material is properly labelled.

Another method for monitoring the operation of the sterilizer is to use a biological indicator. This is the best method for assuring adequacy of the sterilization procedure because the method actually monitors the death of heat-resistant endospores. Typically, spores of *Bacillus stearothermophilus* are used for monitoring the effectiveness of steam sterilizers. The indicator consists of a strip of filter paper impregnated with *B. stearothermophilus* spores. The test strip is exposed to the sterilization procedure and viability is tested by placing the strip in a nutrient solution. After incubation for 24 hours the tube containing the spore strip and nutrient solution is examined for growth. The lack of growth indicates successful sterilization; the occurrence of any growth indicates a failure of the sterilization system.

Photograph showing sterility check with special heat-sensitive tape. Left flask is before sterilization and right flask is after autoclaving. The heat sterilization causes the letters on the tape to appear, indicating that the media has been autoclaved.

ization process, the medium is exposed to steam at a temperature of 121° C (which corresponds to 15 pounds per square inch pressure) for 15 minutes in an autoclave.

An autoclave is an apparatus in which objects or materials may be sterilized by steam under pressure at temperatures over 100° C.

Canning

Canning is the use of heat followed by maintenance of anaerobic conditions (FIG. 11-4). In this preservation method the high temperature exposure kills all the microorganisms in the product, the can or jar acts as a physical barrier to prevent recontamination of the product, and the anaerobic conditions prevent oxidation of the chemicals in the food. Exposure to 115° C for at least 15 minutes is generally considered necessary in home canning to ensure killing of endospore formers in moderate acid (pH 4.5-5.3) to low acid (pH 5.3-7.0) foods (Table 11-1).

Canning is a food preservation method in which suitably prepared foods are placed in glass or metal containers that are heated, exhausted, and hermetically sealed.

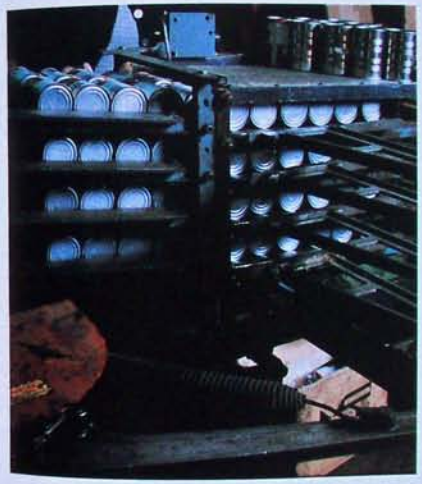


FIG. 11-4 Canning is widely used to preserve foods and keep them free of pathogenic microorganisms. Canning involves heat killing of microorganisms and hermetic sealing under anaerobic conditions to prevent recontamination and spoilage.

Particular concern is given in canning to ensuring sterility of such foods because of the possible growth of *Clostridium botulinum* and the seriousness of the disease botulism. Somewhat lower temperatures, for example, exposure to 100° C for 10 minutes, are often employed in the home canning of acidic (pH less than 4.5) foods. This can be done in part because of the lowered thermal resistance of microorganisms under acidic conditions and because of the fact that *C. botulinum* is unable to grow at low pH values.

The canning industry typically heats food at 121° C for 2.52 minutes. This is 12 times the *D* value for the endospores of *C. botulinum* and therefore reduces the probability of the survival of *C. botulinum* endospores to 10^{-12}. Thus, if there were one spore in every can, the probability of contamination remaining after processing should be reduced to one in every trillion cans. Heating at 121° C for 2.52 minutes therefore should ensure the safety of canned foods with respect to possible contamination with *C. botulinum*. Several commercial canning operations that have not adhered to the necessary standards and whose operations resulted in outbreaks of botulism have been put out of business. Most problems occur with home canning, where a lack of knowledge or care can result in insufficient heating.

TABLE 11-1

Classification of Canned Foods and Their Processing Requirements

ACIDITY CLASS	pH	REPRESENTATIVE FOODS	SPOILAGE AGENTS	PROCESSING
Nonacid-low acid	6-7	Beans, peas, carrots, beets, asparagus, potatoes, poultry, beef, fish, low acid tomatoes	Mesophilic, *Clostridium* spp.	High temperature (121° C)
Acidic-moderately acid	4-5	Tomatoes, pears, peaches, oranges, apricots, apples, pineapples, strawberries, sauerkraut	Aciduric bacteria, fungi	Boiling water (100° C)
Highly acid	3	Pickles, relish, vinegar	Yeasts and other fungi	Boiling water (100° C)

HISTORICAL PERSPECTIVE

Development of Canning

Canning to preserve foods has its origin in 1795 when the French government offered a prize of 12,000 francs for the development of a practical method of food preservation. In 1809 Francois (Nicolas) Appert succeeded in preserving meats in glass bottles that had been kept in boiling water for varying periods of time. Appert was issued a patent for his process in 1810. As early as 1820 the commercial production of canned foods was begun in the United States by W. Underwood and T. Kensett. Appert's discovery that foods could be preserved for prolonged periods of time when they were heated and stored in the absence of oxygen came at the same time that the questions of spontaneous generation and the role of microorganisms in fermentation and putrefaction were being debated by the premier scientists of the day. Spallanzani in 1765 showed that beef broth that had been boiled for an hour and sealed did not spoil. Appert applied to foods the results of Spallanzani's experiments. Not being a scientist, Appert probably did not understand why his method worked or its long-range significance. It was not until almost a half century later that Pasteur, in disproving the theory of spontaneous generation, provided the scientific basis for understanding why Appert's canning method works. Pasteur pointed out that Appert's method, even when modified by using temperatures below 100° C and relatively short incubation times, was a practical method for preventing undesirable ferments. Today, the method of canning, begun almost two centuries ago, is a widely used method for preserving foods.

Low Temperatures

Low temperatures limit the rates of microbial reproduction and thus can be used to prevent or to limit microbial growth (FIG. 11-5). Refrigeration and freezing are widely used to restrict microbial growth. Samples collected in hospital wards for microbiological analysis sometimes are chilled to prevent microbial growth before reaching the clinical microbiologist. Many foods are kept in the refrigerator or freezer to prevent microbial spoilage. Most mesophilic microorganisms grow extremely slowly at refrigerator temperatures (5° C). Although most pathogenic microorganisms are unable to grow in refrigerated foods, *C. botulinum* type E will grow and produce toxin. Psychrophilic microorganisms are also able to grow slowly at 5° C. Thus, although refrigeration extends the shelf life of a product, it does not do so indefinitely.

FIG. 11-5 Refrigeration is used to limit microbial growth. Many foods and products, including microbiological media, shown in this photograph, are preserved by refrigeration.

Freezing at temperatures of −20° C or lower precludes microbial growth entirely. Freezing does not kill most microorganisms, although some microbial death may occur during freezing and during thawing as a result of ice crystal damage to microbial membranes. In fact, freezing at extremely low temperatures is routinely used for preserving microorganisms in type culture collections. Therefore, when food is thawed, the microorganisms associated with that food can grow, leading to food spoilage and potential accumulation of microbial pathogens and toxins if the food is not promptly prepared or consumed. Not all food products can be preserved by freezing because of the damage that may occur to the food as a result of ice formation. Desiccation of frozen foods (freezer burn), although not a microbial spoilage process, causes serious quality defects.

Low temperatures limit rates of microbial reproduction. Temperatures below −20° C preclude microbial growth entirely.

Once thawed, it is generally not advisable to refreeze food products. Freezing, thawing, and refreezing disrupts the texture of the food and in addition permits invasion of the food by microorganisms that are normally restricted to the food surfaces. When thawed a second time, refrozen food products are more prone to microbial spoilage than foods that are allowed to thaw only once.

NEWSBREAK

Refrigeration Fails to Protect Against *Yersinia enterocolitica*

In 1978, containers of chocolate milk were delivered to a school in upstate New York. The milk was refrigerated over a holiday weekend, but it was contaminated with *Yersinia enterocolitica*, a disease-causing bacterium that can grow at refrigerator temperatures. Incubation over the weekend meant that each container of chocolate milk was a culture of *Yersinia*. Fortunately, the ability of a human pathogen to grow at refrigerated temperatures is limited to few species like *Yersinia enterocolitica*. Otherwise, refrigeration would not be useful for preserving foods. Unfortunately, the children who drank the milk containing *Yersinia enterocolitica* developed abdominal pain and other symptoms that normally are characteristic of appendicitis. In all, 200 children were afflicted. One by one they reported to physicians, some of whom diagnosed these as cases of appendicitis, rather than the actual cause—a *Yersinia* infection. Appendicitis is not an infectious disease and would not afflict 200 children simultaneously. Ten appendectomies were performed before the cause of the disease was recognized.

Removal of Water—Desiccation

Many foods are preserved by **desiccation** (removal of water). Water is required for microbial growth. By eliminating water or keeping surfaces dry, microbial growth can be prevented. Exposed wood surfaces are often painted to keep the wood dry enough to preclude microbial growth. Canvas and other textiles are preserved in temperate zones by the lack of water in the air, but in tropical zones these same materials are subject to biodeterioration because the humidity is sufficiently high to permit microbial growth.

Because water is required for microbial growth, many foods can be preserved by desiccation.

Although lack of available water prevents microbial growth, it does not necessarily accelerate the death rate of microorganisms. Some microorganisms, therefore, can be preserved by drying. Active dried yeast is used for baking purposes. After the addition of water, the yeasts begin to carry out active metabolism. **Freeze-drying** (lyophilization) is a common means of removing water that can be used for preserving microbial cultures (FIG. 11-6). During freeze-

FIG. 11-6 Lyophilization, or freeze-drying, is used to preserve microbial cultures. The instrument used for this process uses a high vacuum and low temperature so that water sublimes (goes from the solid frozen state directly to a gas). This removes water from the specimen without disrupting cellular structures, allowing viability to be maintained.

drying, water is removed by sublimation, that is, water is converted directly from the solid to the gas phase. This process generally eliminates damage to microbial cells from the expansion of ice crystals.

Lack of water prevents microbial growth but does not accelerate the microbial death rate, making possible preservation by drying.

Whereas some microorganisms are relatively resistant to drying, other microorganisms are unable to survive desiccating conditions for even a short period of time. The ability to withstand drying can have important pathogenic implications. *Mycobacterium tuberculosis* is a classic example of an organism capable of withstanding severe desiccation and still remaining infective. In contrast, *Treponema pallidum*, the bacterium that causes syphilis, is extremely sensitive to drying and dies almost instantly in the air or on a dry surface.

Some microorganisms produce specialized spores that can withstand the desiccating conditions of the atmosphere. Such spores generally have thick walls that retain moisture within the cell. Many fungal spores can be transmitted over long distances through the atmosphere; some spores even travel from one continent to another. The transmission of fungal spores through the air is a serious problem in agriculture because it permits the spread of fungal diseases of plants from one field to another.

NEWSBREAK

Salem Witch Hunts

In the 1690s, witch hunts were a regular practice in Salem and surrounding regions of New England. People accused of being witches were burned at the stake. In most cases, these people were accused of casting spells or performing supernatural acts. Recent scientific evidence suggests that the accusors were suffering from ergotism, a form of food poisoning that is characterized by hallucinations. The disease occurs when fungi grow on grains and produce chemicals called ergot alkaloids. Apparently, excessive rain wet the grains and without other means of food preservation, the fungi were able to grow on them. The spoilage of grains was widespread, which explains the occurrence of mass hallucinations. The accusors were hallucinating, which is why they thought they saw witches.

Radiation

High-energy, short-wavelength radiation disrupts DNA molecules. Exposure to such radiation may cause mutations, many of which are lethal. Exposure to **gamma radiation** (short wavelengths of 10^{-3} to 10^{-1} nanometers), **X-radiation** (wavelengths of 10^{-3} to 10^{-2} nanometers), and **ultraviolet radiation** (ultraviolet light with wavelengths of 100 to 400 nanometers) increases the death rate of microorganisms and is used in various sterilization procedures to kill microorganisms. Gamma and X-radiation have high penetrating power and are able to kill microorganisms by inducing or forming toxic free radicals (ions). Therefore gamma and X-radiations are referred to as ionizing radiations. Free radicals are highly reactive chemical species that can lead to polymerization and other chemical reactions disruptive to the biochemical organization of microorganisms. Viruses and other microorganisms are inactivated by exposure to ionizing radiation (FIG. 11-7).

Exposure to radiation may cause microbial mutations, induces the formation of toxic free radicals, increases microbial death rates, and is used as a sterilization method.

Sensitivities to ionizing radiation vary. Nonreproducing (dormant) stages of microorganisms tend to be more resistant to radiation than growing organisms. For example, endospores are more resistant than the vegetative cells of many bacterial species.

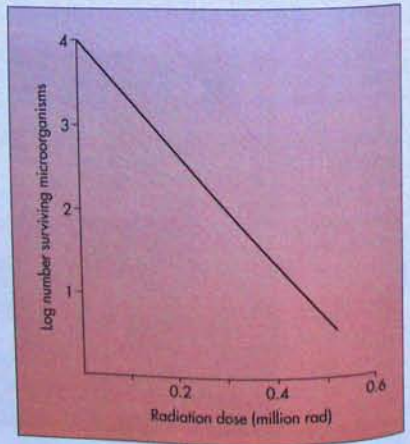


FIG. 11-7 Ionizing radiation effectively kills microorganisms, including viruses.

HIGHLIGHT

Safety of Irradiated Food

Research on irradiation as a food preservation technology began after World War II when the U.S. Army began a series of experiments irradiating fresh foods for troops in the field. Since 1963, the United States Food and Drug Administration (FDA) has passed rules permitting irradiation to curb insects in food and microorganisms in spices, control parasites in pork, and retard spoilage in fruits and vegetables. On May 2, 1990, the FDA approved the use of irradiation as a safe and effective means to control a major source of foodborne illness—*Salmonella* and other foodborne bacteria in raw chicken, turkey, and other poultry. Food safety experts believe that up to 60% of all poultry sold in the United States is contaminated with *Salmonella* and that perhaps all chicken may be contaminated with *Campylobacter* organisms. Eating poultry contaminated with these organisms may cause disease, with symptoms ranging from a simple stomach ache to incapacitating stomach and intestinal disorders, occasionally resulting in death.

Although the FDA has concluded that irradiation of food is safe, the public remains frightened by any use of radiation. They are fearful that irradiated foods may be contaminated and carry dangerous radioactivity. They associate radiation with atomic bombs and nuclear reactor accidents like Chernobyl and Three Mile Island. Action groups have formed to block the distribution of foods sterilized by irradiation. Three states (Maine, New York, and New Jersey) have banned or issued moratoriums on the sale of irradiated foods. Irradiation opponents charge that the FDA, the World Health Organization, and the nuclear power industry are conspiring to promote the technique as a way to dispose of nuclear waste.

To counter fears about radiation, the FDA points out that irradiation does not make food radioactive. The specified exposure times and energy levels of radiation sources approved for food cannot induce radioactivity in the food. Food irradiation does not leave a residue that is harmful to people. It removes potentially harmful pathogens and food spoilage microorganisms. During the irradiation process, the genetic material of bacteria is damaged such that they can no longer survive or multiply. No radioactive material is ever added to the product. The same technique is used to sterilize many disposable medical devices.

The FDA requires that irradiated foods be labeled as such so that consumers know what they are buying. A mandatory logo was added in 1986. It consists of a solid circle, representing an energy source, above two petals, which represent food. Like the FDA, the World Health Organization concludes that irradiation can substantially reduce food poisoning. It sees the use of irradiation as a means of reducing food cost because it can reduce food spoilage. The first major food irradiation plants are currently under construction. The success of these plants will depend on consumer acceptance of the products. You can expect to see foods labeled with the FDA-required labels soon on the shelves of your food market.

Exposure to 0.3 to 0.4 Mrads (million units of radiation) is necessary to cause a tenfold reduction in the number of viable bacterial endospores. An exception is the bacterium *Micrococcus radiodurans*, which is particularly resistant to exposure to ionizing radiation. Vegetative cells of *M. radiodurans* tolerate as much as 1 Mrad of exposure to ionizing radiation with no loss of viability. It appears that efficient DNA repair mechanisms are responsible for the high degree of resistance to radiation exhibited by this bacterium.

Ionizing radiation is used to pasteurize or sterilize some products. Most commercially produced plastic Petri plates are sterilized by exposure to gamma radiation. Foods also sometimes are sterilized in this manner. Most such sterilization procedures employ gamma radiation from ^{60}Co or ^{137}Ce. Bacon, for example, can be sterilized by using radiation doses of 4.5 to 5.6 Mrads.

Unlike gamma radiation, **ultraviolet light** (UV) does not have high penetrating power. It is useful for killing microorganisms only on or near the surface of clear solutions. The strong germicidal wavelength of 260 nanometers coincides with the absorption maximum of DNA, suggesting that the principal mechanism by which ultraviolet light exerts its lethal effect is through the disruption of the DNA. Microorganisms have several mechanisms that can repair the alterations in the DNA that are caused by exposure to ultraviolet light, limiting the effectiveness of using UV exposure to control microbial populations. Exposure to ultraviolet light sometimes is used to maintain the sterility of some surfaces. In some hospitals, benchtops are maintained bacteria-free when not in use by using an ultraviolet lamp. The dangers involved in human exposure to excess ultraviolet radiation include blindness if light is viewed directly.

Ultraviolet light kills microorganisms only on or near the surface of clear solutions by disrupting their DNA.

Long wavelengths of **infrared radiation** (wavelengths of 10^3 to 10^5 nanometers) and **microwave radiations** (wavelengths greater than 10^6 nanometers) also have poor penetrating power. Infrared and microwave radiations do not appear to kill microorganisms directly. Absorption of such long wavelength radiation, however, results in increased temperature. Exposure to infrared or microwave radiations can thus indirectly kill microorganisms by exposing them to temperatures that are higher than their maximal growth temperatures. Because microwaves generally do not kill microorganisms directly, there is some concern in the food industry that cooking with microwave ovens may not adequately kill microorganisms that contaminate food products.

CONTROL OF MICROBIAL GROWTH BY ANTIMICROBIAL AGENTS

Chemical inhibitors are widely used to prevent the spread of disease-causing microorganisms. They are also used to preclude the growth of microorganisms that cause spoilage of foods or biodeterioration of industrial products. Chemicals that kill microorganisms or prevent the growth of microorganisms are called **antimicrobial agents.**

Antimicrobial agents are chemicals that kill or prevent the growth of microorganisms.

Various types of antimicrobial agents are employed to control microbial growth. Many antimicrobial agents are designed to block active metabolism and prevent the organism from generating the macromolecular constituents needed for reproduction. Because resting stages are metabolically dormant and are not reproducing, they are not affected by such antimicrobial agents. Hence, growing microorganisms are more sensitive than dormant stages, such as spores. Similarly, viruses are more resistant than other microorganisms to antimicrobial agents because they are metabolically dormant outside host cells.

Antimicrobial agents are classified according to their application and spectrum of action (Table 11-2). Microorganisms vary in their sensitivity to particular antimicrobial agents. The suffix -cidal is generally added to indicate the ability of that particular agent to kill microorganisms. A sporicidal agent, for example, kills bacterial spores, a bactericidal agent kills bacteria, and so forth. Other chemical agents are tagged with the suffix -static, indicating that growth of the microorganism is stopped (but the cells are not necessarily killed). A bacteriostatic agent inhibits the growth of bacteria; a fungistatic agent prevents the growth of fungi.

Antimicrobial chemicals are also classified according to their intended use (Table 11-3, p. 322). In particular, antimicrobial agents are differentiated based on whether they can be safely ingested or applied to body tissues. The term ***disinfectant*** refers to antimicrobial substances that kill microorganisms on inanimate objects. Generally, disinfectants are too harsh to be used on living tissues such as skin. Under appropriate conditions, a disinfectant may produce complete sterilization, that is, disinfectants may kill all microorganisms. In such cases they are termed *sterilizing agents*. However, disinfectants may not inactivate spores. A **sanitizer** represents a particular kind of disinfectant that is used to reduce numbers of bacteria to levels judged safe by public health officials.

TABLE 11-2

Terms Used to Describe Actions of Antimicrobial Agents

TERM	ACTION	EXAMPLES
Algicide	Agent that kills algae	Copper sulfate
Bactericide	Agent that kills bacteria	Chlorhexidine, ethanol
Biocide	Agent that kills living organisms	Hypochlorite (bleach)
Fungicide	Agent that kills fungi	Ethanol, zinc pyrithione
Germicide	Chemical agent that specifically kills pathogenic microorganisms	Formaldehyde, silver, mercury
Sporicide	Agent that kills bacterial endospores	Glutaraldehyde
Virucide	Inactivates viruses so that they lose the ability to replicate	Cationic detergents (Cepacol, Zephriam)
Bacteriostatic	Inhibits the growth and reproduction of bacteria	Sorbate, benzoate
Fungistatic	Inhibits the growth and reproduction of fungi	Zinc oxide, calcium propionate

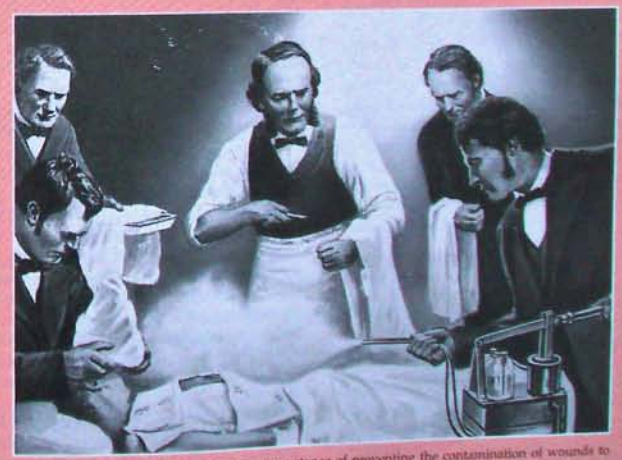
HISTORICAL PERSPECTIVE

Discovery of Antiseptics

The use of antiseptics to prevent infections was introduced by Joseph Lister, an English Quaker and physician, who revolutionized surgical practice in 1867 by introducing antiseptic principles (see Figure). The discovery in the early 1850s of anesthesia and its administration to patients made surgery much easier but, of course, did nothing to reduce the incidence of postsurgical disease, which was often as high as 90%, especially in military hospitals.

As a surgeon, Lister investigated various problems relating to inflammation and pus formation and the coagulation of blood in wound healing. Lister knew that in the 1840s, Ignaz Semmelweis, a Hungarian physician who worked in maternity wards in Vienna, showed that physicians who went from one patient to another without washing their hands were responsible for transmitting childbed fever (puerperal fever). In an attempt to minimize postoperative infections, Lister tried to have his wards kept as clean as possible and to perform his surgeries in an environment that was also as clean as possible, but his patients still contracted hospital bacterial infections at an alarming rate.

Lister was also aware that Pasteur in the 1860s demonstrated that microorganisms in the air caused fermentation and putrefaction and reasoned that these same microorganisms could also cause wound infections. Therefore, if wounds could be kept free of microorganisms, he thought, they would not become infected. Lister experimented with solutions of carbolic acid (phenol) as an antiseptic during surgery to kill the microorganisms. He first used bandages soaked in carbolic acid to dress wounds due to compound fractures to diminish the likelihood of infection. Later he used a carbolic acid spray in addition to direct application of this compound during surgical procedures. Lister quickly achieved amazing results and in 1867 was able to report marked decreases in rates of infection and deaths due to infection. He eventually discarded the practice of spraying after 17 years of trials as unnecessary, but he retained the use of direct application. Lister's frequent modifications of his system and the fact that many physicians and surgeons would not accept the germ theory of disease caused many years of delay before Lister's innovations received widespread application. The evolution of aseptic surgery, preventing access by germs to the operative site by the use of sterilization for gowns, drapes, and instruments was simply the logical extension of Lister's work, making his achievement one of the first great triumphs of applied bacteriology in medicine.



Joseph Lister (1827-1912) recognized the importance of preventing the contamination of wounds to curtail the development of infection. He developed antiseptic methods for preventing infection using carbolic acid (phenol) to treat wounds.

TABLE 11-3

Terms Used to Describe Antimicrobial Agents Based on Their Application

TERM	DESCRIPTION	EXAMPLES
Antibiotic	Agent produced by microorganisms that inhibits or kills other microorganisms	Penicillin, erythromycin, tetracycline, cephalosporin
Antiseptic	Agent that kills or prevents the growth of microorganisms on living tissues	Mercurochrome, gentian violet, hydrogen peroxide, tincture of iodine, phenolics, ethanol
Disinfectant	Agent that kills microorganisms on inanimate objects	Hypochlorite (bleach), formaldehyde, glutaraldehyde
Sanitizer	A disinfectant that is used to reduce numbers of bacteria to levels judged safe by public health officials	Ethanol

Antiseptics kill or prevent the growth of microorganisms on living tissues. The term *antiseptic* literally means a substance that opposes sepsis, that is, a substance that works against putrefaction or decay. Such agents have minimal toxicity to human tissues and can safely be applied to body surfaces. They may be toxic, however, if ingested. Antiseptics are widely used to prevent disease by eliminating viable microorganisms from surface body tissues. Their use in preventing disease is discussed in detail later in this chapter.

Antibiotics, which are substances produced by microorganisms that inhibit or kill other microorganisms, are used to treat infections within the body. They were first discovered by Alexander Fleming who observed that the bacterium *Staphylococcus aureus* would not grow in the vicinity of the fungus *Penicillium* because this fungus produces the antibiotic penicillin. Since the introduction of penicillin in medicine about 50 years ago, antibiotics have revolutionized medical practice. Today many antimicrobials are produced synthetically and the term *antimicrobic* is often used instead of *antibiotic*. The role of antimicrobics in medicine is discussed in Chapter 17.

Disinfectants are antimicrobial substances that kill microorganisms on inanimate objects. Sanitizers reduce bacterial numbers to safe levels. Antiseptics kill or prevent the growth of microorganisms on living tissues. Antibiotics and other antimicrobics are used to treat infections within the body.

Food Preservatives

Food preservatives are antimicrobial agents that prevent the growth of microorganisms in food products. They preserve food products against spoilage or biodeterioration. They are important for increasing the shelf life (preventing spoilage) of the food and for preventing the growth of disease-causing microorganisms within the food. The use of food preservatives is widespread and represents an important means of preserving foods and other products. A variety of food preservatives are used to protect different food products against specific microorganisms (Table 11-4). There is great public concern over the addition of any chemicals to foods because of the finding that some chemicals that were used as food additives, such as red dye number 2, are potential carcinogens. Still it must be remembered that the effective preservation of food prevents spoilage and the transmission of foodborne diseases. In the United States the FDA is responsible for determining and certifying the safety of food additives and must approve any chemicals that are added to foods as preservatives.

Food preservatives are antimicrobial agents that protect a food against microbial spoilage or the growth of disease-causing microorganisms.

Salt and Sugar

The addition of salt or sugar to a food reduces the amount of available water and alters the osmotic pressure. High salt concentrations, such as exist in saturated brine solutions, are bacteriostatic—that is, they prevent the growth of bacteria. Salting is effectively used for the preservation of fish, meat, and other foods. However, because of the association of high levels of salt in the diet with high blood pressure and heart disease, there is currently great interest in lowering the salt content of foods. Sugars, such as sucrose, also act as preservatives and are effective in preserving fruits, candies, condensed milk, and other foods. Some foods, including maple syrup and honey, are preserved naturally by their high sugar content.

Acids

Various carboxylic acids are inhibitors of microbial growth. Lactic, acetic, propionic, citric, benzoic, and sorbic acids or their salts are effective food preservatives. An examination of the lists of food additives in the various foods in your pantry will rapidly convince you of the wide use of organic acids as preservatives. The effectiveness of a particular organic acid depends on the pH of the food. For example, at the same pH, citric acid is less effective than lactic acid, which in turn is less effective than acetic acid.

TABLE 11-4

Some Representative Chemical Food Preservatives

PRESERVATIVES	MAXIMUM	TARGET ORGANISMS	FOODS
Propionic acid and propionates	0.32%	Fungi	Bread, cakes, some cheeses
Sorbic acid and sorbates	0.2%	Fungi	Cheeses, syrups, jellies, cakes
Benzoic acid and benzoates	0.1%	Fungi	Margarine, cider, relishes, soft drinks, catsup
Sulfur dioxide, sulfites, bisulfites, metabisulfites	200—300 ppm	Microorganisms	Dried fruits, grapes, molasses
Ethylene and propylene oxides	700 ppm	Fungi	Spices
Sodium diacetate	0.132%	Fungi	Bread
Sodium nitrite	200 ppm	Bacteria	Cured meats, fish
NaCl	None	Microorganisms	Meats, fish
Sugar	None	Microorganisms	Preserves, jellies
Wood smoke	None	Microorganisms	Meats, fish

Propionates are primarily effective against filamentous fungi. The calcium and sodium salts of propionic acid are used as preservatives in bread, cake, and various cheeses. Lactic and acetic acids are effective preservatives that form naturally in some food products. Cheeses, pickles, and sauerkraut contain concentrations of lactic acid that normally protect the food against spoilage. Vinegar is dilute acetic acid, which is an effective inhibitor of bacterial and fungal growth. Acetic acid is used to pickle meat products and is added as a preservative to various other products, including mayonnaise and catsup. Both of these preservatives, however, will prevent surface fungal growth on a food only if molecular oxygen is excluded.

Benzoates, including sodium benzoate, methyl *p*-hydroxybenzoate (methylparaben), and propyl-hydroxybenzoate (propylparaben), are extensively used as food preservatives in such products as fruit juices, jams, jellies, soft drinks, salad dressings, fruit salads, relishes, tomato catsup, and margarine. They are also used as preservatives in many pharmaceutical preparations.

Sorbic acid, used primarily as calcium, sodium, or potassium salts (for example, sodium sorbate) is more effective as a preservative at pH 4-6 than the benzoates. Sorbates inhibit fungi and bacteria, such as *Salmonella*, *Staphylococcus*, and *Streptococcus* species. Sorbates are frequently added as preservatives to cheeses, baked goods, soft drinks, fruit juices, syrups, jellies, jams, dried fruits, margarine, and various other products.

Boric acid is used as a preservative in eyewash and other products. The limited toxicity of boric acid to human tissues makes it suitable for such applications. Boric acid is also used in urine collection jars to prevent bacterial growth between the time of collection and analysis.

Nitrates and Nitrites

Nitrates and nitrites are added to cured meats to preserve the red meat color and protect against the growth of food spoilage and poisoning microorganisms. Nitrates are effective inhibitors of *Clostridium botulinum* in meat products such as bacon, ham, and sausages. Recently, however, there is great concern over the addition of nitrates and nitrites to meats because these salts can react with secondary and tertiary amines to form nitrosamines, which are highly carcinogenic.

Disinfectants and Antiseptics

Various chemicals are antimicrobial agents that are used as disinfectants and antiseptics (Table 11-5). Disinfectants are used for reducing the numbers of microorganisms on the surfaces of inanimate objects such as floors and walls. Many household cleaning agents contain disinfectants. Disinfectants are also used to limit microbial populations within liquids; for example in swimming pool water. Disinfectants, however, are not considered safe for application to human tissues or for internal consumption. If they are used to destroy microorganisms from a consumable product, such as drinking water, their concen-

TABLE 11-5

Summary of Chemical Agents Used as Disinfectants and Antiseptics

ANTIMICROBIAL AGENT	DESCRIPTION
Phenolics	Phenol is no longer used as a disinfectant or antiseptic because of its toxicity to tissues. Derivatives of phenol such as *o*-phenylphenol, hexylresorcinol, and hexachlorophene are used as disinfectants and antiseptics.
Halogens	Chlorination is extensively used to disinfect water; drinking water, swimming pools, and waste treatment plant effluent are disinfected by chlorination. Organobromine compounds are used to disinfect spas, swimming pools, and cooling towers. Iodine is an effective antiseptic; iodophors are used as disinfectants and antiseptics; the soaps used for surgical scrubs often contain iodophors.
Alcohols	Alcohols are bactericidal and fungicidal but are not effective against endospores and some viruses; ethanol and isopropanol are commonly used as disinfectants and antiseptics. Thermometers and other instruments are disinfected with alcohol, and swabbing of the skin with alcohol is done before injections.
Aldehydes	Formaldehyde is used as a preservative and disinfectant; glutaraldehyde is used to sterilize some surgical equipment.
Heavy metals	Heavy metals such as silver, copper, mercury, and zinc have antimicrobial properties and are used in disinfectant and antiseptic formulations. Silver nitrate was used to prevent gonococcal eye infections. Mercurochrome and Merthiolate are applied to skin after minor wounds. Zinc is used in antifungal antiseptics. Copper sulfate is used as an algicide.
Dyes	Several dyes, such as gentian violet, inhibit microorganisms and are used as antiseptics for treating minor wounds.
Surface-active agents	Soaps and detergents are used to remove microorganisms mechanically from the skin surface. Anionic detergents (laundry powders) remove microorganisms mechanically; cationic detergents, which include quaternary ammonium compounds, have antimicrobial activities. Quaternary compounds (quats) are used as disinfectants and antiseptics.
Oxidizing agents	Ethylene oxide is an excellent sterilizing agent, especially for objects that would be destroyed by heat; ethylene oxide sterilizers are used for the disinfection of plastics and linens. Ozone is a powerful oxidizing agent; ozonation may replace chlorination for the disinfection of drinking water; hydrogen peroxide is a mild antiseptic that is effective against anaerobic bacteria.
Acids	Organic acids can control microbial growth and are frequently used as preservatives. Sorbic, benzoic, lactic, and propionic acids are used to preserve foods and pharmaceuticals. Benzoic, salicylic, and undecylenic acids are used to control fungi that cause diseases such as athlete's foot.

tration must be reduced before the product is consumed.

Concentration and **contact time** are critical factors that determine the effectiveness of disinfectants and antiseptic agents against a particular microorganism. Microorganisms vary in their sensitivity to particular antimicrobial agents. Generally, growing microorganisms are more sensitive than organisms in dormant stages, such as spores. Many antimicrobial agents are aimed at blocking active metabolism and preventing the organism from generating the macromolecular constituents needed for reproduction. Because resting stages are metabolically dormant and are not reproducing, they are not affected by such antimicrobial agents. Similarly, viruses are more resistant than other microorganisms to antimicrobial agents because they are metabolically dormant outside host cells.

Antimicrobial agents are chemicals that kill or prevent the growth of microorganisms.

Disinfectants obviously should have high germicidal activity. They should rapidly kill a wide range of microorganisms, including spores. The agent should be chemically stable and effective in the presence of organic compounds and metals. The ability to penetrate into crevices is desirable. It is essential that a disinfectant not destroy the materials to which it is applied. Furthermore, it should be inexpensive and aesthetically acceptable.

Effective disinfectants kill a wide range of microorganisms, including spores; are chemically stable; work in the presence of organic compounds and metals; penetrate crevices; are safe on surfaces; and are inexpensive and aesthetically pleasing.

Two factors must be evaluated in determining the effectiveness of antiseptics: the agent must produce effective antimicrobial activity and must not be toxic to living tissues. A particularly meaningful approach for comparing antiseptics that encompasses the antimicrobial activity and the toxicity to tissues is the generation of a toxicity index. In the tissue toxicity test, germicides are tested for their ability to kill bacteria and their toxicity to chick-heart tissue cells. The toxicity index is defined as the ratio of the greatest dilution of the product that can kill the animal cells in 10 minutes to the dilution that can kill the bacterial cells in the same period of time and under identical conditions. For example, if a substance is toxic to chick-heart tissue at a dilution of 1:1,000 and bactericidal for *Staphylococcus aureus* at a dilution of 1:10,000, the toxicity index would be 1,000/10,000 or 0.1. Typical toxicity values for iodine solution and Merthiolate are 0.2 and 3.3, respectively. Ideally, an antiseptic should have a toxicity index of less than 1.0, that is, it should be more toxic to bacteria than to human tissue.

Antimicrobial activity and lack of toxicity to living tissue must be determined for antiseptics by generating a toxicity index.

Halogens—Chlorine, Bromine and Iodine

The halogens—chlorine, bromine, and iodine—are effective microbicidal elements that are widely used as disinfectants. Chlorine kills microorganisms by disrupting membranes and inactivating enzymes (FIG. 11-8). Various inorganic and organic forms of chlorine are used for disinfection purposes. Hypochlorite solutions are commonly used in disinfecting and deodorizing procedures. Sodium hypochlorite (Clorox bleach) is widely used as a household disinfectant. Food processing plants and restaurants also use calcium and sodium hypochlorite solutions to disinfect utensils. In some hospitals, hypochlorite is used to disinfect rooms, surfaces, and nonsurgical instruments.

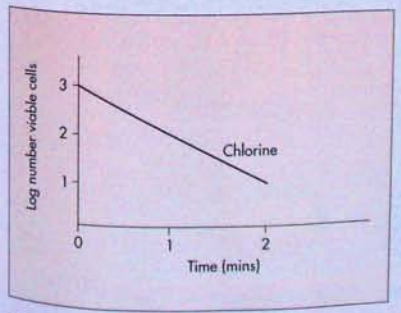


FIG. 11-8 Chlorine rapidly kills microorganisms and is widely used for the disinfection of water.

Chlorine kills microorganisms by disrupting membranes and inactivating enzymes.

The germicidal action of chlorine is based on the formation of hypochlorous acid when it is added to water. Hypochlorous acid releases an active form of oxygen that reacts with cellular biochemicals. Chlorine gas condensed into liquid form is widely used for such disinfection. **Chlorination** (treatment with chlorine) or **chloramination** (treatment with chloramines) is the standard treatment for disinfecting drinking water in most communities. It is also used to disinfect effluents from sewage treatment plants to minimize the spread of pathogenic microorganisms. Because most forms of chlorine are inactivated in the presence of organic matter, this disinfection process does not completely eliminate microorganisms from the water.

The disinfection of water is very important because the same water supplies that are used as drinking water often are also used for human waste disposal. The outfall from one city's sewage treatment plant often flows downstream to the drinking water intake of another city. When the disinfection of drinking water is inadequate, other measures, such as boiling before use, must be employed to ensure water safety. For example, when flooding washes untreated sewage directly into the water supply, such alternative disinfection procedures become necessary. Outbreaks of diseases such as cholera and typhoid fever often occur if drinking water supplies are not completely disinfected.

Campers often use organic compounds containing chlorine to disinfect water. Their campsite water supply may be contaminated with fecal matter and associated human pathogens. Halazone (parasulfone dichloramidobenzoic acid) and succinchlorimide are examples of such chlorine-containing compounds. These organic chlorides, which are quite stable in tablet form, become active when placed in water. A halazone concentration of 4 to 8 milligrams per liter (mg/L) safely disinfects water containing *Salmonella typhi*, the bacterium that causes typhoid fever, within 30 minutes. Succinchlorimide at a concentration of 12 mg/L will disinfect water within 20 minutes. NASA uses iodine in space vehicles to treat potable water.

Chlorine is also used to disinfect swimming pools. Liquid chlorine and hypochlorite solutions are frequently used for such purposes. A residual chlorine level of 0.5 mg/L will achieve control of microbial populations and prevent the multiplication of pathogens in swimming pools. Such levels of chlorine are relatively harmless to human tissues, al-

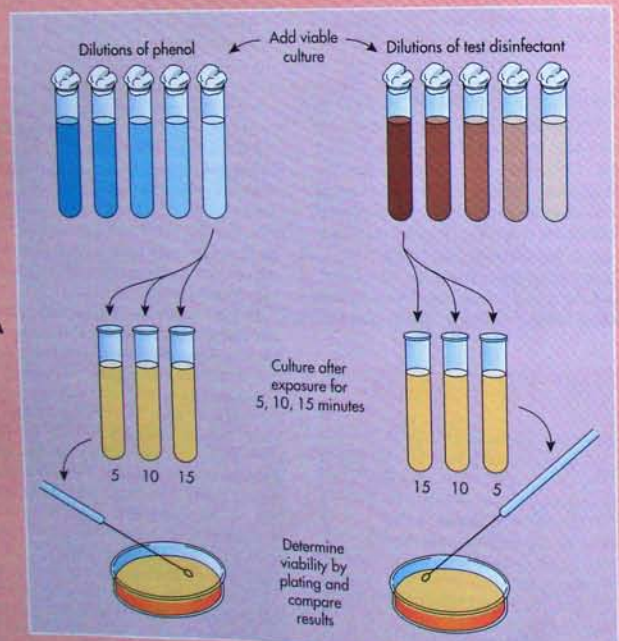
METHODOLOGY

Evaluation of the Effectiveness of Disinfectants

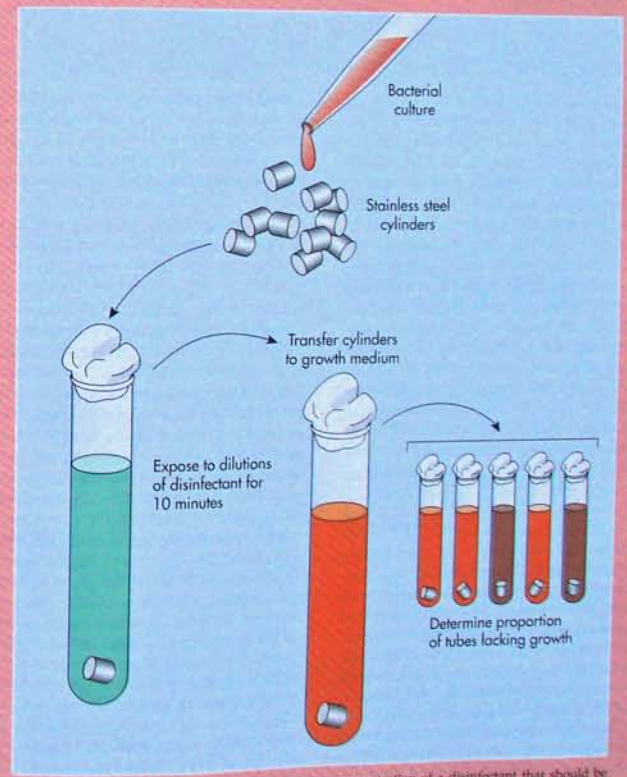
Concentration and contact time are critical factors that determine the effectiveness of an antimicrobial agent against a particular microorganism. Several standardized test procedures have been employed for evaluating the effectiveness of disinfectants. The classic test procedure used until a few decades ago is the phenol coefficient (FIG. *A*). Phenol, which is also known as carbolic acid, was the antimicrobial chemical used by Joseph Lister for preventing infections following compound fractures and for antiseptic surgical practice. The phenol coefficient test compares the activity of a given product with the killing power of phenol under the same test conditions.

To determine the phenol coefficient, dilutions of phenol and the test product are added separately to test cultures of *Staphylococcus aureus* or *Salmonella typhi*. The tests are run in liquid culture. After exposure for 5, 10, and 15 minutes, a sample from each tube is collected and transferred to a nutrient broth medium. After incubation for 2 days, the tubes from the different disinfectant dilutions are examined for visible evidence of growth. The phenol coefficient is defined as the ratio of the highest dilution of a test germicide that kills the test bacteria in 10 minutes but not in 5 minutes to the dilution of phenol that has the same killing effect. For example, if the greatest dilution of a test disinfectant producing a killing effect was 1:100 and the greatest dilution of phenol showing the same result was 1:50, the phenol coefficient would be 100/50 or 2.0.

The phenol coefficient indicates the relative toxicity of various disinfectants but does not establish the appropriate concentration that should be used for disinfecting surfaces. The Association of Official Analytical Chemists (AOAC) use-dilution method, which has replaced the phenol coefficient as the standard method for evaluating the effectiveness of disinfectants, establishes appropriate dilutions of a germicide for actual conditions (FIG. *B*). In this procedure, disinfectants are tested against *Staphylococcus aureus* strain ATCC 6538, *Salmonella choleraesuis* strain ATCC 10708, and *Pseudomonas aeruginosa* strain ATCC 15442. Small stainless steel cylinders are contaminated with specified numbers of the test bacteria. After drying, the cylinders are placed into a series of specified dilutions of the test disinfectant. At least 10 replicates of each organism at the test dilutions of the disinfectant are used. The cylinders are *(1)* exposed to the disinfectant for 10 minutes, *(2)* allowed to drain, *(3)* transferred to appropriate culture media, and *(4)* incubated for 2 days. After incubation the tubes are examined for growth of the test bacteria. No growth occurs if the disinfectant is effective at the test concentration. An acceptable use-dilution is one that kills all test organisms at least 95% of the time.



The phenol coefficient determines the relative effectiveness of a disinfectant to that of phenol for killing microorganisms.



The use dilution method establishes the appropriate concentration of a disinfectant that should be used.

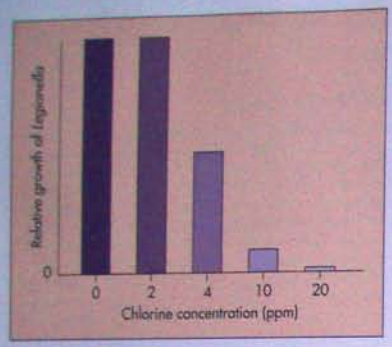


FIG. 11-9 Chlorination is used to control *Legionella* in cooling towers; chlorine effectively kills *Legionella*.

though prolonged exposure can cause irritation to the eyes and will bleach swimsuits.

Chlorination of water in air-conditioning cooling towers is important to control populations of *Legionella pneumophila*, the bacterium that causes Legionnaire's disease (FIG. 11-9). Outbreaks of Legionnaire's disease have frequently been traced to aerosols released from cooling towers that are then dispersed through the air. It has also been suggested that disinfection of home water heaters may be necessary to prevent the multiplication of *L. pneumophila* and its subsequent release in aerosols produced by shower heads. When excessive levels of *L. pneumophila* are detected, shock treatment with calcium hypochlorite at a dose of 50 mg/L per day will lower the concentration of this disease-causing bacterium to acceptable levels.

Generally, bromine is too toxic to be used safely around people. However, organic-bromine complexes are used to disinfect the water in swimming pools, hot tubs, and whirlpools.

Iodine is a very effective antiseptic agent because it is bactericidal and sporicidal so that it kills all types of bacteria, including endospores. It is also effective against many protozoa. Iodine is used in alcohol solution (tincture of iodine) and in combination with organic molecules (an iodophor). Iodine combined with polymers such as polyvinylpyrrolidone is a particularly effective antiseptic because the iodine is released slowly. Iodine is frequently applied to minor wounds to kill microorganisms on the skin, thereby preventing infection of the wound. Normally it does not seriously harm human tissues but tincture of iodine stains tissue and may cause local skin irritation and occasional allergic reactions.

Iodophors are less irritating than tincture of iodine and do not stain the skin surface. Antiseptic iodophors are used routinely for preoperative skin cleansing and disinfection. Wescodyne and Betadine are frequently used for preoperative disinfection of skin and laboratory paraphernalia. In surgical procedures, the surgical staff often scrubs with soap impregnated with iodine, and the patient's skin in the area of the incision is normally treated with iodine before beginning the surgical procedure. Betadine and Isodine are frequently used for this purpose. A standard surgical scrub with a 10% solution (1% available iodine) decreases the cutaneous bacterial population by 85% and is particularly effective against Gram-negative bacteria.

Phenolics

Phenol (carbolic acid) is probably the oldest recognized disinfectant. Its use as a germicide in operating rooms was introduced by Joseph Lister in 1867. Phenol and its chemical derivatives (phenolics) disrupt plasma membranes, inactivate enzymes, and denature proteins, thereby exerting antimicrobial activities. Phenolics are particularly useful as disinfectants because they are very stable when heated or dried. They also retain their activity in the presence of organic material. Phenolics are commonly used for the disinfection of hospital floors and walls.

Phenolics disrupt plasma membranes, inactivate enzymes, and denature proteins. They are stable when heated or dried and remain active in the presence of organic material.

Cresols, which are phenolic derivatives of coal tars, are good disinfectants. Although cresols are only moderately effective against bacterial spores and are disruptive to human tissues, they are useful for disinfecting inanimate objects. The active ingredient in Lysol, a commonly used household disinfectant, is the cresol *o*-phenylphenol. In hospitals the commercial phenolics One-Stroke Vesthene and Wexide are used to disinfect emergency rooms and operating rooms (FIG. 11-10). The distinctive aroma of these phenolics gives many hospitals their characteristic smell. In addition to their use for disinfecting floors and walls, phenolics are incorporated into telephone poles, railroad ties, and other wood products to prevent microbial deterioration of the wood.

Several phenolic compounds are widely used antiseptics. Resorcinol (meta-dihydroxybenzene) is only about one third as active as phenol, but it is both bactericidal and fungicidal. Resorcinol is used in the treatment of acne, ringworm, eczema, psoriasis, dermatitis, and other cutaneous lesions. It is usually applied as a 10% ointment or lotion. Hexylresorcinol is commonly used in mouthwashes and in over-the-counter drugs used for treating sore throats. Thymol is used in vaginal deodorants at a concentration of

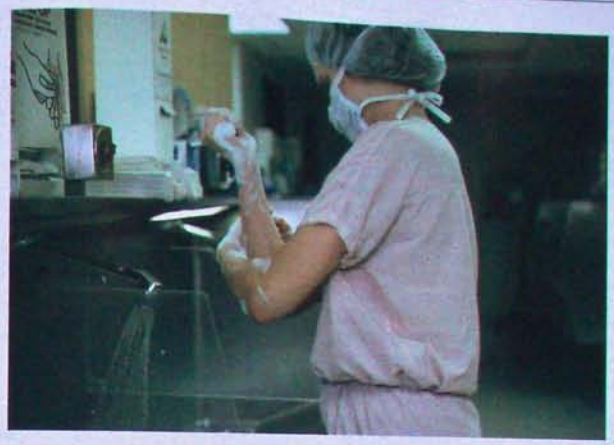


FIG. 11-10 Hospital staff scrub to disinfect skin and reduce the chances of tranferring pathogens.

1% because of its antibacterial and antifungal activities.

Hexachlorophene is one of the most useful of the phenol derivatives. Combined with a soap, it is a highly effective skin disinfectant. Unlike most phenolic compounds, hexachlorophene has no irritating odor and has a high residual action. Hexachlorophene is more effective against Gram-positive than against Gram-negative bacteria. A 3% solution of hexachlorophene will kill *Staphylococcus* within 30 seconds, but up to 24 hours may be required to kill Gram-negative bacteria. Because most bacteria on the skin are Gram-positive, hexachlorophene, the active ingredient in pHisoHex, once was commonly used by surgeons, physicians, and other health care workers. It was also used in the 1960s for daily bathing of newborns to prevent fatal *Staphylococcus* infections. However, it was found that frequent bathing of infants with hexachlorophene could lead to neurological damage and therefore this practice was largely discontinued. Over-the-counter preparations of hexachlorophene were banned in the United States by the FDA, but hexachlorophene is still used for limited purposes in hospitals.

The use of pHisoHex as a surgical scrub has largely been replaced by the use of povidone iodine (scrub soap form of Betadine) and Hibiclens (chlorhexidine gluconate). These two scrubs are effective against many microorganisms commonly encountered in hospitals. The antimicrobial action of iodine has already been discussed. Hibiclens is chemically different from other antimicrobials that are commonly used in the United States. It maintains a high level of antimicrobial activity in the presence of organic matter, such as blood, and does not irritate or dry the skin. **Chlorhexidine** is not a phenol, although its structure and application resemble hexachlorophene. It is frequently used for disinfection of skin and mucous membranes as an alternative to hexachlorophene. It is combined with a detergent or alcohol for surgical hand scrubs and preoperative skin preparation in patients. In such applications, it works more rapidly than hexachlorophene and is equally persistent on the skin. The skin does not absorb it, and no toxicity has been reported. Its killing effect is related to damage to the plasma membrane. It is effective against most vegetative bacteria, but not against spores.

Detergents

Detergents also are effective for removing microorganisms from floors and walls. One end of a detergent molecule is hydrophilic and mixes well with water. The other end is hydrophobic and is attracted to nonpolar organic molecules. If the detergents are electrically charged, they are termed *ionic*. Anionic (negatively charged) detergents are only mildly bactericidal. Anionic detergents are used as laundry detergents to remove soil and debris. They also reduce numbers of microorganisms associated with the item being washed. Cationic (positively charged) detergents are highly bactericidal, that is, they kill bacteria. In particular, cationic detergents are effective against *Staphylococcus* and various viruses. This qual-

ity makes them excellent candidates for disinfecting agents for hospital use.

The most widely used cationic detergents are **quaternary ammonium compounds (quats).** These compounds have four organic groups bonded to a nitrogen atom. Some examples of commonly used quats are Ceepryn (cetylpyridinium chloride), Phemerol (benzethonium chloride), and Zephiran (benzalkonium chloride). These chemicals are commonly used in antiseptic scrubs and mouthwashes. They are effective against fungi and Gram-positive bacteria. Their bactericidal action appears to be based on the disruption of plasma membrane and enzyme function. The quaternary ammonium compounds that are effective antimicrobial agents are used in concentrations that are not irritating to human tissues. Several quaternary ammonium cationic detergents are used as antiseptic agents. These compounds are relatively nonirritating to human tissues at concentrations that are inhibitory to microorganisms. However, they act slowly and are inactivated by soaps. They are also adsorbed by cotton and other porous materials, which can severely interfere with their effectiveness as antiseptics. Many mouthwash formulations contain quats, as do storage solutions for contact lenses. Many hospitals also use quats for disinfecting floors and walls, as antiseptics, and for surgical scrubs.

Quaternary ammonium compounds have four organic groups bonded to a nitrogen atom. Their bactericidal action is based on disrupting plasma membranes and enzymes.

There are, however, some problems associated with the use of quats as disinfectants. Their antimicrobial activity is lowered if they are absorbed by porous or fibrous materials such as gauze bandages. Hard water containing calcium or magnesium ions interferes with their action. Also, they can cause metal objects to rust. More importantly, rather than killing *Pseudomonas* species, quats actually support the growth of these bacteria. As such, quats are not used in operating theaters because of the danger that they will permit *Pseudomonas* to survive and infect surgical wounds.

Alcohols

Alcohols are among the most effective and heavily relied on agents for disinfection (FIG. 11-11). **Alcohols** denature proteins, disrupt membrane structure, and act as a dehydrating agent, all of which contribute to their effectiveness as an antiseptic. Even viruses are inactivated by alcohol. Methanol, ethanol, and isopropanol are commonly used for disinfection. Of the three, isopropyl alcohol has the highest bactericidal activity and therefore is the most widely used.

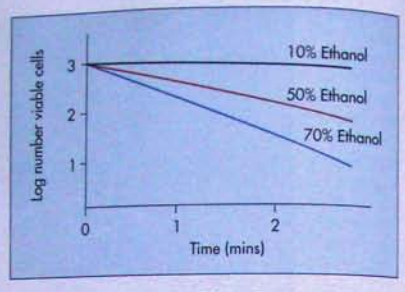


FIG. 11-11 Alcohol is a widely used antiseptic for killing microorganisms on skin. Its effectiveness is concentration dependent. The most effective concentration is about 70% (higher concentrations are less effective).

In practice, a solution of 70% to 80% alcohol in water is generally employed, although isopropyl alcohol is effective in solutions of up to 99%. On the skin, 70% ethanol kills nearly 90% of the cutaneous bacterial population within 2 minutes. Before puncturing the skin with a hypodermic syringe, the area is generally wiped with alcohol. Cabinet surfaces are frequently disinfected by wiping with alcohol. Some medical instruments are left soaking in alcohol to maintain their sterility. Oral thermometers are often wiped with alcohol to kill disease-causing microorganisms that otherwise might be transmitted from one patient to another. Even though brief exposure to alcohol is not sufficient to achieve sterility, it reduces the numbers of microorganisms to levels that make infection unlikely.

Alcohols denature proteins and disrupt membrane structure.

Aldehydes

Formaldehyde and glutaraldehyde are useful for disinfecting medical instruments. These **aldehydes** kill microorganisms by denaturing proteins. Instruments can be sterilized by placing them into a 20% solution of formaldehyde in 70% alcohol for 18 hours. Formaldehyde, however, leaves a residue and it is necessary to rinse the instruments in sterile water before use. A solution of glutaraldehyde at pH 7.5 kills *Staphylococcus* within 5 minutes and *Mycobacterium tuberculosis* within 10 minutes, but endospores may survive for up to 12 hours. The use of glutaraldehyde is limited by its expense. It is used for sterilizing specialized medical instruments such as bronchoscopes.

Aldehydes denature proteins, thus killing microorganisms.

Acids

Several acids are used as antiseptics. Acetic acid at a concentration of 5% is bactericidal and at lower concentrations is bacteriostatic. It is occasionally used at a concentration of 1% in surgical dressings. *Pseudomonas aeruginosa* is particularly susceptible to acetic acid, and this acid may be employed in burn therapy. It is used in vaginal douches to suppress fungal and protozoan infections of the vaginal tract.

Undecylenic acid is active against various fungi, including fungi that cause superficial mycoses. It is usually compounded with zinc but may also be used alone. Compounded undecylenic acid contains 2% to 5% undecylenic acid and 20% zinc undecylenate. This antiseptic agent is very useful for the treatment of ringworm. Undecylenic acid is the active ingredient in Desenex, which is used to treat athelete's foot and other fungus infections of the skin. Benzoic acid and salicylic acid used in combination also inhibit fungal growth. Whitfield's ointment contains benzoic acid and salicylic acid in a ratio of 2:1. This ointment is used to prevent fungal growth on the feet, as occurs in athlete's foot.

Ethylene Oxide

Ethylene oxide has several applications as a sterilizing agent. The ethylene portion of the molecule reacts with proteins and nucleic acids. **Ethylene oxide** kills all microorganisms and endospores. It is toxic and explosive in its pure form, so it is usually mixed with a nonflammable gas such as carbon dioxide or nitrogen.

A special autoclave-type sterilizer is used for ethylene oxide sterilization. Several hours of exposure to 12% ethylene oxide at 60° C is used for sterilization. Its remarkable penetrating power is one reason why ethylene oxide was chosen to sterilize spacecraft sent to land on the moon and planets—using heat to sterilize the electronic gear on these vehicles was not practical.

Because of their ability to sterilize without heat, gases like ethylene oxide are also widely used on medical supplies and equipment that cannot withstand steam sterilization. Examples include disposable sterile plasticware such as syringes and Petri plates, linens, sutures, lensed instruments, artificial heart valves, heart-lung machines, and mattresses. Many large hospitals have ethylene oxide chambers, some large enough to sterilize mattresses, as part of their sterilizing equipment. Additionally, some foods such as nuts and spices are sterilized by exposure to ethylene oxide.

Ethylene oxide sterilizes without heat, making it useful for sterilizing materials that cannot withstand high temperatures.

Hydrogen Peroxide

Hydrogen peroxide (H_2O_2) is an effective antiseptic. The molecule is unstable and degrades into water and oxygen. Anaerobic bacteria are particularly sensitive to peroxides because they do not have catalase, an enzyme that degrades peroxides. Hydrogen peroxide concentrations of 0.3% to 6.0% are used as antiseptics. A 3% solution is often used to cleanse and disinfect wounds. Although its germicidal action is brief, the effectiveness of hydrogen peroxide against anaerobic bacteria is important because several deadly anaerobic bacteria often are associated with soils that may contaminate wounds. Higher concentrations of 6.0% to 25.0% can be used in sterilization. Such treatment is useful for surgical implants and contact lenses because it leaves no residual toxicity after a few minutes of exposure. H_2O_2 is not effective against Gram-positive bacteria, including staphylococci found on skin.

Ozone

Ozone is a strong oxidizing agent that kills microorganisms by oxidizing cellular biochemicals. In some communities, ozone replaces chlorination as the primary means of disinfecting drinking water. Unlike chlorination, **ozonation** leaves no residue. The current cost of ozonation limits its widespread introduction for disinfection purposes. However, ozonation costs may eventually come down, particularly with the advantage of leaving no residue.

Dyes

Various dyes that are bacteriostatic or bactericidal are widely used as antiseptics. For example, crystal violet, which is also called gentian violet, is a potent bacteriostatic agent for Gram-positive bacteria. It is bactericidal at concentrations of less than 1:10,000. The mechanism of action of this compound against Gram-positive bacteria appears to be very similar to that of penicillin. It blocks a final step in the synthesis of cell wall material. Crystal violet has been used for the treatment of vaginal tract infections because the protozoan *Trichomonas* and the fungus *Candida albicans*, two common etiologic agents of vaginitis, are very sensitive to this dye.

Heavy Metals

Microorganisms are inhibited by heavy metals such as mercury, silver, and copper. Mercuric chloride was once used as a disinfectant solution, but because it is inactivated by organic matter, it is not widely used anymore. Copper sulfate is effective as an algicide. This compound is frequently added to swimming pools and aquaria to control algal growth.

Heavy metals are also used in antiseptic formulations. Mercury, zinc, and silver are examples of heavy

FIG. 11-12 Photograph of oligodynamic action of heavy metals.

metals used to kill microorganisms. The inhibitory effect of heavy metals is termed **oligodynamic action** (FIG. 11-12). Heavy metal ions can react with sulfhydral groups (—SH) on proteins, which inactivates them.

Silver nitrate has classically been applied to the eyes of newborn human infants to kill possible microbial contaminants. It is particularly important in the prevention of the transmission of gonococcal infections from mother to newborn. Silver combines with proteins and disrupts bacterial surface structures. Silver nitrate and silver sulfadiazine are used to treat severe burns. Preventing infections of tissues exposed by burns is critical to the recovery of the patient.

Organic mercury compounds are effective antiseptics for the treatment of minor wounds and as preservatives in serums and vaccines. The organic mercurials are bacteriostatic and relatively nontoxic. Mercurochrome (merbromin) was the first organic mercurial antiseptic to be introduced. Mercurochrome actually has limited bacteriostatic action and the lowest therapeutic index of the commercial mercurial antiseptics. Metaphen and Merthiolate are more effective.

Salts of zinc are used as mild antiseptics. Zinc pyrithione is used as an antidandruff agent. Calamine lotion, for example, contains zinc oxide. Calamine lotion is used in the treatment of ringworm, impetigo, and various other cutaneous diseases. White lotion, which contains zinc sulfate at a concentration of 4%, is also used to treat skin diseases and infections. A mixture of a long-chain fatty acid and the zinc salt of the acid is commonly used as an antifungal powder or ointment. It is particularly effective in the treatment of athlete's foot. The zinc salt also acts as an astringent and aids in healing superficial lesions, as does zinc oxide paste. Zinc oxide paste also is commonly recommended for treating diaper rash and its concurrent bacterial or fungal infections.

TABLE 11-6

Some Representative Antibiotics

ANTIBIOTIC	MECHANISM OF ACTION	TARGET ORGANISMS	SOME USES
Penicillin G	Inhibits bacterial cell wall synthesis	Gram-positive bacteria	Streptococcal sore throat, gonorrhea, syphilis
Ampicillin	Inhibits bacterial cell wall synthesis	Gram-positive and Gram-negative bacteria	Middle ear infections, urinary tract infections caused by *Enterococcus faecalis*, infections caused by some strains of *Escherichia coli*
Methicillin	Inhibits bacterial cell wall synthesis	Gram-positive and Gram-negative bacteria	Penicillinase-producing *Staphylococcus aureus* infections
Cephalosporins	Inhibits bacterial cell wall synthesis	Gram-positive and Gram-negative bacteria	Urinary tract and other infections caused by *Escherichia coli*, middle ear infections and meningitis caused by *Haemophilus influenza*
Streptomycin	Inhibits bacterial protein synthesis	Gram-negative bacteria	Bubonic plague, tularemia
Neomycin	Inhibits bacterial protein synthesis	Gram-negative bacteria	Sometimes used as a topical ointment for general cuts and abrasions of the skin
Chloramphenicol	Inhibits bacterial protein synthesis	Gram-positive and Gram-negative bacteria	Meningitis caused by *Haemophilus influenza* or *Neisseria meningitidis*, typhoid fever
Tetracycline	Inhibits bacterial protein synthesis	Gram-positive and Gram-negative bacteria	Pneumonia caused by *Mycoplasma*, nongonococcal urinary tract infections
Bacitracin	Inhibits bacterial cell wall synthesis	Gram-positive bacteria	Topical ointment for general cuts and abrasions of the skin
Erythromycin	Inhibits bacterial protein synthesis	Gram-positive and Gram-negative bacteria	Whooping cough, diphtheria, diarrhea caused by *Campylobacter* and pneumonia caused by *Legionella* or *Mycoplasma*
Rifampicin	Inhibits bacterial RNA synthesis	Gram-positive bacteria and some Gram-negative bacteria	Tuberculosis and Hansen disease (leprosy)
Nystatin	Damages plasma membrane	Yeast	*Candida albicans* infections of skin and vagina
Griseofulvin	Inhibits mitosis	Fungi	Tinea (ringworm) of hair and nails
Amphotericin B	Damages plasma membrane	Fungi	Histoplasmosis, cryptococcal meningitis

ANTIBIOTICS

Antibiotics are antimicrobial substances that were originally produced by other microorganisms. Many antibiotics today are synthetically manufactured by pharmaceutical companies. However, the original parent compound was isolated from a microorganism. For example, penicillin, the first antibiotic to be discovered, was isolated from a fungus, *Penicillium chrysogenum*. Streptomycin was first isolated from the bacterium *Streptomyces griseus*.

Antibiotics are used clinically to prevent or treat human diseases caused by microorganisms (Table 11-6). A more complete description of antibiotics and their use in combatting human infections is presented in Chapter 18. Antibiotics are also used in controlling microorganisms in nonhuman animals. They are routinely added to animal feeds to protect swine, cattle, poultry, and other farm animals from microorganisms. The addition of antibiotics to animal feeds stimulates the growth of the animals by controlling infections, especially in an animal's intestinal tract. This probably contributes to faster weight gain by the animals, which in turn reduces the amount and cost of feeding them. They are especially useful in preventing outbreaks of disease when large numbers of animals are housed together, such as in chicken coops. There is concern, however, that the widespread use of antibiotics in animal husbandry will lead to greater abundances of antibiotic-resistant microorganisms and that residues of antibiotics will cause problems in individuals who consume these food products.

SUMMARY

Control of Microorganisms by Physical Environmental Factors (pp. 311-320)

- Environmental factors influence rates of microbial growth and death. Some environmental conditions increase microbial growth and others decrease or end microbial growth. Microbial populations can be controlled by modifying the physical conditions under which they live. Each microbial species has a specific tolerance range for specific environmental conditions.

Physical Exclusion or Removal of Microorganisms (pp. 311-312)

- Filtration is an effective method for achieving sterility of liquids and gases.

High Temperatures (pp. 312-315)

- High temperatures can be used to kill all microorganisms in a sample. This is heat sterilization. Decimal reduction time describes the heat killing of microorganisms. It is the time required for a tenfold reduction in the number of viable cells at a given temperature. The D value decreases as the temperature is raised above maximal. The D value for heat-resistant, endospore-forming microorganisms is used for processing in the canning industry.
- Pasteurization uses relatively brief exposures to moderately high temperatures to reduce the number of viable microorganisms and to eliminate human pathogens. Pasteurization reduces the number of microorganisms but does not sterilize.
- Sterilization uses high temperatures to kill all viable microorganisms. Autoclaving, using steam under pressure, is used to sterilize many materials by exposure at 121° C at 15 pounds per square inch pressure for 15 minutes. Dry heat sterilization requires higher temperatures and longer exposure times.
- Canning uses elevated heat followed by conditions that ensure the maintenance of aerobic conditions to preserve foods.

Low Temperatures (p. 316)

- Low temperatures limit the rates of microbial reproduction and can be used to prevent or limit microbial growth. Freezing at −20° C or lower precludes microbial growth entirely. Refrigeration at 5° C limits the rate of microbial growth.

Removal of Water—Dessication (pp. 317-318)

- Desiccation removes the water required for microbial growth; it does not necessarily increase the death rate. Some microorganisms can be preserved by drying. Water can be removed by sublimation in freeze-drying (lyophilization).
- Microorganisms are unable to grow at low water activity. Adding salt limits microbial growth and increases the shelf life of some foods. Drying is used to

preserve many foods, such as powdered milk, dried fruits, and cereals.

Radiation (pp. 318-320)

- Microorganisms can be killed by exposure to certain forms of radiation. Gamma and X-radiation have high penetrating power and kill microorganisms. Ultraviolet light does not have high penetrating power but kills microorganisms on surfaces. Infrared and microwave radiations have poor penetrating power and do not appear to kill microorganisms directly. The absorption of long wavelength radiation results in increased temperature, killing microorganisms.

Control of Microbial Growth by Antimicrobial Agents (pp. 320-333)

- Chemical inhibitors are used to prevent the spread of disease-causing microorganisms and to preclude the growth of microorganisms that cause the spoilage of foods or biodeterioration of industrial products.

Types of Antimicrobial Agents (pp. 320-322)

- Antimicrobial agents are classified according to their application and spectrum of action. Biocides are agents that kill living organisms or inactivate viruses. Germicides are chemical agents that kill microorganisms. Virucides inactivate viruses; bactericides kill bacteria; algicides kill algae; and fungicides kill fungi.

Food Preservatives (pp. 322-323)

- Chemical additives are used in the preservation of food and other products. The addition of salt or sugar to food reduces the amount of available water and alters the osmotic pressure, creating bacteriostatic conditions.
- Various low molecular weight carboxylic acids are effective inhibitors of microbial growth. Propionates are effective against filamentous fungi and are used in milk and bread dough products. Lactic and acetic acids are effective, naturally occurring preservatives in such food products as cheeses, pickles, and sauerkraut. Benzoates are used as preservatives in fruit juices, jams, jellies, soft drinks, salad dressings, catsup, margarine, and pharmaceuticals. Sorbic acid inhibits fungi and bacteria at pH 4-6. Boric acid is used as a preservative in eyewash and other products.
- Nitrites and nitrates are added to cured meats to preserve red meat color and protect against the growth of food spoilage and poisoning microorganisms.

Disinfectants and Antiseptics (pp. 323-333)

- Disinfectants are antimicrobial substances that kill or prevent the growth of microorganisms and are used on inanimate objects. Disinfectants must have high germicidal activity; rapidly kill a wide range of microorganisms, including spores; be chemically stable and effective in the presence of organic compounds and metals; be able to penetrate crevices; and be inexpensive and aesthetically acceptable.
- Concentration and contact time are critical factors in determining effectiveness of antimicrobial agents; standardized tests evaluate the effectiveness of disinfectants.
- Antiseptics are antimicrobial agents with relatively low toxicities to human tissues so that they can be applied to the skin.
- Antiseptics are used for surface applications to biological tissue but are not necessarily safe for consumption. A toxicity index is the ratio of the greatest dilution of the product that can kill animal cells in 10 minutes to the dilution that can kill bacterial cells in the same period of time and under identical conditions. Antiseptics should have a toxicity index of less than 1.0.
- Chlorine kills microorganisms by disrupting membranes and inactivating enzymes. Chlorination is the standard treatment for disinfecting drinking water and effluents from sewage treatment plants.
- Iodine is bactericidal and sporicidal and is also effective against protozoa. Iodophors are used for preoperative skin cleansing and disinfection.
- Phenolics disrupt plasma membranes, inactivate enzymes, and denature proteins; they are very stable when heated or dried and retain activity in the presence of organic material; they are in common household disinfectants and are used in hospital wards and operating theaters. Phenolics include resorcinol and hexachlorophene. Resorcinol is bactericidal and fungicidal and is used in the treatment of acne, ringworm and other skin infections. Hexachlorophene combined with soap is a highly effective skin disinfectant and deodorant.
- Detergents, particularly quaternary ammonium compounds, are effective disinfectants used for removing microorganisms from floors and walls. Anionic detergents are used as laundry detergents to remove soil and thus lower the numbers of associated microorganisms. Quaternary ammonium cationic detergents are used as antiseptic agents.
- Alcohols are the most effective and most used agents for sterilization and disinfection; they denature proteins and disrupt membrane function.
- Aldehydes, such as formaldehyde and glutaraldehyde, are used for disinfecting and sterilizing medical instruments.
- Acids used as antiseptic agents include acetic acid and undecylenic acid.
- Peroxides, in the form of hydrogen peroxide, are effective nontoxic antiseptics because they are unstable and degrade into a reactive form of oxygen that is toxic to microorganisms. They are used with surgical implants and contact lenses.
- Dyes, such as crystal violet, block the final step in the synthesis of cell wall material.
- Ethylene oxide sterilization is used in hospitals to disinfect materials that cannot withstand steam sterilization.
- Ozone is a strong oxidizing agent that kills microorganisms by oxidizing cellular biochemicals. Ozonation is used to disinfect drinking water.
- Heavy metals, —mercury, silver, and copper— inhibit microorganisms. Heavy metals are inhibitory to microbial growth because of their oligodynamic action.

Antibiotics (p. 333)

- Antibiotics are antimicrobial substances produced by microorganisms.
- Antibiotics are used to treat disease in humans and nonhuman animals.

CHAPTER REVIEW

REVIEW QUESTIONS

1. Define sterilization.
2. Define disinfection.
3. Explain how you could disinfect different types of materials.
4. Describe how you would sterilize several different types of materials.
5. Define antisepsis and explain its relation to the elimination or suppression of microbial growth.
6. What is a germicide and what is its relation to the elimination or suppression of microbial growth?
7. How is microbial growth affected by the type of microorganism and environmental conditions?
8. What physical methods can be employed to control microbial growth?
9. What factors determine the effectiveness of a disinfection agent?
10. How are disinfectants evaluated?
11. What is pasteurization?
12. How does refrigeration increase the shelf life of milk?
13. Why don't you have to refrigerate sugar-coated cereal?
14. What is a phenol coefficient?
15. Which heavy metals are used as disinfectants or antiseptics?
16. How are the halogens—chlorine, bromine, and iodine—used as disinfectants?

CRITICAL THINKING QUESTIONS

1. How can you determine if a disinfectant or antiseptic is bacteriostatic or bactericidal? How can you determine the appropriate disinfectant to use for cleaning the floor of a hospital? How can you determine the appropriate antiseptic for treating minor abrasions?
2. What are the possible consequences of irradiation of food? Is it safe to eat perishable food that has been irradiated to extend its shelf life? What precautions, if any, should be taken with irradiated food?
3. What factors would you have to take into account in search for a new antiseptic? A new disinfectant? A new food preservative?
4. If your local water treatment plant shuts down because of a natural disaster, how could you treat the water at home to ensure that it is safe to drink?
5. Many health food stores market foods that are free of preservatives. What special precautions must be used with such foods? Are such foods safe for consumption?
6. What special precautions are taken during surgical procedures? Why does the surgical staff scrub if they are going to wear gloves? Are the gloves, gowns, and masks worn by the surgical staff aimed at protecting the patient or the physicians and nurses?

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